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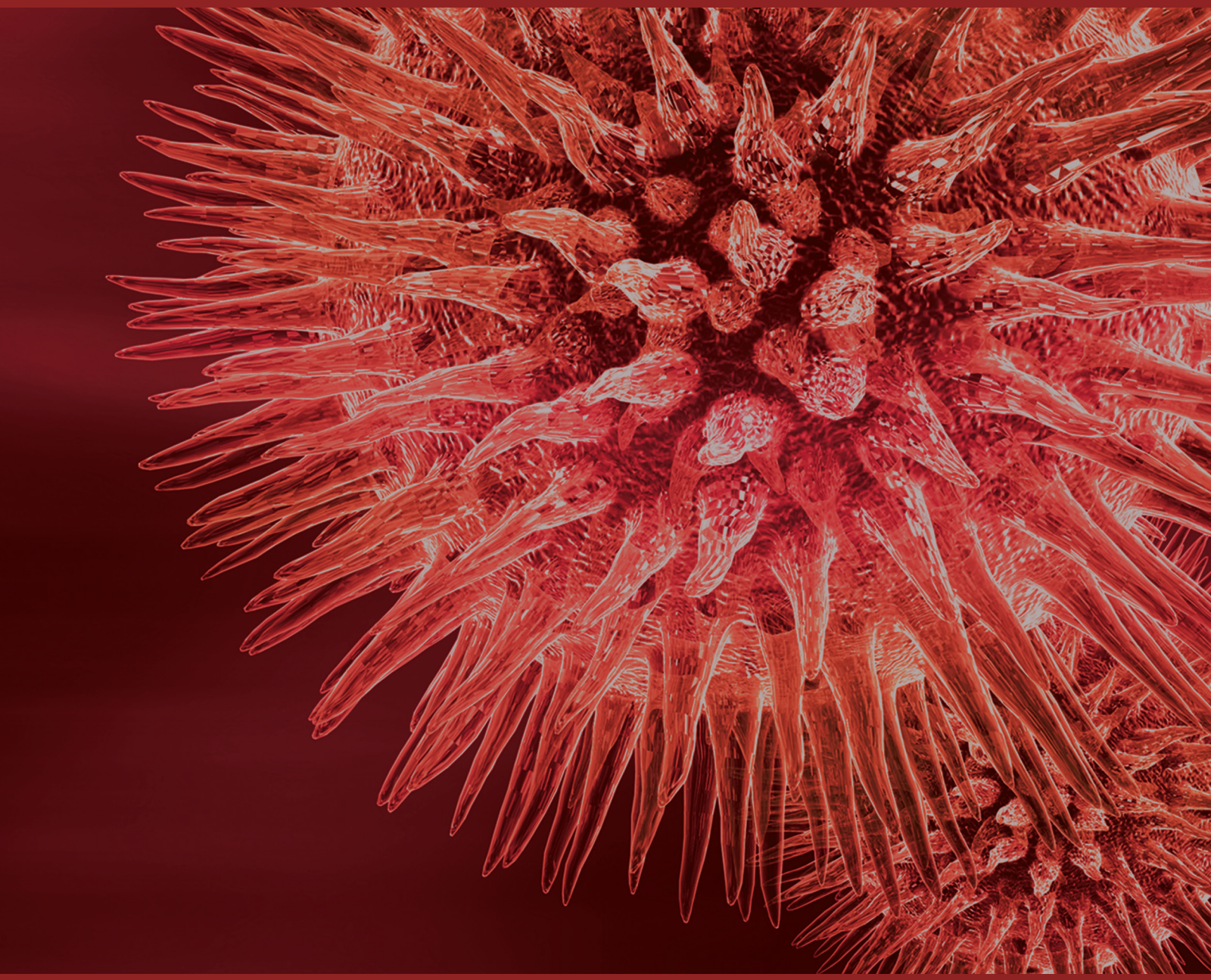
Microbial Enzymes and Their Applications in Industries and Medicine 2014

Guest Editors: Periasamy Anbu, Subash C. B. Gopinath, Bidur Prasad Chaulagain, Thean-Hock Tang, and Marimuthu Citartan



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Editorial

Microbial Enzymes and Their Applications in Industries and Medicine 2014

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Production of microbial enzymes is a necessary event in the industrial sectors, due to the high and superior performances of enzymes from different microbes, which works well under a wide range of varied physical and chemical conditions. Microbial enzymes serve as a potential replacement, in absence or insufficiency of human enzymes. Further, microbial enzymes are the preferred source of industrial enzymes as they can be produced in large quantities in a short period of time and have shorter generation times, and genetic manipulations can be performed more easily on bacterial cells to increase the enzyme production. Still the industries are looking for new microbial strains in order to produce different enzymes to fulfill the enzyme requirements. This special issue covers ten articles including two review articles, highlighting the importance and applications of biotechnologically relevant microbial enzymes.

In the work carried out by J. R. Wang et al., they have optimized the codon usage in order to enhance the expression level of α-amylase gene from *Bacillus licheniformis* in *Pichia pastoris*. A total of 328 nucleotides were altered and the G + C content was improved from 47.6 to 49.2%. The optimized gene has demonstrated a higher level of expression in *P. pastoris* after methanol induction for 168 h in 5- and 50-L bioreactor with the maximum activity of 8100 and 11000 U/mL, compared to the wild type by 2.31- and 2.62-fold. The enzyme is viewed as a potential candidate for α-amylase production in industrial use.

L. M. Colla et al. have carried out studies on the partial characterization of lipase acquired from solid-state fermentation by the species of *Aspergillus*. The fungal strains isolated from diesel contaminated soil and selected as the liapse producer. Two types of fermentation were adopted, whereby lipase produced from the submerged fermentation showed an optimum activity at 37°C and pH 7.2. In another fermentation mode, the solid-state fermentation, the optimal temperature and pH for the solid-state fermentation are 35°C and pH 6.0, respectively. Out of the two fermentation modes, lipase produced from the submerged fermentation has more stability at higher temperature than the solid-state fermentation. Lipase from the submerged fermentation still retains 72% of residual activity after one hour of exposure at 90°C. Furthermore, 80% of the residual activity is retained at the acidic pH while lipase obtained from the solid-state fermentation results in residual activity of 60% at the alkaline pH.

N. E.-A. El-Naggar et al. have isolated L-asparaginase producing actinomycete from soil samples. In this study, the isolated strain was identified as *Streptomyces olivaceus* based on the morphological, physiological, biochemical, and molecular analysis. Further, the enzyme production was optimized by response surface method to improve the level of enzyme synthesis. The authors have screened fifteen variables using Plackett-Burman experimental design. The most significant independent variables affecting enzyme production were further optimized by the face-centered central

composite design-response surface methodology. Finally, the strain was able to produce a significant level of L-asparaginase enzyme in optimized media.

The review article by F. D. Inácio et al. focused on proteases of wood rot fungi with emphasis on the *Pleurotus* genus. In this review article, the authors have highlighted the characteristics of the *Pleurotus* genus such as easy cultivation techniques, high yield, low nutrient requirements, and its adaptation. In addition, the authors have also highlighted the uses and applications of the proteases in various industries such as biotechnology and medicine.

Another study with amylase is by I. Ali et al.; they performed purification and characterization of polyextremophilic extracellular α-amylase from a halophilic strain, *Aspergillus penicillioides,* to be coused with detergents. Purification was performed by ammonium sulfate precipitation and Sephadex G100 gel filtration methods and the purified enzyme has an apparent molecular weight of 42 kDa. The purified enzyme showed higher specificity with the substrate, starch, with an optimal activity at pH 9, 80°C in the presence of NaCl and $CaCl_2$. Authors have shown retaining amylase activity as 80% with different laundry detergents, claimed as better activity than commercial amylase.

The review article by S. C. B. Gopinath et al. is on the biotechnological aspects of keratinase production and their future perspectives in industries. Because of ever increasing industrialized food and agriculture market there is high biomass production of the tough biomass keratin which will be biopollutant to the earth and needs to be addressed in a more ecofriendly way. The review highlights the production of keratinase from reliable sources that can be easily managed. The authors put emphasis on microbial keratinase because it is less expensive and more precise than those conventionally used chemicals to treat keratin. The paper discusses different perspective of enzymatic keratinases which can be obtained from fungi, bacteria, and actinomycetes. The attraction to this article for readers is due to the expansion of information on various aspects of keratinase like descriptions on keratinophilic fungi, keratin-degrading bacterial isolates, secretion of microbial keratinases, optimization of keratinase activities, purification process, accelerating microbial keratinase production through biotechnological approach, and developing keratinase sensing technologies in future.

In the article by A. Badhan et al., they have utilized enzyme fingerprinting to identify cell wall components resistant to total tract digestion. Acetyl xylan esterases were identified as the key component to the improved ruminal digestion. Polysaccharide-lignin cross-linked cell wall polymers were identified as the principal components indigested fiber residues in the feces. Enzyme pretreatment was carried out following the analysis of the structural information obtained from the enzymatic fingerprinting and FTIR to enhance glucose yield from barley straw and alfalfa hay. Statistical experimental design was used to analyze the prehydrolysis effects of recombinant acetyl xylan esterases (AXE16B_ASPNG and AXE16A_ASPNG), polygalacturonase (PGA28A_ASPNG), and α-arabinofuranosidase (ABF54B_ASPNG) all from *Aspergillus niger*, feruloyl esterase (FAE1a) from *Anaeromyces mucronatus* (expressed in *E. coli*), and endoglucanase GH7 (EGL7A_THITE) from *Thielavia terrestris* (produced in *Aspergillus niger*). Degradation of plant structural polysaccharides is inducted by the fungal fibrinolytic hemicellulases and auxiliary enzymes. Moreover, *in vitro* saccharification of alfalfa and barley straw by mixed rumen enzymes was improved. The model also estimated glucose yield that improves by 75% following pretreatment with polygalacturonase (PGA28A_ASPNG) and α-arabinofuranosidase (ABF54B_ASPNG) in 1 : 1 ratio. A hundred percent improvement was obtained after prehydrolysis of barley straw with a mixture of EGL7A_THITE (50%) and FAE1a (50%). Microassay and statistical experimental design can be integrated to predict effective enzyme pretreatments that can enhance plant cell wall digestion by mixed rumen enzymes. This study also has the potential to develop specific enzyme pretreatments for forages, depending on their structure and composition.

B. K. Dash et al. have isolated a new *Bacillus subtilis* strain (BI 19) from the soil and studied the molecular characterizations for the enhanced production of extracellular amylase. In their study, the phylogenetic tree was constructed on the basis of 16S rDNA gene sequences and revealed that this strain clustered with the closest members of *Bacillus* sp. The effect of various fermentation conditions on amylase production from this strain, through shake-flask culture, was investigated. Rice flour was found to be a cheap natural carbon source to induce amylase production. In addition, a combination of peptone and tryptone as organic and ammonium sulfate as inorganic nitrogen sources gave highest yield. Further, maximum production was obtained with the following optimal conditions: 24 h of incubation, 37°C, and pH 8.0. Further increments in the amylase productions were noticed in the presence of Tween 80 and sodium lauryl sulfate. Their results suggest that newly isolated *B. subtilis* BI 19 could be exploited for industrial production of amylase at relatively low cost and time.

S. G. Karp et al. on their original research report discuss the optimization of laccase production and its role in delignification. They have worked on the statistical optimization of laccase production by a strain of *Pleurotus ostreatus* and its delignification properties of sugarcane bagasse. With the determination of the mathematical model of laccase production through the response surface method they have analyzed the role of various factors and came to conclusion that yeast extract as an organic nitrogen source is very important along with appropriate concentration of copper sulfate and ferulic acid and the incubation period in solid-state fermentation for the delignification of sugar bagasse. The authors are successful in reducing the lignin content of sugar bagasse around one-third to one-fourth of the original lignin biomass. Such kind of experiment is important for delignification of biomass for future use in other industrial applications like production of biobased fuel to other organic products like food and beverages to paper and textile industries. The advantages of enzymatic delignification over the conventional physicochemical methods will be less health hazardous to ecosystem in future. The other advantages of laccase enzyme optimization may include relatively higher product yields and fewer side reactions and increased reactor

efficiency due to mild reaction conditions and lesser energy requirements.

L. P. Lee et al. have isolated many *Bacillus* species from oil spillage area in Malaysia. In this study, the isolated strains were screened for lipase and protease enzymes using lipid and gelatin, respectively, as the substrates. In addition, the comparison analyses of lipase and protease activities were also analyzed. The result showed that most of the strains were able to produce both enzymes at significant levels. The simultaneous secretion of both the lipase and protease is a means of survival. The isolated *Bacillus* species which harbor lipase and protease enzymes could render potential industrial based applications and solve the environmental problems.

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Research Article

Codon Optimization Significantly Improves the Expression Level of α-Amylase Gene from *Bacillus licheniformis* in *Pichia pastoris*

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α-Amylase as an important industrial enzyme has been widely used in starch processing, detergent, and paper industries. To improve expression efficiency of recombinant α-amylase from *Bacillus licheniformis* (*B. licheniformis*), the α-amylase gene from *B. licheniformis* was optimized according to the codon usage of *Pichia pastoris* (*P. pastoris*) and expressed in *P. pastoris*. Totally, the codons encoding 305 amino acids were optimized in which a total of 328 nucleotides were changed and the G+C content was increased from 47.6 to 49.2%. The recombinants were cultured in 96-deep-well microplates and screened by a new plate assay method. Compared with the wild-type gene, the optimized gene is expressed at a significantly higher level in *P. pastoris* after methanol induction for 168 h in 5- and 50-L bioreactor with the maximum activity of 8100 and 11000 U/mL, which was 2.31- and 2.62-fold higher than that by wild-type gene. The improved expression level makes the enzyme a good candidate for α-amylase production in industrial use.

1. Introduction

α-Amylases (E.C.3.2.1.1) are classified as a member of family 13 of the glycosyl hydrolases and catalyze the hydrolysis of internal α-1,4-O-glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products [1]. α-Amylases are one of the most important industrial enzymes that have a wide variety of applications in starch processing, paper industries, detergent, and so on [2, 3]. α-Amylases are ubiquitous enzymes produced by plants, animals, and microorganisms. Although there are many sources of α-amylases, microorganisms are the most important sources of α-amylases for industrial purposes due to advantages such as less time and space required for production, cost effectiveness, and ease of process modification and optimization [4].

In recent years, many kinds of α-amylase have been isolated from various microorganisms, such as bacteria and fungi. Among bacteria, *Bacillus* sp. is widely used for α-amylase production to meet industrial needs. *Bacillus subtilis* (*B. subtilis*), *Bacillus stearothermophilus* (*B. stearothermophilus*), and *Bacillus licheniformis* (*B. licheniformis*) are known to be good producers of α-amylase and these have been widely used for commercial production of the enzyme for various applications [5]. So far, a number of α-amylase genes have been isolated and characterized from *Bacillus* sp., including *B. licheniformis*, *B. stearothermophilus,* and *B. subtilis* [6–8]. In previous studies, a gene encoding α-amylase from *B. licheniformis* was cloned and expressed in *E. coli* and *B. subtilis* [9]. However, the low expression level does not allow the recombinant protein to be applied practically and economically in industry. For commercial exploitation of the recombinant α-amylase, it is essential to achieve high yield of the protein.

The methylotrophic yeast *P. pastoris* has many advantages as a host for production of recombinant heterologous proteins, such as high cell density, high levels of productivity,

ease of genetic manipulation, the ability to perform complex posttranslational modifications, and very low secretion levels of endogenous proteins [10]. To improve heterologous expression of genes, many strategies have been developed in *P. pastoris*. It includes high copy number of heterologous gene, appropriate signal peptide in expression vector, high efficient transcriptional promoters, and optimization of cell cultivation [11–13]. However, these optimization strategies did not universally result in high protein production for every recombinant protein as expected. It has now been shown that the difference of codon usage between the native gene sequence and expression host has significant impact on the expression level of recombinant protein [14, 15]. Therefore, the codon optimization is a promising technique for increasing foreign protein expression level.

In this study, we describe the high-level expression of *B. licheniformis* α-amylase (BlAmy) in *P. pastoris* and this is the first report about high cell density fermentation for production of recombinant *B. licheniformis* α-amylase (rBlAmy) in 5- and 50-L bioreactor. Furthermore, the α-amylase gene from *B. licheniformis* (*BlAmy*) was modified and expressed according to its preferred condon usage in *P. pastoris*. To our knowledge, this is also the first report to improve *B. licheniformis* α-amylase (BlAmy) production by codon optimization strategies in *P. pastoris*.

2. Materials and Methods

2.1. Strains, Plasmids, Reagents, and Media. The *P. pastoris* strain GS115 and the expression vector pPIC9K were purchased from Invitrogen (Carlsbad, CA, USA). The *E. coli* strain Top 10 is routinely conserved in our laboratory. Restriction enzymes, T_4-DNA ligase, and Pfu DNA polymerase were purchased from Sangon Biotech (Shanghai, China). All other chemicals used were analytical grade reagents unless otherwise stated. Yeast extract peptone dextrose (YPD) medium, buffered glycerol complex (BMGY) medium, and buffered methanol complex (BMMY) medium were prepared according to the manual of *Pichia* Expression Kit (Version F, Invitrogen). Fermentation Basal Salts (BSM) Medium and PTM1 Trace Salts used for fermentation were prepared according to the *Pichia* Fermentation Process Guidelines (Invitrogen).

2.2. Codon Optimization and Synthesis of the Gene. The codon usage of *BlAmy* (GenBank M38570) from *B. licheniformis* was analyzed using Graphical Codon Usage Analyser (http://gcua.schoedl.de/) and was optimized by replacing the codons predicted to be less frequently used in *P. pastoris* with the frequently used ones by (http://www.dna20.com/). Signal peptide was analyzed by SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). The optimized gene (*BlAmy-opt*) was synthesized by Sangon (Shanghai, China).

2.3. Vector Construction. The synthetic gene encoding the mature region of α-amylase without the predicted signal sequence was digested by EcoRI and NotI and then ligated into pPIC9K, forming pPIC9K-*BlAmy-opt*. The native α-amylase gene (*BlAmy*) from *B. licheniformis* was cloned into pPIC9K using primers BlF (5′-CATCGAATTCGCAAATCTTAATGGGACGCTG-3′) and BlR (5′-CATAGCGGCCGCCTATCTTTGAACATAAATTGA-3′), resulting in the recombinant plasmid pPIC9K-*BlAmy*. The recombinant plasmids were checked by DNA sequencing.

2.4. Transformation of P. pastoris and Screening Transformants. *P. pastoris* GS115 was transformed with 10 μg of SacI-linearized pPIC9K-*BlAmy-opt* and pPIC9K-*BlAmy* vector by electrotransformation, according to Invitrogen's recommendations. Transformants were initially selected by MD medium (1.34% yeast nitrogen base, 4×10^{-5}% biotin, 2% dextrose) plates and then checked by colony PCR. The insertion copy number of transformants was determined by their resistance to G418 and transformants with the same copy number were selected. The recombinants carrying *BlAmy* and *BlAmy-opt* were screened by a new plate assay method. The recombinants from 1.0, 2.0, and 4.0 mg/mL G418-YPD plate were picked and cultured in 96-deep-well microplates containing 200 μL/well BMGY medium at 30°C for 24 h. After this, the cells were harvested by centrifugation, resuspended, and cultured in 400 μL/well BMMY medium. After 24 h, plates were subjected to centrifugation again and supernatants were used in subsequent activity assays. Qualitative α-amylase activity was determined by a halo plate assay containing 3% (w/v) agar and 5% (w/v) soluble starch (Sangon, China). Supernatants (20 μL) were loaded into wells and plates were incubated at 60°C for 30 min. The clones were selected according to the size of the halos and their activities were checked by shaking flask fermentation.

2.5. Expression of BlAmy-opt and BlAmy in P. pastoris Shake-Flask Cultures. The transformants were selected and cultivated in shaking flask. The seeds were inoculated in 10 mL of BMGY medium in a 100 mL shake flask and incubated at 30°C and 250 rpm until the culture reached an OD600 = 2.0–6.0. The cells were harvested by centrifugation and resuspended in 50 mL of BMMY medium and incubated at 30°C and 200 rpm. The methanol induction temperature was set at 30°C, and 0.7% (v/v) methanol was fed at 24 h intervals for 5 days. The activities of the α-amylase were checked at 24, 48, 72, 96, 120, 144, and 168 h. The colony with the highest activity was selected as the strain to ferment in 5- and 50-L bioreactor.

2.6. High Cell Density Fermentation. The transformed strain showing the highest α-amylase activity in shake-flask culture was cultivated in high cell density fermentor. High cell density fermentation was carried out in 5- and 50-L bioreactor (Baoxing Co., Shanghai, China). The cultivation conditions and medium composition was the same as the previous described method [16]. Inoculum was cultured in BMGY medium. Cells were grown for 18–20 h at 30°C on shaker of 200 rpm. Then, 10% (v/v) of the inoculum was inoculated into the 5- and 50-L bioreactors containing 2- and 20-L basal

salt medium, made of 0.47 g/L $CaSO_4 \cdot 2H_2O$, 9.1 g/L K_2SO_4, 7.5 g/L $MgSO_4 \cdot 7H_2O$, 6.2 g/L KOH, 13.35 mL/L H_3PO_4 (85%), 20.0 g/L glycerol, and 1.5 mL *Pichia* trace metal 1 (PTM1). One liter PTM1 consists of 6 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3 g $MnSO_4 \cdot H_2O$, 0.5 g $CoCl_2$, 20 g $ZnCl_2$, 0.02 g H_3BO_3, 0.2 g $Na_2MnO_4 \cdot 2H_2O$, 65 g $FeSO_4 \cdot 7H_2O$, 0.2 g biotin, and 30 mL 6 N H_2SO_4. The temperature was controlled at 30°C and the pH was maintained at 5.0 using NH_4OH (28%) and H_3PO_4 (10%). For 5 L bioreactor, the agitation rate was set at 600 rpm and the aeration rate was 30 L/min. For 50 L bioreactor, the agitation rate was set at 500 rpm and the aeration rate was 40 L/min. When glycerol was used up, as indicated by an increase in dissolved oxygen (DO), 0.5% (v/v) methanol was added to induce expression α-amylase. Feeding of methanol was linked to the dissolved oxygen (DO). When the initial methanol 0.5% (v/v) was depleted (indicated by an abrupt increase in DO), 100% methanol solution containing 1.2% (v/v) PTM1 was added automatically. The concentration of methanol was kept stable by monitoring the dissolved oxygen (OD) content and maintaining it at greater than 20%. The enzyme activity of the supernatant and dry cell weight were monitored throughout the cultivation.

2.7. Purification, Deglycosylation, and SDS-PAGE Analysis of Recombinant BlAmy. After fermentation, cells from the cultures were removed by centrifuging at 6000 ×g for 10 min. The supernatant was concentrated by ultrafiltration using a Millipore set-up according to the manufacturer's instructions with a membrane of 10 kDa cut-off. The supernate containing recombinant BlAmy was purified by 2 mL Ni^{2+}-chelating chromatography according to the manuals (Biorad, USA). The elution buffer containing purified recombinant BlAmy was used for further analysis. Purified recombinant BlAmy was deglycosylated using 300 U of Endo H for 3 h at 37°C according to the manufacturer's instructions (NEB, USA). The deglycosylated and untreated were analyzed by SDS-PAGE. SDS-PAGE was carried out on a 12% running gel and stained with Coomassie Blue.

2.8. Assay of α-Amylase Activity and Protein Determination. α-Amylase activity was assayed according to the method described by previous studies [17]. One unit of α-amylase was defined as the amount of amylase needed to complete the liquefaction of 1 mg of starch into dextrin per minute at 70°C and pH 6.0. The protein content was determined according to the Bradford method using BSA as standard.

2.9. Characterization of the Recombinant BlAmy and Deglycosylated Recombinant BlAmy. The relative enzyme activity was determined at various pH values using 100 mM buffers, pH ranging from 4.0 to 11.0. Buffers used as standard were sodium acetate buffer (pH 4–6), sodium phosphate buffer (pH 6–8), and sodium carbonate buffer (pH 9–11). To evaluate the pH stability, aliquots of enzyme samples were incubated at 30°C for 24 h with respective pH buffers. Remaining enzyme activity was measured under standard assay protocol and calculated considering the initial activity. The optimal temperature of the enzyme was determined by measuring the enzyme activity at various temperatures (40–100°C) in 100 mM of sodium phosphate buffer, pH 7.0. Thermal stability was determined by incubating the purified enzyme in 100 mM of sodium phosphate buffer (pH 7.0) for 1 h at the desired temperatures (60–100°C) followed by measuring the residual activity.

3. Results and Discussion

3.1. Sequence Optimization and De Novo Synthesis of BlAmy. *P. pastoris* has been routinely used as a heterologous expression system because of its efficient secretion, high expression level, and high cell density [10]. However, the bias of codon usage between the native gene sequence and *P. pastoris* has significant impact on the expression level of recombinant protein. Codon optimization by using frequently used codons in the host is an efficient measure to improve the expression level of heterologous gene. Generally, this is accomplished by replacing all codons with preferred codons, eliminating AT-rich stretches and adjusting the G+C content [18, 19]. Analysis of the DNA sequence of native α-amylase gene (BlAmy) using Graphical Codon Usage Analyser revealed that some amino acid residues were encoded by codons that are rarely used in *P. pastoris*, codons like GGC (Gly), GCG (Ala), AGC (Ser), TCG (Ser), and CCG (Pro); most of them are shared less than 15% of usage percentage, which may result in a much lower expression level in *P. pastoris*. In order to achieve a high-level expression of BlAmy in *P. pastoris*, the codons of BlAmy were replaced with those more frequently used by *P. pastoris* (Table 1). The codon adaptation index (CAI) of the native *BlAmy* was improved from 0.74 to 0.86. Furthermore, the G+C content was increased from 47.6 to 49.2%, which was closer to the G+C content of other high-expression genes in *P. pastoris*. The nucleotides A, T, G, and C dispersed evenly in the synthesized gene to eliminate AT- or GC-rich motifs, codons containing both AT and GC were selected when the differences between the codon frequencies were not significant. Totally, the codons encoding 305 amino acids were optimized in which a total of 328 nucleotides were changed (Table 1). The optimized gene (*BlAmy-opt*) shared 77% of nucleotide sequence identity with that of the native gene (*BlAmy*) (Figure 1).

3.2. Vector Construction and Selection of Producing Clones. The recombinant plasmids pPIC9K-*BlAmy-opt* and pPIC9K-*BlAmy* were linearized and transformed into *P. pastoris* GS115 and several thousands of transformants were obtained on MD plates. In this study, the putative multicopy inserts were selected for expression by screening with the same concentration of G418. The positive clones (from 1.0, 2.0, and 4.0 mg/mL G418-YPD plate) were cultured in 96-deep-well microplates and further screened by a new halo plate assay (Figure 2). According to the size of the halos, twenty clones (ten isolated from recombinants carrying *BlAmy-opt* and ten from recombinants carrying *BlAmy*, resp.) from 2.0 mg/mL G418-YPD plate were selected for shake-flask cultures. The plate assay is a simple, rapid, and well adapted method for screening of large number of samples [20]. The diameter of

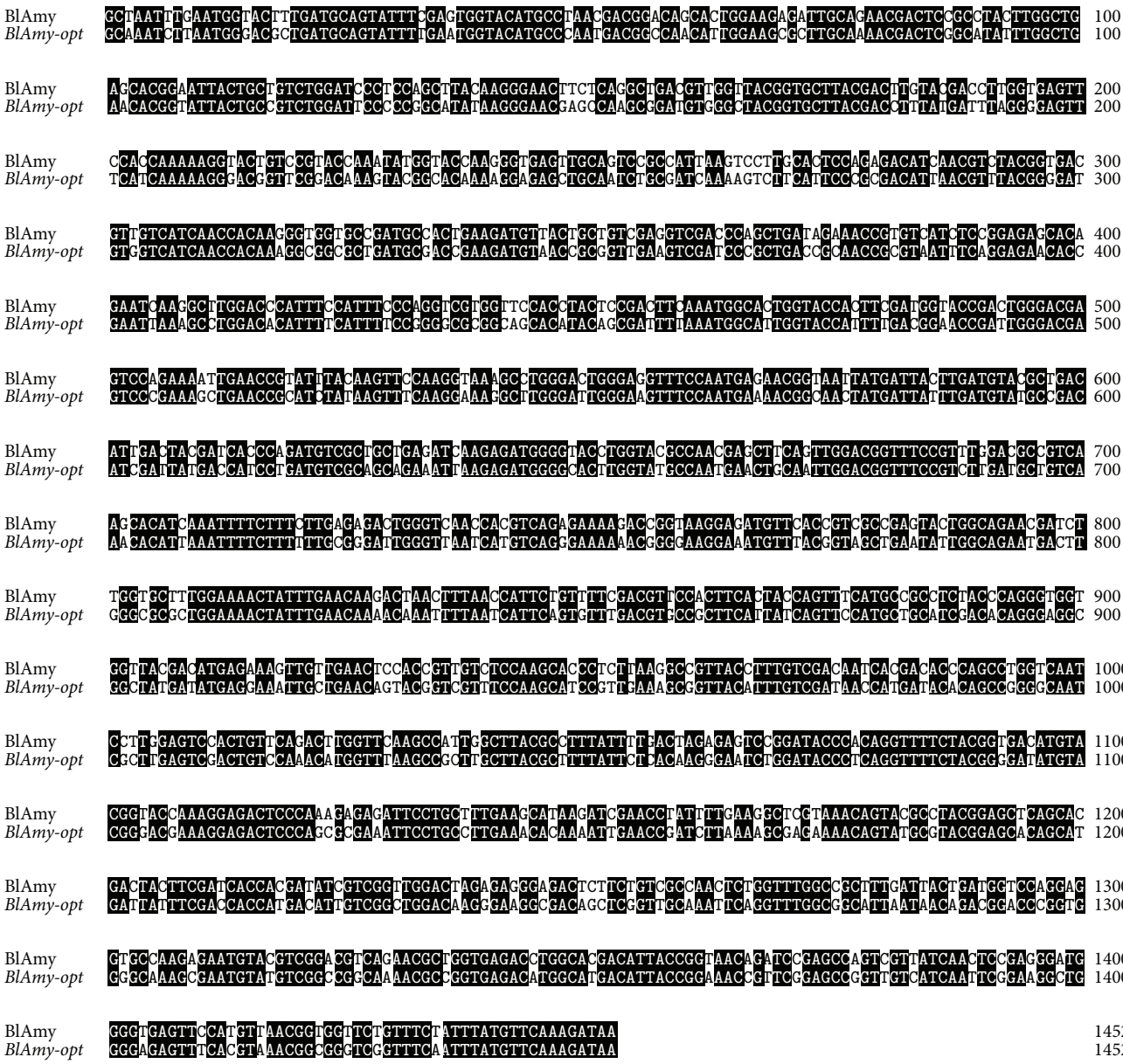

Figure 1: Sequence comparison between the original (*BlAmy*) and the optimized (*BlAmy-opt*) genes. Identical residues are marked in black background.

the halo zone is very useful for predicting the enzyme yield as an aid to select strains with a high level of α-amylase production.

3.3. Expression of BlAmy-opt and BlAmy in P. pastoris at Shaking Flask Level. Twenty colonies with larger halos were selected and cultivated in shaking flask. In shaking flask, the α-amylase activity increased gradually and reached the highest activity after 144 h of cultivation. After 144 h of cultivation under inducing conditions, the α-amylase activity of the supernatant from different clones carrying *BlAmy-opt* varied between 310 and 420 U/mL (enhanced 5.25-fold compared with the wild strain *Bacillus licheniformis*), while the recombinants carrying *BlAmy* varied between 150 and 230 U/mL (enhanced 2.87-fold compared with the wild strain *Bacillus licheniformis*), respectively. Two clones (one carrying *BlAmy-opt*, the other carrying *BlAmy*) from YPD plate containing 2.0 mg/mL showed the highest α-amylase activity of 420 U/mL and 230 U/mL in shaking flask culture was chosen for high cell density fermentation.

3.4. High Cell Density Fermentation. To obtain a large amount of active protein, fed-batch studies were carried out in 5- and 50-L fermentor. Upon methanol induction, the maximum α-amylase activity and protein concentration produced by recombinant strain GS115 carrying *BlAmy-opt* reached 8100 U/mL and 8.3 g/L, respectively, in the 5-L fed batch bioreactor (Figure 3(a)). Compared with

Table 1: Comparison of the codon usage for wild-type and synthetic α-amylase gene targeted at *P. pastoris* for expression.

AA	Codon	Host fraction	*BlAmy*	*BlAmy-opt*
Gly	GGG	0.10	11	0
	GGA	0.32	12	11
	GGT	0.44	6	34
	GGC	0.14	16	0
Glu	GAG	0.43	7	21
	GAA	0.57	18	4
Asp	GAT	0.58	19	11
	GAC	0.42	18	26
Val	GTG	0.19	4	0
	GTA	0.15	4	0
	GTT	0.42	12	14
	GTC	0.23	12	18
Ala	GCG	0.06	9	0
	GCA	0.23	10	0
	GCT	0.45	10	19
	GCC	0.26	7	17
Arg	AGG	0.16	4	0
	AGA	0.48	3	15
	CGG	0.05	3	0
	CGA	0.10	3	0
	CGT	0.16	2	7
	CGC	0.05	7	0
Lys	AAG	0.53	11	20
	AAA	0.47	17	8
Ser	AGT	0.15	2	0
	AGC	0.09	4	0
	TCG	0.09	8	0
	TCA	0.19	4	0
	TCT	0.29	3	9
	TCC	0.20	5	17
Stop	TAA	0.53	1	1
Asn	AAT	0.49	11	5
	AAC	0.51	14	20
Met	ATG	1.00	7	7
Ile	ATA	0.19	1	0
	ATT	0.50	13	10
	ATC	0.30	6	10
Thr	ACG	0.11	7	0
	ACA	0.24	13	0
	ACT	0.40	3	12
	ACC	0.25	4	15
Trp	TGG	1.00	17	17
Cys	TGT	0.65	0	0
	TGC	0.35	0	0
Tyr	TAT	0.46	18	5
	TAC	0.55	12	25
Leu	TTG	0.33	11	23
	TTA	0.16	3	0
	CTG	0.16	6	0
	CTA	0.11	0	0
	CTT	0.16	7	5
	CTC	0.08	1	0
Phe	TTT	0.54	16	5
	TTC	0.46	4	15
Gln	CAG	0.39	9	15
	CAA	0.61	11	5
His	CAT	0.57	17	6
	CAC	0.43	7	18
Pro	CCC	0.15	3	0
	CCG	0.09	8	0
	CCA	0.41	1	9
	CCT	0.35	3	6

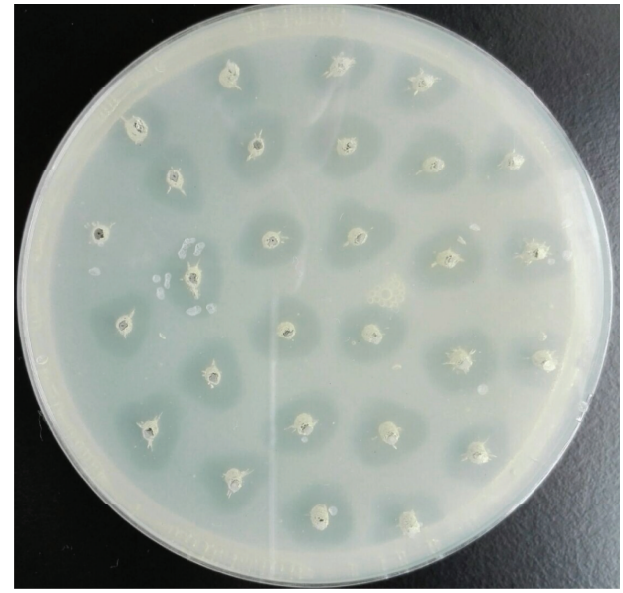

Figure 2: Screening of strains with higher level of α-amylase production by plate assay. The agar plates contain 3% (w/v) agar and 5% (w/v) soluble starch.

the expression of the native gene in *P. pastoris* (3500 U/mL), the expression level of codon optimized gene was increased by 2.31-fold (Figure 3(a)). The maximum α-amylase activity and protein concentration of recombinant strain GS115 carrying *BlAmy-opt* obtained in the 50-L fed-batch bioreactor were 11000 U/mL and 12.2 g/L, respectively (Figure 3(b)). Compared with the expression of the native gene in *P. pastoris* (4200 U/mL), the expression level of codon optimized gene was increased by 2.62-fold (Figure 3(b)). The recombinant protein accounted for 86% of the total protein in the medium as estimated by the Software Quantity One (Figure 4(a)).

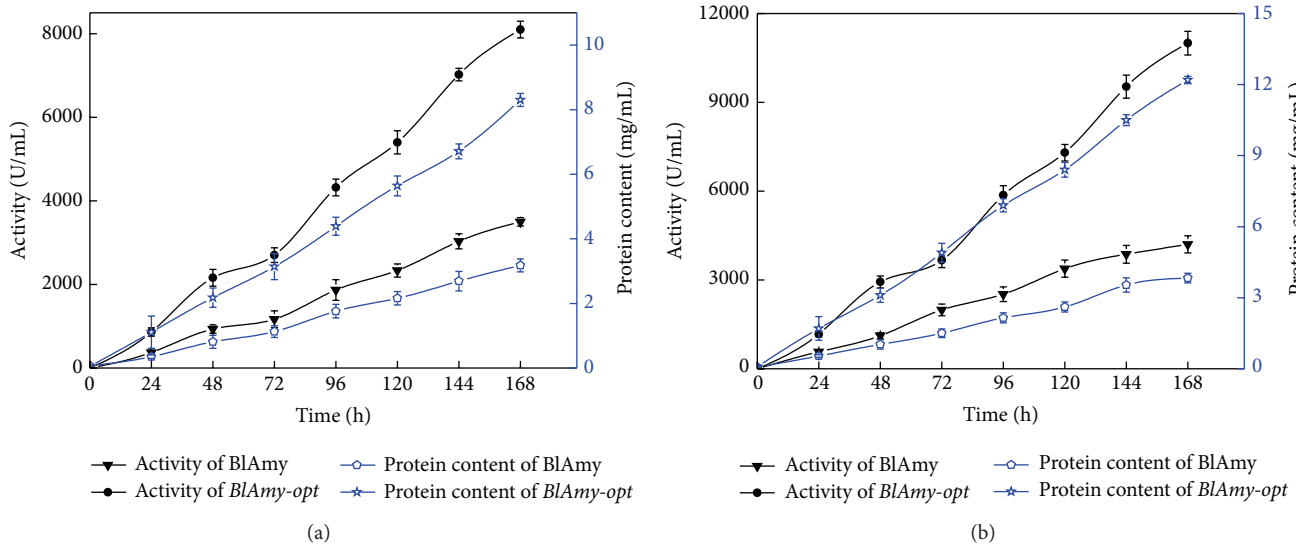

Figure 3: α-Amylase activity and total protein content in basal salt medium at 30°C and pH 5.0 during fed-batch fermentation in 5-L (a) and 50-L (b) bioreactor. α-Amylase activity was determined by starch-iodine color method; the protein content was determined according to the Bradford method using BSA as standard. All measurements were carried out in triplicate.

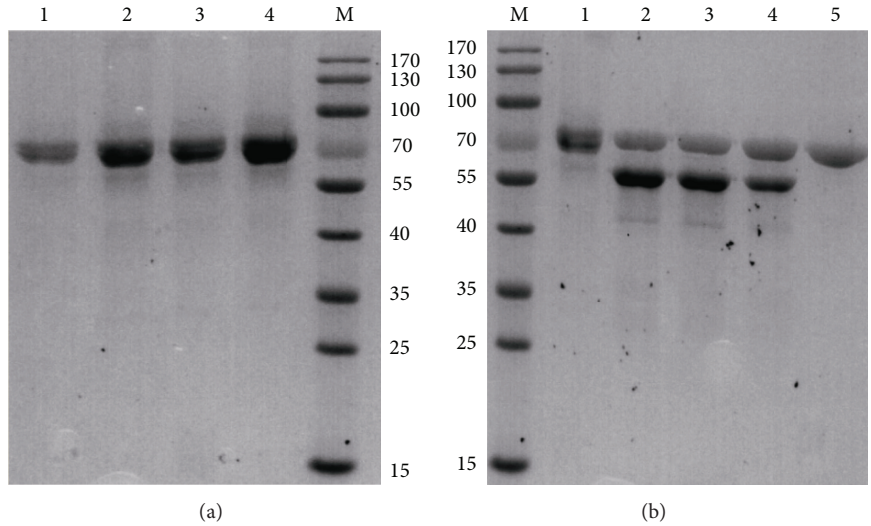

Figure 4: SDS-PAGE analysis of recombinant BlAmy. (a) SDS-PAGE of recombinant BlAmy in fermentation broth from 5- and 50-L bioreactor after methanol induced for 168 h. M: protein MW markers; lane 1 and lane 2: recombinant BlAmy and *BlAmy-opt* from 5-bioreactor, respectively. Lane 3 and lane 4: recombinant BlAmy and *BlAmy-opt* from 50-bioreactor, respectively. (b) Analysis of purified recombinant BlAmy and *N*-deglycosylated recombinant BlAmy by Endo H. Lane 1: purified recombinant BlAmy; lane 2, lane 3, and lane 4: the *N*-deglycosylated recombinant BlAmy and Endo H; lane 5: Endo H.

As an easy and simple system, *P. pastoris* is now widely used for heterologous production of recombinant proteins [21]. Due to the difference of codon usage between the native gene sequence and expression host, researchers have used codon optimization to increase the expression level of heterologous genes in *P. pastoris*. By codon optimization, the expression of xylanase gene from *Thermotoga maritime* and *Aspergillus sulphureus* was improved 2.8- and 5-fold, respectively [18, 22]. The optimization of glucanase gene from *B. licheniformis* and *Fibrobacter succinogenes* resulted in a 10- and 2.34-fold increase of target protein production [15, 23]. In this study, the *BlAmy-opt* was expressed in *P. pastoris* at a significantly higher level (12.2 g/L) with α-amylase activity of 11000 U/mL in 50-L fermentor after 168 h induction through codon optimization. These results showed that codon optimization is an effective method to increase the expression of foreign protein in *P. pastoris*. Meanwhile, the codon optimized recombinant α-amylase has a great potential use in industrial application due to its high-expression level.

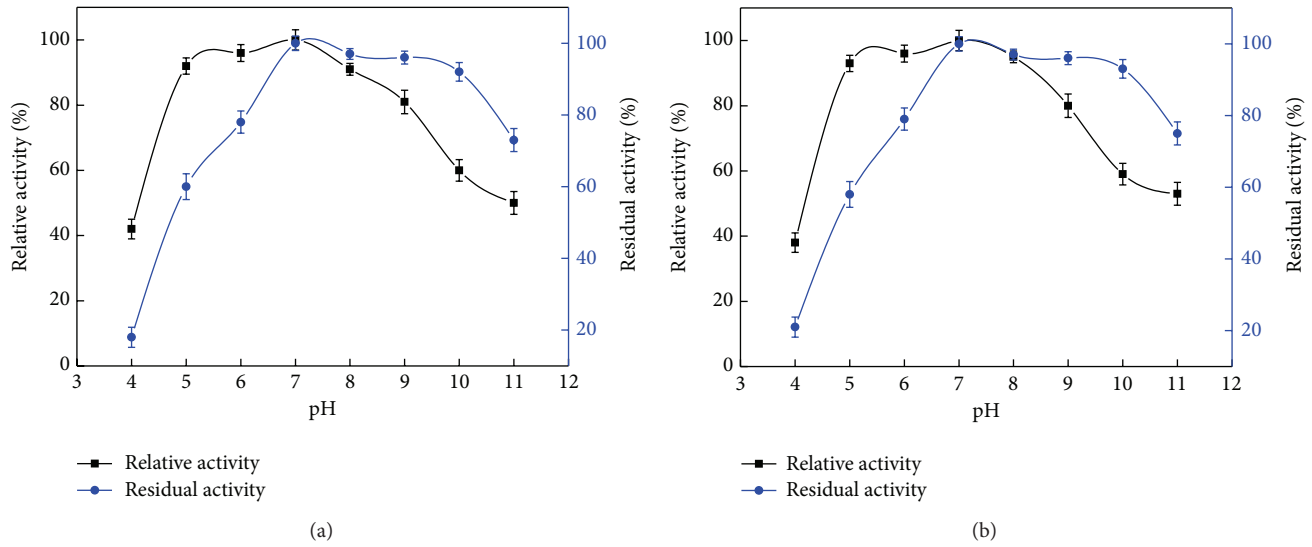

Figure 5: Influence of pH on activity and stability of recombinant BlAmy (a) and deglycosylated recombinant BlAmy (b). α-Amylase activity was determined by starch-iodine color method. Optimal pH was determined by assessing the activity of the purified recombinant BlAmy at pH 4.0–11.0. The relative activity at different pH values was calculated by setting pH 7.0 as 100%. The pH stability was determined by measuring the residual enzyme activities after incubating purified recombinant BlAmy at various pH for 24 h at 30°C. The residual activity was calculated by taking the activity of purified recombinant BlAmy without buffer treatment as 100%. All measurements were carried out in triplicate.

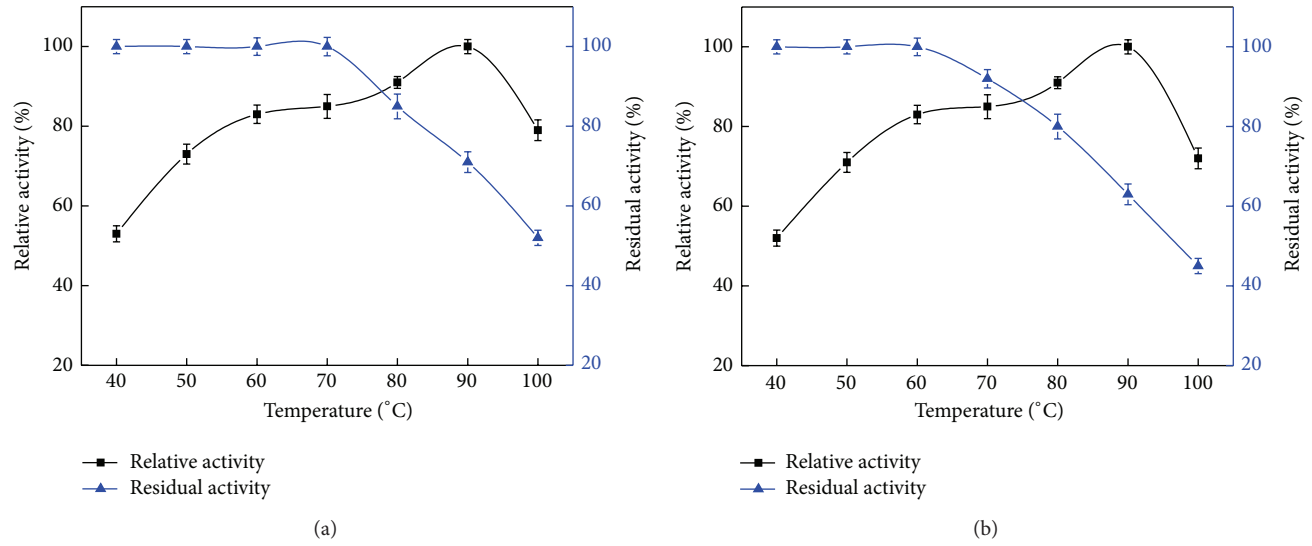

Figure 6: Influence of temperature on activity and stability of recombinant BlAmy (a) and deglycosylated recombinant BlAmy (b). α-Amylase activity was determined by starch-iodine color method. The optimum temperature of purified recombinant BlAmy was measured at different temperatures ranging from 40 to 100°C. The relative activity at different temperatures was calculated by setting 90°C as 100%. The thermal stability was studied by incubating lipase at various temperatures (40–100°C) in sodium phosphate buffer (pH 7.0) up to 1 h. The residual enzyme activity was measured at 70°C and the residual activity was calculated by taking the nonheated lipase activity as 100%. All measurements were carried out in triplicate.

3.5. SDS-PAGE Analysis of Recombinant BlAmy. As shown in Figure 4(a), the purified recombinant BlAmy showed two forms of BlAmy with molecular masses close to 70 kDa, which is about 11 kDa larger than 58.5 kDa, the calculated molecular weight of the nonglycosylated BlAmy. As shown in Figure 4(b), Endo H treatment of recombinant BlAmy resulted in a shift in the protein band on SDS-PAGE and yielded a single band of 58 kDa, suggesting that the two forms of recombinant BlAmy contained different degree of glycosylation.

3.6. Characterization of the Recombinant BlAmy and Deglycosylated Recombinant BlAmy. The influence of pH on recombinant *BlAmy-opt* and deglycosylated recombinant BlAmy activity and stability are presented in Figure 5. The activity

of recombinant *BlAmy-opt* and deglycosylated recombinant BlAmy were measured over a pH range of 4.0–11.0. As shown in Figure 5(a), the recombinant *BlAmy-opt* remained active at a pH range of 5.0–9.0 and showed maximum activity at pH 7.0, which is similar to the deglycosylated recombinant BlAmy (Figure 5(b)). In the pH stability study, the recombinant *BlAmy-opt* and deglycosylated recombinant BlAmy are stable at abroad range of pH values between pH 6.0 and 10.0 after 24 h incubation at 30°C, retaining over 78% of its initial activity.

The activity of recombinant *BlAmy-opt* and deglycosylated recombinant BlAmy were also determined at different temperatures. As shown in Figures 6(a) and 6(b), the recombinant *BlAmy-opt* and deglycosylated recombinant BlAmy showed an optimum activity at 90°C and activity dropped above 100°C. Thermostability was examined by incubating the recombinant *BlAmy-opt* and deglycosylated recombinant BlAmy at different temperatures for 1 h, and the residual activity was measured at 70°C under the conditions mentioned above. The activity of the recombinant *BlAmy-opt* was almost not affected by a temperature below 70°C, but it decreased dramatically when the temperature was above 90°C. The thermostability of BlAmy was higher than deglycosylated recombinant BlAmy. BlAmy showed 71 and 53% residual activity after 1 h incubation at 90 and 100°C, whereas deglycosylated recombinant BlAmy showed only 62 and 43%.

4. Conclusions

In this study we report the high-level expression of BlAmy in *P. pastoris*. The results showed that *P. pastoris* is an excellent host to production of BlAmy. To our knowledge, this is the first report about high cell density fermentation for production of recombinant BlAmy in 5- and 50-L bioreactor. Meanwhile, we developed a new simple and quick plate assay for screening of strains with higher level of α-amylase production. The most striking success in this study was that we improved the expression of BlAmy in *P. pastoris* by rewriting native *BlAmy* according to *P. pastoris* preferred codon usage. The results showed that codon optimization is an effective method to increase the expression of foreign protein in *P. pastoris*. Meanwhile, the results presented here will greatly contribute to improving production of recombinant BlAmy and offer a greater value in various industrial applications.

Conflict of Interests

The authors declared that they have no conflict of interests.

Authors' Contribution

Jian-Rong Wang and Yang-Yuan Li contributed equally to this paper.

Acknowledgments

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References

[1] R. Gupta, P. Gigras, H. Mohapatra, V. K. Goswami, and B. Chauhan, "Microbial α-amylases: a biotechnological perspective," *Process Biochemistry*, vol. 38, no. 11, pp. 1599–1616, 2003.

[2] Š. Janeček, B. Svensson, and E. A. MacGregor, "α-Amylase: an enzyme specificity found in various families of glycoside hydrolases," *Cellular and Molecular Life Sciences*, vol. 71, no. 7, pp. 1149–1170, 2013.

[3] W. D. Crabb and C. Mitchinson, "Enzymes involved in the processing of starch to sugars," *Trends in Biotechnology*, vol. 15, no. 9, pp. 349–352, 1997.

[4] S. Sivaramakrishnan, D. Gangadharan, K. M. Nampoothiri, C. R. Soccol, and A. Pandey, "α-amylases from microbial sources—an overview on recent developments," *Food Technology and Biotechnology*, vol. 44, no. 2, pp. 173–184, 2006.

[5] P. M. de Souza and P. O. Magalhães, "Application of microbial α-amylase in industry—a review," *Brazilian Journal of Microbiology*, vol. 41, no. 4, pp. 850–861, 2010.

[6] H. Liu, J. K. Yang, and Y. J. Yan, "Cloning and expression of an α-amylase gene from *Bacillus subtilis*," *Biotechnology*, vol. 5, no. 3, pp. 104–108, 2007 (Chinese).

[7] D. M. Ren, C. G. He, Z. W. Qian, X. C. Deng, and Q. Y. Yang, "Cloning and expression of two α-amylase gene from *Bacillus stearothermophilus* in *Ecoli*," *Chinese Journal of Biotechnology*, vol. 3, no. 5, pp. 197–202, 1987 (Chinese).

[8] T. Yuuki, T. Nomura, H. Tezuka et al., "Complete nucleotide sequence of a gene coding for heat- and pH-stable α-amylase of Bacillus licheniformis: comparison of the amino acid sequences of three bacterial liquefying α-amylases deduced from the DNA sequences," *The Journal of Biochemistry*, vol. 98, no. 5, pp. 1147–1156, 1985.

[9] P. Joyet, M. Guerineau, and H. Heslot, "Cloning of a thermostable α-amylase gene from *Bacillus licheniformis* and its expression in *Escherichia coli* and *Bacillus subtilis*," *FEMS Microbiology Letters*, vol. 21, no. 3, pp. 353–358, 1984.

[10] S. Macauley-Patrick, M. L. Fazenda, B. McNeil, and L. M. Harvey, "Heterologous protein production using the *Pichia pastoris* expression system," *Yeast*, vol. 22, no. 4, pp. 249–270, 2005.

[11] R. Ramón, P. Ferrer, and F. Valero, "Sorbitol co-feeding reduces metabolic burden caused by the overexpression of a Rhizopus oryzae lipase in *Pichia pastoris*," *Journal of Biotechnology*, vol. 130, no. 1, pp. 39–46, 2007.

[12] M. Yu, S. Wen, and T. Tan, "Enhancing production of *Yarrowia lipolytica* lipase Lip2 in *Pichia pastoris*," *Engineering in Life Sciences*, vol. 10, no. 5, pp. 458–464, 2010.

[13] X.-F. Wang, X.-G. Shen, Y.-C. Sun et al., "Production of *Yarrowia lipolytica* lipase LIP2 in *Pichia pastoris* using the nitrogen source-regulated *FLD1* promoter," *Journal of Chemical Technology and Biotechnology*, vol. 87, no. 4, pp. 553–558, 2012.

[14] A.-S. Xiong, Q.-H. Yao, R.-H. Peng et al., "High level expression of a synthetic gene encoding *Peniophora lycii* phytase in methylotrophic yeast *Pichia pastoris*," *Applied Microbiology and Biotechnology*, vol. 72, no. 5, pp. 1039–1047, 2006.

[15] D. Teng, Y. Fan, Y.-L. Yang, Z.-G. Tian, J. Luo, and J.-H. Wang, "Codon optimization of *Bacillus licheniformis* β-1,3-1,4-glucanase gene and its expression in *Pichia pastoris*," *Applied Microbiology and Biotechnology*, vol. 74, no. 5, pp. 1074–1083, 2007.

[16] J.-R. Wang, Y.-Y. Li, S.-D. Xu, P. Li, J.-S. Liu, and D.-N. Liu, "High-level expression of pro-form lipase from *Rhizopus oryzae* in *Pichia pastoris* and its purification and characterization," *International Journal of Molecular Sciences*, vol. 15, no. 1, pp. 203–217, 2013.

[17] Y. Liu, S. Fan, X. Liu et al., "A highly active alpha amylase from *Bacillus licheniformis*: directed evolution, enzyme characterization and structural analysis," *Journal of M icrobiology and Biotechnology*, vol. 24, no. 2, pp. 898–904, 2014.

[18] H. Y. Jia, G. S. Fan, Q. J. Yan, Y. C. Liu, Y. Yan, and Z. Q. Jiang, "High-level expression of a hyperthermostable *Thermotoga maritima* xylanase in *Pichia pastoris* by codon optimization," *Journal of Molecular Catalysis B: Enzymatic*, vol. 78, pp. 72–77, 2012.

[19] J. Yang and L. Liu, "Codon optimization through a two-step gene synthesis leads to a high-level expression of *Aspergillus niger lip2* gene in *Pichia pastoris*," *Journal of Molecular Catalysis B: Enzymatic*, vol. 63, no. 3-4, pp. 164–169, 2010.

[20] L. N. Ten, W.-T. Im, M.-K. Kim, M. S. Kang, and S.-T. Lee, "Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates," *Journal of Microbiological Methods*, vol. 56, no. 3, pp. 375–382, 2004.

[21] D.-M. Lan, N. Yang, W.-K. Wang, Y.-F. Shen, B. Yang, and Y.-H. Wang, "A novel cold-active lipase from *Candida albicans*: cloning, expression and characterization of the recombinant enzyme," *International Journal of Molecular Sciences*, vol. 12, no. 6, pp. 3950–3965, 2011.

[22] Y. Li, B. Zhang, X. Chen, Y. Chen, and Y. Cao, "Improvement of *Aspergillus sulphureus* endo-β-1,4-xylanase expression in *Pichia pastoris* by codon optimization and analysis of the enzymic characterization," *Applied Biochemistry and Biotechnology*, vol. 160, no. 5, pp. 1321–1331, 2010.

[23] H. Huang, P. Yang, H. Luo et al., "High-level expression of a truncated 1,3-1,4-β-D-glucanase from *Fibrobacter succinogenes* in *Pichia pastoris* by optimization of codons and fermentation," *Applied Microbiology and Biotechnology*, vol. 78, no. 1, pp. 95–103, 2008.

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Research Article

Improvement in Saccharification Yield of Mixed Rumen Enzymes by Identification of Recalcitrant Cell Wall Constituents Using Enzyme Fingerprinting

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Identification of recalcitrant factors that limit digestion of forages and the development of enzymatic approaches that improve hydrolysis could play a key role in improving the efficiency of meat and milk production in ruminants. Enzyme fingerprinting of barley silage fed to heifers and total tract indigestible fibre residue (TIFR) collected from feces was used to identify cell wall components resistant to total tract digestion. Enzyme fingerprinting results identified acetyl xylan esterases as key to the enhanced ruminal digestion. FTIR analysis also suggested cross-link cell wall polymers as principal components of indigested fiber residues in feces. Based on structural information from enzymatic fingerprinting and FTIR, enzyme pretreatment to enhance glucose yield from barley straw and alfalfa hay upon exposure to mixed rumen-enzymes was developed. Prehydrolysis effects of recombinant fungal fibrolytic hydrolases were analyzed using microassay in combination with statistical experimental design. Recombinant hemicellulases and auxiliary enzymes initiated degradation of plant structural polysaccharides upon application and improved the *in vitro* saccharification of alfalfa and barley straw by mixed rumen enzymes. The validation results showed that microassay in combination with statistical experimental design can be successfully used to predict effective enzyme pretreatments that can enhance plant cell wall digestion by mixed rumen enzymes.

1. Introduction

Rising grain prices heightened concerns over the use of food as feed for livestock production and the negative impacts of annual crops on carbon sequestration and biodiversity has prompted research into finding ways to increase the use of fibrous forage in ruminant diets. Plant cell walls can constitute a primary source of nutritional energy for ruminants. However for many types of forage, less than 50% of the cell wall fraction is digested and utilized by the ruminant host [1]. Substantial benefits would be realized if a greater percentage of this potential energy was made available for fermentation in the rumen through an increase in the digestibility of the cell wall fraction.

Fiber digestion in ruminants occurs primarily in the rumen and cecum. Generally, the amount of fiber digested in the lower tract is relatively small, with the rumen being the primary site of digestion. The ruminal microbial population secrets diverse hydrolases to degrade and ferment structural carbohydrates in plant cell walls. The physical and chemical nature of forages can present a barrier to their complete digestion in the rumen [2]. Therefore, prior knowledge about the structural aspects of cell wall polymers that limit digestion is critical to identifying efficient enzymatic pretreatments. In this study enzyme fingerprinting was used in combination with Flourier infrared spectroscopy (FTIR) to identify recalcitrant factors that limit fiber digestion by mixed rumen enzymes.

Exogenous enzymes have been used to remove antinutritional factors from feeds, to increase the digestibility of existing nutrients, and to supplement the activity of the endogenous enzymes. To date, they have primarily been used in poultry and swine production [3, 4]. In cattle, the addition of cellulases and xylanases directly to feeds has been shown to increase the *in vivo* numbers of fibrolytic rumen bacteria that utilize the secondary products of cellulose digestion [5]. Feedstuffs are structurally complex; each substrate presents its own set of recalcitrant components that limit the extent of feed digestion in the rumen. Ultimately, enzyme pretreatments should be designed specifically to overcome the constraints limiting digestion of different types of forages. There have been number of reports recently focusing on development of synthetic formulation of lignocellulolytic enzymes and chemical pretreatments for biomass use in biofuel production. Synthetic enzyme mixtures for ammonia fiber expansion (AFEX) treated corn stover deconstruction have been reported [6, 7]. Similarly optimized synthetic mixture of enzymes from *Trichoderma reesei* for hydrolysis of steam exploded wheat straw [8] and enzyme formulations to enhance performance of commercial enzymes against alkaline pretreated barley straw and alfalfa have been recently reported [9].

In this study we sought to use a microassay procedure in combination with statistical experimental design to predict the optimized synergy between enzyme prehydrolysis and maximum solubilization of cellulose by mixed rumen enzymes (rumen endogenous enzymes). The optimized enzyme pretreatment conditions were then validated using a scale-up assay. A similar approach using a combination of statistical design and microplate technique for enzymatic hydrolysis with comparable protein to biomass load and reaction volumes has been reported previously [9, 10]. The present work describes the use of a technique to specifically assay very small quantities of enzymes, enabling the screening of a large number of recombinant enzymes from novel sources for their ability to enhance the digestion of plant cell walls by mixed rumen enzymes. It is expected that the method described here will facilitate the development of enzyme cocktails for use as ruminant feed additives.

2. Material and Method

2.1. Source of Enzymes, Production, and Biochemical Characterization of Recombinant Enzymes. The source of recombinant enzymes, their expression, biochemical characterisation (Table 1), along with details about commercial enzymes, and the methods used to prepare mixed rumen enzymes have been previously reported [9].

2.2. Statistical Design. Simplex-lattice designs were created using Design-Expert software (Version 8.0; Stat-Ease, Inc., Minneapolis, MN; http://www.statease.com) as described earlier [9] with slight modifications. Details of augmented special quadratic design for experiment number 1 (six components) and experiment number 2 (seven components) with 28 and 41 separate assays are shown in Tables 2, 3, 4, and 5, respectively. The relative abundance of core enzymes (i.e., Accellerase 1500 and Accellerase XC) was set to vary from 25% to 75%, while upper and lower limits for fungal enzymes were set between 50% and 100% in assay mixtures for experiment 1, whereas, in order to optimize the efficiency of enzymatic prehydrolysis, upper and lower limits for all enzymes were set to vary from 100% to 0% in experiment 2.

2.3. Experimental Detail

2.3.1. Experiment Number 1: Enzyme Fingerprinting of Alkaline Peroxide Pretreated (AP) Barley Silage and Total Tract Indigested Fiber Residues (TIFR). Enzymatic fingerprinting of AP treated barley silage and total tract indigestible fiber residue (TIFR) was used in this study to gain insight into the recalcitrant components in plant cell walls that may respond to enzyme pretreatment and enhance the activity of mixed rumen enzymes. Alkaline peroxide treatment was used for selective delignification of cell walls in order to enable enzymes to access inner core cellulose and hemicellulose which would otherwise have remained inaccessible.

(a) Alkaline Peroxide Pretreatment of Barley Silage and TIFR. Heifers (five) were fed a barley silage-based diet (70 : 30 barley silage to barley grain) with approximately 65% of dietary neutral detergent fiber coming from barley silage, as described earlier [11]. Samples ($n = 5$) of barley silage were collected over the course of the feeding experiment, freeze dried and ground though a 1.0 mm screen. Representative faecal samples were collected for three days (once a day) from each heifer as reported previously and washed (6-7 times) in 50 mM citrate buffer to remove solubles and to recover the final fiber residue. The material obtained after washing was termed total tract indigested fiber residue (TIFR), with the three samples being pooled. Barley silage was also washed through cheese cloth to obtain a similar particle size. Washed TIFR and barley silage were freeze dried and pretreated with alkaline peroxide using the procedure described earlier [9].

(b) Enzymatic Fingerprinting of Alkaline Peroxide Treated Barley Silage and TIFR. Enzymatic digestion of AP treated barley silage and TIFR was carried out in microassays as reported earlier [9]. Respective enzyme volumes containing defined protein contents for each reaction mixture were calculated according to statistical design detailed in Tables 2 and 3 and dispensed into a substrate slurry as described previously [9]. Samples were incubated at 50°C for 48 h on a rotating shaker at 10 rpm. After incubation, tubes were centrifuged at 1,500 ×g for 5 min and the supernatants (100 μL) were heated at 90°C for 10 min to inactivate enzymes prior to determination of liberated glucose and xylose.

2.3.2. Experiment Number 2: Effect of Enzymatic Prehydrolysis on Sugar Release from Alfalfa Hay and Barley Straw Exposed to Mixed Rumen Enzymes In Vitro. Enzyme prehydrolysis to enhance glucose yield from plant cell walls by mixed rumen enzymes was developed based on relative abundance data from the enzyme fingerprinting conducted in experiment 1 and differential Fourier transform infrared spectroscopy (FTIR) analysis of barley silage versus TIFR. The main

Table 1: Sources of gene information, materials for production of recombinant enzymes, and properties of recombinant enzymes used in this study.

Clone ID	Entry name	Enzyme activity	CAZy family	Organism	mycoCLAP entry name	JGI ID	UniProt ID	Production host	pH optimum	Temp opt
Anig_Anig200605C	ABF54B_ASPNG	Alpha-N-arabinofuranosidase	GH54	*Aspergillus niger* N400	ABF54B_ASPNG	200605	P42255[a]	*Aspergillus niger* pyl1	4.5	50
Anig_Anig214598C	PGA28A_ASPNG	Endopolygalacturonase	GH28	*Aspergillus niger* N400	PGA28A_ASPNG	214598	Q9P4W4[a]	*Aspergillus niger* pyl1	4	60
Asnl94096	AXE16B_ASPNG	Acetylesterase	CE16	*Aspergillus niger* N400	To be submitted	194096	G3Y7Z1[a]	*Aspergillus niger* pyl1	5	60
Asn7870	AXE16A_ASPNG	Acetylesterase	CE16	*Aspergillus niger* N400	To be submitted	54865	G3Y497[a]	*Aspergillus niger* pyl1	6	55
Anig_TterXXXX7G	EGL7A_THITE	Endoglucanase	GH7	*Thielavia terrestris*	To be submitted	54138	G2QZA7[b]	*Aspergillus niger* pyl1	6	55
—	FAE 1a	Ferulic acid esterase	CE1	*Anaeromyces mucronatus*	—	—	F2YCB6[c]	*E. coli*	7.2	37

[a]The ID number corresponds to the protein ID of *Aspergillus niger* v3.0 annotation, http://genome.jgi-psf.org/Aspni5; [b]the ID number corresponds to the protein ID of *Thielavia terrestris* v3.0 annotation, http://genome.jgi-psf.org/Thite2. [c]ID for FAE 1a is a primary (citable) accession number (http://www.uniprot.org/uniprot/F2YCB6).

Table 2: Experimental design for comprehensive digestion of barley silage.

Std	Run	Component 1 A: acell 1500	Component 2 B: acell XC	Component 3 C: E-BGLUC	Component 4 D: FAE	Component 5 E: AXE16A_ASPNG	Component 6 F: EGL7A_THITE	Response 1 Glucose rel	Response 2 Xylose rel
12	1	0.250	0.500	0.250	0.000	0.000	0.000	153.436	36.7534
4	2	0.250	0.250	0.000	0.500	0.000	0.000	166.702	30.6087
10	3	0.500	0.250	0.000	0.000	0.250	0.000	172.536	50.8231
24	4	0.292	0.292	0.292	0.042	0.042	0.042	182.126	48.4112
3	5	0.250	0.250	0.500	0.000	0.000	0.000	135.056	32.2167
20	6	0.250	0.250	0.000	0.250	0.000	0.250	190.597	52
1	7	0.750	0.250	0.000	0.000	0.000	0.000	152.397	39.9119
28	8	0.333	0.333	0.083	0.083	0.083	0.083	177	54
18	9	0.250	0.250	0.250	0.000	0.000	0.250	203.223	69.6018
15	10	0.250	0.500	0.000	0.000	0.000	0.250	178.849	65.2948
8	11	0.500	0.250	0.250	0.000	0.000	0.000	135.456	37.5574
11	12	0.500	0.250	0.000	0.000	0.000	0.250	251.492	78.2159
25	13	0.292	0.292	0.042	0.292	0.042	0.042	172.776	44.9655
14	14	0.250	0.500	0.000	0.000	0.250	0.000	175.093	57.3124
13	15	0.250	0.500	0.000	0.250	0.000	0.000	179.968	49.4449
17	16	0.250	0.250	0.250	0.000	0.250	0.000	156.313	58.2887
9	17	0.500	0.250	0.000	0.250	0.000	0.000	169.659	44.1616
22	18	0.542	0.292	0.042	0.042	0.042	0.042	209.776	52.2014
26	19	0.292	0.292	0.042	0.042	0.292	0.042	190	63.6294
23	20	0.292	0.542	0.042	0.042	0.042	0.042	190	52
7	21	0.500	0.500	0.000	0.000	0.000	0.000	215.61	50
21	22	0.250	0.250	0.000	0.000	0.250	0.250	182.605	53.6945
27	23	0.292	0.292	0.042	0.042	0.042	0.292	200	62
6	24	0.250	0.250	0.000	0.000	0.000	0.500	198.828	65.6968
5	25	0.250	0.250	0.000	0.000	0.500	0.000	215.21	77.9288
16	26	0.250	0.250	0.250	0.250	0.000	0.000	182.845	37.5
2	27	0.250	0.750	0.000	0.000	0.000	0.000	180.208	61.16
19	28	0.250	0.250	0.000	0.250	0.250	0.000	163.506	49.6172

objective of this experiment was to formulate mixed rumen enzymes in combination with recombinant enzymes in ratios that enhance plant cell wall digestion . Recombinant glycosyl hydrolases (GH) (endoglucanase GH7 (EGL7A_THITE)) and auxiliary enzymes, that is, esterase (AXE16A_ASPNG, AXE16B_ASPNG, FAE 1a), were used with barley straw, whereas hemicellulase polygalacturonase (PGA28A_ASPNG) and α-arabinofuranosidase (ABF54B_ASPNG) were used with alfalfa hay.

Alfalfa hay and barley straw were first ground to pass through a 1 mm screen and then were suspended separately at a final concentration of 0.5% in 50 mM sodium citrate (pH 5.0, containing 5 μg/mL tetracycline, 5 μg/mL cycloheximide, and 0.02% sodium azide). While the slurry was kept in suspension using a paddle reservoir designed for dispensing pharmaceutical beads (Biomek FXP, Model VP 756C-1P100, V&P Scientific, Inc., San Diego, CA), a total of 200 μL (duplicate) of substrate slurry was dispensed into a mini-Eppendorf tube as described previously [9]. Defined protein content of each constituent enzyme for every reaction mixture (prehydrolysis) was calculated and dispensed according to the experimental design (Tables 3 and 4). Control samples were incubated without enzymes for 48 h at 50°C (total protein load 15 mg protein per g of glucan). After incubation, enzymes were inactivated by heating at 90°C for 15 min. Samples were allowed to cool and were subsequently centrifuged (1,500 ×g for 3 min) three times with 50 mM sodium citrate (pH 5.0, containing 5 μg/mL tetracycline, 5 μg/mL cycloheximide, and 0.02% sodium azide). Residues were added to mixed rumen enzymes at final concentration of 15 mg protein per g of glucan and incubated for further 48 h at 50°C. After incubation, the tubes were centrifuged at 1,500 ×g for 3 min to separate the solid residue from the supernatants (100 μL) which were transferred into Costar 96-well plates and heated at 100°C for 10 min to inactivate enzymes.

2.4. Glucose and Xylose Assay, Scale-Up Assay, Total Glucan Content, and Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR FT-IR) of Barley Silage and TIFR. Released glucose and xylose and total glucan contents of barley silage and TIFR were determined in a scale-up assay as previously reported [9]. ATR FT_IR analysis was also performed as documented earlier [12].

Table 3: Experimental design for comprehensive digestion of TIFR.

Std	Run	Component 1 A: acell 1500	Component 2 B: acell XC	Component 3 C: E-BGLUC	Component 4 D: FAE	Component 5 E: AXE16A_ASPNG	Component 6 F: EGL7A_THITE	Response 1 Glucose rel	Response 2 Xylose rel
12	1	0.250	0.500	0.250	0.000	0.000	0.000	65.7698	36.3515
4	2	0.250	0.250	0.000	0.500	0.000	0.000	64.731	27.1631
10	3	0.500	0.250	0.000	0.000	0.250	0.000	90.6233	59.6095
24	4	0.292	0.292	0.292	0.042	0.042	0.042	91.8221	44.6784
3	5	0.250	0.250	0.500	0.000	0.000	0.000	78.6361	31.585
20	6	0.250	0.250	0.000	0.250	0.000	0.250	87.5866	49.5597
1	7	0.750	0.250	0.000	0.000	0.000	0.000	84.0703	42.3813
28	8	0.333	0.333	0.083	0.083	0.083	0.083	87	47.0904
18	9	0.250	0.250	0.250	0.000	0.000	0.250	89.8242	54.4985
15	10	0.250	0.500	0.000	0.000	0.000	0.250	94.2994	53.6371
8	11	0.500	0.250	0.250	0.000	0.000	0.000	85.9084	37.0406
11	12	0.500	0.250	0.000	0.000	0.000	0.250	100.053	56.2213
25	13	0.292	0.292	0.042	0.292	0.042	0.042	90.4635	40
14	14	0.250	0.500	0.000	0.000	0.250	0.000	77.0378	56.6807
13	15	0.250	0.500	0.000	0.250	0.000	0.000	95.7379	41.9219
17	16	0.250	0.250	0.250	0.000	0.250	0.000	117.315	63.1126
9	17	0.500	0.250	0.000	0.250	0.000	0.000	90.5434	35.2029
22	18	0.542	0.292	0.042	0.042	0.042	0.042	93	52.7182
26	19	0.292	0.292	0.042	0.042	0.292	0.042	84.39	58.5758
23	20	0.292	0.542	0.042	0.042	0.042	0.042	90.7032	53.2351
7	21	0.500	0.500	0.000	0.000	0.000	0.000	86.9473	44.3338
21	22	0.250	0.250	0.000	0.000	0.250	0.250	82.7917	50.536
27	23	0.292	0.292	0.042	0.042	0.042	0.292	85.7485	53
6	24	0.250	0.250	0.000	0.000	0.000	0.500	98.8546	59.6095
5	25	0.250	0.250	0.000	0.000	0.500	0.000	60.2557	60
16	26	0.250	0.250	0.250	0.250	0.000	0.000	64.3314	31.8147
2	27	0.250	0.750	0.000	0.000	0.000	0.000	93.2605	46.6884
19	28	0.250	0.250	0.000	0.250	0.250	0.000	84.7096	48.2389

2.5. Data Analysis. Glucose and xylose released in each assay served as the response for experimental design in experiment 1, while in experiment 2 assay responses were expressed as a percentage yield of glucose in prehydrolyzed samples relative to the controls, where the residues were not prehydrolyzed. For experiments 1 and 2, ANOVA calculations were conducted and are reported in Tables 6 and 7, respectively.

3. Results and Discussions

One increasingly important aspect of modern livestock production is the use of feed additives that aim to improve the efficiency of feed utilization and thereby contribute to the sustainability of meat and milk production. In monogastrics, exogenous enzymes have been used to remove antinutritional factors from feeds, to increase the digestibility of existing nutrients, and to complement the activity of endogenous enzymes [3, 4]. Digestion of plant cell walls to volatile fatty acids by ruminal microorganisms is a key step in the derivation of energy from recalcitrant substrates such as cereal straws by ruminants. Sufficient intake of digestible forage with an appropriate profile of nutrients is critical for optimal ruminant production. Hence, identification of those plant cell wall components that resist rumen digestion is vital for developing effective and efficient additives that improve the utilization of forages by ruminants. In this study, we used enzymatic fingerprinting of undigested fiber residue that has passed through the digestive tract (TIFR) to identify major undigested components of feed. We used two commercial enzymes (Accellerase 1500 and Accellerase XC) as core enzyme preparations as these two preparations are comprehensive and are routinely used for cell wall digestion. An enzyme cocktail containing 49% Accellerase 1500, 25% Accellerase XC, 25% of endoglucanase EGL7A_THITE, and 1% of β-glucosidase E-BGLUC activity resulted in the highest yield of glucose and xylose from AP treated barley silage (Figure 1(a)). Interestingly, enzyme fingerprinting of AP treated TIFR from cattle fed barley silage showed the highest sugar yield for the enzyme mix containing supplemental acetyl xylan esterase AXE16B_ASPNG (25%) and β-glucosidase E-BGLUC (25%) activity in addition to Accellerase 1500 (25%) and Accellerase XC (25%) (Figure 1(b)). These results suggest that effective digestion of AP treated TIFR increases with supplemental acetyl xylan

Table 4: Experimental design for comprehensive digestion of alfalfa hay by rumen mix enzymes after 48 h of enzyme prehydrolysis.

Std	Run	Component 1 A: EGL7A_THITE	Component 2 B: AXE16A_ASPNG	Component 3 C: ASN ACEA	Component 4 D: AXE16B_ASPNG	Component 5 E: FAE	Component 6 F: ABF54B_ASPNG	Component 7 G: PGA28A_ASPNG	Response 1 Glucose release %
10	1	50.000	0.000	0.000	50.000	0.000	0.000	0.000	145
24	2	0.000	0.000	0.000	50.000	0.000	50.000	0.000	149
19	3	0.000	0.000	50.000	50.000	0.000	0.000	0.000	134
12	4	50.000	0.000	0.000	0.000	0.000	50.000	0.000	144
41	5	0.000	0.000	0.000	0.000	100.000	0.000	0.000	108
40	6	0.000	0.000	0.000	100.000	0.000	0.000	0.000	135
29	7	57.143	7.143	7.143	7.143	7.143	7.143	7.143	153
9	8	50.000	0.000	50.000	0.000	0.000	0.000	0.000	166
7	9	0.000	0.000	0.000	0.000	0.000	0.000	100.000	141
26	10	0.000	0.000	0.000	0.000	50.000	50.000	0.000	126
32	11	7.143	7.143	7.143	57.143	7.143	7.143	7.143	143
6	12	0.000	0.000	0.000	0.000	0.000	100.000	0.000	141
28	13	0.000	0.000	0.000	0.000	0.000	50.000	50.000	176
13	14	50.000	0.000	0.000	0.000	0.000	0.000	50.000	143
22	15	0.000	0.000	50.000	0.000	0.000	0.000	50.000	139
31	16	7.143	7.143	57.143	7.143	7.143	7.143	7.143	145
15	17	0.000	50.000	0.000	50.000	0.000	0.000	0.000	145
36	18	14.286	14.286	14.286	14.286	14.286	14.286	14.286	148
37	19	100.000	0.000	0.000	0.000	0.000	0.000	0.000	148
8	20	50.000	50.000	0.000	0.000	0.000	0.000	0.000	160
27	21	0.000	0.000	0.000	0.000	50.000	0.000	50.000	149
21	22	0.000	0.000	50.000	0.000	0.000	50.000	0.000	137
11	23	50.000	0.000	0.000	0.000	50.000	0.000	0.000	131
35	24	7.143	7.143	7.143	7.143	7.143	7.143	57.143	146
33	25	7.143	7.143	7.143	7.143	57.143	7.143	7.143	136
38	26	0.000	100.000	0.000	0.000	0.000	0.000	0.000	131
30	27	7.143	57.143	7.143	7.143	7.143	7.143	7.143	139
17	28	0.000	50.000	0.000	0.000	0.000	50.000	0.000	141
5	29	0.000	0.000	0.000	0.000	100.000	0.000	0.000	109
2	30	0.000	100.000	0.000	0.000	0.000	0.000	0.000	134
23	31	0.000	0.000	0.000	50.000	50.000	0.000	0.000	132
16	32	0.000	50.000	0.000	0.000	50.000	0.000	0.000	128
4	33	0.000	0.000	0.000	100.000	0.000	0.000	0.000	
3	34	0.000	0.000	100.000	0.000	0.000	0.000	0.000	138
20	35	0.000	0.000	50.000	0.000	50.000	0.000	0.000	154
14	36	0.000	50.000	50.000	0.000	0.000	0.000	0.000	129
34	37	7.143	7.143	7.143	7.143	7.143	57.143	7.143	147
25	38	0.000	0.000	0.000	50.000	0.000	0.000	50.000	139
18	39	0.000	50.000	0.000	0.000	0.000	0.000	50.000	138
39	40	0.000	0.000	100.000	0.000	0.000	0.000	0.000	138
1	41	100.000	0.000	0.000	0.000	0.000	0.000	0.000	153

Table 5: Experimental design for comprehensive digestion of barley straw by rumen mix enzymes after 48 h of enzyme prehydrolysis.

Std	Run	Component 1 A: EGL7A_THITE	Component 2 B: AXE16A_ASPNG	Component 3 C: ASN ACEA	Component 4 D: AXE16B_ASPNG	Component 5 E: FAE	Component 6 F: ABF54B_ASPNG	Component 7 G: PGA28A_ASPNG	Response 1 Glucose release %
14	1	0.000	0.500	0.500	0.000	0.000	0.000	0.000	158
31	2	0.071	0.071	0.571	0.071	0.071	0.071	0.071	139
18	3	0.000	0.500	0.000	0.000	0.000	0.000	0.500	268
19	4	0.000	0.000	0.500	0.500	0.000	0.000	0.000	
36	5	0.143	0.143	0.143	0.143	0.143	0.143	0.143	160
7	6	0.000	0.000	0.000	0.000	0.000	0.000	1.000	139
12	7	0.500	0.000	0.000	0.000	0.000	0.500	0.000	122
39	8	0.000	0.000	1.000	0.000	0.000	0.000	0.000	270
21	9	0.000	0.000	0.500	0.000	0.000	0.500	0.000	89
2	10	0.000	1.000	0.000	0.000	0.000	0.000	0.000	268
40	11	0.000	0.000	0.000	1.000	0.000	0.000	0.000	183
32	12	0.071	0.071	0.071	0.571	0.071	0.071	0.071	154
34	13	0.071	0.071	0.071	0.071	0.071	0.571	0.071	162
26	14	0.000	0.000	0.000	0.000	0.500	0.500	0.000	131
6	15	0.000	0.000	0.000	0.000	0.000	1.000	0.000	95
1	16	1.000	0.000	0.000	0.000	0.000	0.000	0.000	249
24	17	0.000	0.000	0.000	0.500	0.000	0.500	0.000	181
8	18	0.500	0.500	0.000	0.000	0.000	0.000	0.000	246
17	19	0.000	0.500	0.000	0.000	0.000	0.500	0.000	203
35	20	0.071	0.071	0.071	0.071	0.071	0.071	0.571	
41	21	0.000	0.000	0.000	0.000	1.000	0.000	0.000	203
27	22	0.000	0.000	0.000	0.000	0.500	0.000	0.500	207
9	23	0.500	0.000	0.500	0.000	0.000	0.000	0.000	297
4	24	0.000	0.000	0.000	1.000	0.000	0.000	0.000	230
10	25	0.500	0.000	0.000	0.500	0.000	0.000	0.000	206
23	26	0.000	0.000	0.000	0.500	0.500	0.000	0.000	191
15	27	0.000	0.500	0.000	0.500	0.000	0.000	0.000	243
20	28	0.000	0.000	0.500	0.000	0.500	0.000	0.000	305
33	29	0.071	0.071	0.071	0.071	0.571	0.071	0.071	236
28	30	0.000	0.000	0.000	0.000	0.000	0.500	0.500	337
29	31	0.571	0.071	0.071	0.071	0.071	0.071	0.071	254
30	32	0.071	0.571	0.071	0.071	0.071	0.071	0.071	247
5	33	0.000	0.000	0.000	0.000	1.000	0.000	0.000	235
3	34	0.000	0.000	1.000	0.000	0.000	0.000	0.000	341
37	35	1.000	0.000	0.000	0.000	0.000	0.000	0.000	262
11	36	0.500	0.000	0.000	0.000	0.500	0.000	0.000	370
16	37	0.000	0.500	0.000	0.000	0.500	0.000	0.000	296
13	38	0.500	0.000	0.000	0.000	0.000	0.000	0.500	214
22	39	0.000	0.000	0.500	0.000	0.000	0.000	0.500	271
25	40	0.000	0.000	0.000	0.500	0.000	0.000	0.500	276
38	41	0.000	1.000	0.000	0.000	0.000	0.000	0.000	375

Table 6: ANOVA calculations of F-value, P value, R^2, adjusted R^2, predicted R^2, and adequate precision as calculated by the Design-Expert software for enzymatic fingerprinting of barley silage and tract indigested fiber residue (TIFR).

Source	Enzyme source	F-value	P value	R-square	Adjusted R-square	Predicted R-square	Difference between Adj and Pred R-square	Adequate precision
AP pretreated barley silage feed	Accell1500 + AccellXC + EGL7A_THITE + E-BGLUC	129.1	<0.0001	0.98	0.97	0.86	0.11	52.3
AP pretreated TIFR	Accell1500 + AccellXC + AXE16B_ASPNG + E-BGLUC	16.60	<0.0001	0.88	0.83	0.71	0.12	16.8

Table 7: ANOVA calculations of F-value, P value, R^2, adjusted R^2, predicted R^2, and adequate precision as calculated by the Design-Expert software for enzymatic prehydrolysis of alfalfa hay and barley straw on final saccharification yield from rumen mix enzyme digestion.

Feedstock	Enzyme source	F-value	P value	R-square	Adjusted R-square	Predicted R-square	Difference between Adj and Pred R-square	Adequate precision
Alfalfa	Enzyme pretreatments followed by rumen enzyme mix	129.1	<0.0001	0.98	0.97	0.86	0.11	52.3
Barley	Enzyme pretreatments followed by rumen enzyme mix	16.60	<0.0001	0.88	0.83	0.71	0.12	16.8

esterase as well as β-glucosidase activity. With 22–50% of xylose residues being acetylated at the 0–2 and or 0–3 positions, acetylation has been reported to be an important factor influencing the digestibility of plant cell walls in ruminants [13]. In addition, arabinoxylan one of the main components in hemicellulose that forms the backbone structure of β-1, 4-linked xylose with arabinose side chains has been reported to be ester-linked to p-coumaric and ferulic acid and cross-linked to lignin via ferulic acid [14, 15].

Relatively lower yields were observed when the enzyme mix contained a higher percentage of core enzymes (only Accellerase 1500 and Accellerase XC, Tables 2 and 3). However, assays with high xylanase levels (Accellerase XC) produced higher glucose and xylose yield as compared to assays with high endoglucanase (Accellerase 1500) both from barley silage and TIFR (Tables 2 and 3). These results reflect the layered structure of cellulose and xylan chains within plant cell walls as xylan hydrolysis significantly improved the activity of cellulases against cellulose. Comparative analysis of results from enzymatic fingerprinting experiment for AP treated barley silage versus AP treated TIFR demonstrates that TIFR still contains a significant amount of residual sugars that could be released if digested with a suitable enzyme cocktail (Figure 1). Comprehensive saccharification of AP pretreated barley silage released 252 mg/g of glucose and 78 mg/g of xylose while 117 mg/g of glucose and 63 mg/g of xylose were released from AP TIFR. Thus, significant glucose (40%) and xylose (80%) were still recoverable from TIFR even after it had been subject to digestion within the intestinal tract of cattle (Figure 1). These results also suggest that cattle feces have considerable potential as a feedstock for biofuel production. Using manure as a feedstock for bioethanol production addresses some of the serious concerns raised against first generation biofuels in terms of their impact on biodiversity, competition for fuel versus food and carbon emissions.

The high abundance of xylose in AP treated TIFR and critical requirement of acetyl xylan esterase reflects (Figure 1(b)) the recalcitrant nature of xylan and its cross-linked nature within the cell wall architecture. An abundance of undigested xylan and esterified hemicellulose components were also supported by differential spectra of barley silage versus TIFR by FTIR analysis. Peaks within the range of 1020 cm^{-1} to 1130 cm^{-1} corresponded to undigested arabinoglucuronoxylan, xyloglucan, arabinan, and pectin [16] and were reflective of an abundance of cross-linked hemicellulose within TIFR (Figure 2). Lignin was also concentrated in TIFR as indicated by spectral differences at 1508 cm^{-1} (aromatic skeletal vibration in lignin), 1541 cm^{-1} (C–H deformations; asymmetrical in $-CH_3$ and $-CH_2$), 1653 cm^{-1} (adsorbed O–H and conjugated C–O), and 1688 cm^{-1} (C=O in lignin) (Figure 2) [17]. Cross-linked esterified xylan and pectin in undigested residue were evident at peak 1714 cm^{-1} (C=O from xylan), 1738 cm^{-1} to 1747 cm^{-1} (unconjugated C=O stretch in xylan from acetic acid ester and pectin) [16, 17]. Our results are consistent with the notion that cross-linked xylan or ferulate-polysaccharide-lignin complexes are in part responsible for the recalcitrance of cellulose microfibrils [18, 19]. Similar results have been reported previously [9, 12] for barley straw where esterified pectin or xylan cross-linked to lignin was identified as the major factor responsible for the recalcitrance of these forages to mixed rumen enzymes as well as to commercial enzymes preparations.

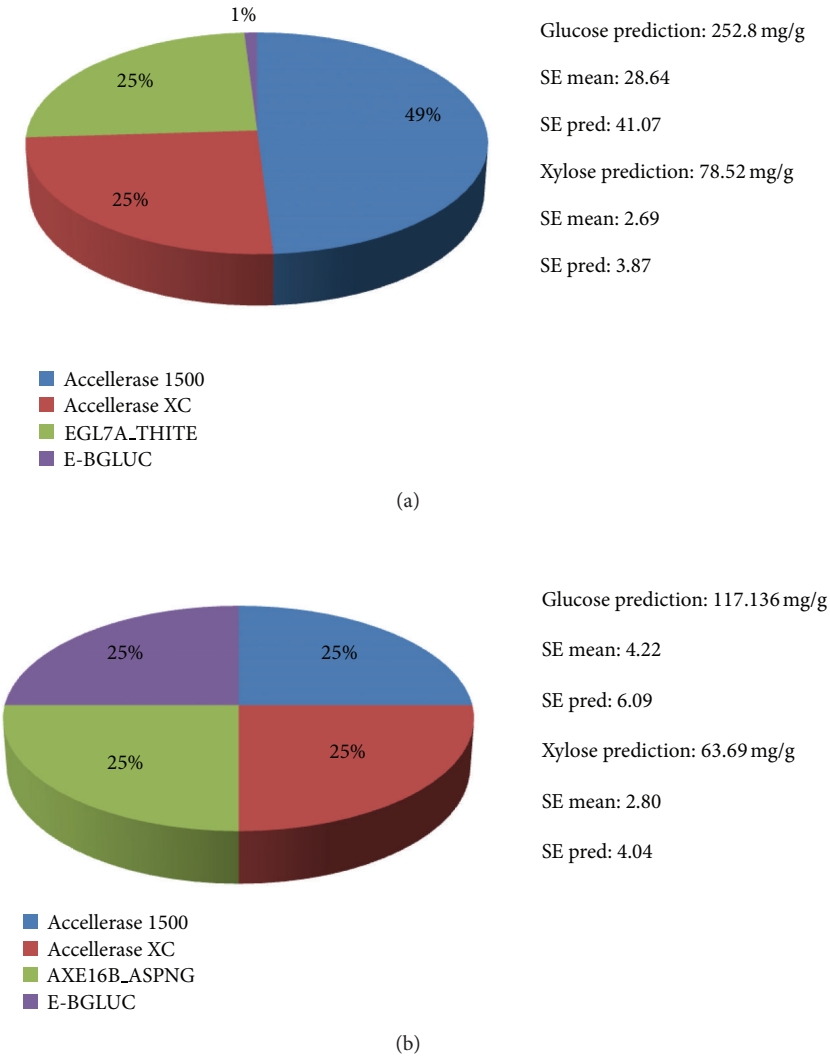

Figure 1: Enzyme fingerprinting of barley silage (a) and tract indigested fiber residues (TIFR) (b) for glucose and xylose released.

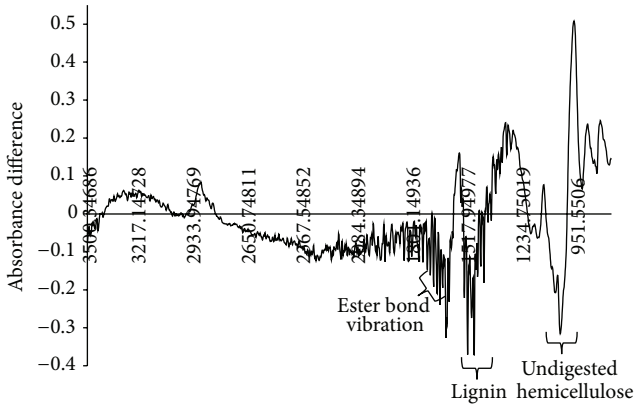

Figure 2: FTIR spectral difference for feed versus tract indigested fiber residues (TIFR) showing major undigested plant cell wall components after rumen digestion.

We hypothesize that prehydrolysis of the forage with efficient auxiliary enzymes like esterases prior to consumption may increase fiber digestibility in ruminants by reducing recalcitrant cross-linked xylan content. This would be beneficial to ruminant production in the form of increased efficiency of meat and milk production.

Based on the results from the enzyme fingerprinting (experiment 1) we selected recombinant enzymes (namely acetyl xylan esterase AXE16A_ASPNG and AXE16B_ASPNG, polygalacturonase PGA28A_ASPNG, arabinofuranosidase ABF54B_ASPNG, and ferulic acid esterase FAE 1a) and endoglucanase EGL7A_THITE for prehydrolysis with an aim to increase the sugar yield from substrates exposed to mixed rumen enzymes. We specifically selected barley straw as the substrate in experiment 2 with the expectation that it would represent even a more recalcitrant forage source than barley silage. The model predicted a significant increase in glucose

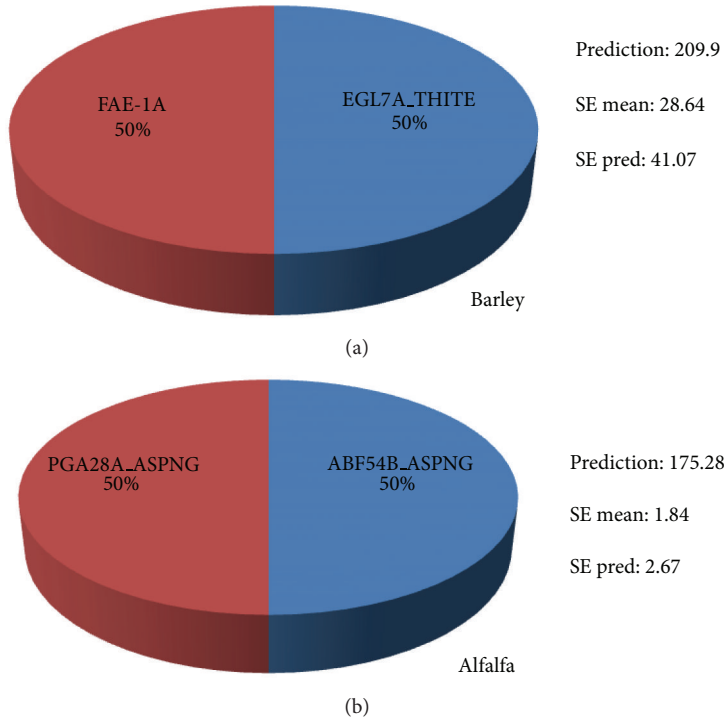

Figure 3: Optimization of enzyme ratios for enzyme prehydrolysis to aid high relative glucose yield from mixed rumen enzymes digestion of barley straw (a) and alfalfa hay (b).

yield as result of enzymatic prehydrolysis of alfalfa hay and barley straw prior to digestion by mixed rumen enzymes (Figure 3). Prehydrolysis of barley straw with a mixture of endoglucanase GH 7 (EGL7A_THITE) and feruloyl esterase (FAE 1a: 1 : 1) prior to exposure to mixed rumen enzymes resulted in a 100% increase in glucose release as compared to the untreated control (Figure 3(a)), while for alfalfa hay, a 75% higher glucose yield was predicted by the model as a result of enzymatic prehydrolysis of alfalfa with a 1 : 1 ratio of polygalacturonase (PGA28A_ASPNG) and arabinofuranosidase (ABF54B_ASPNG) prior to digestion by mixed rumen enzymes (Figure 3(b)). These results are in agreement with major structural disparity between alfalfa and barley plant cell walls. The carbohydrates within barley plant cell walls are mainly cellulose and hemicellulose with a negligible amount of pectin [20], whereas alfalfa cell wall contains pectin and xylan in roughly similar proportions, each accounting for 15–20% of total cell wall carbohydrates [21]. Effectiveness of esterase (FAE 1a) as a prehydrolysis for barley straw digestion by mixed rumen enzymes is in accordance with earlier reports regarding esterified cross-linkages being the major factor limiting the hydrolysis of barley straw by rumen microbes [9]. However, the hydrolysis of hemicellulose in alfalfa by mixed rumen enzymes was enhanced by the addition of polygalacturonase (PGA28A_ASPNG) and arabinofuranosidase (ABF54B_ASPNG). These results suggested that multienzyme mixtures have potential as feed additives by initiating degradation of plant structural polysaccharides prior to ingestion by the ruminant animal.

Predictions made by our micromodel were also validated in scale-up assays that used a solid load of 2% w/v of barley straw or alfalfa hay. The effect of enzyme prehydrolysis on the subsequent enhancement of cell wall hydrolysis was studied by sequential or simultaneous addition of recombinant enzymes to mixed rumen enzymes for barley straw and alfalfa hay. Results of hydrolysis of barley straw and alfalfa hay by mixed rumen enzymes after 48 h of prehydrolysis by endoglucanase EGL7A_THITE (50%) and ferulic acid esterase FAE 1a (50%) for barley straw and polygalacturonase PGA28A_ASPNG (50%) and arabinofuranosidase ABF54B_ASPNG (50%) for alfalfa hay confirmed that these mixtures increased the release of glucose and xylose ($P < 0.05$) as a result of prehydrolysis (Figures 4(a) and 4(b)). Supplementation of rumen mixed enzymes with endoglucanase EGL7A_THITE (50%) and ferulic acid esterase FAE 1a (50%) during the digestion of barley straw and polygalacturonase PGA28A_ASPNG (50%) and arabinofuranosidase ABF54B_ASPNG (50%) with alfalfa hay enhanced ($P < 0.05$) digestion as compared to mixed rumen enzymes alone (Figures 4(c) and 4(d)). A direct relationship was observed between xylan conversion (fraction of available xylan converted) and glucose conversion (fraction of available glucan conversion) during the hydrolysis of plant cell walls (Figure 5). However, a stronger correlation between xylan and glucan digestion with added auxiliary enzymes for optimized mixed rumen enzymes (Figure 5) suggested improved glucan conversion, perhaps due to better xylan saccharification.

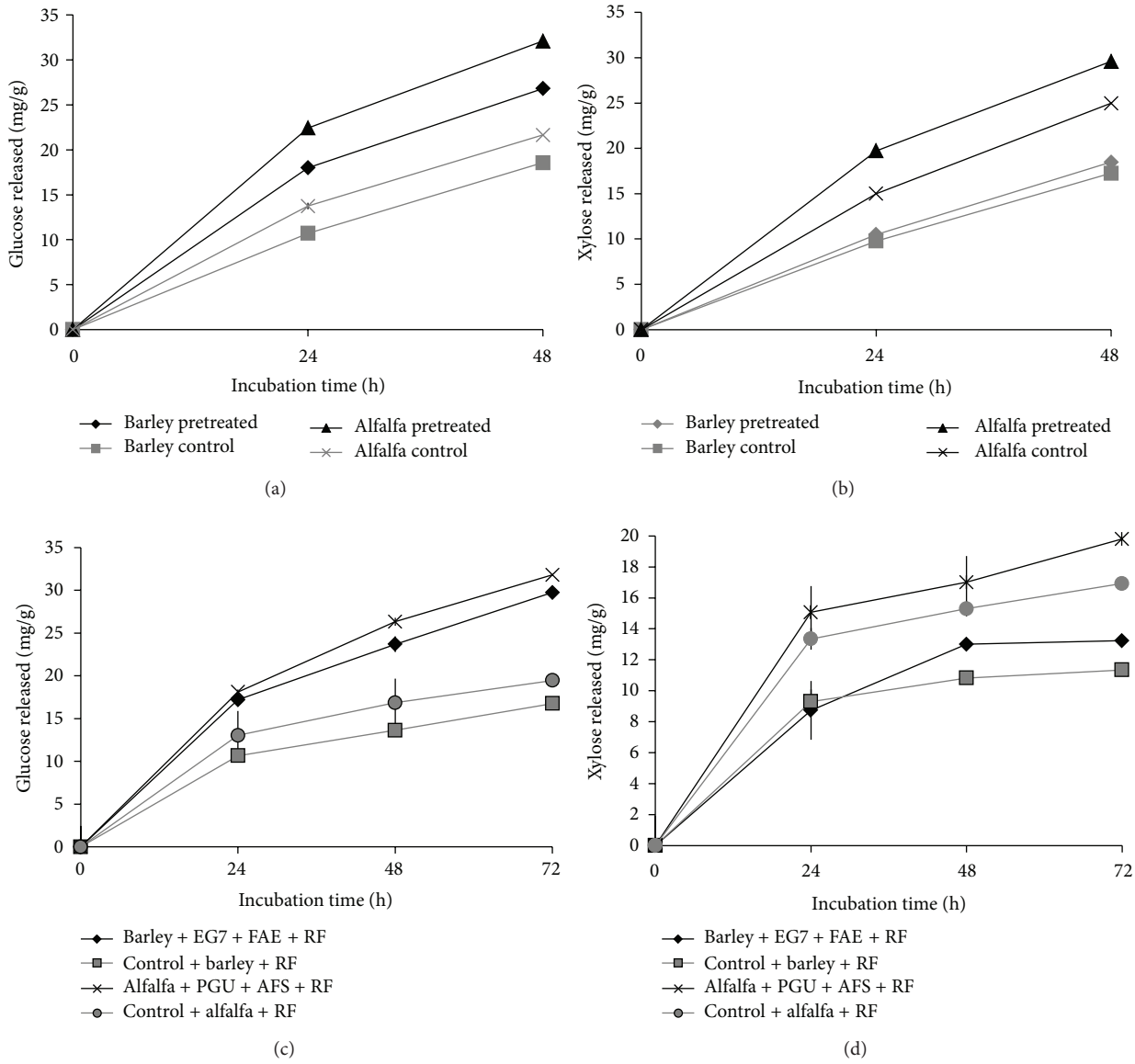

Figure 4: Glucose and xylose released as result of sequential ((a), (b)) and simultaneous ((c), (d)) hydrolysis of barley straw and alfalfa (biomass load: 2%, 30 mg of protein load/g of glucan in 5 mL reaction volume). Error bars (often invisible) represent SD of the mean ($n = 8$).

Comprehensive digestion of cell wall requires a battery of carbohydrases. Moreover, yields of recombinant enzymes from expression systems are often low. Hence, a microassay for screening novel enzymes against diverse biomass with ability to study synergy among hydrolases at low protein load is critical for development of enzyme formulations as additives to enhance ruminal digestion. In this study we successfully developed a microassay in combination with experimental design, to screen a number of recombinant enzymes at low protein loads, to enhance ruminal digestion of barley straw and alfalfa through augmentation of natural enzymatic activity in the rumen. Development of enzyme formulations that further enhance the utilization of low quality cellulosic feedstocks will ensure the sustainability of the beef industry in an environment of increasing demand for human food.

4. Conclusion

Enzyme fingerprinting was successfully used to identify principal recalcitrant constituent of barley silage. Cross-linked hemicelluloses as well as layered structure of cellulose and xylan were identified as prime recalcitrant factors to digestion. Partial digestion of hemicellulose in alfalfa hay and barley straw prior to ingestion with a cocktail of auxiliary enzymes significantly improved the hydrolysis of cellulose by mixed rumen enzymes. These results strengthen the rational of enzyme pretreatments targeting particular forage types.

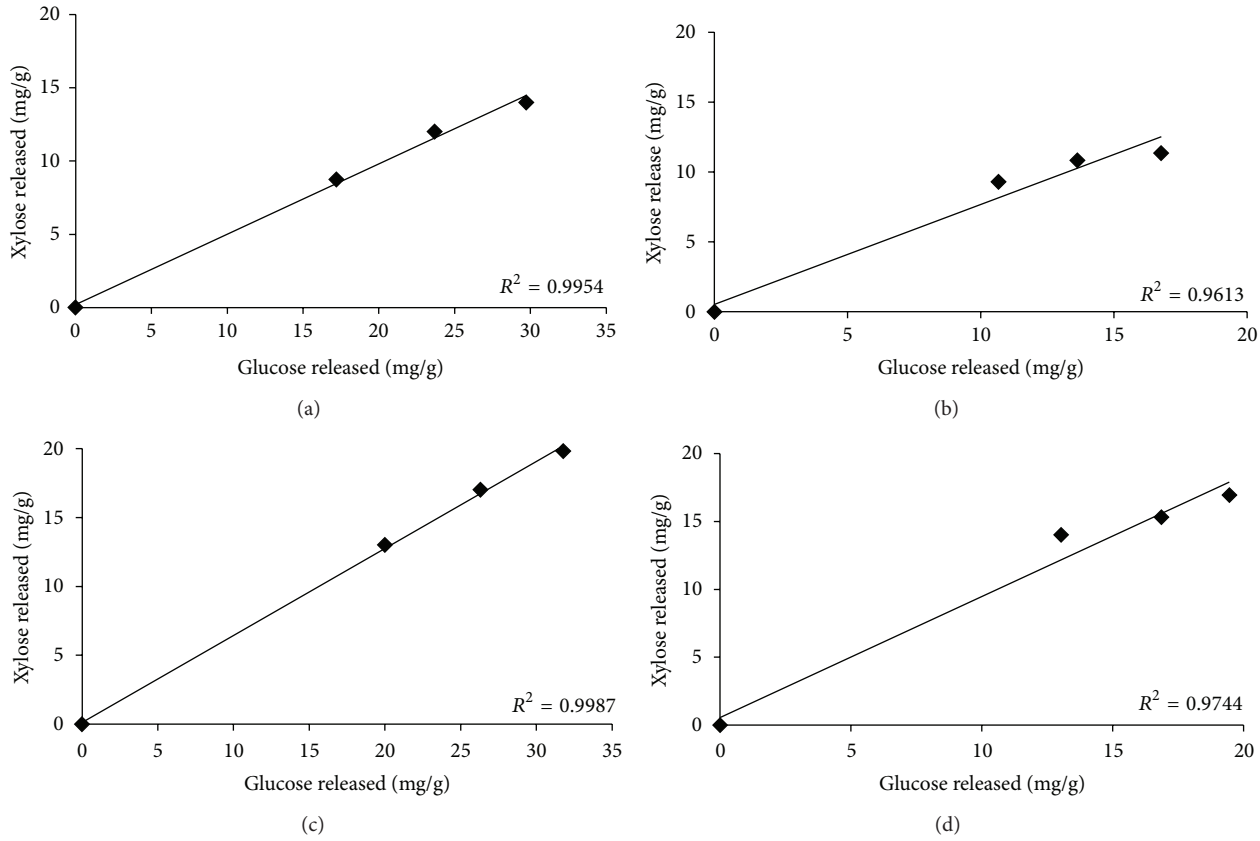

Figure 5: Change in glucan conversion plotted against change in xylan conversion for simultaneous hydrolysis of barley straw (a) alfalfa hay (c) by optimized enzyme mix and their respective controls ((b), (d); only rumen mix enzymes). Optimized enzyme mix composition used for barley straw and alfalfa hay digestion was identical to those used in Figure 3. Final enzymes load of 30 mg/g glucan with 2% solid load in 5 mL reaction volume was used.

These same approaches could be used to improve the value of animal waste as a feedstock for biofuel production.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] R. D. Hatfield and J. Ralph, "Modelling the feasibility of intramolecular dehydrodiferulate formation in grass walls," *Journal of the Science of Food and Agriculture*, vol. 79, no. 3, pp. 425–427, 1999.

[2] G. A. Varga and E. S. Kolver, "Microbial and animal limitations to fiber digestion and utilization," *The Journal of Nutrition*, vol. 127, no. 5, pp. 819S–823S, 1997.

[3] H. L. Classen, H. Graham, J. Inborr, and M. R. Bedford, "Growing interest in feed enzymes to lead to new products," *Feedstuffs*, vol. 63, pp. 22–24, 1991.

[4] M. R. Bedford, "Mode of action of feed enzymes," *The Journal of Applied Poultry Research*, vol. 2, pp. 85–92, 1993.

[5] V. L. Nsereko, K. A. Beauchemin, D. P. Morgavi et al., "Effect of a fibrolytic enzyme preparation from *Trichoderma longibrachiatum* on the rumen microbial population of dairy cows," *Canadian Journal of Microbiology*, vol. 48, no. 1, pp. 14–20, 2002.

[6] G. Banerjee, S. Car, J. S. Scott-Craig, M. S. Borrusch, and J. D. Walton, "Rapid optimization of enzyme mixtures for deconstruction of diverse pretreatment/biomass feedstock combinations," *Biotechnology for Biofuels*, vol. 3, article 22, 2010.

[7] D. Gao, S. P. S. Chundawat, C. Krishnan, V. Balan, and B. E. Dale, "Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover," *Bioresource Technology*, vol. 101, no. 8, pp. 2770–2781, 2010.

[8] H. Billard, A. Faraj, N. Lopes Ferreira, S. Menir, and S. Heiss-Blanquet, "Optimization of a synthetic mixture composed of

major *Trichoderma reesei* enzymes for the hydrolysis of steam-exploded wheat straw," *Biotechnology for Biofuels*, vol. 5, article 9, 2012.

[9] A. Badhan, Y. Wang, R. Gruninger et al., "Formulation of enzyme blends to maximize the hydrolysis of alkaline peroxide pretreated alfalfa hay and barley straw by rumen enzymes and commercial cellulases," *BMC Biotechnology*, vol. 14, article 31, 2014.

[10] S. P. S. Chundawat, V. Balan, and B. E. Dale, "High-throughput microplate technique for enzymatic hydrolysis of lignocellulosic biomass," *Biotechnology and Bioengineering*, vol. 99, no. 6, pp. 1281–1294, 2008.

[11] D. Vyas, E. J. McGeough, S. M. McGinn, T. A. McAllister, and K. A. Beauchemin, "Effect of *Propionibacterium* spp. on ruminal fermentation, nutrient digestibility, and methane emissions in beef heifers fed a high-forage diet," *Journal of Animal Science*, vol. 92, no. 5, pp. 2192–2201, 2014.

[12] A. Badhan, L. Jin, Y. Wang et al., "Expression of a fungal ferulic acid esterase in alfalfa modifies cell wall digestibility," *Biotechnology for Biofuels*, vol. 7, no. 1, article 39, 2014.

[13] R. B. Hespell and M. A. Cotta, "Degradation and utilization by Butyrivibrio fibrisolvens H17c of xylans with different chemical and physical properties," *Applied and Environmental Microbiology*, vol. 61, no. 8, pp. 3042–3050, 1995.

[14] N. Carpita and M. McCann, "The cell wall," in *Biochemistry and Molecular Biology of Plants*, B. Buchanan, W. Gruissmen, and R. Jones, Eds., pp. 52–108, John Wiley & Sons, Somerset, NJ, USA, 2000.

[15] Y. Sun and J. Cheng, "Hydrolysis of lignocellulosic materials for ethanol production: a review," *Bioresource Technology*, vol. 83, no. 1, pp. 1–11, 2002.

[16] M. Kacuráková, P. Capek, V. Sasinková, N. Wellner, and A. Ebringerová, "FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses," *Carbohydrate Polymers*, vol. 43, no. 2, pp. 195–203, 2000.

[17] G. Zhou, G. Taylor, and A. Polle, "FTIR-ATR-based prediction and modelling of lignin and energy contents reveals independent intra-specific variation of these traits in bioenergy poplars," *Plant Methods*, vol. 7, no. 1, article 9, 2011.

[18] Y. Wang, B. M. Spratling, D. R. Zobell, R. D. Wiedmeier, and T. A. McAllister, "Effect of alkali pretreatment of wheat straw on the efficacy of exogenous fibrolytic enzymes," *Journal of Animal Science*, vol. 82, no. 1, pp. 198–208, 2004.

[19] M. J. Selig, E. P. Knoshaug, W. S. Adney, M. E. Himmel, and S. R. Decker, "Synergistic enhancement of cellobiohydrolase performance on pretreated corn stover by addition of xylanase and esterase activities," *Bioresource Technology*, vol. 99, no. 11, pp. 4997–5005, 2008.

[20] K. Pahkala, M. Kontturi, A. Kallioinen et al., "Production of bioethanol from barley straw and reed canary grass: a raw material study," in *Proceedings of the 15th European Biomass Conference and Exhibition*, pp. 154–157, Berlin, Germany, 2007.

[21] B. S. Dien, H.-J. G. Jung, K. P. Vogel et al., "Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass," *Biomass and Bioenergy*, vol. 30, no. 10, pp. 880–891, 2006.

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Research Article

Molecular Identification of a Newly Isolated *Bacillus subtilis* BI19 and Optimization of Production Conditions for Enhanced Production of Extracellular Amylase

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A study was carried out with a newly isolated bacterial strain yielding extracellular amylase. The phylogenetic tree constructed on the basis of 16S rDNA gene sequences revealed this strain as clustered with the closest members of *Bacillus* sp. and identified as *Bacillus subtilis* BI19. The effect of various fermentation conditions on amylase production through shake-flask culture was investigated. Rice flour (1.25%) as a cheap natural carbon source was found to induce amylase production mostly. A combination of peptone and tryptone as organic and ammonium sulfate as inorganic nitrogen sources gave highest yield. Maximum production was obtained after 24 h of incubation at 37°C with an initial medium pH 8.0. Addition of surfactants like Tween 80 (0.25 g/L) and sodium lauryl sulfate (0.2 g/L) resulted in 28% and 15% increase in enzyme production, respectively. Amylase production was 3.06 times higher when optimized production conditions were used. Optimum reaction temperature and pH for crude amylase activity were 50°C and 6.0, respectively. The crude enzyme showed activity and stability over a fair range of temperature and pH. These results suggest that *B. subtilis* BI19 could be exploited for production of amylase at relatively low cost and time.

1. Background

Amylase represents a group of extracellular enzymes (consisting of α-amylase, β-amylase, and glucoamylase) that act on starch or oligosaccharide molecules in a random manner and hydrolyze into diverse products including dextrins and progressively smaller polymers composed of glucose units [1]. They have most widely been reported to occur in micro-organisms (fungi, yeast, bacteria, and actinomycetes), although they are found in plants and animals [2]. In present day they have found applications in all the industrial processes such as in food, detergents, textiles, pharmaceutical, paper and fine chemical industries for the hydrolysis of starch [3–5]. Amylase has great significance in present-day biotechnology having approximately 25–30% of the world enzyme market [6]. These extensive potentials of amylase to be used in broad range of industries have placed greater stress on researchers to search for more efficient amylase production.

The genus *Bacillus* has been becoming a reliable option to find out novel and promising bacteria for the production of amylase and other extracellular enzymes. Different species of *Bacillus*, most notably, *B. subtilis*, *B. licheniformis, B. amyloliquefaciens,* and *B. stearothermophilus,* are reported to produce approximately 60% of commercially available enzymes [7]. Short fermentation cycle, capacity to secrete proteins into the extracellular medium, safe handling, eco-friendly behavior, easy manipulation to obtain enzymes of desired characteristics, high enzymatic activity in a wide range of conditions (extreme pH, temperature, osmolarity, pressure,

etc.), and simple and cost effective production have made this genus as bacterial workhorses for the production of a variety of enzymes as well as fine biochemicals for decades [8, 9]. Different *Bacillus* species have similar growth patterns and enzyme profiles but depending upon the strain their general properties (thermostability, pH profile, pH stability, etc.) and optimized fermentation conditions may vary [10]. Thus it is really challenging to obtain a strain that can produce amylase meeting specific industrial demands.

There is no local production and thereby availability of amylase in Bangladesh. As a result, most of the existing and growing starch based industries are using expensive chemicals for starch hydrolysing based purposes. Keeping in mind the growing demand of amylases by different industrial sectors this study was carried out to obtain laboratory scale fermentation of amylases in shake flask culture by newly isolated *B. subtilis* BI19 along with optimization of medium components and culture conditions for enhanced production, thereby to understand its potential for biotechnological application.

2. Materials and Methods

2.1. Isolation and Screening of Amylolytic of Bacteria. The soil samples were collected from different areas of Savar, Dhaka, in the month of August, 2011. Bacteria were isolated by serial dilution and spread plate method in nutrient agar (NA) (Oxoid, UK). Before spreading diluted soil samples were heated at 90°C for 15 min. Isolated pure cultures were primarily screened for amylase activity by employing zone clearing technique on starch agar plate containing 1% starch (BDH, England) fortified with NA [11]. Then they were assessed for potency index (PI) according to Ball and McGonagle (1978) [12]. Higher PI value indicates the greater ability of an isolate to produce extracellular enzyme [13].

2.2. 16S rDNA Sequence Analysis for Identification of Bacteria. DNA was extracted from single colony by alkaline lysis [14, 15]. Extracted DNA was stored at −20°C for further molecular analyses. 16S rDNA amplification and sequencing was performed as described by Rahman et al. (2014a, b) [14, 15]. Primers used to amplify 16S rDNA sequence were forward: 63F 5′CAGGCCTAACACATGCAAGTC [16] and reverse: 1389R 5′ACGGGCGGTGTGTACAAG [17] in a PCR thermal cycler (ICycler 170-8740, USA). The amplified DNA was visualized by gel electrophoresis and sequenced. The 16S rDNA sequence was analyzed using Chromas LITE (Version 2.01); the most similar bacterial species was found in the GenBank by using BLAST search (http://www.ncbi.nlm.nih.gov/). Neighbor-joining phylogenetic trees were constructed based on 16S rDNA sequences using ClustalW.

2.3. Preparation of Inoculum. Vegetative inoculums were used in the present studies. Fifty (50) mL of inoculum medium containing nutrient broth 13 g/L, pH 7.4, was transferred to a 250 mL Erlenmeyer flask and was sterilized in an autoclave (CL-40M, Japan) at 15 lbs/inch^2 pressure at 121°C for 20 min. After cooling at room temperature, a loopful of freshly grown bacterial culture was aseptically transferred to it. The flask was incubated overnight at 37°C and 150 rpm in a rotary shaking incubator (Stuart SI 500, UK).

2.4. Submerged Fermentation for Amylase Production. Amylase production was carried out in basal medium containing 1.0% starch, 1% peptone, 0.8% $(NH_4)_2SO_4$, 0.2% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2 \cdot 2H_2O$, 1.4% K_2HPO_4, and 0.6% KH_2PO_4 (a slight modification of Sarikaya and Gürgün, 2000; a single nitrogenous source instead of two was used) [18]. One (1) mL (2%) of 24 h grown inoculums was cultivated in 250 mL Erlenmeyer flasks containing 50 mL (w/v) of medium with an initial pH 7.0. The cultures were shaken at 150 rpm in an orbital shaker incubator at 37°C for at least 72 h unless otherwise stated. After incubation, fermented broth was centrifuged in a refrigerated centrifuge machine (Hitachi CF16RXII, Japan) at 8000 rpm for 15 min at 4°C. Cell free supernatant was collected and preserved for the estimation of amylase activity. To optimize the medium components various carbon sources, organic and inorganic nitrogen sources, and added surfactants and polyhydroxy alcohols were varied in different concentrations in the basal medium one at a time while other ingredients were kept constant.

2.5. Enzyme Assay. Amylase was determined by using soluble starch, 1% (w/v), as substrate in 0.05 M sodium phosphate buffer (pH 6.5) essentially according to Gomes et al. (2001) [19]. The reaction mixture containing 1.8 mL substrate solution and 0.2 mL suitably diluted enzyme solution was incubated at 50°C for 10 min. The reaction was stopped by adding 3 mL dinitrosalicylic acid (DNS). The reducing sugar released was determined by the method of Miller (1959) [20]. The absorbance was measured at 540 nm with spectrophotometer (Jenway 6305, USA). One unit (U) of enzyme activity is defined in all cases as the amount of enzyme releasing 1 μg of reducing sugar as maltose per minute, under assay conditions.

$$\text{Enzyme Activity (U/mL/min)} = \frac{\text{Sugar released } (\mu g) \times \text{Total volume of reactive media (mL)} \times \text{Dilution factor (DF)}}{\text{Molecular weight of maltose} \times \text{Enzyme used (mL)} \times \text{Time of incubation (min)}}. \quad (1)$$

2.6. Partial Characterization of Crude Amylase. Enzyme samples were incubated for 10 min at temperatures ranging from 30 to 90°C in 0.05 M sodium phosphate buffer (pH 6.5). Thermal stabilities were determined by heating enzyme without the substrate fractions at various temperatures between 30 and 60°C for 1 h. At 10 min intervals, aliquots of 1 mL of

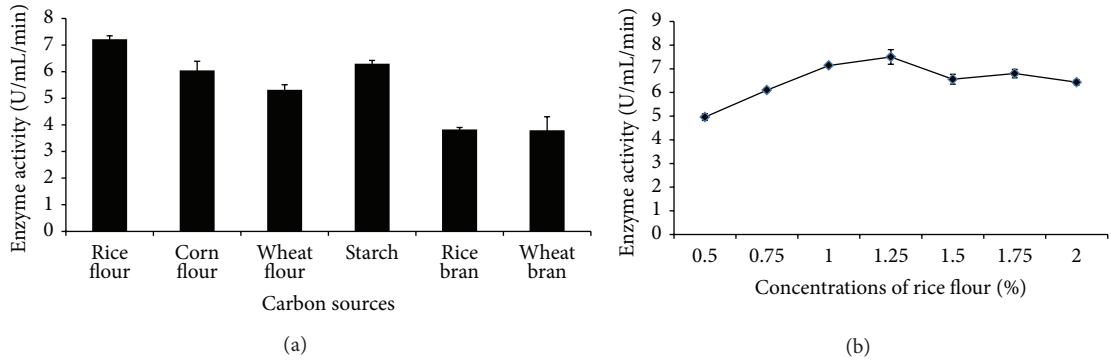

Figure 1: The production of amylase by *Bacillus subtilis* BI19 in shake flask fermentation. (a) Effect of different carbon sources and (b) effect of different concentrations of rice flour. Each value is an average of three replicates, pH 7.0, incubation period 72 h, incubation temperature 37°C, agitation speed 150 rpm, and inoculums volume 2%.

the incubated enzyme were assayed for activity. The optimum pH for the enzyme activity was determined in different pH (4.0–9.0). The pH stability was determined by incubating the enzyme in 0.05 M sodium phosphate buffer (pH 6.5) with different pH values for 2 h at room temperature (25°C).

2.7. Statistical Analysis. A statistical package (SPSS version 11.0, SPSS Inc., Chicago, IL) was used for the data analysis. Each experiment was run in triplicate. Mean values and standard deviations were calculated.

3. Results and Discussion

3.1. Isolation, Screening, and Identification of Amylolytic Bacteria. Isolation and selection of suitable organism are essential for the production of extracellular amylases. Members of genus *Bacillus* were found to be better producer of different types of amylase [21, 22]. In this connection, a total of 35 morphologically well-formed single colonies were selected from different soil samples on the basis of their morphological differences in NA plates. Among them 19 strains were found to be positive as amylase producers. Finally, BI19 strain was selected as the best amylase producer according to highest potency index value. On the basis of multiple sequence alignments to rooted phylogenic tree with branch length (UPGMA) of 16S rDNA sequence by CLUSTALW, the strain BI19 exhibited high level (99%) of similarity with the known sequences in the public databases in NCBI and BLAST results and identified as *Bacillus subtilis* (accession number FJ527663).

3.2. Optimization of Carbon Sources, Organic Nitrogenous, and Inorganic Nitrogenous Sources for Amylase Production. The cell growth and production of amylase by *Bacillus* sp. is reported to be dependent on the strain, composition, and concentration of media, methods of cultivation, cell growth, nutrient requirements, pH, temperature, time of incubation, and thermostability [22, 23]. Thus to enhance the final production level it is essential to screen various medium components and cultural conditions associated with the growth of the inoculum [24].

The production of amylase by *B. subtilis* reported to be effected by various carbon sources [5]. In our study, rice flour, starch and corn flour were found to be the stimulator of amylase production (Figure 1(a)). It may be due to starch and rice flour metabolized slowly by the bacterium as complex carbohydrate sources with increasing accumulation of inducible amylase in the fermentation medium [25]. Rice flour at a concentration of 1.25% (w/v) supported optimal enzyme production, followed by a decline at higher concentrations (Figure 1(b)). This can be attributed to the high viscosity of culture broth at such concentrations that interferes with O_2 transfer leading to limitations of dissolved O_2 for growth of bacteria [26]. Hence, these starch-rich rice and corn flours may prove useful and cheaper alternative natural sources of carbon and energy for the bacterial production of amylase.

The nature and relative concentration of different complex nitrogenous sources in the growth medium are both important in the synthesis of amylase. Like lower levels, higher levels of nitrogen are equally detrimental causing enzyme inhibition [27]. Various complex nitrogen sources were added separately and in combination as replacement of peptone (1%) to the original medium to assess their effects on the final production (Figure 2(a)). It has been previously found that organic nitrogen sources like peptone and yeast extract usually have stimulating effects [28] and our findings are similar to them. Yeast extract also reported to serve as good organic nitrogen source for α-amylase synthesis from *B. amyloliquefaciens* [27]. Bozic et al. (2011) [29] found casein to be the best nitrogen source for α-amylase production from *B. subtilis* IP 5832. Albeit peptone as single replacement was significant; all the combination of peptone, tryptone, and yeast extract gave better results for amylase production in this experiment. Nusrat and Rahman (2007) [21] reported similar results for α-amylase production by *B. licheniformis* and *B. subtilis*. As a single organic nitrogen source, 1.2% of peptone was found to produce maximum amylase (7.82 U/mL/min) (Figure 2(b)). Inorganic nitrogen sources likely ammonium salts have been reported to induce amylase production [4]. Our findings are in good agreement with these studies.

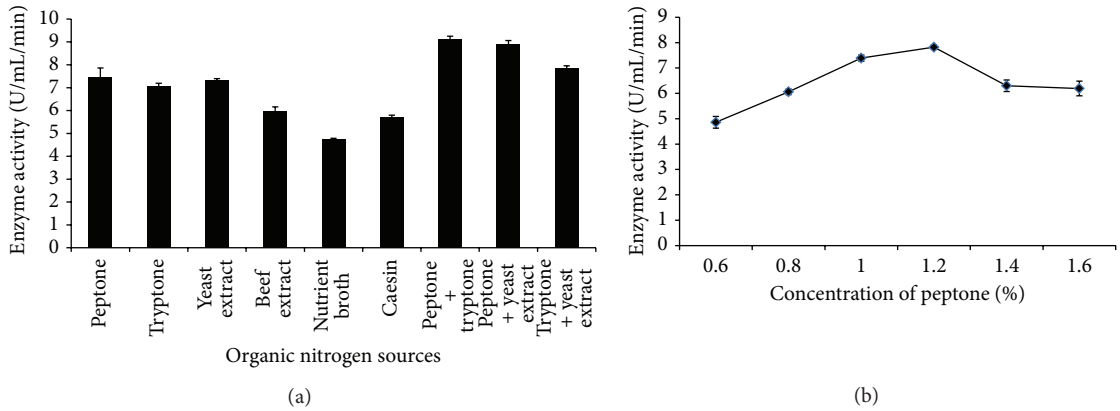

Figure 2: The production of amylase by *Bacillus subtilis* BI19 in shake flask fermentation. (a) Effect of different organic nitrogen sources and (b) effect of different concentrations of peptone.

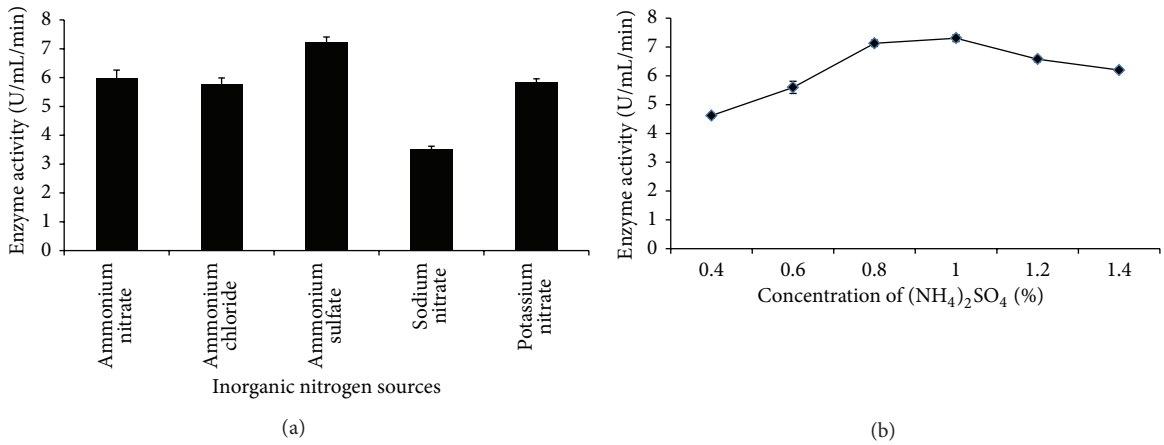

Figure 3: The production of amylase by *Bacillus subtilis* BI19 in shake flask fermentation. (a) Effect of different inorganic nitrogen sources and (b) effect of different concentrations of ammonium sulfate.

Presence of 1% $(NH_4)_2SO_4$ was found to give maximum yield (7.31 U/mL/min) of amylase in this experiment (Figures 3(a) and 3(b)). The decline in amylase production at increased nitrogen concentration could be due to the lowering of pH of the production medium or induction of protease, which suppresses the amylolytic activity [23]. Swain et al. (2006) [30] reported to find suppressed α-amylase production by newly isolated *B. subtilis* when 1% ammonium sulphate was used in the fermentation medium which is contrary with our findings.

3.3. Effect of Added Glucose, Surfactants, and Polyhydroxy Alcohols on Production of Amylase. Addition of free glucose in the fermentation medium was found to suppress amylase production greatly as shown in Figure 4(a). The inhibitory effect of glucose on α-amylase synthesis increased with the increase of glucose concentration in the medium. Addition of 2% glucose resulted in about 54% of production loss (3.33 U/mL/min). Similar results were found by Nusrat and Rahman (2007) [21]. The most possible reason may the suppression of synthesis of carbohydrate degrading enzymes by readily metabolizable substrates such as glucose and fructose (mediated by the protein encoded by the CreA gene) [31].

Addition of surfactants and polyhydroxy alcohols in production medium reported to increase amylase secretion [32]. In this study, amylase production was found to increase by 28%, 15%, and 12%, respectively, in culture medium over control due to addition of Tween 80 (0.025%), sodium lauryl sulfate (0.02%), and sorbitol (0.3%) whereas glycerol (0.3%) and mannitol (0.3%) were found to suppress the production (Figure 4(b)). This increase in production might be due to increase in cell membrane permeability [33] and/or modification (swelling) of starch [34]. Palit and Banerjee (2001) [35] found the similar result with *B. circulans*.

3.4. Optimization of Incubation Temperature, Initial Medium pH, Incubation Period, and Agitation Speed for Amylase Production. The influence of temperature on amylase production is related to the growth of the organism. The temperature range of 35–45°C reported to be preferred for the biosynthesis of amylases by *Bacillus* strains [3, 4]. *B. amyloliquefaciens*,

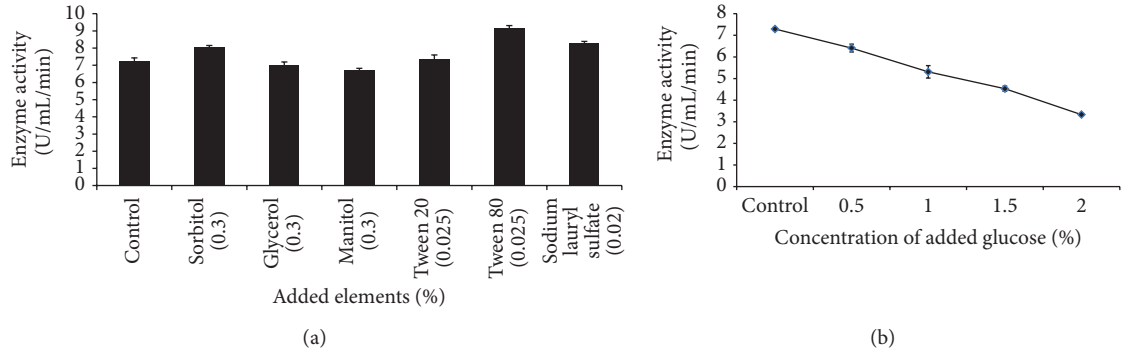

Figure 4: The production of amylase by *Bacillus subtilis* BI19 in shake flask fermentation. (a) Effect of addition of different concentrations of glucose in basal medium and (b) effect of addition of various elements in basal medium.

B. subtilis, *B. licheniformis,* and *B. stearothermophilus* are among the most commonly used *Bacillus* sp. reported to produce amylase at temperatures 37–60°C [36]. In the present study, production of amylases was found to be optimum as the fermentation was carried out at 37°C (Table 1). Further increase in the temperature gave insignificant production which might be due to the very sensitiveness of the organism to temperature [3].

The pH ranges from 6.0 to 7.0 have been reported for normal growth and enzyme activity in *Bacillus* strain isolated from soil [37]. When pH is altered below or above the optimum, activity decreased due to denaturation of proteins [38]. It is evident from the results of the present study that amylase production by the *B. subtilis* BI19 is better at neutral to alkaline range of pH (Table 1). The production was found to be optimum (7.77 U/mL/min) when the initial pH of the fermentation medium was maintained at 8.0. Further increase or decrease in pH resulted in gradual reduction of amylase production. These findings agree with those studies reported for *B. thermoleovorans* NP54 [39], *B. licheniformis* [40], *B. subtilis* JS-2004 [41], and *B. brevis* [42].

Optimization of incubation period was found to be very critical for maximum production of amylase [9]. In this study, the production of amylases was highest (8.67 U/mL/min) at 24 h after inoculation and decreased rapidly thereafter (Table 1). It might be due to that the organism entered in the stationary phase and fermentation approached its end point [43]. Maximum studies revealed that the production of amylase increased up to the level of 72 h of incubation [21]. Possible reason for amylase inactivation after 24 h might be due to release of high levels of intracellular proteases and/or secondary metabolites in the culture medium at the end of exponential phase. The present work is more encouraging as there was reduction in time period that can save energy requirement of the fermentation conditions and provide relatively efficient handling. Similar results were supported by Abate et al. (1999) [44].

Proper agitation is a basic need in order to achieve a good mixing, mass, and heat transfer in submerged fermentation [45]. In our study, amylase production was found to be increased steadily with the increase of agitation speed up to 150 rpm and the range of 140–170 rpm was found optimum (Table 1). Our findings are in good agreement with Nusrat and Rahman (2007) [21] and Sarikaya and Gürgün (2000) [18].

3.5. Effect of Volume of Fermentation Medium and Inoculums Size on Production of Amylase. Volume of fermentation medium plays critical roles in air and nutrient supply, growth of microorganisms, and production of enzyme [46]. In our study, maximum production was obtained at 50 mL of fermentation medium (Table 1). As the volume of the medium increased, the production was decreased most probably due to reduction in the agitation rate of medium that took place with high volume of fermentation medium leading to reduction in air supply as well as insufficient supply of nutrients required for biomass and enzyme synthesis [47].

Inoculum level reported to play critical role in submerged production [31]. In this investigation, production level was found to increase with increase in size of inoculum and found to be optimal at 2% (v/v) (Table 1). Further increase in the inoculum size greatly decreased the production that might be due to rapid growth of bacteria and rapid consumption of essential nutrient sources by bacteria in the initial stages. Malhotra et al. (2000) [39] also reported the similar findings.

3.6. Effect of Temperature on Activity and Stability of Crude Amylase. The effect of temperature on amylase activity was assayed at different temperatures ranging from 30 to 90°C at optimum pH 8.0. The optimum temperature for amylase activity was found to be between 40 and 60°C (Figure 5(a)). Amylase retained 100% relative activity when incubated at 50°C and, as temperature increased from 60 to 80°C, the activity was swiftly declined. At 80°C, the activity was the least (12%) and no activity was found at 90°C. These findings are comparable with that reported for the production of α-amylase using *B. amyloliquefaciens* by Demirkan (2011) [48]. In our study, crude amylase was heated at different temperatures for 1 h followed by testing its activity. The results showed that room temperature (25°C) was suitable for a long term stability of enzyme activity retaining 100% relative activity (Figure 5(b)). The enzyme retained above 60% relative activity even after heating at 50°C for 1 h. Thus these results concluded that the crude enzyme is moderately

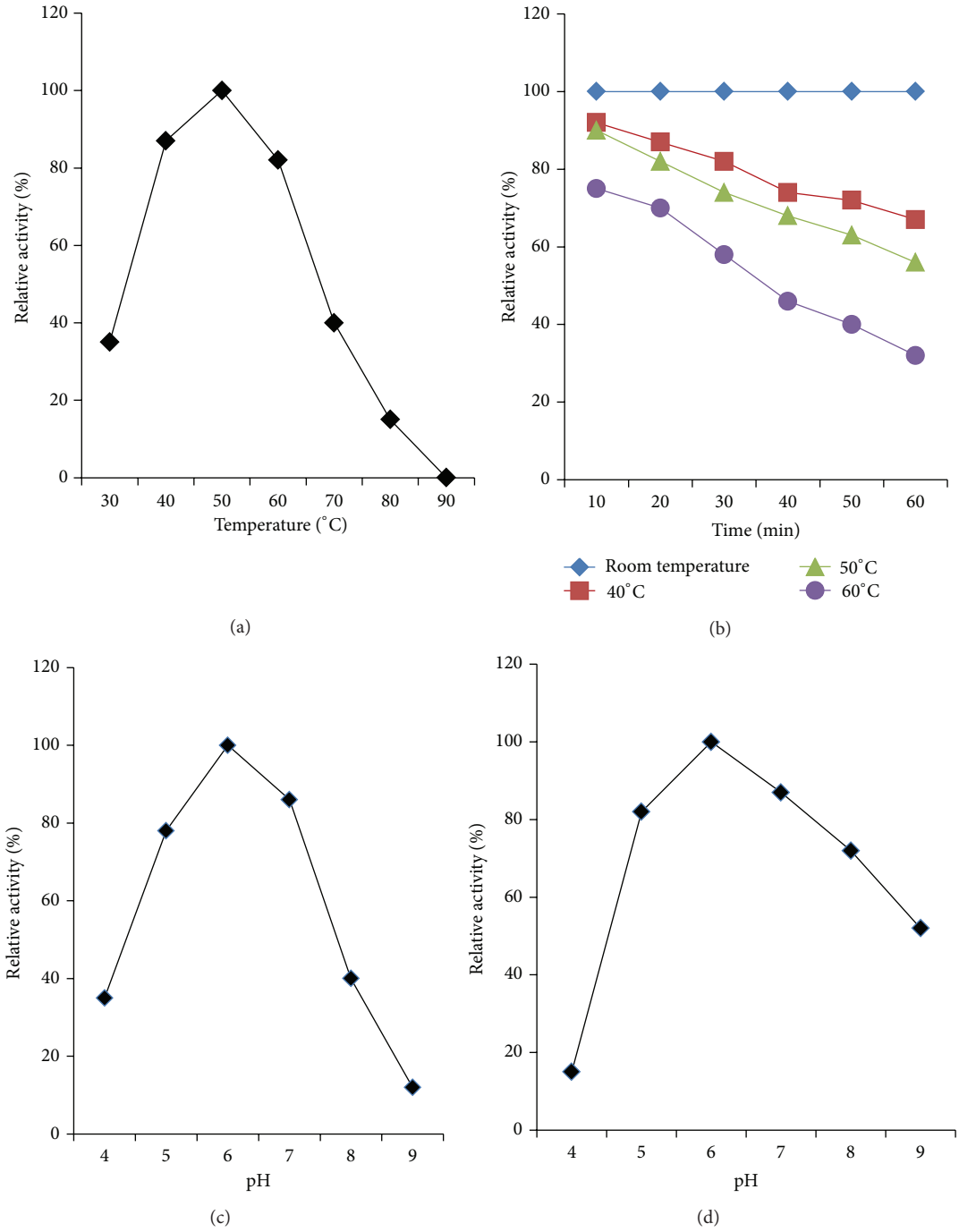

Figure 5: Partial characterization of crude amylase. (a) Effect of temperature on activity of crude amylase. (b) Effect of temperature on stability of crude amylase. (c) Effect of pH on activity of crude amylase. (d) Effect of pH on stability of crude amylase.

temperature stable. These findings agree with that reported by Yang and Liu, 2004 [49].

3.7. Effect of pH on Activity and Stability of Crude Amylase. A good industrial catalyst should be stable under the toughest operating conditions and for long durations [50]. When the crude amylase was treated at different pH, maximum activity was obtained at slightly acidic pH 6.0, retaining 100% activity as stated in Figure 5(c). There was a dramatic decline over activity when pH changes from 7.0 to 9.0 (retaining only 10% activity). The amylase retained more than 80% of its original activity between pH 5.0 and 7.0. The present study indicates that this enzyme prefers slightly acidic and/or alkaline pH for optimal activity. Similar preferred conditions for amylase activity have been found in previous studies [48]. Gupta et al. (2003) [51] stated this value as within the range of values

Table 1: Effect of different culture conditions for production of amylase by *Bacillus subtilis* BI19 in shake-flask fermentation.

Culture conditions	Amylase activity (U/mL/min)	Relative activity (%)
Incubation temperature (°C)		
30	4.12 ± 0.31	56.82
32	5.59 ± 0.19	77.10
35	6.78 ± 0.94	93.51
37	7.25 ± 0.13	100.00
40	6.83 ± 0.45	94.20
42	5.20 ± 0.53	71.72
45	4.91 ± 0.45	67.72
Initial pH		
6.0	4.75 ± 0.47	52.90
6.5	5.37 ± 0.81	69.11
7.0	7.19 ± 0.19	92.53
7.5	7.51 ± 0.26	96.65
8.0	7.77 ± 0.10	100.00
8.5	6.60 ± 0.43	84.94
9.0	4.99 ± 0.53	64.22
Incubation period (h)		
12	3.61 ± 0.09	41.63
24	8.67 ± 0.08	100.00
36	7.44 ± 0.21	85.81
48	7.38 ± 0.14	85.12
60	7.29 ± 0.18	84.08
72	7.17 ± 0.07	82.70
Agitation speed (rpm)		
100	2.43 ± 0.23	33.20
120	3.94 ± 0.07	53.82
140	6.87 ± 0.22	93.85
150	7.32 ± 0.15	100.00
160	7.04 ± 0.17	96.17
170	6.73 ± 0.09	91.93
180	5.81 ± 0.23	79.37
Fermentation medium (mL)		
25	4.62 ± 0.52	63.55
50	7.27 ± 0.01	100.00
75	6.54 ± 0.19	89.96
100	6.04 ± 0.18	83.08
125	5.88 ± 0.07	80.88
150	5.09 ± 0.43	70.01
175	4.14 ± 0.31	56.95
Inoculum size (v/v %)		
1	5.98 ± 0.11	82.48
2	7.25 ± 0.61	100.00
3	7.00 ± 0.12	96.55
4	6.66 ± 0.19	91.86
5	4.27 ± 0.18	58.90

Data represent as mean for three replicates ± standard deviation.

for most starch degrading bacterial enzymes. The enzyme was found to be stable over a wide pH range (5.0 to 8.0) (Figure 5(d)). More than 60% residual activity was obtained at this range. From pH 4.0 to 6.0, there was a massive retention of about 60% activity. The possible reason may be the inhibition of enzyme active site by changing in the concentration of hydrogen ions.

4. Conclusion

Optimization of medium components and culture conditions for enhancing final production of amylase by *B. subtilis* BI19 and partial characterization of the crude amylase were carried out in this study. The most emerging and significant findings were the ability of this strain to utilize rice flour as sole carbon source to produce maximum level of amylase after only 24 h of incubation. Enzyme synthesis and secretion were affected greatly by addition of surfactants (positive inducer) and readily metabolizable glucose (negative inducer) in the basal medium. The optimum enzyme production by the bacterial isolate was found at 37°C, whereas maximum enzyme activity was found at 60°C and pH 5.0–7.0. Further research will be planned and carried out for improvement of the stain, purification of crude amylase, determination of encoded gene sequence of amylase, and further scaling up using fermenter.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] W. W. Windish and N. S. Mhatre, "Microbial amylases," in *Advances in Applied Microbiology*, W. U. Wayne, Ed., vol. 7, pp. 273–304, Academic Press, New York, NY, USA, 1995.

[2] M. J. E. C. van der Maarel, B. van der Veen, J. C. M. Uitdehaag, H. Leemhuis, and L. Dijkhuizen, "Properties and applications of starch-converting enzymes of the α-amylase family," *Journal of Biotechnology*, vol. 94, no. 2, pp. 137–155, 2002.

[3] A. Pandey, P. Nigam, C. R. Soccol, V. T. Soccol, D. Singh, and R. Mohan, "Advances in microbial amylases," *Biotechnology and Applied Biochemistry*, vol. 31, no. 2, pp. 135–152, 2000.

[4] K. Das, R. Doley, and A. K. Mukherjee, "Purification and biochemical characterization of a thermostable, alkaliphilic, extracellular α-amylase from *Bacillus subtilis* DM-03, a strain isolated from the traditional fermented food of India," *Biotechnology and Applied Biochemistry*, vol. 40, no. 3, pp. 291–298, 2004.

[5] B. Prakash, M. Vidyasagar, M. S. Madhukumar, G. Muralikrishna, and K. Sreeramulu, "Production, purification, and characterization of two extremely halotolerant, thermostable, and alkali-stable α-amylases from *Chromohalobacter* sp. TVSP 101," *Process Biochemistry*, vol. 44, no. 2, pp. 210–215, 2009.

[6] M. A. K. Azad, J.-H. Bae, J.-S. Kim et al., "Isolation and characterization of a novel thermostable α-amylase from Korean pine seeds," *New Biotechnology*, vol. 26, no. 3-4, pp. 143–149, 2009.

[7] A. Burhan, U. Nisa, C. Gökhan, C. Ömer, A. Ashabil, and G. Osman, "Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6," *Process Biochemistry*, vol. 38, no. 10, pp. 1397–1403, 2003.

[8] E. S. Demirkan, B. Mikami, M. Adachi, T. Higasa, and S. Utsumi, "α-Amylase from *B. amyloliquefaciens*: purification, characterization, raw starch degradation and expression in *E. coli*," *Process Biochemistry*, vol. 40, no. 8, pp. 2629–2636, 2005.

[9] P. Deb, S. A. Talukdar, K. Mohsina, P. K. Sarker, and S. M. A. Sayem, "Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001," *SpringerPlus*, vol. 2, article 154, 2013.

[10] M. Gavrilescu and Y. Chisti, "Biotechnology —a sustainable alternative for chemical industry," *Biotechnology Advances*, vol. 23, no. 7-8, pp. 471–499, 2005.

[11] R. M. Atlas, L. C. Parks, and A. E. Brown, *Laboratory Manual of Experimental Microbiology*, Mosby-Year Book Inc, St. Louis, Mo, USA, 1995.

[12] C. Ball and M. P. McGonagle, "Development and evaluation of a potency index screen for detecting mutants of *Penicillium chrysogenum* having increased penicillin yield," *Journal of Applied Bacteriology*, vol. 45, no. 1, pp. 67–74, 1978.

[13] L. M. Hamilton, C. T. Kelly, and W. M. Fogarty, "Production and properties of the raw starch-digesting α-amylase of *Bacillus* sp. IMD 435," *Process Biochemistry*, vol. 35, no. 1-2, pp. 27–31, 1999.

[14] M. M. Rahman, M. Basaglia, E. Vendramin et al., "Bacterial diversity of a wooded riparian strip soil specifically designed for enhancing the denitrification process," *Biology and Fertility of Soils*, vol. 50, no. 1, pp. 25–35, 2014.

[15] M. M. Rahman, M. Basaglia, F. Lorenzo et al., "A wooded riparian strip set up for nitrogen removal can affect the water flux microbial composition," *Italian Journal of Agronomy*, vol. 9, no. 1, article 548, 2014.

[16] J. R. Marchesi, T. Sato, A. J. Weightman et al., "Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA," *Applied and Environmental Microbiology*, vol. 64, no. 6, pp. 795–799, 1998.

[17] A. M. Osborn, E. R. B. Moore, and K. N. Timmis, "An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics," *Environmental Microbiology*, vol. 2, no. 1, pp. 39–50, 2000.

[18] E. Sarikaya and V. Gürgün, "Increase of the alpha-amylase yield by some *Bacillus* strains," *Turkish Journal of Biology*, vol. 24, pp. 299–308, 2000.

[19] I. Gomes, M. Sultana, K. Uddin, J. Gomes, W. Steiner, and D. J. Gomes, "Nutrient composition and fermentation conditions for a-amylase production by *Bacillus amyloliquefaciens*," *Bangladesh Journal of Microbiology*, vol. 18, no. 2, pp. 141–150, 2001.

[20] G. L. Miller, "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Analytical Chemistry*, vol. 31, no. 3, pp. 426–428, 1959.

[21] A. Nusrat and S. R. Rahman, "Comparative studies on the production of extracellular α-amylase by three mesophilic *Bacillus* isolates," *Bangladesh Journal of Microbiology*, vol. 24, no. 2, pp. 129–132, 2007.

[22] K. Khajeh, H. Naderi-Manesh, B. Ranjbar, A. A. Moosavi-Movahedi, and M. Nemat-Gorgani, "Chemical modification of lysine residues in *Bacillus* α-amylases: effect on activity and stability," *Enzyme and Microbial Technology*, vol. 28, no. 6, pp. 543–549, 2001.

[23] A. Tonkova, " Microbial starch converting enzymes of the α-amylase family," in *Microbial Biotechnology in Horticulture*, R. C. Ray and O. P. Wards, Eds., pp. 421–472, Science Publishers, Enfield, NH, USA, 2006.

[24] K. Kathiresan and S. Manivannan, "α-amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil," *African Journal of Biotechnology*, vol. 5, no. 10, pp. 829–832, 2006.

[25] P. V. D. Aiyer, "Effect of C:N ratio on alpha amylase production by *Bacillus licheniformis* SPT 27," *African Journal of Biotechnology*, vol. 3, no. 10, pp. 519–522, 2004.

[26] R. Rukhaiyar and S. K. Srivastava, "Effect of various carbon substrates on a-amylase production from *Bacillus* species," *World Journal of Microbiology and Biotechnology*, vol. 10, pp. 76–82, 1995.

[27] N. Sharma, R. Vamil, S. Ahmad, and R. Agarwal, "Effect of different carbon and nitrogen sources on α-amylase production from *Bacillus amyloliquefaciens*," *International Journal of Pharmaceutical Sciences and Research*, vol. 3, no. 4, pp. 1161–1163, 2012.

[28] C. J. Hewitt and G. L. Solomons, "The production of α-amylase (E.C.3.2.1.1.) by *Bacillus amyloliquefaciens*, in a complex and a totally defined synthetic culture medium," *Journal of Industrial Microbiology*, vol. 17, no. 2, pp. 96–99, 1996.

[29] N. Bozic, J. Ruiz, J. Lopez-Santin, and Z. Vujcic, "Optimization of the growth and α-amylase production of *Bacillus subtilis* IP 5832 in shake flask and laboratory fermenter batch cultures," *Journal of the Serbian Chemical Society*, vol. 76, no. 7, pp. 965–972, 2011.

[30] M. R. Swain, S. Kar, G. Padmaja, and R. C. Ray, "Partial characterization and optimization of production of extracellular α-amylase from B*acillus subtilis* isolated from culturable cow dung microflora," *Polish Journal of Microbiology*, vol. 55, no. 4, pp. 289–296, 2006.

[31] L. L. Lin, C. C. Chyau, and W. H. Hsu, "Production and properties of a raw-starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23," *Biotechnology and Applied Biochemistry*, vol. 28, no. 1, pp. 61–68, 1998.

[32] S. Arnesen, S. Havn Eriksen, J. Ø. Olsen, and B. Jensen, "Increased production of α-amylase from *Thermomyces lanuginosus* by the addition of Tween 80," *Enzyme and Microbial Technology*, vol. 23, no. 3-4, pp. 249–252, 1998.

[33] J. L. U. M. Rao and T. Satyanarayana, "Enhanced secretion and low temperature stabilization of a hyperthermostable and Ca^{2+}-independent α-amylase of *Geobacillus thermoleovorans* by surfactants," *Letters in Applied Microbiology*, vol. 36, no. 4, pp. 191–196, 2003.

[34] S. N. Moorthy, "Physicochemical and functional properties of tropical tuber starches: a review," *Starch/Staerke*, vol. 54, no. 12, pp. 559–592, 2002.

[35] S. Palit and R. Banerjee, "Optimization of extraction parameters for recovery of aamylase from the fermented bran of *Bacillus circulans* GRS313," *Brazilian Archives of Biology and Technology*, vol. 44, no. 1, pp. 147–152, 2001.

[36] S. Mishra, S. B. Noronha, and G. K. Suraishkumar, "Increase in enzyme productivity by induced oxidative stress in *Bacillus subtilis* cultures and analysis of its mechanism using microarray data," *Process Biochemistry*, vol. 40, no. 5, pp. 1863–1870, 2005.

[37] M. V. Ramesh and B. K. Lonsane, "Regulation of alpha-amylase production in *Bacillus licheniformis* M27 by enzyme end-products in submerged fermentation and its overcoming in solid state fermentation system," *Biotechnology Letters*, vol. 13, no. 5, pp. 355–360, 1991.

[38] T. Hayashi, T. Abiba, and K. Horikosh, "Properties of a new alkaline maltohexose forming amylases," *Applied Microbiology and Biotechnology*, vol. 28, pp. 281–285, 1988.

[39] R. Malhotra, S. M. Noorwez, and T. Satyanarayana, "Production and partial characterization of thermostable and calcium-independent α-amylase of an extreme thermophile *Bacillus thermooleovorans* NP54," *Letters in Applied Microbiology*, vol. 31, no. 5, pp. 378–384, 2000.

[40] T. Krishnan and A. K. Chandra, "Purification and characterization of α-amylase from *Bacillus licheniformis* CUMC305," *Applied and Environmental Microbiology*, vol. 46, no. 2, pp. 430–437, 1983.

[41] M. Asgher, M. J. Asad, S. U. Rahman, and R. L. Legge, "A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing," *Journal of Food Engineering*, vol. 79, no. 3, pp. 950–955, 2007.

[42] V. T. Tsvetkov and E. I. Emanuilova, "Purification and properties of heat stable α-amylase from *Bacillus brevis*," *Applied Microbiology and Biotechnology*, vol. 31, no. 3, pp. 246–248, 1989.

[43] P. Hillier, D. A. J. Wase, A. N. Emery, and G. L. Solomons, "Instability of α-amylase production and morphological variation in continuous culture of *Bacillus amyloliquefaciens* is associated with plasmid loss," *Process Biochemistry*, vol. 32, no. 1, pp. 51–59, 1997.

[44] C. M. Abate, G. R. Castro, F. Siñeriz, and D. A. S. Callieri, "Production of amylolytic enzymes by *Bacillus amyloliquefaciens* in pure culture and in co-culture with *Zymomonas mobilis*," *Biotechnology Letters*, vol. 21, no. 3, pp. 249–252, 1999.

[45] O. C. Amadi and B. N. Okolo, "Influence of stirrer speed on the morphology of *Aspergillus carbonarius* var (Bainier) Thom IMI 366159 during raw starch digesting amylase production," *Asian Journal of Biological Sciences*, vol. 6, no. 2, pp. 84–95, 2013.

[46] V. Ivanova, D. Yankov, L. Kabaivanova, and D. Pashkkoulov, "Simultaneous biosynthesis and purification of two extra cellular *Bacillus* hydrolases in aqueous two alpha amylases," *Journal of Biochemical Engineering*, vol. 8, no. 1, pp. 61–81, 2001.

[47] S. Narang and T. Satyanarayana, "Thermostable alpha-amylase production by an extreme thermophile Bacillus thermooleovorans," *Letters in Applied Microbiology*, vol. 32, no. 1, pp. 31–35, 2001.

[48] E. Demirkan, "Production, purification, and characterization of α-amylase by *Bacillus subtilis* and its mutant derivates," *Turkish Journal of Biology*, vol. 35, no. 6, pp. 705–712, 2011.

[49] C.-H. Yang and W.-H. Liu, "Purification and properties of a maltotriose-producing α-amylase from *Thermobifida fusca*," *Enzyme and Microbial Technology*, vol. 35, no. 2-3, pp. 254–260, 2004.

[50] A. Pal and F. Khanum, "Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation," *Bioresource Technology*, vol. 101, no. 19, pp. 7563–7569, 2010.

[51] R. Gupta, P. Gigras, H. Mohapatra, V. K. Goswami, and B. Chauhan, "Microbial α-amylases: a biotechnological perspective," *Process Biochemistry*, vol. 38, no. 11, pp. 1599–1616, 2003.

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Research Article

Production and Characterization of Lipases by Two New Isolates of *Aspergillus* through Solid-State and Submerged Fermentation

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Due to the numerous applications of lipases in industry, there is a need to study their characteristics, because lipases obtained from different sources may present different properties. The aim of this work was to accomplish the partial characterization of lipases obtained through submerged fermentation and solid-state fermentation by two species of *Aspergillus*. Fungal strains were isolated from a diesel-contaminated soil and selected as good lipases producers. Lipases obtained through submerged fermentation presented optimal activities at 37°C and pH 7.2 and those obtained through solid-state fermentation at 35°C and pH 6.0. The enzymes produced by submerged fermentation were more temperature-stable than those obtained by solid-state fermentation, presenting 72% of residual activity after one hour of exposition at 90°C. Lipases obtained through submerged fermentation had 80% of stability in acidic pH and those obtained through solid-state fermentation had stability greater than 60% in alkaline pH.

1. Introduction

Lipases (triacylglycerol acyl-hydrolases EC3.1.1.3) are enzymes capable of hydrolyzing the ester bonds of insoluble substrates in water at the substrate-water interface [1]. The main industrial applications of lipases are in detergents [2], medicines [3], and foods [1]. The maturation of cheeses [4], the synthesis of aromas [5], and the production of lipids with high levels of unsaturated fatty acids [6, 7] are examples of the application in food industry. The production of methyl-esters of fatty acids (biodiesel) [8] is the most recent and mentioned application nowadays.

As revised by Treichel et al. [9] many researchers worldwide direct their activities to the screening of new lipase-producing microorganisms and, subsequently, to the optimization of the medium composition and operational variables. All these efforts are justified by the great versatility of lipase applications. Due to the numerous applications of lipases in industry, there is a need to study their characteristics, because lipases obtained from different sources may have different properties [10].

The optimum activity of enzymes depends on the integrity of its structure; therefore factors that may affect these, such as pH, temperature, chemical agents, autolysis (proteases), and ionic strength, affect the enzyme's maximum activity [11]. Temperature has a significant effect on the kinetic energy of enzyme molecules and substrates and causes a greater number of productive collisions per unit of time. The inactivation of enzymatic activity can result from the absorption of excessive energy which causes the disruption or denaturation of the enzyme's tertiary structure due to changes in bonds, such as hydrogen bonds, disulfide bonds, and hydrophobic interactions [12]. The pH affects the stability of enzymes by changing the electrostatic interactions of their protein structure, causing changes in the amino acids' ionization status, which defines the secondary and tertiary structures of protein and therefore its activity and stability [13, 14].

According to Glogauer et al. [15], determination of the pH stability of enzymes is important for identifying nondenaturing pH values of buffers for purification, storage, and reaction steps. With regard to temperature, in an enzymatic process there is a critical play between thermostability and the effect of temperature on activity. It is necessary to identify a reaction temperature that at the same time allows a reasonably high rate of reaction and keeps the rate of denaturation at a reasonably low level.

Given the importance of characterizing lipases obtained from new sources in order to determine their application, the aim of this work was to characterize the lipases produced by *Aspergillus flavus* and *Aspergillus niger* through submerged and solid-state fermentation, respectively, according to the optimum temperature and pH, and to determine the stability of enzymes in relation to temperature and pH.

2. Material and Methods

2.1. Microorganisms: Isolation, Maintenance, and Inoculum Preparation. The filamentous fungi *Aspergillus* (strain O-8) and *Aspergillus* (strain O-4) were isolated from diesel-contaminated soil and previously selected as a good producer of lipase through submerged [16] and solid-state fermentation [17], respectively. The contaminated soil was collected after a case of leaking diesel from a storage tank to the fuel station, which occurred in the city of Passo Fundo, RS, Brazil.

The isolates were submitted to genetic identification through Phred/Phrap and Consed, using the methodology cited by Smaniotto et al. [18], at the Center of Nuclear Energy in Agriculture (Cena) from University of São Paulo (USP), Brazil. Sequences were compared to 18S rRNA data obtained from GenBank (http://www.ncbi.nlm.nih.gov/).

The isolate O-8 was identified as *Aspergillus flavus* strain DAOM (99% identity, GenBank accession number: JN938987.1) and the isolate O-4 was identified as *Aspergillus niger* DAOM (100% identity, GenBank accession number: KC545858.1).

After isolation, the microorganisms were kept in tubes with potato dextrose agar (PDA) inclined under 4°C refrigeration, with periodic replications every 3 months.

The inoculum preparation of *Aspergillus flavus* to submerged fermentation was carried out by inoculation of the fungi in Petri dishes with 30 mL of solidified PDA and incubation at 30°C for 5 days. The inoculation was accomplished using 10 mm of diameter circular areas containing spores growth prepared in Petri dishes [19].

The inoculum preparation of *Aspergillus niger* to solid-state fermentation was carried out by inoculating the fungus in 1 L Erlenmeyer's flasks containing 30 mL of solidified PDA medium and incubated at 30°C for 5 days. A spore suspension was obtained by adding 20 mL of a 0.1% Tween to the inoculum after incubation and by scraping the spores with a Drigalski loop. The fermentation media were inoculated with 2.10^6 spores/g [19].

2.2. Culture Medium to Solid-State and Submerged Fermentation. The culture conditions of lipase production in submerged and solid-state fermentation had been previously optimized [19].

The medium to submerged fermentation was prepared with 10% (w/v) of wheat bran, which was boiled at 100°C for 30 min. Afterwards, the medium was filtered and the soluble extract added to 10% (v/v) of saline solution, 45 g/L of yeast extract as nitrogen source and 20 g/L of soybean oil as inducer. The composition of saline solution [20] was 2 g/L KH_2PO_4, 1 g/L $MgSO_4$, and 10 mL/L of trace solution containing (mg/L) $FeSO_4 \cdot 7H_2O$ (0.63), $MnSO_4$ (0.01), $ZnSO_4$ (0.62). The medium was autoclaved at 103 kPa for 20 min and the pH adjusted to 7.0 using HCl 1.5 mol/L or NaOH 1 mol/L. After inoculation, the cultures were incubated for 4 days at 30°C with agitation of 160 min^{-1}.

The medium for solid-state fermentation was prepared under previously optimized conditions [20] with 85% of soybean or wheat bran and 15% of rice husk. The medium was added to 71% (v/w) of saline solution [20] and 2% of sodium nitrate as nitrogen source. The medium was autoclaved at 103 kPa for 20 min and subsequently added to 2% olive oil as an inducer of lipase production. The pH was adjusted to 4.5 by the addition of a 1.5 mol/L solution of H_2SO_4 and moisture was adjusted to 60% by adding sterile distilled water. Fermentations were carried out in 300 mL Erlenmeyer's flasks containing 50 g of the medium, which were incubated at 30°C for 96 h after inoculation. The fermented brans were kept at −20°C until use.

2.3. Achievement of Enzymatic Extracts. After the production of lipase by submerged and solid-state fermentation, procedures for obtaining the enzymatic extracts were conducted, which are described below.

The fermented medium obtained under submerged fermentation by the fungi *Aspergillus flavus* was filtered in cotton for the retention of hyphae and frozen at −20°C, being after used in the determinations of enzymatic activities [19].

The extraction of lipase from the fermented bran obtained in solid-state fermentation by the fungi *Aspergillus niger* was carried out by adding 10 mL buffer with pH established in each methodology at 1 g of fermented medium, followed by agitation of 160 min^{-1} for 30 min at 37°C. The extract was cotton-filtered and used as enzyme extract in subsequent reactions [19].

2.4. Effect of pH and Temperature on the Optimal Activity of Enzymatic Extracts. The enzymatic extracts without the cells of microorganisms were submitted to tests to determine the influence of pH and temperature on the enzymatic activities.

The optimum activity of enzymatic extracts produced through submerged fermentation was determined by a Central Composite Design (CCD) composed of 4 factorial points, 4 axial points, and 3 central points (Table 1). The levels of variables ranged from 28°C to 42°C for temperature and 6.3 to 7.7 for pH. Optimum pH and temperature for activity of the enzymes produced through solid-state fermentation were determined using a 3^2 Full Factorial Design (FFD) (Table 2). The variables levels were 5 to 7 for the pH and 30°C to 40°C for the temperature.

Table 1: Central composite design (CCD) used to determine the influence of pH and temperature on the optimal activity of lipase obtained through submerged fermentation by *Aspergillus flavus* (strain O-8) and results of lipolytic activity (U/mL). Results of mean and standard deviation.

Experiment	pH (X_1)*	Temperature (X_2)	Lipolytic activity (U/mL)
1	6.5 (−1)	30°C (−1)	3.15 ± 0.13
2	7.5 (+1)	30°C (−1)	3.72 ± 0.01
3	6.5 (−1)	40°C (+1)	3.21 ± 0.07
4	7.5 (+1)	40°C (+1)	4.02 ± 0.08
5	6.3 (−1.414)	35°C (0)	3.54 ± 0.05
6	7.7 (+1.414)	35°C (0)	4.23 ± 0.02
7	7.0 (0)	28°C (−1.414)	3.82 ± 0.01
8	7.0 (0)	42°C (+1.414)	4.37 ± 0.03
9	7.0 (0)	35°C (0)	4.30 ± 0.12
10	7.0 (0)	35°C (0)	4.26 ± 0.01
11	7.0 (0)	35°C (0)	4.03 ± 0.03

*Conditions of pH obtained using 0.2 M phosphate buffer in the enzymatic activity determination.

Table 2: Full Factorial Design (3^2) used to determine the influence of pH and temperature on optimum activity of lipase obtained through solid-state fermentation by *Aspergillus niger* (strain O-4) and results of lipolytic activity (U/g). Results of mean and standard deviation.

Experiment	pH (X_1)*	Temperature (°C) (X_2)	Lipolytic activity (U/g)
1	5 (−1)	30 (−1)	12.20 ± 0.96
2	6 (0)	30 (−1)	40.62 ± 1.90
3	7 (+1)	30 (−1)	15.49 ± 1.92
4	5 (−1)	35 (0)	34.14 ± 2.52
5	6 (0)	35 (0)	42.82 ± 1.65
6	7 (+1)	35 (0)	35.41 ± 1.92
7	5 (−1)	40 (+1)	10.53 ± 0.96
8	6 (0)	40 (+1)	34.58 ± 1.65
9	7 (+1)	40 (+1)	22.13 ± 0.96

*Conditions of pH obtained using 0.2 M phosphate buffer in the steps of enzyme extraction of the fermented bran and in the enzymatic activity determination.

The enzyme activity was determined using the method standardized by Burkert et al. [21] which is based on titration with NaOH of fatty acids released by the action of lipase in the extract on the triacylglycerols of olive oil emulsified in arabic gum. The following were added to 250 mL flasks: 2 mL buffer prepared according to the objective of the test, 5 mL of emulsion prepared with 75 mL of 7% arabic gum, and 25 mL of olive oil. Next, 1 mL of enzyme extract was added to this system and it was incubated at temperatures described in the experimental design for 30 min. After incubation, the reaction was stopped by adding 15 mL of acetone : ethanol : water (1 : 1 : 1) and the released fatty acids were titrated with a solution of 0.01 mol/L NaOH using phenolphthalein as indicator. One unit of activity was defined as the amount of enzyme that releases 1 μmol of fatty acid per minute per mL of enzyme extract of submerged fermentation (1 U = 1 μmol/min·mL) or per g of fermented brand (1 U = 1 μmol/min·g) of solid-state fermentation, under the test conditions.

2.5. Temperature Stability of Enzymatic Extracts. Thermostability of lipases obtained through submerged and solid-state fermentation was measured by incubating the enzyme extract at 35°C to 90°C. Aliquots were periodically taken to measure lipolytic activity, using the optimum temperature and pH for enzyme activity, obtained as mentioned in Section 2.4, to each enzymatic extract. The experiments were duplicated.

For the enzymes obtained through solid-state fermentation it was possible to calculate the Arrhenius thermal deactivation and activation energy for thermal destruction constants (E_a). Therefore, the data of enzymatic activity at each temperature tested were used to calculate the residual lipase activity (RA) over time. The constant of thermal deactivation (k_d) at each temperature was calculated by linear regression of the data of Ln (RA) versus time, according to the Arrhenius kinetic model, considering that inactivation of the enzyme obeys first-order kinetics, as in the following. Consider

$$\frac{d[E]}{[E]} = -k_d \cdot dt. \tag{1}$$

After integration,

$$\mathrm{Ln}\frac{[E]_2}{[E]_1} = -k_d \cdot \Delta t. \tag{2}$$

Considering that the enzyme concentration ([E]) is directly proportional to the enzymatic reaction speed,

$$\frac{[E]_2}{[E]_1} = AR. \tag{3}$$

We get the following:

$$\mathrm{Ln}\,(AR) = -k_d \cdot \Delta t. \tag{4}$$

From the thermal deactivation constants at each temperature, the half-lives ($t_{1/2}$) were obtained (5) which corresponds to the time required, at the temperature tested, so that 50% of the initial enzyme concentration is inactivated:

$$t_{1/2} = \frac{0.693}{k_d}. \tag{5}$$

The activation energy (E_a) for thermal destruction of the enzyme was calculated from (6). The value of E_a was obtained from the inclination of the regression line of ln K versus $1/T$:

$$\ln K = \ln A - \frac{E_a}{RT}, \tag{6}$$

where [E] = enzyme concentration, AR = residual activity of the enzyme, t = time (min), k_d = thermal deactivation constant, A = Arrhenius factor (depending, among other things, on the contact area), E_a = activation energy, R = ideal gases constant (8.314 J mol^{-1}K^{-1}), and T = absolute temperature (K).

2.6. pH Stability of Enzymatic Extracts. The effect of pH on the stability of enzymes obtained through submerged fermentation was determined by treating 1 mL of enzyme extract with 2 mL of buffer solutions at pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, and 9.0 for 24 hours at 25°C. The buffers used were 0.1 mol/L citrate (pH 3.5), 0.2 mol/L acetate (pH 4.0 to 5.5), 0.2 mol/L phosphate (pH 6.0 to 8.0), and 0.2 mol/L glycine (pH 9 and 10). Enzyme activity in initial and final times was carried out at optimum temperature and pH for enzyme activity, obtained from the results of the assays of Section 2.4. The experiments were duplicated.

The stability of enzymes obtained through solid-state fermentation was assessed through their extraction from the fermented medium using the following buffer solutions: 0.1 mol/L citrate (pH 3.5), 0.2 mol/L acetate (pH 4.0, 4.5, 5.0, and 5.5), 0.2 mol/L phosphate (pH 6.0, 6.5, 7.0, 7.5, and 8.0), and 0.2 mol/L glycine (pH 9 and 10). The extracts were kept at 25°C for 24 h and the residual lipolytic activity was determined at optimum temperature and pH for enzyme activity, according to the results obtained from the assays of Section 2.4.

3. Results and Discussion

In the submerged culture fermentation the microorganisms grow in a liquid medium in which the nutrients are dissolved. In solid-state fermentation the microorganisms grow on the surface of a solid matrix in which the nutrients are adsorbed, and the moisture does not exceed the water retention capacity of this matrix [22, 23]. These differences between production methods as well as the differences between the microorganisms used in fermentation processes can lead to obtaining lipases with different characteristics.

3.1. Effect of pH and Temperature on the Optimal Activity of Enzymatic Extracts. The pH and temperature have great influence on the enzyme activity, being important to define these parameters for the characterization of the enzymes obtained. After fungal growth in culture media of submerged and solid-state fermentations, enzymatic extracts were obtained as described in Section 2.3 and used in the assays mentioned in Section 2.4. The results of enzymatic activities were presented in Tables 1 and 2, which also shows the experimental conditions of the experimental designs used to determine optimum temperatures and pH of the enzymes produced through submerged and solid-state fermentations, respectively.

The highest lipolytic activities in the solid-state fermentation (Table 2) may be due to the characteristics of this type of cultivation when compared with submerged cultivation. In the solid state fermentation, the concentration of the final product is higher and the fungus has the appropriate characteristics, as tolerance to low water activity and production of enzymes through hyphae [23]. Furthermore, lipase production was performed by different microorganisms, although both are of the genus *Aspergillus*.

The analysis of variance of lipolytic activity obtained in each experimental design demonstrated, that in both cases, the $F_{\text{calculated}}$ obtained in the analysis of regression models were higher than the $F_{\text{tabulated}}$ value ($F_{\text{calculated}}$ of 6.43 and $F_{\text{tabulated}}$ of 2.85 for Composite Central Design of submerged fermentation; $F_{\text{calculated}}$ of 32.2 and $F_{\text{tabulated}}$ of 2.80 for Full Factorial Design of solid-state fermentation), which means that the variation caused by the models is significantly greater than the unexplained variation [24].

Equations (7) and (8) show the regression models for the enzymes obtained through submerged fermentation (SmF) and solid-state fermentation (SSF), respectively. The correlation coefficients between experimental data and models were of 81.7% and 94.02%, which validates the mathematical models obtained [25, 26]:

$$\text{AL}_{\text{SmF}} = 4.19 + 0.28 \cdot X_1 - 0.254 \cdot {X_1}^2 + 0.142 \cdot X_2 - 0.17 \cdot {X_2}^2, \quad (7)$$

$$\text{AL}_{\text{SSF}} = 49.2 + 2.7 \cdot X_1 - 17.7 \cdot {X_1}^2 - 14.9 \cdot {X_2}^2. \quad (8)$$

The estimated effects of variables of CCD on lipolytic activity showed that linear and quadratic effects of pH were significant ($P < 0.05$). The temperature had significance levels very close to 0.05, of $P = 0.057$ and $P = 0.054$, for the linear and quadratic effects, respectively. Thus, the effect of temperature was considered for the expression of the model. Both variables showed positive linear effects, and the effect of pH was greater than the effect of temperature (0.561 and 0.284, resp.).

Linear and quadratic effects of pH were significant ($P = 0.02$ and $P < 0.01$, resp.) and positive on the activity of lipases obtained through solid-state fermentation (5.4 and −35.4, resp.). On the other hand, only the quadratic effect of temperature (−29.7) had significant influence (<0.01) on the lipolytic activity.

The use of response surface methodology has the advantage of allowing the estimation of the effects of experimental variables on the response variable within the limits stipulated by the experimental design. Thus, the optimal values of the experimental variables can be calculated from the mathematical modeling of the data, when the response surface is validated by analysis of variance. The optimal pH and temperature were calculated by equating the first derivative of the lipolytic activity as a function of the pH and temperature to zero in both mathematical models. The maximum lipolytic activities of lipases obtained through submerged fermentation were obtained at the levels +0.55 and +0.417 from pH and temperature, respectively, corresponding to pH 7.2 and temperature of 37°C. The maximum lipolytic activities of lipases obtained through solid-state fermentation were obtained at the levels +0.076 and zero (0) to pH and temperature, respectively, corresponding to pH 6.0 and temperature of 35°C. These results could differ a little from the maximum results obtained in Tables 1 and 2, because they were obtained from the mathematical models generated from the experimental data.

Figures 1(a) and 1(b) show the response surface representing the mathematical models of (7) and (8) of the enzymes obtained through submerged and solid-state fermentation, respectively.

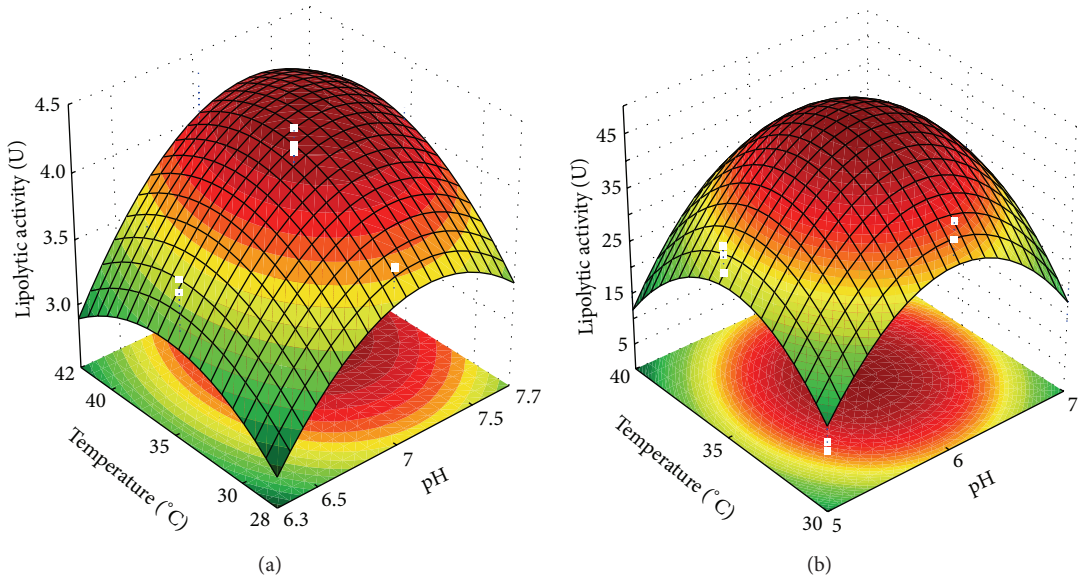

Figure 1: Response surface of the influence of pH and temperature on the lipolytic activity of enzymes produced (a) through submerged fermentation by *Aspergillus flavus* (strain O-8) from CCD and (b) through solid-state fermentation by *Aspergillus niger* (strain O-4) from the 3^2 Full Factorial Design.

The optimum pH and temperature found in this study for the activity of fungal lipases are similar to results reported in academic literature, in which maximum lipase activities were obtained at around 30°C and 40°C and pH 6.0 to 8.0 [27].

Maldonado [10] obtained maximum lipolytic activities at pH 7.0 and 37°C for crude and purified lipase of *Geotrichum candidum*. Baron et al. [28] found that there is a pH range (6.0 and 8.0) where enzyme activity is maximal and that the activity depends not only on the pH of the medium but also on the type of buffer used. Freire et al. [29] reported that the optimum pH and temperature for lipases produced by *Penicillium* sp. were 7.0 and 37°C. Benjamin and Pandey [30] reported that the lipases produced by *Candida rugosa* showed optimum activity at pH 7.0 and temperature 40°C. Pastore et al. [31] characterized the lipases produced by *Rhizopus* sp. and found maximum activity at pH 6.0 and 6.5 and 40°C. However, Diaz et al. [32] characterized the lipases of the fungus *Rhizopus homothallicus* obtained by solid-state and submerged bioprocesses, obtaining maximum activity at pH 7.5 and 30 to 40°C, respectively.

Santos et al. [33] and Lotrakul and Dharmsthiti [34] characterized lipases that obtained maximum activities at temperatures higher than those obtained in this study: 45°C to 50°C for the lipase produced in solid-state fermentation by *Trichosporon* spp., and 45°C for the lipase from *Aeromonas sobria* isolated from raw milk, respectively. Shangguan et al. [35] studied a lipase obtained from *A. fumigatus* that presented optimum pH and temperature of 8.5 and 65°C, respectively.

3.2. Temperature Stability. These experiments were conducted to evaluate the stability of enzymes obtained through two fermentation processes. After maintenance of enzymatic extracts at temperatures ranging from 35°C to 90°C for different periods of time (Section 2.5), the enzymatic activity was evaluated at pH and temperatures optimized in the previous step (Section 2.4) to each fermentation process.

The temperature stability of lipases produced through submerged fermentation by *Aspergillus flavus* was initially assessed at 40 to 80°C, as shown in Figure 2(a). The enzymes were stable between 40°C and 50°C, with residual activity greater than 90% for 7 hours. An initial inactivation of the enzymes between 70°C and 80°C was observed and subsequently they became stable, with residual activity of 80% and 65% at 80°C and 70°C, respectively. The enzymes did not exhibit first-order thermal destruction kinetic behavior (Arrhenius), despite being more stable at 80°C than 70°C.

In order to confirm the data obtained, tests were carried out at 70°C, 80°C, and 90°C for 8 h, and the results (shown in Figure 2(b)) confirm the aforementioned behavior, with an initial enzyme inactivation, caused by thermal shock, and later stability. Furthermore, the enzymes exhibited greater stability at higher temperatures in the first hour of testing. The lipases were stable at 70°C to 90°C, with mean residual activities of around 72% after the first hour of incubation.

Figure 3(a) shows the thermal destruction kinetics of the enzyme produced through solid-state fermentation between 35°C and 90°C, which follows the pattern of first-order thermal destruction predicted by the Arrhenius model. Table 3 shows the thermal deactivation constants between 35°C and 90°C, obtained from angular coefficients of the curves shown in Figure 3, as well as the determination coefficients of regression and the half-life of enzymes at each temperature. The enzyme had higher thermal stability at 35°C and 40°C, which can be observed from the high half-lives ($t_{1/2}$), around 6 and 4.3 h, respectively. Above 50°C, the half-life considerably decreased to 29 min between 60°C and 70°C. Figure 3(b) shows the graph of ln (k_d) as a function

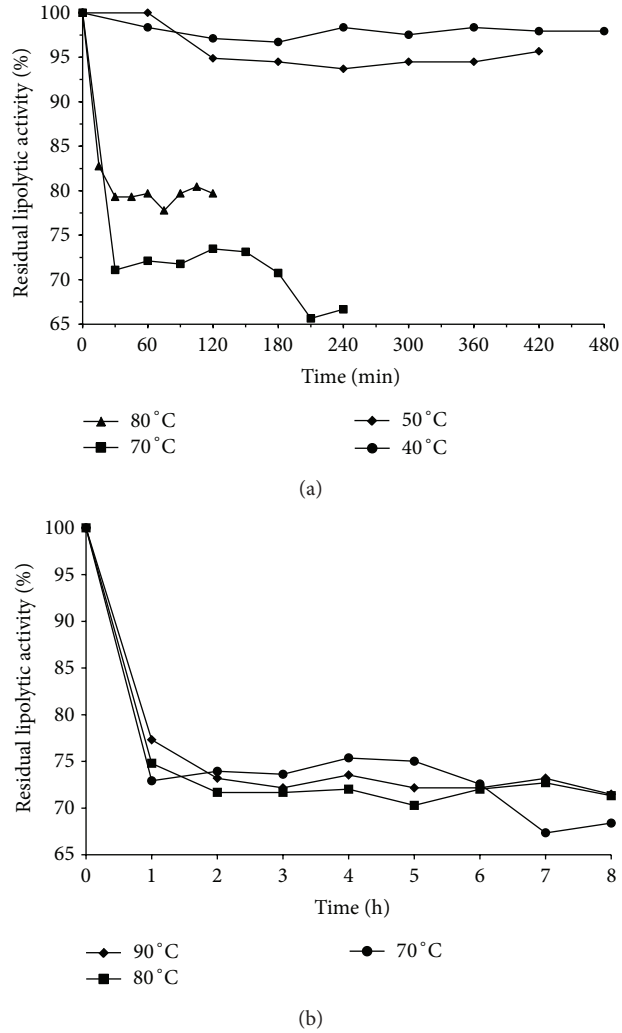

Figure 2: Thermal stability of lipases produced in submerged fermentation using *Aspergillus flavus* (strain O-8): (a) 40°C, 50°C, 70°C, and 80°C; (b) 70°C, 80°C, and 90°C.

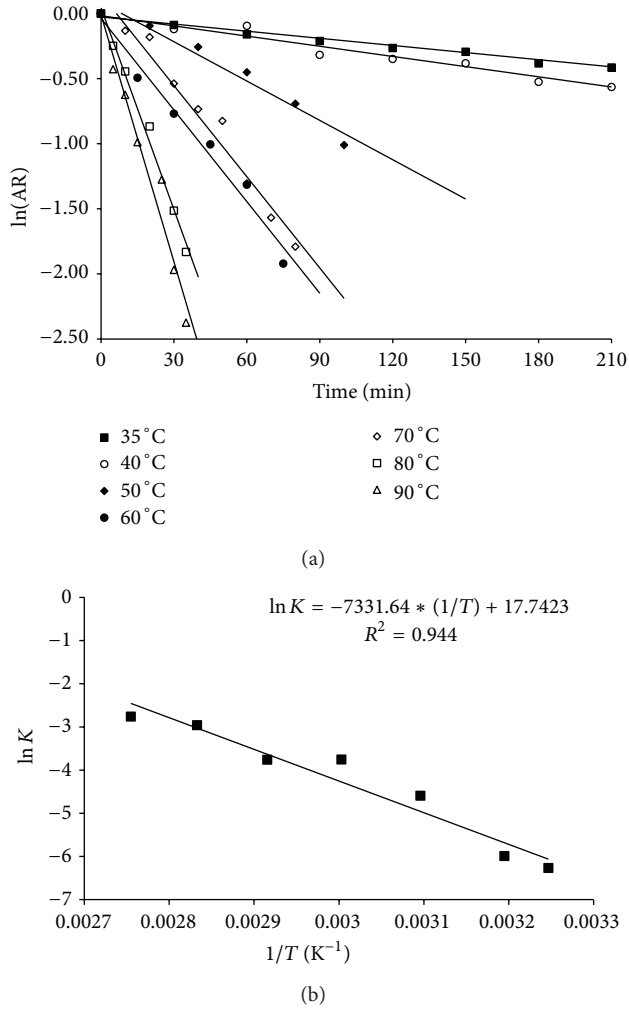

Figure 3: Kinetics of thermal destruction of the enzymatic extracts produced by *Aspergillus niger* in solid-state fermentation: (a) at temperatures of 35°C to 90°C. *AR*: enzyme residual activity, (b) linear regression of the thermal deactivation constants obtained at 35°C to 90°C (ln of data) as function of inverse of absolute temperature for calculating the energy of thermal deactivation of the enzyme.

Table 3: Thermal deactivation constant (k_d), correlation coefficients of regressions (R^2), and times of half-lives ($t_{1/2}$) of enzymatic extracts of solid-state fermentation at temperatures of 35°C to 90°C.

Temperature (°C)	k_d (min^{-1})	R^2	$t_{1/2}$ (min)
35	0.0019	0.985	364.74
40	0.0027	0.954	256.67
50	0.0100	0.966	69.30
60	0.0234	0.976	29.62
70	0.0233	0.971	29.74
80	0.0516	0.991	13.43
90	0.0630	0.968	11.00

of absolute temperature (K), used to calculate the energy of thermal deactivation, which was 60.33 kJ/mol for the studied enzyme.

Lipase obtained with solid-state fermentation showed higher energy of deactivation than those obtained by Diaz et al. [32] and Lima et al. [36], which were 30 and 34.2 kJ/mol for the lipases produced by *Rhizopus homothallicus* and *Bacillus megaterium*, respectively. However, they had lower deactivation energy than the ones obtained by Maldonado [10], which were 330, 140, and 182 kJ/mol for the lipases produced by *Geotrichum candidum* in media containing peptone, hydrolyzed yeast, and macerated clarified corn water, respectively. The activation energy reflects the dependence of the thermal deactivation constant with respect to temperature [37, 38], and the higher the constant, the greater the variation of the thermal deactivation constant with the temperature variation.

Razak et al. [39] reported that fungal lipases in general are unstable above 40°C, with moderate stability, contrary to what has been observed in lipases produced by bacteria such as *Bacillus* [40, 41] and *Pseudomonas* [42], which are

thermostable above 60 C. However, the lipases produced by *Rhizopus* sp. maintained 50% or more of their activity when heated for 60 min between 40 and 55°C [31] and lipases produced by Geotrichum-like R59 showed thermostability, with maximum residual activity after incubation at 60°C for 1 h [43].

Shu et al. [44] reported that the lipases produced by *Antrodia cinnamomea* had 50% residual activity between 25°C and 40°C. Lipase from *Aspergillus niger* NCIM 1207 was stable at 40°C for 3 h; however the treatment at 50°C for 1 h caused 52% loss of activity [45]. Baron et al. [28] showed that the lipases produced by *Penicillium corylophilum* were completely inactivated after 30 min at 60°C. However, the lipases produced by *Rhizopus* sp. had moderate thermostability, with 70% residual activity at temperatures of 40 to 55°C [31]. Ginalska et al. [43] showed that the lipases produced by *Geotrichum* sp. had 100% residual activity after 1 hour of incubation at 60°C, and 50% residual activity at 70°C for 45 min. Furthermore, Sharma et al. [14] reported that the lipase produced by *Bacillus* sp. RSJ-1 presented 90 and 70% residual activity after treatment at 50°C for 120 and 240 min, respectively. Lipase obtained from Iftikhar et al. [46] showed that lipases retained 80% of its activity at 25–30°C by wild and 100% of its activity at 20–50°C by mutant strain of *R. oligosporus*. By further increase in the incubation temperature, the activity of the enzyme was greatly inhibited.

Compared with the aforementioned enzymes, lipases obtained in this study through submerged fermentation had higher thermostability and may have applications in industrial processes that require high temperatures. Enzymatic processes that occur at higher temperatures have higher reaction rates [11]. It may be possible to use thermostable lipases in the synthesis of biopolymers, pharmaceuticals, agrochemicals, cosmetics, biodiesel, and aromas [47]. According to Diaz et al. [32] even for identical lipases produced by different methods of cultivation (submerged and solid-state), there may be thermostability differences caused by the binding of nonprotein compounds derived from the culture medium through noncovalent bonds to the lipases, changing their physical and chemical properties.

Enzyme thermostability may be affected by production conditions, such as the producer microorganism, the method of cultivation, and the medium used [10]. Thermostability is the result of the protein's amino acid sequence, which provides a more rigid conformation to the enzyme [11] through intramolecular interactions, with the internalization of hydrophobic residues and superficial exposure of hydrophilic residues [45]. Lipase thermostability may also be affected by the presence of compounds such as short-chain alcohols, metals, and ions as Ca^{+2} and Mg^{+2} which bind to the surface of enzymes whose binding sites are generally formed by negatively charged groups [48]. According to Iyer and Ananthanarayan [11] thermal stabilization of lipases may be caused by the presence of divalent ions, anions (SO_4^{-2} > Cl^- > Br^- > NO_3^- > ClO_4^-), or cations (NH_4^+ > K^+ > Na^+ > Mg^{+2} > Ca^{+2} > Ba^{+2}). Mg^{+2} and SO_4^{-2} were present in the lipase production culture medium, which, if not consumed by the fungus for growth and synthesis, remain soluble after the separation of cells, and become part of the lipolytic extract. That may explain the thermostability of the produced enzymes. However, if this enzyme extract containing lipases were purified for further use, causing the removal of these ions of the culture medium, the study of the stability of the purified protein would be needed.

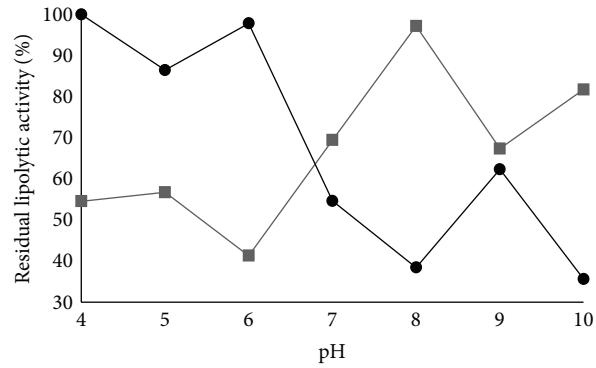

Figure 4: pH stability of lipases produced (●) by *Aspergillus flavus* (strain O-8) through submerged fermentation and (■) by *Aspergillus niger* (strain O-4) through solid-state fermentation.

3.3. pH Stability. The pH stability of enzymatic extracts obtained through submerged and solid-state fermentation was determined according to Section 2.6 treating these extracts with different buffers for 24 h and after the enzymatic activity was determined using the optimized pH and temperature for the enzymes of each fermentation process (Section 2.4).

Figure 4 shows the residual lipolytic activity as a function of pH for the enzymes produced through solid-state and submerged fermentation. Lipases produced through submerged fermentation by *Aspergillus flavus* were stable at pH ranging from 3.5 to 6.5 for 24 h, with residual activities greater than 80%. At pH 7 to 10 there was a reduction in the stability of enzymes with residual activity of around 50%.

Lipase produced through solid-state fermentation by *Aspergillus niger* had greater stability at pH greater than 7.0, with residual activity greater than 60%. In acidic pH (4 to 6), the stability of the enzyme after 24 h was around 50%. It was found that the enzyme showed optimal activity at acidic pH (6.0), while the highest stability was observed with alkaline pH.

This behavior is similar to that reported by Mhetras et al. [45], who reported that lipases produced by *Aspergillus niger* NCIM 1207 were stable when pH was alkaline (pH 8 to 11) despite having had optimum activity at an acidic pH. Sharma et al. [14] reported that the lipases produced by *Bacillus* sp. RSJ-1 had 84 and 82% residual activity, respectively, after 2 h at pH 8 and 9. The lipases produced by *Candida* sp. were stable at pH ranging from 7.5 to 8.5 for 15 min [27].

4. Conclusion

Lipase produced by the *Aspergillus flavus* (strain O-8) through submerged fermentation had maximum activities

at 37°C and pH 7.2. The thermal stability was 72% after 1 h of exposure to temperatures of 70 to 90°C and pH stability greater than 80% in acidic pH, which are desirable traits for industrial application. On the other hand, lipases produced through solid-state fermentation with *Aspergillus niger* (O-4) had optimum temperature and pH around 35°C and pH 6.0 and stability at room temperature (63.6% and 26.8% of residual activity after 1 h of exposure to 50 and 60°C, resp.), lower than that observed with enzymes obtained through submerged fermentation. The pH stability was higher in alkaline pH, with residual activity greater than 60% after 24 h of exposure.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] R. Sharma, Y. Chisti, and Y. C. Banerjee, "Production, purification, characterization, and applications of lipases," *Biotechnology Advances*, vol. 19, no. 8, pp. 627–662, 2001.

[2] N. Saisubramanian, N. G. Edwinoliver, N. Nandakumar, N. R. Kamini, and R. Puvanakrishnan, "Efficacy of lipase from *Aspergillus niger* as an additive in detergent formulations: a statistical approach," *Journal of Industrial Microbiology and Biotechnology*, vol. 33, no. 8, pp. 669–676, 2006.

[3] F. Hasan, A. A. Shah, and A. Hameed, "Industrial applications of microbial lipases," *Enzyme and Microbial Technology*, vol. 39, no. 2, pp. 235–251, 2006.

[4] C. Dupuis, C. Corre, and P. Boyaval, "Lipase and esterase activities of *Propionibacterium freudenreichii* subsp. freudenreichii," *Applied and Environmental Microbiology*, vol. 59, no. 12, pp. 4004–4009, 1993.

[5] R. Ben Salah, H. Ghamghui, N. Miled, H. Mejdoub, and Y. Gargouri, "Production of butyl acetate ester by lipase from novel strain of *Rhizopus oryzae*," *Journal of Bioscience and Bioengineering*, vol. 103, no. 4, pp. 368–372, 2007.

[6] M. V. Reshma, S. S. Saritha, C. Balachandran, and C. Arumughan, "Lipase catalyzed interesterification of palm stearin and rice bran oil blends for preparation of zero trans shortening with bioactive phytochemicals," *Bioresource Technology*, vol. 99, no. 11, pp. 5011–5019, 2008.

[7] D. Wang, Y. Xu, and T. Shan, "Effects of oils and oil-related substrates on the synthetic activity of membrane-bound lipase from *Rhizopus chinensis* and optimization of the lipase fermentation media," *Biochemical Engineering Journal*, vol. 41, no. 1, pp. 30–37, 2008.

[8] E. Y. Park, M. Sato, and S. Kojima, "Fatty acid methyl ester production using lipase-immobilizing silica particles with different particle sizes and different specific surface areas," *Enzyme and Microbial Technology*, vol. 39, no. 4, pp. 889–896, 2006.

[9] H. Treichel, D. de Oliveira, M. A. Mazutti, M. di Luccio, and J. V. Oliveira, "A review on microbial lipases production," *Food and Bioprocess Technology*, vol. 3, no. 2, pp. 182–196, 2010.

[10] R. R. Maldonado, *Production, purification and characterization of lipase of Geotrichum candidum obtained from industrial media [dissertation]*, Universidade Estadual de Campinas—Unicamp, Campinas, Brazil, 2006.

[11] P. V. Iyer and L. Ananthanarayan, "Enzyme stability and stabilization—aqueous and non-aqueous environment," *Process Biochemistry*, vol. 43, no. 10, pp. 1019–1032, 2008.

[12] F. M. Gomes, A. V. de Paula, G. S. Silva, and H. F. de Castro, "Determinação das propriedades catalíticas em meio aquoso e orgânico da lipase de *Candida rugosa* imobilizada em celulignina quimicamente modificada por carbonildiimidazol," *Química Nova*, vol. 29, no. 4, pp. 710–718, 2006.

[13] E. Rajakumara, P. Acharya, S. Ahmad, R. Sankaranaryanan, and N. M. Rao, "Structural basis for the remarkable stability of *Bacillus subtilis* lipase (Lip A) at low pH," *Biochimica et Biophysica Acta*, vol. 1784, no. 2, pp. 302–311, 2008.

[14] R. Sharma, S. K. Soni, R. M. Vohra, L. K. Gupta, and J. K. Gupta, "Purification and characterisation of a thermostable alkaline lipase from a new thermophilic *Bacillus* sp. RSJ-1," *Process Biochemistry*, vol. 37, no. 10, pp. 1075–1084, 2002.

[15] A. Glogauer, V. P. Martini, H. Faoro et al., "Identification and characterization of a new true lipase isolated through metagenomic approach," *Microbial Cell Factories*, vol. 10, article 54, 2011.

[16] L. M. Colla, A. L. Primaz, S. Benedetti et al., "Selection of lipase-producing microorganisms through submerged fermentation," *Zeitschrift für Naturforschung*, vol. 65, no. 7-8, pp. 483–488, 2010.

[17] L. M. Colla, K. Rezzadori, S. K. Câmara et al., "A solid-state bioprocess for selecting lipase-producing filamentous fungi," *Zeitschrift für Naturforschung*, vol. 64, no. 1-2, pp. 131–137, 2009.

[18] A. Smaniotto, A. Skovronski, E. Rigo et al., ""Synthetic lipase" production from a newly isolated *Sporidiobolus pararoseus* strain by submerged fermentation," *Brazilian Journal of Microbiology*, vol. 43, no. 4, pp. 1490–1498, 2012.

[19] L. M. Colla, *Biotechnological optimization of lipases production and its correlation with biossurfactantes production [Ph.D. thesis]*, Federal University of Rio Grande-FURG, Rio Grande, RS, Brazil, 2009.

[20] T. E. Bertolin, J. A. V. Costa, and G. D. L. Pasquali, "Glucoamylase production in batch and Fed-batch solid state fermentation: effect of maltose or starch addition," *Journal of Microbiology and Biotechnology*, vol. 11, no. 1, pp. 13–16, 2001.

[21] J. F. M. Burkert, F. Maugeri, and M. I. Rodrigues, "Optimization of extracellular lipase production by *Geotrichum* sp. using factorial design," *Bioresource Technology*, vol. 91, no. 1, pp. 77–84, 2004.

[22] W. Schmidell, E. Aquarone, W. Borzani et al., *Biotecnologia Industrial*, vol. 2, Editora Edgard Blücher, São Paulo, Brazil, 2001.

[23] M. Raimbault, "General and microbiological aspects of solid substrate fermentation," *Electronic Journal of Biotechnology*, vol. 1, no. 13, pp. 174–188, 1998.

[24] R. V. Muralidhar, R. R. Chirumamila, R. Marchant, and P. Nigam, "A response surface approach for the comparison of lipase production by *Candida cylindracea* using two different carbon sources," *Biochemical Engineering Journal*, vol. 9, no. 1, pp. 17–23, 2001.

[25] Y. Teng and Y. Xu, "Culture condition improvement for whole-cell lipase production in submerged fermentation by *Rhizopus chinensis* using statistical method," *Bioresource Technology*, vol. 99, no. 9, pp. 3900–3907, 2008.

[26] R. Kaushik, S. Saran, J. Isar, and R. K. Saxena, "Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipase production by *Aspergillus carneus*," *Journal of Molecular Catalysis B: Enzymatic*, vol. 40, no. 3-4, pp. 121–126, 2006.

[27] D. Fu, M. Yu, T. Tan, and X. Zhou, "Separation, characterization and catalytic properties of Lip2 isoforms from *Candida* sp. 99–125," *Journal of Molecular Catalysis B: Enzymatic*, vol. 56, no. 2-3, pp. 115–121, 2009.

[28] A. M. Baron, V. M. Z. Turra, D. A. Mitchell et al., "Produção e caracterização de lipases de Penicillium corylophilum IOC 4211," in *Proceedings of 15th National Symposium of Fermentations*, Recife, Brazil, 2005.

[29] D. M. Freire, P. M. Gomes, E. P. S. Bon et al., "Lipase production by a new promissing strain *Penicillium restrictum*," *Revista de Microbiologia*, vol. 28, no. 1, pp. 6–12, 1997.

[30] S. Benjamin and A. Pandey, "Isolation and characterization of three distinct forms of lipases from candida rugosa produced in solid state fermentation," *Brazilian Archives of Biology and Technology*, vol. 44, no. 2, pp. 213–221, 2001.

[31] G. M. Pastore, V. S. R. Costa, and M. G. B. Koblitz, "Purificação parcial e caracterização bioquímica de lipase extracelular produzida por nova linhagem de *Rhizopus* sp," *Food Science and Technology*, vol. 23, no. 2, pp. 135–140, 2003.

[32] J. C. M. Diaz, J. A. Rodríguez, S. Roussos et al., "Lipase from the thermotolerant fungus *Rhizopus homothallicus* is more thermostable when produced using solid state fermentation than liquid fermentation procedures," *Enzyme and Microbial Technology*, vol. 39, no. 5, pp. 1042–1050, 2006.

[33] J. A. Santos, C. O. M. Amaral, T. A. S. Araújo et al., "Produção, extração e caracterização parcial de lipase extracelular de Trichosporon sp pelo sistema de duas fases aquosas PEG-sais fosfatos," in *Proceedings of the National Symposium of Fermentations XV*, Recife, Brazil, 2005.

[34] P. Lotrakul and S. Dharmsthiti, "Purification and characterization of lipase from *Aeromonas sobria* LP004," *Journal of Biotechnology*, vol. 54, no. 2, pp. 113–120, 1997.

[35] J.-J. Shangguan, Y.-Q. Liu, F.-J. Wang et al., "Expression and characterization of a novel lipase from *Aspergillus fumigatus* with high specific activity," *Applied Biochemistry and Biotechnology*, vol. 165, no. 3-4, pp. 949–962, 2011.

[36] V. M. G. Lima, D. A. Mitchell, and N. Krieger, "Caracterização cinética e bioquímica da lipase de *Bacillus megaterium*," in *Proceedings of the 15th National Symposium of Fermentations*, Recife, Brazil, 2005.

[37] P. Atkins and L. Jones, *Princípios de Química: Questionando a Vida Moderna e o Meio Ambiente*, Editora Bookman, Porto Alegre, Brazil, 2001.

[38] O. R. Fennema, *Introduccion a La Ciência de los Alimentos*, Reverte, Barcelona, Spain, 2000.

[39] C. N. A. Razak, A. B. Salleh, R. Musani, M. Y. Samad, and M. Basri, "Some characteristics of lipases from thermophilic fungi isolated from palm oil mill effluent," *Journal of Molecular Catalysis B: Enzymatic*, vol. 3, no. 1–4, pp. 153–159, 1997.

[40] E. H. Ghanem, H. A. Al-Sayed, and K. M. Saleh, "An alkalophilic thermostable lipase produced by a new isolate of *Bacillus alcalophilus*," *World Journal of Microbiology and Biotechnology*, vol. 16, no. 5, pp. 459–464, 2000.

[41] N. Nawani, N. S. Dosanjh, and J. Kaur, "A novel thermostable lipase from a thermophilic *Bacillus* sp.: characterization and esterification studies," *Biotechnology Letters*, vol. 20, no. 10, pp. 997–1000, 1998.

[42] N. Kulkarni and R. V. Gadre, "Production and properties of an alkaline, thermophilic lipase from *Pseudomonas fluorescens* NS2W," *Journal of Industrial Microbiology and Biotechnology*, vol. 28, no. 6, pp. 344–348, 2002.

[43] G. Ginalska, R. Bancerz, and T. Korniłłowicz-Kowalska, "A thermostable lipase produced by a newly isolates *Geotrichum*-like strain, R59," *Journal of Industrial Microbiology and Biotechnology*, vol. 31, no. 4, pp. 177–182, 2004.

[44] C. H. Shu, C. J. Xu, and G. C. Lin, "Purification and partial characterization of a lipase from *Antrodia cinnamomea*," *Process Biochemistry*, vol. 41, no. 3, pp. 734–738, 2006.

[45] N. C. Mhetras, K. B. Bastawde, and D. V. Gokhale, "Purification and characterization of acidic lipase from *Aspergillus niger* NCIM 1207," *Bioresource Technology*, vol. 100, no. 3, pp. 1486–1490, 2009.

[46] T. Iftikhar, M. Niaz, R. Jabeen, and I. U. Haq, "Purification and characterization of extracellular lipases," *Pakistan Journal of Botany*, vol. 43, no. 3, pp. 1541–1545, 2011.

[47] H. Horchani, H. Mosbah, N. B. Salem, Y. Gargouri, and A. Sayari, "Biochemical and molecular characterisation of a thermoactive, alkaline and detergent-stable lipase from a newly isolated *Staphylococcus aureus* strain," *Journal of Molecular Catalysis B: Enzymatic*, vol. 56, no. 4, pp. 237–245, 2009.

[48] K. Chakraborty and R. P. Raj, "An extra-cellular alkaline metallolipase from Bacillus licheniformisMTCC 6824: purification and biochemical characterization," *Food Chemistry*, vol. 109, no. 4, pp. 727–736, 2008.

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Review Article

Proteases of Wood Rot Fungi with Emphasis on the Genus *Pleurotus*

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Proteases are present in all living organisms and they play an important role in physiological conditions. Cell growth and death, blood clotting, and immune defense are all examples of the importance of proteases in maintaining homeostasis. There is growing interest in proteases due to their use for industrial purposes. The search for proteases with specific characteristics is designed to reduce production costs and to find suitable properties for certain industrial sectors, as well as good producing organisms. Ninety percent of commercialized proteases are obtained from microbial sources and proteases from macromycetes have recently gained prominence in the search for new enzymes with specific characteristics. The production of proteases from saprophytic basidiomycetes has led to the identification of various classes of proteases. The genus *Pleurotus* has been extensively studied because of its ligninolytic enzymes. The characteristics of this genus are easy cultivation techniques, high yield, low nutrient requirements, and excellent adaptation. There are few studies in the literature about proteases of *Pleurotus* spp. This review gathers together information about proteases, especially those derived from basidiomycetes, and aims at stimulating further research about fungal proteases because of their physiological importance and their application in various industries such as biotechnology and medicine.

1. Introduction

Enzymes are increasingly required in the commercial and industrial fields. For this reason, there is an intense search for new enzymes with particular properties that are desirable for certain commercial applications [1]. There are a limited number of known enzymes that are used commercially and consequently, the enzymes that are available are not used in large quantities. Approximately 75% of industrial enzymes are hydrolases, and the enzymes which degrade proteins account for 65% of the enzymes that are marketed worldwide [2].

Proteases catalyze hydrolytic reactions, in which protein molecules are degraded into peptides and amino acids. Their properties are very diverse because the group is large and complex [3]. The study of proteases is of note in enzymology because of its biotechnological relevance. Proteases are a special group of enzymes because of their importance in the metabolism of organisms, their biochemical functions in metabolic pathways and cellular signaling, the importance of protease inhibitors, and their use in fine chemicals and the pharmaceutical industry [4].

Most of the proteases used industrially are microbial and especially bacterial origin and these are preferred for their desired characteristics in biotechnology and their lower cost. Proteases which are of plant and animal origin, except for some specific uses, do not meet industrial demand. The industrial production of microbial proteases is favored due to the fact that they have a short generation time, because

of the ease of genetically manipulating microorganisms, and because of the diversity of species available in nature, many of which are still unexplored [2, 3].

Because of their potential therapeutic use, genes from protease bacteria, fungi, and viruses have been cloned and sequenced in order to increase the production of enzymes by recombinant DNA technology, to study the role of enzymes in pathogenicity and to cause changes in the properties of proteases to improve their commercial usage. In industries, proteases contribute to the development of processes and products with high added value. As biological catalysts, they offer advantages in relation to the use of chemical catalysts for numerous reasons, such as high catalytic activity, high specificity, and their availability in economically viable quantities [5]. However, the cost of production of proteases is the greatest barrier to their industrial application. Consequently, researches have been conducted to find low cost proteases useful in commercial and industrial sectors [6].

Bacteria produce the majority of proteases of microbial origin. The genus *Bacillus* produces proteases which are mainly neutral and alkaline [7]. However, the proteases of fungal origin, *Aspergillus* [8] and *Penicillium* [5], as well as being widely studied, appear in greater variety. A species can produce neutral, acidic, or alkaline proteases, as is the case of *Aspergillus oryzae* [7]. Proteases from basidiomycetes have unique properties and deserve further study. Although scientific research regarding the structural and functional characteristics of proteases from basidiomycetes started more than 30 years ago, the diversity and complexity of action of these enzymes has resulted in recent studies of xylotrophic basidiomycetes as a new source of proteases [9].

The *Pleurotus* species are highly appreciated in cooking for their refined flavour and they have also been investigated because they contain bioactive, antitumor, anti-inflammatory, hypocholesterolemic, antiviral, antibiotic, antioxidant, antidiabetic, immunomodulatory, antitumor, antihyperlipidemic, and hepatoprotective compounds, among others [3, 10–12].

The genus *Pleurotus* is also known for its ability to degrade lignin through the production of ligninolytic enzymes, particularly laccase [11, 13]. Several studies have been performed with laccase of *Pleurotus* spp. [14, 15], linking the metabolism of ligninolytic enzymes with the presence of proteases [16]. Besides laccase, the production of numerous hydrolytic enzymes by such organisms has also been reported [17], and interesting studies of the proteases produced by *Pleurotus* spp. have been described, resulting in the need for further research on these properties of this genus. The aim of this review was to gather information on proteolytic enzymes, including their most relevant and current industrial applications, as well as to gather the characteristics of proteases obtained from basidiomycetes, especially from the genus *Pleurotus*.

2. Uses and Applications of Proteases

2.1. In the Detergent Industry. The use of proteases as a detergent dates back to 1914, when the "Burnus" brand of detergent was produced, which contained sodium carbonate and pancreatic extract [7]. Proteases can be separated into two major groups according to their ability to cleave N- or C-terminal peptide bonds (exopeptidases) or internal peptide bonds (endopeptidases), the latter being those which are most important industrially. They are also classified according to their optimum pH for activity (acid, neutral, or alkaline) and substrate specificity (collagenase, elastase, keratinase, etc.). Based on their mechanism of action and the functional groups in the active site, proteases can be classified into four main groups: serine, cysteine, aspartate, and metalloprotease [9].

There are several industries that benefit from the catalytic properties of proteases, such as pharmaceutical, chemical, food processing, detergents, leather processing, and others. Their use in bioremediation processes has also been explored. Their properties, such as substrate specificity, optimum temperature and pH for activity, stability, and catalytic mechanism, differ greatly because this group is quite diverse [9, 18].

The proteases used in detergents need to have stability in wide ranges of pH and at high temperatures, as well as compatibility with oxidizing agents. Interest in proteases that are active in a wide temperature range has been increasing because garments made from synthetic fibers are sensitive to high temperatures [19]. However, although bacterial proteases are commonly used in detergents, the high cost of the cell separation process, that is, obtaining cell-free enzyme preparations, limits their use. In this context, enzymes of fungal origin have advantages because they are mainly extracellular. Furthermore, the use of proteases as a basis for detergents is preferable to conventional synthetic products because they have greater cleaning capacity, improved performance at low wash temperatures, and reduce pollution because they are natural. Thus, there is always a demand for enzymes with improved efficiency that can improve the performance of detergents containing enzymes [7].

2.2. In the Pharmaceutical and Food Industries. Many proteases are related to the processes of infection caused by viruses, bacteria, and fungi, which are central to the interaction with the host cell. Proteolytic reactions are finely regulated and the variety of mechanisms involve high substrate specificity, ATP-directed protein degradation, restricted access to the active site, activation cascade, and selective and highly specific protein modification, as can be seen in the activation of zymogenic forms of enzymes by limited proteolysis [18, 20].

The involvement of proteases in the mechanisms that cause diseases has caused them to become a target for developing therapeutic agents against diseases such as AIDS, cancer, Chagas disease, hepatitis, malaria and candidiasis, as well as inflammatory, immune, respiratory, cardiovascular, and neurodegenerative disorders [7, 18].

Natural inhibitors play a role in the regulation of the proteolytic activity in cells; hence knowledge about the interaction of proteases with their substrates and their specificity is an essential tool for the development of synthetic inhibitors that can be used to control diseases in which proteases are involved [21]. There is an emerging market for enzyme inhibitors in countries like India, China, Japan, South Korea, Taiwan, Canada, Australia, and New Zealand [22]. Studies of

the protein structure of peptidases through X-ray diffraction have made the development of proteolytic inhibitors possible by molecular modeling [21]. The first successful examples of protease inhibitors were the inhibitors of the aspartic protease of HIV-1, which were developed by the modeling technique. The peptidase of HIV cleaves the polyproteins of the virus into structural proteins, which are essential for the production of mature, infectious viral particles [23].

The accumulation of fibrin in the blood can lead to thrombosis, which can cause heart attacks and other cardiovascular diseases [24]. Many products currently used in thrombolytic clinical therapy have undesirable side effects, such as intestinal bleeding in oral treatments, low specificity to fibrin, and relatively high costs [25–27]. Consequently, the growing interest in obtaining fibrinolytic proteases at a reduced cost and with the appropriate medical characteristics has led researchers to intensify their studies and, in recent decades, a number of fibrinolytic enzymes were isolated and characterized. Enzymes with fibrinolytic capacity have been obtained from snake venom, insects, marine animals, algae, fermented products, and microorganisms that are safe for humans and animals (food grade) [28–39].

Recently, the fibrinolytic activity of proteases produced by microorganisms has attracted greater medical and commercial interest. Microorganisms are important sources of thrombolytic agents, though few of them have GRAS status ("generally recognized as safe", i.e., totally safe for humans and animals, and the products obtained from them). Some species of *Bacillus* produce enzymes with thrombolytic activity, such as nattokinase (NK) from *Bacillus natto*, subtilisin DFE, and subtilisin DFE DJ-4 from *Bacillus amyloliquefaciens* [39]. Likewise, *Streptococcus hemolyticus* produces a streptokinase with thrombolytic action [26, 40]. In recent years, the search has intensified for microorganisms producing proteases with fibrinolytic activity and which are "food grade," with the potential for exploiting them as functional additives in food and drugs to prevent or treat thrombosis and other related organic disorders [39].

Other therapeutic agents include proteases that are used in the correction of deficient digestive enzymes. Elastase is used in the treatment of wounds, burns and abscesses [41, 42]. Proteases also play important roles in the production of animal feed, cleaning contact lenses, silver recovery from photographic films and X-ray and in the treatment of domestic and industrial sewage [19, 43].

One of the most important industries in which proteases play an essential role is the food industry. They act as agents for modifying the functional properties of proteins, particularly in the processing of cheese (milk clotting, by the hydrolysis of a specific binding in casein), in obtaining protein hydrolyzates, improving the flavor of some foods and also in baking [19]. Proteases from *Aspergillus oryzae* are used to modify the gluten of wheat flour by facilitating handling and increasing the volume of bread dough. Proteases have been used since ancient times to prepare sauce and other derivatives from soy because this grain has high, good quality protein content. The proteolytic modification of soy proteins helps to improve their functional properties. These enzymes are also used in the synthesis of the artificial sweetener aspartame (through synthesis reactions produced by the thermolysin of *B. thermoprotyolyticus*) and in the maturation (softening) of meat, particularly beef, through the alkaline elastase action of *Bacillus*. The microorganisms most commonly used for the production of proteases in the food industry are from the genus *Bacillus* [7, 18, 26, 40].

The requirements for proteases to act as industrial catalysts vary considerably. The enzymes to be used in the production of detergents and in the food industries need be produced in large quantities and should be efficient without further processing (*in natura*). The proteases already used in the pharmaceutical industries (such as medicines) are produced in small amounts but require extensive purification procedures [18].

3. Proteases of Fungal Origin

The cost of production of proteases is the biggest obstacle to their industrial application. Consequently, the development of new processes to increase the yield of proteases with respect to industrial production, concomitantly with reduced production costs, is highly advantageous from the commercial point of view. Increased productivity has been achieved by selecting hyper-productive strains or by improving the culture media [7]. The global market for industrial enzymes reached about US $4.5 billion in 2012, with a projection of US $7.1 billion for 2018. Research on enzymes has revealed their use in different sectors and their catalytic properties have stimulated their use in industrial production and processes. Market growth has been positively influenced by new products and their advantages over traditional industrial methods [22, 44].

Although the species of microorganisms that are used for industrial production are few in number, 90% of commercialized proteases are obtained from microbial sources. These are preferred to proteases from plants and animals due to their various characteristics, which are more suitable for biotechnological applications, such as activity within a broad range of temperature and pH, thermal stability, and high catalytic activity [18, 41, 45].

Biodiversity is an invaluable resource for biotechnological innovation and it promotes the search for new strains of microorganisms to be used for specific industrial purposes. Because the use of proteases, especially those of the alkaline variety, is expected to rise over the coming decades, the production of microbial proteases represents a good alternative for the development of new methods in order to improve the production of these enzymes, as well as decreasing their price [7, 19]. The increased demand for proteases with specific properties has led biotechnologists to explore new sources of proteases.

Most fungal proteases have neutral to slightly acidic characteristics [19]. Xerophilic fungi often contain proteases of low molecular weight (26 to 50 kDa) [24]. The study of fungal proteases has increased in recent decades, but knowledge about proteases from basidiomycetes is still limited [9]. In 2009, approximately 60% of the enzymes commercialized originated from fungi and only five originate macrofungi (three laccases, one peroxidase, and one phytase) [11].

A few years ago, the proteases produced from micromycetes were predominant in studies regarding the search for new bioactives with economic and medicinal benefits [24]. *Aspergillus* is considered to be the best producer of proteases [8]. In the food industry *A. oryzae* and *A. sojae* are noteworthy for their ability to eliminate bitterness [19]. *Penicillium* and *Rhizopus* are also considered to produce proteases [5, 46] and the proteases from macromycetes recently gained prominence in the search for new enzymes with specific characteristics. Proteases produced from basidiomycetes such as *Agaricus bisporus*, *Armillariella mellea*, *Flammulina velutipes*, *Grifola frondosa*, *Pleurotus ostreatus*, *Pleurotus eryngii*, *Phanerochaete chrysosporium*, *Schizophyllum commune*, and others have been reported [47–50].

There are vast majority of microorganisms that exist in nature have not yet been studied. Thus, the search for new natural molecules with interesting physiological effects, and the need to understand the mechanisms of production and regulation of expression of these bioactives, has resulted in the fact that the cultivation conditions that have already been defined and used successfully for ascomycetes such as *Aspergillus* sp. and *Penicillium* sp. have now been extended to include basidiomycetes in the search for secondary bioactives and metabolites such as enzymes, antibiotics, and organic acids [46].

4. Proteases from Basidiomycetes

Basidiomycetes are fungi important for biological communities because they are excellent at degrading wood. Some genera have been used as food for centuries and they have enormous commercial importance. They are also producers of a group of commonly studied extracellular enzymes (xylanases, cellulases, and ligninolytic enzymes) [51]. Proteases play important roles in the physiology of fungi, acting in processes such as germination and sporulation. These enzymes seem to have a close relationship with the lifestyle of saprophytic fungi, as observed in *Pleurotus citrinopileatus* [43].

It has been found that *P. pulmonarius*, which usually grows on dead timber, secretes subtilisin but does not produce trypsin. *P. ostreatus*, which grows in living hosts, secretes extracellular trypsin throughout its development. The presence of living tissues as hosts may be related to the expression of trypsin-type proteases [24]. Because most of the nitrogen in timber is in the form of proteins, proteases play a very important role in the metabolism of the proteins in the fungi of white rot in wood and it has been observed that depletion of nitrogen in the medium stimulates the secretion of proteases by fungi [3, 24, 52–57].

The mycelial secretion of proteases by saprophytic basidiomycetes has led to the identification of various classes of proteases: subtilases were found in *Pleurotus ostreatus* [58], *Phanerochaete chrysosporium* [59], *Serpula lacrymans* [52], *Schizophyllum commune* [47], and *Coprinus* sp. [60]. Metalloproteinase was reported by Mchenry et al. [61] in *Chondrostereum purpureum* and in *Hypsizygus marmoreus* [62]. The mycelial secretion of aspartate proteases was reported in *P. chrysosporium* [49, 59], *Amanita muscaria* [63], and *Irpex lacteus* [64].

Although they are recognized for their nutritional value and the extraction of bioactive compounds of basidiome and mycelia, mushrooms still possess much unexplored information in relation to some of the enzymes that they produce, such as proteases [6]. Proteases extracted from mushrooms have been purified and characterized [48]. The role of proteases in the regulation of the formation of basidiome in *Hypsizygus marmoreus* was described by Terashita et al. [62] and their regulatory role regarding ligninolytic activity in *P. chrysosporium* and *P. ostreatus*, under nutritional limitation, was highlighted by Dass et al. [59] and Palmieri et al. [16], respectively. *Phanerochaete chrysosporium* has produced an acid protease in solid medium with wood, under ligninolytic conditions. This enzyme showed an isoelectric point that was higher than that of most acid proteases (5.6) and it has been characterized as a glycoprotein aspartate protease [49].

From a selection of 27 strains of basidiomycetes that produce proteases [3], *Lentinula edodes* stood out with the largest halo of proteolytic activity using the method of selection on plates containing casein. In this study, the genus *Pleurotus* ranked second in the production of protease. The authors attributed this proteolytic activity to ability of the fungus to grow on substrates with low nitrogen availability [47]. However, Zorn et al. [65] consider that the existence of nitrogen seems to stimulate the production of proteases by fungi. Media containing soybean, casein, gelatin, corn, and yeast are commonly used to produce protease. Other sources, such as starch, lactose, and barley are also used, but it is known that high concentrations of carbohydrates inhibit the production of enzymes [19]. The purification of a fibrinolytic protease from *Cordyceps militaris* showed characteristics of a 52 kDa subtilisin, which was higher than other fungal proteases. The enzyme rapidly degraded the α and γ chains, but it took longer to degrade the β chains of the fibrin, which was a pattern quite different from the action of proteases derived from snake venom [27]. The fibrinolytic protease activity of basidiomycetes has been recently demonstrated by several authors. Kim et al. [66] purified and characterized a metalloprotease from the mycelium of *Perenniporia fraxinea* with fibrinolytic activity. The cloning, purification, and characterization of proteases from *Pleurotus ostreatus* with similar characteristics were performed by Yin et al. [2], Shen et al. [67], and Joh et al. [68].

Although several studies have performed the purification and characterization of proteases from mycelium, basidiome, or culture filtrate, many aspects of the production of these enzymes have yet to be explored. The process of producing basidiome is laborious and time-consuming; it requires large volumes of substrate, space, and qualified labor and these factors hinder research in the laboratory. Cultivations which are performed in the vegetative phase are more viable for research because they can be kept in the laboratory, performed on a small and medium scale, and important parameters such as temperature, humidity, and agitation can be controlled [18].

Microbial proteases can be produced in various ways and studies have shown that, depending on the culture conditions, different forms of the same protease can be expressed [19].

Figure 1: *Pleurotus pulmonarius*.

Most of the enzymes produced in industry are produced by submerged fermentation [8]. The use of liquid cultures facilitates the purification of bioactives such as enzymes and polysaccharides [3]. The submerged culture of *P. ostreatus* in wheat gluten resulted in the secretion of proteases that noticeably increased the overall solubility of the medium [13].

Submerged media with complex sources provide higher yields of protease compared to simple media, such as casein or gelatin [19]. However, using submerged culture requires greater resources, specific strains, and very controlled conditions, which does not occur in solid state fermentation, which therefore offers advantages in terms of environmental and economic aspects [8]. The solid cultivation of mushrooms and mycelium in order to obtain bioactives and enzymes remains a very viable alternative; waste from agriculture, forestry, or municipal waste are used for the production of enzymes of industrial interest. The combination of different solid substrates sometimes appears to increase the production of protease by fungi [19].

Due to the similarity of the natural habitat of basidiomycetes, these organisms have excelled in the production of enzymes in solid cultures. Solid state fermentation in tomato pulp yielded good colonization and protease production on a large scale using *P. ostreatus* [8]. Furthermore, proteases have been obtained by the solid state fermentation of soybean and wheat bran fibers [69]. The literature includes standardized techniques for the high yield recovery of proteases, as well as immobilization methods and different protocols for proteolytic assays and the purification of proteases [19].

5. Proteases from *Pleurotus* spp.

The genus *Pleurotus* is the second main group of cultivated edible mushrooms in the world, comprising more than 40 species [51]. In descending order of worldwide production, the seven most produced edible mushrooms are *Agaricus bisporus*, *Pleurotus* spp., *Lentinula edodes*, *Auricularia* spp., *Volvariella volvacea*, *Flammulina velutipes*, and *Tremella fuciformis* [11]. However, species of the genus *Pleurotus* present advantages when compared with others mushrooms. For example, they can be cultivated in different substrates and temperatures. They are also rich in essential amino acids and vitamins [70]. *Pleurotus* can be grown artificially without major problems and it grows in a disorderly manner in tropical and subtropical regions [51]. This genus is a part of lignolytic organisms and several studies have reported the lignolytic capacity of its species [65]. Several bioactive compounds have been extracted from crude extracts, mycelia, and basidiome of *Pleurotus* spp. for study, such as polysaccharides, hemicelluloses, peptides, glycoproteins, lipids, hydrolytic enzymes, and others [12].

Extracts of the basidiome and mycelia of *Pleurotus* spp. have been used as medicines and as nutritional supplements for human health. Several studies have reported its nutritional, immunomodulatory, antioxidant, antitumor, and hypoglycemic properties, among others. *P. ostreatus* has been effective in alleviating the effects of hepatotoxicity in rats and it protects the liver, heart, and brain against oxidative stress [51].

In recent years, examples of the main genera of cultured basidiomycetes have been studied for their positive therapeutic effects. Hepatoprotective effect was observed for *P. pulmonarius*, *P. ferulae*, and *P. tuber-regium*, which were also active against human cancer cells [71]. Moreover, species of the genus have been used in the processes of bioremediation, delignification, and disinfection of effluent [17].

Different strains of *Pleurotus* spp. (Figure 1) exhibit specific behaviors, which vary depending on the conditions where they are cultivated, including environmental factors such as types of substrates and supplementation. A study of three strains of *P. eryngii* using sawdust and rice straw as a substrate for cultivation showed significant differences between the strains regarding growth rates, number of days for the first harvest, biological efficiency, and other parameters [72]. *P. eryngii* is considered as one of the best species of the genus due to its consistency and because it has a longer lifetime than all the other species of *Pleurotus*. While most of the fungi in the Agaricales order show steady growth in tree trunks, *P. eryngii* grows well in subtropical pastures and grows excellently during cold periods [72].

The artificial cultivation of *P. eryngii* on farms using automatic devices and sawdust has been performed successfully in Korea. The species has also been effective in lowering the levels of blood glucose, the inhibition of tumor cells, and

antioxidant activity [73]. *P. citrinopileatus* contains polysaccharides that have antihyperglycemic and antitumor effects [43]. Medicinal properties have also been observed in *P. tuber-regium*, which is also edible and grows well in tropical and subtropical regions. Several of its bioactive substances have been identified, such as glycoproteins, polysaccharides, and phytochemicals with pharmacological action [74].

Basidiome of *P. pulmonarius* has shown antitumor, antioxidant, and anti-inflammatory properties, suggesting the therapeutic effects of its metabolites in the treatment against some diseases, such as cancer [75]. *P. sajor-caju* and *P. ostreatus* have also been investigated for their antioxidant capacity and both species share a similar amino acid profile. However, despite similarities with the properties of *Pleurotus* spp., there are many studies of *P. ostreatus* at the expense of other species of the genus [51]. As stated, there is still little knowledge about the proteases derived from mushrooms, mainly the *Pleurotus* genus [11].

Studies of the *P. ostreatus*, *P. eryngii*, *P. citrinopileatus*, and *P. chrysosporium* species have showed that the *Pleurotus* genus is a producer of proteases that seem to participate in the complex ligninolytic mechanism, degrading the laccase enzyme at certain stages of fungal growth [16, 43, 50, 59, 73].

P. pulmonarius has important antimicrobial, anti-inflammatory, antioxidant, and antitumor properties; however, there is still little material in the literature regarding its production of proteases and their characterization. Nevertheless, it is known that the proteases secreted by this species do not appear to participate in the regulation of peroxidases, as has been reported for *P. ostreatus* proteases [16, 24, 75, 76]. In addition, no degradation of ligninolytic enzymes was observed when they came into contact with proteases from *Phanerochaete chrysosporium* [49].

In a comparison of six species of basidiomycetes, *P. eryngii* excelled in the production of protease. Pleurerin, the protease extracted from fruiting bodies of *P. eryngii* with anti-HIV-1 action, has presented the characteristics of an aspartic protease due to its N-terminal sequence, which is different from other aspartic fungal proteases [50]. Most proteases of the genus *Pleurotus* feature the characteristics of alkaline subtilases. There are six families of serine proteases, which are based on their amino acid sequence [58]. A study of proteases from 43 species of basidiomycetes showed a predominance of serine protease [9]. An alkaline protease was found in the basidiome of *P. citrinopileatus* and it showed a similarity in amino acid sequence with *Agaricus bisporus*, *Epichloë typhina*, and *Penicillium oxalicum* fungi [43]. Aspartic proteases are divided into 16 groups and are rarer in *Pleurotus* [2].

The fibrinolytic proteases of *Pleurotus* spp. have received attention in recent years because those searching for new proteases with fibrinolytic capacity are interested in nontoxic and edible fungi [77]. A monomeric protease with fibrinolytic activity was purified 29.3-fold from the basidiome of *P. eryngii* produced in corn cob. The protease in question showed a high capacity for degrading fibrin and demonstrated a possible application as a thrombolytic agent. The hydrolysis of α and β chains of fibrinogen occurred in less than 10 min. The enzyme showed characteristics of a serine protease similar to subtilisin, as has been reported for most proteases from the genus [73].

In a study by Liu et al. [77] the fibrinolytic and fibrinogenolytic enzyme from *P. pulmonarius* grown in submerged state were efficient in degrading the α (3 min) and β (45 min) chains of fibrinogen, followed by γ after 10 h incubation. The enzyme was purified 147-fold and presented good stability at human body temperature, which enables it to be used as an alternative in thrombolytic treatments, including oral applications, because it is an edible fungus. Apart from fibrin degradation, the enzyme was also able to act as a plasminogen activator, which is not common in the literature. A fibrinolytic metalloproteinase purified from mycelia of *P. ostreatus* showed a high similarity with the fibrinolytic proteases from the basidiome of the same fungus, which suggests the need for studies related to therapeutic treatments for thrombosis from the mycelium, which can be obtained more quickly and easily than the fruiting bodies [67].

Hemolytic proteases have been reported less frequently in the genus *Pleurotus*. The hemolysin of the basidiome of *P. nebrodensis* showed apoptosis-inducing activity and antiproliferative cancer cells and also anti-HIV-1 activity, interfering in some way in the permeability of the cell membranes and preventing virus infection [78]. Activity against cancer cells has also been verified for proteases of *P. ostreatus*. Hemolysin extracted from *P. eryngii* was effective against leukemia cells [79] and showed antimicrobial effect for *Bacillus* sp. [80]. However, these enzymes are not stable at temperatures higher than 40°C, which hinders their possible application as a medicament in the form of food because they would be made inactive by cooking or when passing through the intestinal tract [80, 81].

Keratinases are a class of proteases that have received attention in the past. They consist of proteases that are capable of degrading substrates that are rich in insoluble keratins, such as wool, hair, and nail. Because of this, keratinases are used in environmental and technological processes [82]. However, regarding proteases in general, there is little existing research on keratinases of basidiomycetes and there are few reports about keratinases produced by the genus *Pleurotus* spp.

The secretome of *P. sapidus* has shown protease production and ligninolytic enzymes, but some points remained unidentified, indicating that new enzymes with potential biotechnological applications should be studied and identified in the species [65]. The secretome of *P. ostreatus* has shown the presence of proteases with a potential role in the regulation of other extracellular enzymes [13]. Metalloproteases are enzymes that are finely associated with physiological processes and they have been explored in studies of bacteria and mammals; however, there have been very few studies of the metalloproteases of basidiomycetes [68]. *Pleurotus* spp. has been greatly cultivated for research related to medicine and also for the consumption of its fruiting bodies, which have agreeable flavors [24].

Although the process of the emergence of the fruiting body of basidiomycetes is still not fully known, the expression of a metalloprotease in the early stages of the formation of a basidiome of *P. ostreatus* has been verified, although

Table 1: Characteristics of proteases *Pleurotus* spp.

Species	Molecular weight (kDa)	Optimum pH	Optimum temperature	Kind of protease	References
P. ostreatus	43				[2]
P. ostreatus var. *florida*	38.7	7.5	37°C	Serine proteinase	[13]
P. citrinopileatus	28	10	50°C	Serine proteinase	[43]
P. eryngii	11.5	5	45°C	Aspartic protease	[50]
P. ostreatus	32	6.5	35°C	Metalloprotease	[67]
P. eryngii	14	5	30–40°C	Serine proteinase	[73]
P. ostreatus	18.2	7.4	40°C	Metalloprotease	[77]
P. nebrodensis	27				[78]
P. eryngii	17		37°C		[80]
P. ostreatus				Serine proteinase/metalloprotease	[83]
P. ostreatus	22	6.7			[84]
P. ostreatus	75	7.8		Serine protease	[85]
P. ostreatus	30/19/42.5	7.4/5.6		Serine protease/metalloprotease	[86]
P. sajor-caju	14.5/86			Metalloprotease	[87]
P. ostreatus	97/48.5	5.5–6.5		Cysteine protease	[88]

the enzyme was not expressed in the mycelial stage or in the formation of spores [68]. The increase of proteases in mature hyphae has also been noted in mycelia of *P. pulmonarius*, linking the time of the formation of the basidiome with the presence of such enzymes [70]. Furthermore, the addition of inhibitors of metalloproteinases has prevented the normal process of the formation of fruiting bodies of *Hypsizygus marmoreus* [62]. Vanillic acid has been reported as a good inducer of proteases of *Pleurotus ostreatus* [58]. In just four days of cultivation in solid state fermentation in residues in tomato, *P. ostreatus* produced high levels of protease, surpassing fungi which are considered to be the best producers of this enzyme, such as *Aspergillus* [8].

The most studied species of the genus is *P. ostreatus*. Proteases of *P. ostreatus* with the capacity to coagulate of milk have been purified [83] and despite the classical techniques of production of proteases, recombinant enzymes have been produced in order to find better yields and new specialties [2, 19, 67, 68].

Recent studies of DNA sequence and proteins have come together with the aim of studying enzymatic structures and mechanisms. As already mentioned, due to the vast diversity of proteases, further knowledge of molecular structures in 3D, active sites and mechanisms of catalysis, and enzyme inhibition are increasingly necessary [18]. Based on the N-terminal sequences of proteases of *P. ostreatus*, a primer was developed in order to clone and amplify a DNA sequence that showed homologous regions with a hypothetical protease of *Neurospora crassa* and another of *Phanerochaete chrysosporium*—the first basidiomycete with a completely sequenced genome [58].

A recent study compared the genomes of 33 basidiomycetes and resulted in the idea that the division of fungi into white rot fungi and brown rot fungi in wood cannot be sustained because of the existence of DNA sequences shared between the two groups of fungi that attributes complexity to the mechanisms of degradation of cellulose, hemicellulose, and lignin by basidiomycetes [89]. The three-dimensional

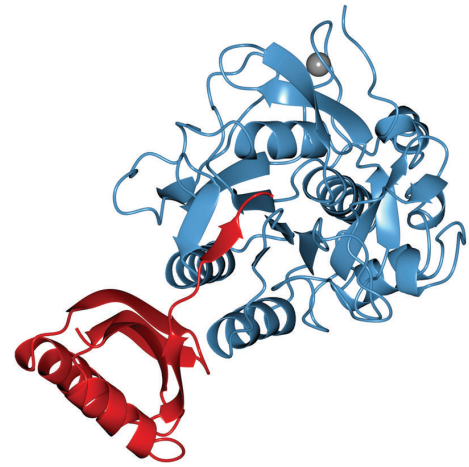

Figure 2: Ribbon model of subtilisin BPN (blue) from *Bacillus amyloliquefaciens* in complex with serine protease inhibitor POIA1 (red) and calcium ion (grey sphere). Figure from pdbid: 1V5I.

structures of proteases and their inhibitors provide rich information about the mechanisms involved in catalysis, and they suggest processes for enzyme inhibition that are still unknown. Using X-ray diffraction, the three-dimensional structure of a serine protease inhibitor of *P. ostreatus* complexed with subtilisin is shown in Figure 2 [84]. Table 1 shows some characteristics of proteases *Pleurotus* spp.

6. Conclusions

There is still much progress to be made in the study of proteases of *Pleurotus* spp. and there is still much to be discovered regarding the genome, proteome, and metabolome of the genus. Several proteases of *Pleurotus* spp. have shown unique characteristics, which require further research. *Pleurotus ostreatus* is one of the few edible mushrooms produced on

an industrial scale. Most of the research to be found in the literature concerns artificially cultivated basidiomycetes. It is known that there is a high demand in industry for proteolytic enzymes with appropriate specificity and stability to temperature, pH, metal ions, and so forth. However, it is common to find that studies of proteases of basidiomycetes recommend that further, more detailed, studies are required to reveal the mechanisms and physiological effects of proteases. Thus, studies of new proteases of the genus *Pleurotus*, especially wild species, are an area of biotechnology that needs to be explored.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] D. J. Daroit, A. P. F. Corrêa, and A. Brandelli, "Production of keratinolytic proteases through bioconversion of feather meal by the Amazonian bacterium *Bacillus* sp. P45," *International Biodeterioration and Biodegradation*, vol. 65, no. 1, pp. 45–51, 2011.

[2] C. Yin, L. Zheng, L. Chen, Q. Tan, X. Shang, and A. Ma, "Cloning, expression, and characterization of a milk-clotting aspartic protease gene (Po-Asp) from *Pleurotus ostreatus*," *Applied Biochemistry and Biotechnology*, vol. 172, no. 4, pp. 2119–2131, 2014.

[3] C. Campos, D. C. Dias, M. P. dos Santos et al., "Seleção de basidiomicetos proteolíticos," *Arquivos de Ciências Veterinárias e Zoologia da UNIPAR*, vol. 14, no. 1, pp. 45–49, 2011.

[4] O. A. Kudryavtseva, Y. E. Dunaevsky, O. V. Kamzolkina, and M. A. Belozersky, "Fungal proteolytic enzymes: Features of the extracellular proteases of xylotrophic basidiomycetes," *Microbiology*, vol. 77, no. 6, pp. 643–653, 2008.

[5] A. M. Shaba and J. Baba, "Screening of *Pleurotus ostreatus* and *Gleophylum sepiarium* strains for extracellular protease enzyme production," *Bayero Journal of Pure and Applied Sciences*, vol. 5, no. 1, pp. 187–190, 2012.

[6] M. Nakamura, A. Iketani, and Y. Shioi, "A survey of proteases in edible mushrooms with synthetic peptides as substrates," *Mycoscience*, vol. 52, no. 4, pp. 234–241, 2011.

[7] D. Said and R. C. L. R. Pietro, *Enzimas como agentes biotecnológicos*, Legis Summa, Ribeirão Preto, Brazil, 2004.

[8] D. Iandolo, A. Piscitelli, G. Sannia, and V. Faraco, "Enzyme production by solid substrate fermentation of *Pleurotus ostreatus* and *Trametes versicolor* on tomato pomace," *Applied Biochemistry and Biotechnology*, vol. 163, no. 1, pp. 40–51, 2011.

[9] J. Sabotič, T. Trček, T. Popovič, and J. Brzin, "Basidiomycetes harbour a hidden treasure of proteolytic diversity," *Journal of Biotechnology*, vol. 128, no. 2, pp. 297–307, 2007.

[10] N. A. Abd Wahab, N. Abdullah, and N. Aminudin, "Characterisation of potential antidiabetic-related proteins from *Pleurotus pulmonarius* (Fr.) Quél. (grey oyster mushroom) by MALDI-TOF/TOF mass spectrometry," *BioMed Research International*, vol. 2014, Article ID 131607, 9 pages, 2014.

[11] J. Erjavec, J. Kos, M. Ravnikar, T. Dreo, and J. Sabotič, "Proteins of higher fungi—from forest to application," *Trends in Biotechnology*, vol. 30, no. 5, pp. 259–273, 2012.

[12] Y. Patel, R. Naraian, and V. K. Singh, "Medicinal properties of pleurotus species (Oyster Mushroom): a review," *World Journal of Fungal and Plant Biology*, vol. 3, no. 1, pp. 1–12, 2012.

[13] N. Eisele, D. Linke, M. Nimtz, and R. G. Berger, "Heterologous expression, refolding and characterization of a salt activated subtilase from *Pleurotus ostreatus*," *Process Biochemistry*, vol. 46, no. 9, pp. 1840–1846, 2011.

[14] P. Giardina, G. Cennamo, C. Greco, G. Palmieri, and G. Sannia, "Protease mediated processing of a Cu-induced laccase in *Pleurotus ostreatus*: a natural approach to improve protein stability," *Progress in Biotechnology*, vol. 21, pp. 113–120, 2002.

[15] M. Téllez-Téllez, F. J. Fernández, A. M. Montiel-González, C. Sánchez, and G. Díaz-Godínez, "Growth and laccase production by *Pleurotus ostreatus* in submerged and solid-state fermentation," *Applied Microbiology and Biotechnology*, vol. 81, no. 4, pp. 675–679, 2008.

[16] G. Palmieri, P. Giardina, C. Bianco, B. Fontanella, and G. Sannia, "Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*," *Applied and Environmental Microbiology*, vol. 66, no. 3, pp. 920–924, 2000.

[17] M. Téllez-Téllez, R. Díaz, C. Sánchez, and G. Díaz-Godínez, "Hydrolytic enzymes produced by *Pleurotus* species," *African Journal of Microbiology Research*, vol. 7, no. 4, pp. 276–281, 2013.

[18] M. B. Rao, A. M. Tanksale, M. S. Ghatge, and V. V. Deshpande, "Molecular and biotechnological aspects of microbial proteases," *Microbiology and Molecular Biology Reviews*, vol. 62, no. 3, pp. 597–635, 1998.

[19] A. Sumantha, C. Larroche, and A. Pandey, "Microbiology and industrial biotechnology of food-grade proteases: a perspective," *Food Technology and Biotechnology*, vol. 44, no. 2, pp. 211–220, 2006.

[20] L. Vandeputte-Rutten and P. Gros, "Novel proteases: common themes and surprising features," *Current Opinion in Structural Biology*, vol. 12, no. 6, pp. 704–708, 2002.

[21] "Enzimas proteolíticas: aplicações biotecnológicas," in *Enzimas em Biotecnologia: Produção, Aplicações e Mercado*, E. P. S. Bom, M. A. Ferrara, M. L. Corvo et al., Eds., Interciência, Rio de Janeiro, Brazil, 2008.

[22] Global Markets for Enzymes in Industrial Applications, 2014, http://www.companiesandmarkets.com/Market/Healthcare-and-Medical/Market-Research/Global-Markets-for-Enzymes-in-Industrial-Applications/RPT1072821.

[23] C. Dash, A. Kulkarni, B. Dunn, and M. Rao, "Aspartic peptidase inhibitors: implications in drug development," *Critical Reviews in Biochemistry and Molecular Biology*, vol. 38, no. 2, pp. 89–119, 2003.

[24] O. A. Kudryavtseva, Y. E. Dunaevsky, O. V. Kamzolkina, and M. A. Belozersky, "Fungal proteolytic enzymes: features of the extracellular proteases of xylotrophic basidiomycetes," *Microbiology*, vol. 77, no. 6, pp. 643–653, 2008.

[25] S.-E. Park, M.-H. Li, J.-S. Kim et al., "Purification and characterization of a fibrinolytic protease from a culture supernatant of *Flammulina velutipes* mycelia," *Bioscience, Biotechnology and Biochemistry*, vol. 71, no. 9, pp. 2214–2222, 2007.

[26] Y. Peng, X. Yang, and Y. Zhang, "Microbial fibrinolytic enzymes: an overview of source, production, properties, and thrombolytic activity in vivo," *Applied Microbiology and Biotechnology*, vol. 69, no. 2, pp. 126–132, 2005.

[27] J.-S. Kim, K. Sapkota, S.-E. Park et al., "A fibrinolytic enzyme from the medicinal mushroom *Cordyceps militaris*," *The Journal of Microbiology*, vol. 44, no. 6, pp. 622–631, 2006.

[28] H. Mihara, H. Sumi, T. Yoneta et al., "A novel fibrinolytic enzyme extracted from the earthworm, *Lumbricus rubellus*," *Japanese Journal of Physiology*, vol. 41, no. 3, pp. 461–472, 1991.

[29] H. Wang and T. B. Ng, "An antifungal protein from ginger rhizomes," *Biochemical and Biophysical Research Communications*, vol. 336, no. 1, pp. 100–104, 2005.

[30] Y.-H. Jia, Y. Jin, Q.-M. Lü, D.-S. Li, W.-Y. Wang, and Y.-L. Xiong, "Jerdonase, a novel serine protease with kinin-releasing and fibrinogenolytic activity from *Trimeresurus jerdonii* venom," *Acta Biochimica et Biophysica Sinica*, vol. 35, no. 8, pp. 689–694, 2003.

[31] S. G. de-Simone, C. Correa-Netto, O. A. C. Antunes, R. B. de-Alencastro, and F. P. Silva Jr., "Biochemical and molecular modeling analysis of the ability of two p-aminobenzamidine-based sorbents to selectively purify serine proteases (fibrinogenases) from snake venoms," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 822, no. 1-2, pp. 1–9, 2005.

[32] M. Y. Ahn, B.-S. Hahn, K. S. Ryu, J. W. Kim, I. Kim, and Y. S. Kim, "Purification and characterization of a serine protease with fibrinolytic activity from the dung beetles, *Catharsius molossus*," *Thrombosis Research*, vol. 112, no. 5-6, pp. 339–347, 2003.

[33] Y. K. Jeong, J. U. Park, H. Baek et al., "Purification and biochemical characterization of a fibrinolytic enzyme from *Bacillus subtilis* BK-17," *World Journal of Microbiology and Biotechnology*, vol. 17, no. 1, pp. 89–92, 2001.

[34] A. H. K. Wong and Y. Mine, "Novel fibrinolytic enzyme in fermented shrimp paste, a traditional Asian fermented seasoning," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 4, pp. 980–986, 2004.

[35] H. Sumi, N. Nakajima, and H. Mihara, "fibrinolysis relating substances in marine creatures," *Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology*, vol. 102, no. 1, pp. 163–167, 1992.

[36] H.-S. Choi and Y.-S. Sa, "Fibrinolytic and antithrombotic protease from *Ganoderma lucidum*," *Mycologia*, vol. 92, no. 3, pp. 545–552, 2000.

[37] H. S. Choi and Y. S. Sa, "Fibrinolytic and antithrombotic protease from *Spirodela polyrhiza*," *Bioscience, Biotechnology and Biochemistry*, vol. 65, no. 4, pp. 781–786, 2001.

[38] H. Sumi, H. Hamada, H. Tsushima, H. Mihara, and H. Muraki, "A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet," *Experientia*, vol. 43, no. 10, pp. 1110–1111, 1987.

[39] H. Sumi, Y. Yanagisawa, C. Yatagai, and J. Saito, "Natto bacillus as an oral fibrinolytic agent: Nattokinase activity and the ingestion effect of *Bacillus subtilis natto*," *Food Science and Technology Research*, vol. 10, no. 1, pp. 17–20, 2004.

[40] D. Collen and H. R. Lijnen, "Staphylokinase, a fibrin-specific plasminogen activator with therapeutic potential?" *Blood*, vol. 84, no. 3, pp. 680–686, 1994.

[41] C. R. Félix, E. F. Noronha, and J. L. Marco, "Proteases: características e aplicações industriais," in *Enzimas Como Agentes Biotecnológicos*, chapter 18, pp. 327–339, 2004.

[42] R. J. FitzGerald and G. O'Cuinn, "Enzymatic debittering of food protein hydrolysates," *Biotechnology Advances*, vol. 24, no. 2, pp. 234–237, 2006.

[43] L. Cui, Q. H. Liu, H. X. Wang, and T. B. Ng, "An alkaline protease from fresh fruiting bodies of the edible mushroom P*leurotus citrinopileatus*," *Applied Microbiology and Biotechnology*, vol. 75, no. 1, pp. 81–85, 2007.

[44] V. N. Jisha, R. B. Smitha, S. Pradeep et al., "Versatility of microbial proteases," *Advances in Enzyme Research*, vol. 1, no. 3, pp. 39–51, 2013.

[45] S. Savitha, S. Sadhasivam, K. Swaminathan, and F. H. Lin, "Fungal protease: production, purification and compatibility with laundry detergents and their wash performance," *Journal of the Taiwan Institute of Chemical Engineers*, vol. 42, no. 2, pp. 298–304, 2011.

[46] M. Papagianni, "Fungal morphology and metabolite production in submerged mycelial processes," *Biotechnology Advances*, vol. 22, no. 3, pp. 189–259, 2004.

[47] J. M. Johnston, E. R. Ramos, R. E. Bilbrey, A. C. Gathman, and W. W. Lilly, "Characterization of ScPrI, a small serine protease, from mycelia of *Schizophyllum commune*," *Mycological Research*, vol. 104, no. 6, pp. 726–731, 2000.

[48] V. Healy, J. O'Connell, T. V. McCarthy, and S. Doonan, "The lysine-specific proteinase from *Armillaria mellea* is a member of a novel class of metalloendopeptidases located in basidiomycetes," *Biochemical and Biophysical Research Communications*, vol. 262, no. 1, pp. 60–63, 1999.

[49] A. Datta, "Purification and characterization of a novel protease from solid substrate cultures of *Phanerochaete chrysosporium*," *The Journal of Biological Chemistry*, vol. 267, no. 2, pp. 728–732, 1992.

[50] H. Wang and T. B. Ng, "Pleureryn, a novel protease from fresh fruiting bodies of the edible mushroom *Pleurotus eryngii*," *Biochemical and Biophysical Research Communications*, vol. 289, no. 3, pp. 750–755, 2001.

[51] P. Chirinang and K.-O. Intarapichet, "Amino acids and antioxidant properties of the oyster mushrooms, *Pleurotus ostreatus* and *Pleurotus sajor-caju*," *ScienceAsia*, vol. 35, no. 4, pp. 326–331, 2009.

[52] S. C. Watkinson, K. S. Burton, and D. A. Wood, "Characteristics of intracellular peptidase and proteinase activities from the mycelium of a cord-forming wood decay fungus, *Serpula lacrymans*," *Mycological Research*, vol. 105, no. 6, pp. 698–704, 2001.

[53] E. V. Crisan and A. Sands, "A nutritional value," in *The Biology and Cultivation of Edible Mushroom*, S. T. Chang and W. A. Hayes, Eds., Academic Press, New York, NY, USA, 1978.

[54] S. I. Kurasawa, T. Sugahara, and J. Hayashi, "Studies of dietary fibre of mushrooms and edible wild plants," *Nutrition Reports International*, vol. 26, no. 2, pp. 167–173, 1982.

[55] P. Mattila, K. Suonpää, and V. Piironen, "Functional properties of edible mushrooms," *Nutrition*, vol. 16, no. 7-8, pp. 694–696, 2000.

[56] S. P. Wasser and A. L. Weis, "Therapeutic effects of substances occurring in higher *Basidiomycetes mushrooms*: a modern perspective," *Critical Reviews in Immunology*, vol. 19, no. 1, pp. 65–96, 1999.

[57] P. Bobek, V. Nosálová, and S. Cerná, "Effect of pleuran (beta-glucan from *Pleurotus ostreatus*) in diet or drinking fluid on colit rats," *Food/Nahrung*, vol. 45, no. 5, pp. 360–363.

[58] V. Faraco, G. Palmieri, G. Festa, M. Monti, G. Sannia, and P. Giardina, "A new subfamily of fungal subtilases: structural and functional analysis of a *Pleurotus ostreatus* member," *Microbiology*, vol. 151, no. 2, pp. 457–466, 2005.

[59] S. B. Dass, C. G. Dosoretz, C. A. Reddy, and H. E. Grethlein, "Extracellular proteases produced by the wood-degrading fungus *Phanerochaete chrysosporium* under ligninolytic and non-ligninolytic conditions," *Archives of Microbiology*, vol. 163, no. 4, pp. 254–258, 1995.

[60] K. A. Shaginyan, I. A. Alekhina, and N. P. Denisova, "Serine proteinase from the higher basidiomycete *Genus coprinus*," *Biokhimiia*, vol. 55, no. 8, pp. 1387–1395, 1990.

[61] J. Z. Mchenry, J. T. Christeller, E. A. Slade, and W. A. Laing, "The major extracellular proteinases of the silverleaf fungus, *Chondrostereum purpureum*, are metalloproteinases," *Plant Pathology*, vol. 45, no. 3, pp. 552–563, 1996.

[62] T. Terashita, Y. Nakaie, T. Inoue, K. Yoshikawa, and J. Shishiyama, "Role of metal proteinases in the fruit-body formation of *Hypsizygus marmoreus*," *Journal of Wood Science*, vol. 44, no. 5, pp. 379–384, 1998.

[63] U. Nehls, A. Bock, W. Einig, and R. Hampp, "Excretion of two proteases by the ectomycorrhizal fungus *Amanita muscaria*," *Plant, Cell and Environment*, vol. 24, no. 7, pp. 741–747, 2001.

[64] Z. Fujimoto, Y. Fujii, S. Kaneko, H. Kobayashi, and H. Mizuno, "Crystal structure of aspartic proteinase from *Irpex lacteus* in complex with inhibitor pepstatin," *Journal of Molecular Biology*, vol. 341, no. 5, pp. 1227–1235, 2004.

[65] H. Zorn, T. Peters, M. Nimtz, and R. G. Berger, "The secretome of *Pleurotus sapidus*," *Proteomics*, vol. 5, no. 18, pp. 4832–4838, 2005.

[66] J.-S. Kim, J.-E. Kim, B.-S. Choi et al., "Purification and characterization of fibrinolytic metalloprotease from *Perenniporia fraxinea* mycelia," *Mycological Research*, vol. 112, no. 8, pp. 990–998, 2008.

[67] M.-H. Shen, J.-S. Kim, K. Sapkota et al., "Purification, characterization, and cloning of fibrinolytic metalloprotease from *Pleurotus ostreatus* mycelia," *Journal of Microbiology and Biotechnology*, vol. 17, no. 5, pp. 1271–1283, 2007.

[68] J.-H. Joh, B.-G. Kim, W.-S. Kong et al., "Cloning and developmental expression of a metzincin family metalloprotease cDNA from oyster mushroom *Pleurotus ostreatus*," *FEMS Microbiology Letters*, vol. 239, no. 1, pp. 57–62, 2004.

[69] L. Thomas, C. Larroche, and A. Pandey, "Current developments in solid-state fermentation," *Biochemical Engineering Journal*, vol. 81, pp. 146–161, 2013.

[70] J. E. S. Vázquez and D. Royse, *La biología y el cultivo de Pleurotus spp.*, Uteha, San Cristóbal de las Casas, México, 2001.

[71] P. Roupas, J. Keogh, M. Noakes, C. Margetts, and P. Taylor, "The role of edible mushrooms in health: evaluation of the evidence," *Journal of Functional Foods*, vol. 4, no. 4, pp. 687–709, 2012.

[72] M. Moonmoon, M. N. Uddin, S. Ahmed, N. J. Shelly, and M. A. Khan, "Cultivation of different strains of king oyster mushroom (*Pleurotus eryngii*) on saw dust and rice straw in Bangladesh," *Saudi Journal of Biological Sciences*, vol. 17, no. 4, pp. 341–345, 2010.

[73] W.-S. Cha, S.-S. Park, S.-J. Kim, and D. Choi, "Biochemical and enzymatic properties of a fibrinolytic enzyme from *Pleurotus eryngii* cultivated under solid-state conditions using corn cob," *Bioresource Technology*, vol. 101, no. 16, pp. 6475–6481, 2010.

[74] B. Huang, W. Lin, P. C. K. Cheung, and J. Wu, "Differential proteomic analysis of temperature-induced autolysis in mycelium of *Pleurotus tuber-regium*," *Current Microbiology*, vol. 62, no. 4, pp. 1160–1167, 2011.

[75] N. Jose, T. A. Ajith, and K. K. Janardhanan, "Antioxidant, anti-inflammatory, and antitumor activities of culinary-medicinal mushroom *Pleurotus pufmonanus* (Fr.) Quel. (Agaricomycetideae)," *International Journal of Medicinal Mushrooms*, vol. 4, no. 4, pp. 59–66, 2002.

[76] C. Ramesh and M. G. Pattar, "Antimicrobial properties, antioxidant activity and bioactive compounds from six wild edible mushrooms of western ghats of Karnataka, India," *Pharmacognosy Research*, vol. 2, no. 2, pp. 107–112, 2010.

[77] X.-L. Liu, X.-Q. Zheng, P.-Z. Qian et al., "Purification and characterization of a novel fibrinolytic enzyme from culture supernatant of *Pleurotus ostreatus*," *Journal of Microbiology and Biotechnology*, vol. 24, no. 2, pp. 245–253, 2014.

[78] H. Lv, Y. Kong, Q. Yao et al., "Nebrodeolysin, a novel hemolytic protein from mushroom *Pleurotus nebrodensis* with apoptosis-inducing and anti-HIV-1 effects," *Phytomedicine*, vol. 16, no. 2-3, pp. 198–205, 2009.

[79] S. Berne, I. Križaj, F. Pohleven, T. Turk, P. Maček, and K. Sepčić, "*Pleurotus* and *Agrocybe* hemolysins, new proteins hypothetically involved in fungal fruiting," *Biochimica et Biophysica Acta*, vol. 1570, no. 3, pp. 153–159, 2002.

[80] P. H. K. Ngai and T. B. Ng, "A hemolysin from the mushroom *Pleurotus eryngii*," *Applied Microbiology and Biotechnology*, vol. 72, no. 6, pp. 1185–1191, 2006.

[81] T. Shibata, M. Kudou, Y. Hoshi, A. Kudo, N. Nanashima, and K. Miyairi, "Isolation and characterization of a novel two-component hemolysin, erylysin A and B, from an edible mushroom, *Pleurotus eryngii*," *Toxicon*, vol. 56, no. 8, pp. 1436–1442, 2010.

[82] R. Gupta, R. Sharma, and Q. K. Beg, "Revisiting microbial keratinases: next generation proteases for sustainable biotechnology," *Critical Reviews in Biotechnology*, vol. 33, no. 2, pp. 216–228, 2013.

[83] G. V. Lebedeva and M. T. Proskuryakov, "Purification and characterization of milk-clotting enzymes from oyster mushroom (*Pleurotus ostreatus* (Fr.) Kumm)," *Applied Biochemistry and Microbiology*, vol. 45, no. 6, pp. 623–625, 2009.

[84] A. W. Bernheimer and L. S. Avigad, "A cytolytic protein from the edible mushroom, *Pleurotus ostreatus*," *Biochimica et Biophysica Acta—General Subjects*, vol. 585, no. 3, pp. 451–461, 1979.

[85] G. Palmieri, C. Bianco, G. Cennamo et al., "Purification, characterization, and functional role of a novel extracellular protease from *Pleurotus ostreatus*," *Applied and Environmental Microbiology*, vol. 67, no. 6, pp. 2754–2759, 2001.

[86] N. Dohmae, K. Hayashi, K. Miki, Y. Tsumuraya, and Y. Hashimoto, "Purification and characterization of intracellular proteinases in *Pleurotus ostreatus* fruiting bodies," *Bioscience, Biotechnology and Biochemistry*, vol. 59, no. 11, pp. 2074–2080, 1995.

[87] H.-H. Shin and H.-S. Choi, "Purification and characterization of metalloproteases from *Pleurotus sajor-caju*," *Journal of Microbiology and Biotechnology*, vol. 9, no. 5, pp. 675–678, 1999.

[88] H.-H. Shin and H.-S. Choi, "Purification and characterization of cysteine protease from *Pleurotus ostreatus*," *Bioscience, Biotechnology and Biochemistry*, vol. 62, no. 5, pp. 1416–1418, 1998.

[89] R. Riley, A. A. Salamov, D. W. Brown et al., "Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 27, pp. 9923–9928, 2014.

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Research Article

Purification and Characterization of a Polyextremophilic α-Amylase from an Obligate Halophilic *Aspergillus penicillioides* Isolate and Its Potential for Souse with Detergents

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An extracellular α-amylase from the obligate halophilic *Aspergillus penicillioides* TISTR3639 strain was produced and enriched to apparent homogeneity by ammonium sulfate precipitation and Sephadex G100 gel filtration column chromatography. The mass of the purified amylase was estimated to be 42 kDa by SDS-PAGE. With soluble starch as the substrate it had a specific activity of 118.42 U·mg^{-1} and V_{max} and K_m values of 1.05 μmol·min^{-1}·mg^{-1} and 5.41 mg·mL^{-1}, respectively. The enzyme was found to have certain polyextremophilic characteristics, with an optimum activity at pH 9, 80°C, and 300 g·L^{-1} NaCl. The addition of $CaCl_2$ at 2 mM was found to slightly enhance the amylase activity, while $ZnCl_2$, $FeCl_2$, or EDTA at 2 mM was strongly or moderately inhibitory, respectively, suggesting the requirement for a (non-Fe^{2+} or Zn^{2+}) divalent cation. The enzyme retained more than 80% of its activity when incubated with three different laundry detergents and had a better performance compared to a commercial amylase and three detergents in the presence of increasing NaCl concentrations up to 300 g·L^{-1}. Accordingly, it has a good potential for use as an α-amylase in a low water activity (high salt concentration) and at high pH and temperatures.

1. Introduction

Hypersaline environments are caused by the evaporation of water and they are also called thallasohaline environment. Due to the evaporation process the sodium chloride (NaCl) concentration rises above 300 psu. Many microbial communities have been found from the sesalterns. Halophilic microorganisms are extremophiles that are able to survive in and may require salt for their growth [1]. Fungi that are isolated from hypersaline environments with a salinity above 100 g·L^{-1} and are able to grow *in vitro* at a 175 g·L^{-1} salt concentration are categorized as halophilic fungi [2, 3]. Very few fungi have been reported yet to inhabit the hypersaline habitats. Their function in these environments is still not fully understood [2]. Unlike other microbes, the fungi can grow independent of salt concentration in saline environments [1]. However, obligate halophilic fungi are those that are unable to grow in the absence of a salt concentration [4].

Extremophilic microorganisms adopt different strategies to survive in extreme available conditions. They harbor different metabolites such as enzymes that can work at extreme conditions [5] and so are of interest for diverse biotechnological applications. Halophiles are currently used in several fermentation processes [6], such as for the production of bioactive compounds [7], biorhodopsin, biosurfactants, food additives, and biocompatible solutes [8]. Extreme halophiles have been increasingly investigated for their hydrolytic enzymes since these have potential uses in several

industrial applications [9, 10]. However, the use of halophilic microorganisms and their metabolites has largely involved halophilic bacteria [11]. Despite the fact that halophilic fungi, especially the obligate strains, are better sources of extracellular enzymes, they have not been investigated very much for their potential in biotechnological applications [3, 12]. α-Amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) is a class of important industrial enzymes that are used in the food, textile, laundry, and pharmaceutical industries [13, 14] and currently form about 25% of the total enzyme market [15]. Although many microbial amylases have been used as an additive in laundry detergents [16], they do not perform well in hard or saline water, which then limits their use in such areas [3, 12].

Recently, the obligate halophilic *Aspergillus penicillioides* TISTR 3639 strain was isolated from an extreme hypersaline environment (a man-made solar saltern) in the Ban Laem district of Phetchaburi province, Thailand [1, 4]. The fungus was found to be positive for extracellular α-amylase activity [3] by plate screening method (Supplementary Figure S1; see Supplementary Material available online at http://dx.doi.org/10.1155/2015/245649). In this study, the purification and characterization of α-amylase from *A. penicillioides* TISTR 3639 were performed. Due to its polyextremophilic properties, the potential of using this enzyme as a laundry detergent additive was then investigated.

2. Materials and Methods

2.1. Growth Conditions for Enzyme Production. The *A. penicillioides* TISTR3639 strain was grown in 150 mL flasks containing 100 mL of production medium (PM) at room temperature (25 ± 2°C) at 150 rpm for 14 d. The PM was made according to Ali et al. [3] with a few modifications such that the composition was composed of 10 g·L^{-1} soluble starch, 3.0 g·L^{-1} mycological peptone, 100 g·L^{-1} NaCl, 8.0 g·L^{-1} $CaCO_3$, 6.6 g·L^{-1} $(NH_4)_2SO_4$, 3.5 g·L^{-1} KH_2PO_4, 0.15 g·L^{-1} $FeSO_4 \cdot 7H_2O$, and 0.10 g·L^{-1} $MgSO_4 \cdot 7H_2O$.

2.2. Amylase Purification. Enrichment to apparent homogeneity of the α-amylase was performed by ammonium sulfate precipitation and Sephadex G100 gel filtration chromatography as previously described [12, 17, 18]. The 14 d grown culture broth (100 mL) was centrifuged at 13,000 ×g at 4°C for 10 min and the supernatant was harvested. The amylase was then precipitated by bringing the supernatant to 90% saturation $(NH_4)_2SO_4$, storing overnight at 4°C, and harvesting the insoluble fraction by centrifugation at 12,000 ×g for 30 min. The pellet was then dissolved in 100 mM Tris-HCl buffer (pH 8) and dialyzed against the same buffer for 48 h. The dialyzate was then subjected to Sephadex G100 gel filtration using a 2.6 cm × 150 cm column, preequilibrated in and then eluted with 25 mM Tris-HCl buffer (pH 8) containing 5 mL·L^{-1} Triton X-100 at a flow rate of 30 mL·h^{-1}. Fractions (5 mL) were collected and each was tested for α-amylase activity and total protein content.

Determination of the purity and molecular weight of the enriched α-amylase was performed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) resolution (150 g·L^{-1} resolving gel) followed by coomassie blue staining, as reported by Hmidet et al. [18]. The purified amylase was mixed at a 1 : 5 volume ratio with the loading buffer (10 mM Tris-HCl, pH 8, 25 g·L^{-1} SDS, 50 mL·L^{-1} β-mercaptoethanol, 10 mL·L^{-1} glycerol, and 0.002 g·L^{-1} bromophenol blue). Prior to loading and electrophoresis, the sample was denatured and reduced by heating at 100°C for 5 min. Gels were stained with 2.5 g·L^{-1} Coomassie Brilliant Blue R250 in 450 mL·L^{-1} ethanol-100 mL·L^{-1} acetic acid and destained with 50 mL·L^{-1} ethanol-7.5 mL·L^{-1} acetic acid. The molecular weight was determined in comparison to the Unstained Precision Plus Protein 161 molecular marker kit (Bio-Rad, USA).

2.3. α-Amylase Assay. α-Amylase activity was determined by the 3,5-dinitrosalicylic acid (DNS) method as described by Miller [19] using 10 g·L^{-1} soluble starch as the substrate. The reaction mixture (0.1 mL enzyme solution, 0.5 mL 0.1 M phosphate buffer, and 5 mg soluble starch) was incubated at 40°C for 10 min. The reaction was then stopped by the addition of 3 mL of DNS and heating in a boiling water bath for 5 min. After cooling, 10 mL of water was added and the absorbance of the reaction mixture was read at 540 nm (A_{540}). One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 μmol of glucose in 1 min.

2.4. Protein Estimation. The amount of protein was estimated by the method of Lowry [20], using bovine serum albumin (BSA) as the standard.

2.5. Characterization of the Enriched α-Amylase. The effect of the pH, temperature, and NaCl concentration on the enriched amylase enzyme activity was evaluated by sequential univariate analysis of the pH, temperature, and NaCl concentration, respectively, and monitoring the relative enzyme activity (the highest activity was referred to as 100%) as the selected parameter. For evaluation of the optimal pH, the reaction mixture was incubated in 0.1 M acetate buffer for pH 5-6 and 0.1 M phosphate buffer for pH 7–12, at a constant 30°C with no added NaCl. For evaluation of the optimal temperature, the reaction mixture was incubated at a temperature range of 40–100°C in 0.1 M phosphate buffer at the optimal pH (as determined above). Finally, for evaluation of the optimal salinity level, the sample mixture in 0.1 M phosphate buffer at the found optimal pH was supplemented with NaCl to a final concentration of 0–500 g·L^{-1} and incubated at the optimal temperature.

The effect of various metal ions, or the enzyme inhibitors β-mercaptoethanol and EDTA, on the enzyme activity was investigated by separately adding $BaCl_2$, $CaCl_2$, $FeCl_2$, $HgCl_2$, $MgCl_2$, and $ZnCl_2$ to the reaction mixture at a final concentration of 2 mM. The relative amylase activity (%) was calculated in comparison to that without any additives.

2.6. Determination of the Kinetic Parameters of the Enriched α-Amylase. The kinetic parameters of the enzyme were determined by incubating the enriched α-amylase with 0.1–40 g·L^{-1} soluble starch (substrate) under the previously found

Figure 1: SDS-PAGE analysis of the enriched α-amylase from *A. penicillioides* TISTR 3639. Lane 1: molecular mass ladder; lane 2 the enriched α-amylase, showing a single band at approximately 42 kDa.

optimum conditions. The V_{max} and K_m values were then calculated from the Lineweaver-Burk plot.

2.7. Compatibility of the Enriched α-Amylase with Commercial Detergents. The compatibility of the enriched α-amylase with three commercial detergents (a liquid detergent (A) and two powdered detergents (B and C) bought from Talad Thai market, Pathumthani, Thailand) was determined in terms of the respective enzyme activities in comparison with that of the enriched enzyme in the absence of the detergents.

The detergent solutions were prepared by dissolving the powdered or liquid detergent in distilled water to 7 g·L^{-1} and heating at 100°C for 90 min to denature any enzyme activity present in the solutions. The cooled solutions were then mixed with the purified amylase at a 1 : 1 volume ratio and incubated for 1 h at 40°C. The residual enzyme activity was calculated in comparison with the control (distilled water instead of the detergents solution), expressed as the relative activity (%) of the control experiment.

2.8. Performance of the Enriched α-Amylase with Commercial Detergents in Varying NaCl Concentrations. For the performance test, the control mixture was made using 0.1 mL of the enriched α-amylase from *A. penicillioides* TISTR3639 in 0.5 mL of 0.1 M phosphate buffer (pH 7) containing 10 g·L^{-1} of soluble starch and 0–50 g·L^{-1} NaCl, while the three detergents (A, B, and C) were assayed as above except for adding the respective detergent instead of the enriched amylase. A commercial α-amylase from *A. oryzae* (Sigma Aldrich, Germany) was also used for comparison.

2.9. Statistical Analysis. Each experiment with the required controls was performed in triplicate and the data are presented as the mean ± one standard deviation (SD). Significance of the differences between means was tested for by analysis of variance (ANOVA) and Duncan's multiple means tests (DMMT) on the parametric or arc-sine square root transformed data using the SPSS software, where a P value of less than 0.05 was considered as significant.

3. Results

3.1. Enrichment of the α-Amylase. The initial 90% saturation $(NH_4)_2SO_4$ cut gave a 2.5-fold increased enzyme specific activity for a 32% yield loss and a 76% total protein reduction (Table 1). Following Sephadex G100 gel filtration, the eluted fraction showing the highest amylase activity was then evaluated for its apparent purity and molecular weight by SDS-PAGE resolution and coomassie blue staining. Only a single protein band was visualized suggesting the apparent enrichment of the enzyme to homogeneity (Figure 1). Overall, the enzyme activity was increased 6.96-fold following the 90% saturation $(NH_4)_2SO_4$ precipitation and Sephadex G100 gel filtration for a loss of 56% activity. The specific activity of the enriched α-amylase was estimated to be 118.4 ± 1.2 U·mg^{-1} (Table 1), with an estimated molecular weight of ~42 kDa by SDS-PAGE analysis (Figure 1).

3.2. Characterization of the Enriched α-Amylase. The effect of increasing the pH (range 5–12) on the enriched α-amylase activity, when assayed at 30°C, is shown in Figure 2(a), where the enzyme activity was higher (>80%) in a neutral to moderately alkaline pH (7–10). The enzyme activity declined above and below pH 9, but this decline was more marked with increasing pH above 9 (64% activity at pH 12) than with decreasing pH (~75 and ~73% at pH 6 and 5, resp.).

Increasing the temperature increased the activity of the enriched α-amylase, as assayed at the more optimal pH 9 (Figure 2(b)), to peak at 80°C (100±0.81%), and then declined at temperatures above 80°C, but it still retained more than 60% of its initial activity at 100°C. Thus, the thermophilic nature of the enzyme at temperatures higher than 60°C was clearly revealed.

With respect to the effect of the salinity, a gradual increase in the enriched α-amylase enzyme activity was observed with increasing NaCl concentrations up to 300 g·L^{-1}, as assayed at pH 9 and 80°C (Figure 3). Although there was a sharp decrease in the enzyme activity above 300 g·L^{-1} NaCl, the enzyme was still able to retain most (89 ± 0.21%) of its initial activity at the nearly saturated salt concentration of 400 g·L^{-1} NaCl.

Finally, the effect of various metal ions and enzyme inhibitors at a concentration of 2 mM on the activity of the enriched α-amylase is summarized in Table 2. Except for $CaCl_2$, which only weakly (but statistically significantly) increased the activity, none of the metal ions and enzyme inhibitors at this concentration were found to increase the enzyme activity. The slight decrease in the enzyme activity with the addition of 2 mM $BaCl_2$, $HgCl_2$, and b-mercaptoethanol was significant all the same, whilst the enzyme was moderately inhibited by the inclusion of $FeCl_2$ or EDTA and strongly inhibited by $ZnCl_2$.

3.3. Enzyme Kinetics. From the Lineweaver-Burk plot (Figure 4), the enriched amylase had a K_m of 5.41 mg·mL^{-1} and

Table 1: Stepwise summary of the enrichment of the α-amylase from *A. penicillioides* TISTR 3639.

Properties	Cell-free supernatant	$(NH_4)_2SO_4$ precipitation	Gel filtration chromatography
Total protein (mg)	2301.1 ± 1.0	785.4 ± 1.1	219.4 ± 1.3
Total activity (U)	39142 ± 1.5 (100%)	34015.8 ± 1.0 (68%)	25891.9 ± 1.1 (44%)
Specific activity ($U \cdot mg^{-1}$)	17 ± 0.0	43.3 ± 0.1	118.4 ± 1.2
Purification fold	1.0	2.5	6.9

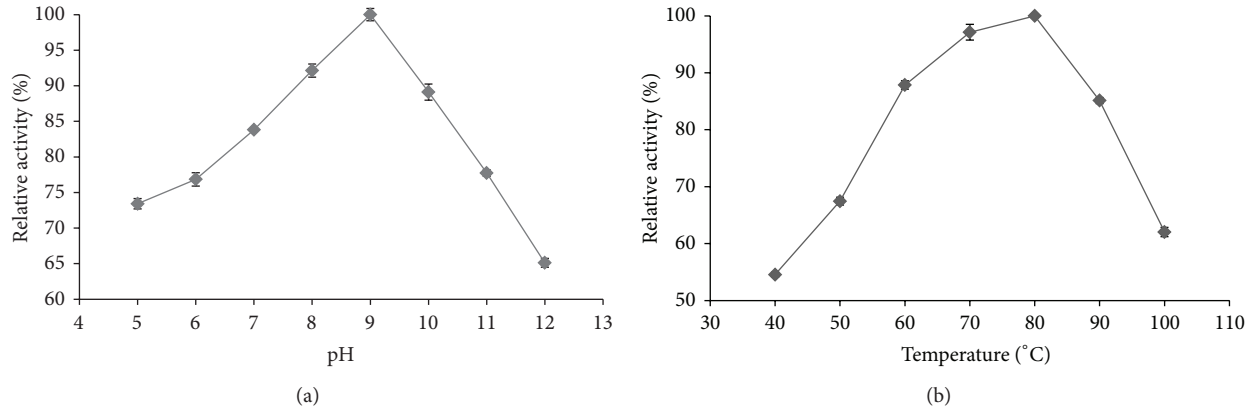

Figure 2: Effect of the (a) pH (at 30°C) and (b) temperature (at pH 9.0) on the activity of the enriched α-amylase from *A. penicillioides* TISTR 3639. Data are shown as the mean relative activity (%) ±1 SD (error bars), derived from three repeats. Means with a different lowercase superscript letter (a, b, and c) are significantly different (ANOVA and DMRT of the transformed data, $P < 0.05$).

Table 2: Effect of various metal ions and additives on the activity of the enriched α-amylase from *A. penicillioides* TISTR 3639.

Additives (2 mM)	Relative activity (%)
None	100^{*f}
$BaCl_2$	97.8 ± 0.8^{e}
$CaCl_2$	104.2 ± 1.7^{g}
$FeCl_2$	78.1 ± 1.5^{c}
$HgCl_2$	97.1 ± 1.4^{e}
$MgCl_2$	99.1 ± 2.2^{ef}
$ZnCl_2$	44.3 ± 0.9^{a}
β-Mercaptoethanol	95.1 ± 1.5^{d}
EDTA	73.4 ± 0.4^{b}

*One hundred percent activity corresponded to the activity of the amylase without additive. Data are shown as the mean relative value ± 1SD, derived from three repeats. Means followed by a different lowercase superscript letter (a, b, c, d, e, f, and g) are significantly different (ANOVA and DMRT of the transformed data, $P < 0.05$).

V_{max} of 1.05 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ with soluble starch as the substrate.

3.4. Compatibility of the Enriched α-Amylase with the Selected Three Commercial Detergents. The compatibility results of the enriched enzyme with three commercial detergents (A, B, and C) as well as the commercial amylase enzyme are summarized in Table 3. The residual activity taken in percentage, as compared with control, showed that the enzyme was seen to retain more than 80% of the activity without any added detergent. The powdered B and C detergents were found to be more compatible with the enriched amylase, by giving the residual enzyme activity of 87% and 94%, respectively, than was liquid detergent A, which was observed to provide 81% amylase residual activity.

Table 3: Effect of various detergents on the residual activity of the enriched α-amylase from *A. penicillioides* TISTR 3639.

Additive	Residual activity (%)
Distilled water (control)	100^{*d}
Detergent A	81 ± 1.5^{a}
Detergent B	87 ± 0.1^{b}
Detergent C	94 ± 1.5^{c}

*One hundred percent activity corresponded to the activity of the amylase without any added detergent. Data are shown as the mean relative value ± 1SD, derived from three repeats. Means followed by a different lowercase superscript letter (a, b, c, and d) are significantly different (ANOVA and DMRT of the transformed data, $P < 0.05$).

3.5. Performance Test with Commercial Detergents. The performance test results of this enriched α-amylase in the presence of the respective commercial detergents are summarized in Figure 5, where the relative activity (%) was found to decrease with increasing salt concentration. However, in similar salt conditions the commercial amylase and the detergents had an inferior activity in comparison to this enriched α-amylase from *A. penicillioides*. At 50 $g \cdot L^{-1}$ NaCl, only 60% of the *A. penicillioides* amylase activity was detected in the commercial amylase and 60–70% in the three detergents.

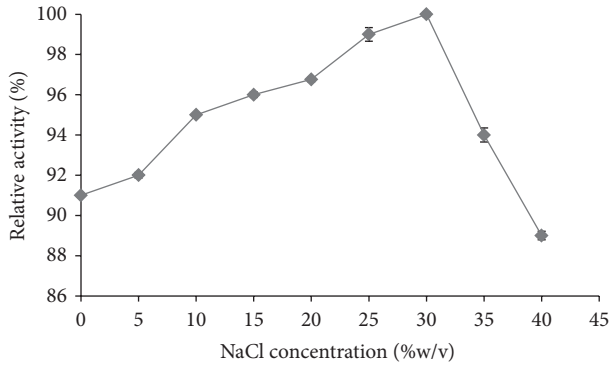

Figure 3: Effect of the NaCl concentration (g·L^{-1}) on the activity of the enriched α-amylase from *A. penicillioides* TISTR 3639 at pH 9 and 80°C. Data are shown as the mean relative activity (%) ±1 SD (error bars), derived from three repeats. Means with a different lowercase superscript letter (a, b, and c) are significantly different (ANOVA and DMRT of the transformed data, $P < 0.05$).

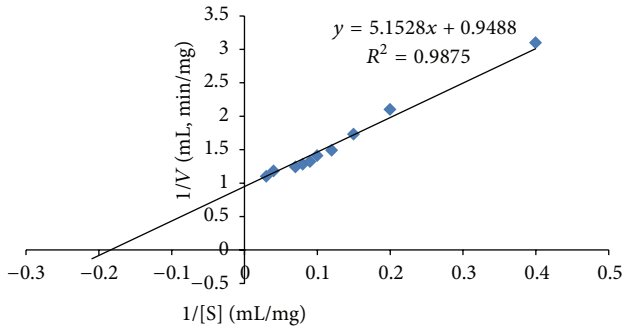

Figure 4: Lineweaver-Burk plot for the determination of the V_{max} and K_m values of the enriched α-amylase from *A. penicillioides* TISTR 3639, at optimum conditions (pH 9 and 80°C), in the presence of different concentrations of soluble starch.

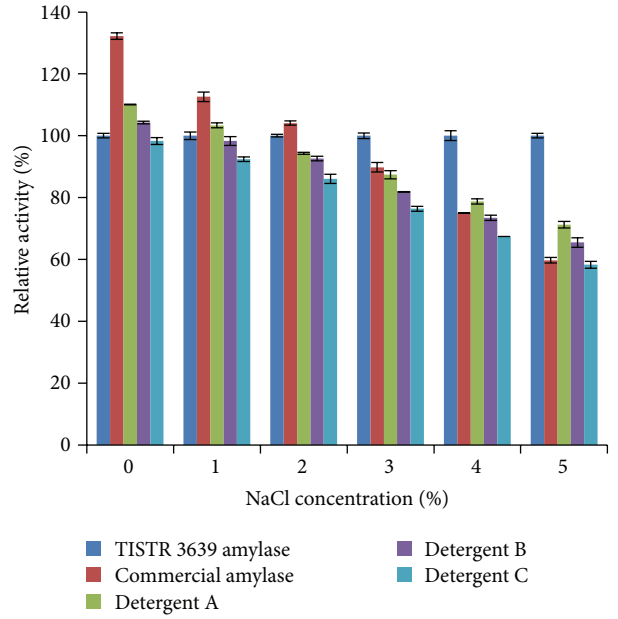

Figure 5: Performance comparison of a commercial amylase, detergents A, B, and C, and the enriched α-amylase (control) from *A. penicillioides* TISTR 3639 at pH 7 and 30°C in the presence of different concentrations of NaCl. The results are expressed as the relative activity (%) to that of the enriched α-amylase from *A. penicillioides* TISTR 3639 without NaCl and are shown as the mean relative activity (%) ±1 SD (error bars), derived from three repeats. Means with a different lowercase superscript letter (a, b, and c) are significantly different (ANOVA and DMRT of the transformed data, $P < 0.05$).

4. Discussion

Many attempts have been made to find suitable fungus strains for the production of amylases with desirable properties [21]. Mesophilic fungi are reported to be the principal amylase producers [16] and especially members of the *Aspergillus* and *Penicillium* genera that appear to be the dominant producing species [22]. Fungal amylases are preferred for use in various industries, including the food and pharmaceutical industries, due to their nontoxic characteristics [3, 12, 23]. Consequentially, *Aspergillus* species, such as *Aspergillus niger* and *Aspergillus oryzae*, are frequently used in the industrial production of amylases [24], but there are few reports on the purification and detailed characterization of α-amylases from halophilic fungi [12]. In addition, to the best of our knowledge, this is the first report of the determination of the potential of an amylase from any obligate halophilic fungus to be used as an additive in laundry detergent.

The molecular mass of amylases from halophilic fungi is mostly reported in the range of 50–75 kDa [25]. In this study for *A. penicillioides* TISTR 3639, it was found slightly smaller with an approximate mass of 42 kDa (Figure 1). Moreover, the specific activity of the amylase was in the moderate range [17], whilst the K_m value (5.41 mg·mL^{-1}) of the enzyme falls in the middle of the range (0.35–11.66 mg·mL^{-1}) reported for amylases from halophilic fungi [26]. The K_m and V_{max} values of the enriched α-amylase of this study (with soluble starch as the substrate) suggested that it has a moderately high affinity for soluble starch and requires a relatively low concentration of this substrate to achieve V_{max}.

The optimal pH profile of this enriched amylase mirrors the pH-dependent growth profile of its producing *A. penicillioides*, where it was previously reported that *A. penicillioides* grew relatively well at a neutral to alkaline pH [1]. This also matches the pH found in most of the hypersaline environments [6]. The enzyme in this study retained almost 80% of its relative activity at an extreme pH (pH 11), but at pH 5 it was slightly lower at less than 75% relative activity, showing that it is potentially more tolerant of alkaline pH. An optimal pH of 9-10 has been reported for many enzymes from halophilic organisms and an alkalophilic property has been considered as the nature of halophilic enzymes [27, 28].

The alkaline amylases from microorganisms have been found to have tremendous applications in detergent industries [15]. However, most halophilic enzymes are denatured and lose their activity at temperatures over 50°C [29], whereas this halophilic and alkalophilic amylase from *A. penicillioides* TISTR 3639 showed an optimal activity at 80°C and was

still relatively stable at 90°C or lower with more than 80% of its initial activity remaining after a 1 h incubation at this temperature (data not shown). Thus, it is extremely thermophilic [30, 31]. Thermophilic amylases are mostly applied in various starch industries [29].

Salinity is a crucial factor in the normal functioning of most enzymes from obligate halophilic microorganisms. Previously, it was found that *A. penicillioides* grew best at a salinity of 100 g·L^{-1} NaCl [1], a salinity level that has been widely reported to favor amylase production in most halophilic microbes [12, 32, 33]. However, the α-amylase from *A. penicillioides* TISTR 3639 still had a high catalytic activity even at extreme salt concentrations (300–400 g/mL^{-1} NaCl), much higher than those of the amylases reported from other extreme halophilic Archaea and Bacteria [10, 33, 34], adding a novelty to this enzyme. This extremophile property of the enzyme suggests the potential to be used in saline waste water management, in bioremediation processes in saline areas [12, 35, 36], and for biofuel production, where halophilic enzymes are reported to work better than normal enzymes [37].

The amylases from halophilic microorganisms have frequently been reported to have polyextremophilic characteristics [10, 12, 33]. They are mostly reported to be thermotolerant with haloalkalophilic properties [25], where the enzyme must have the capability to withstand extreme conditions for several industrial processes [38]. The amylase from *A. penicillioides* TISTR 3639 has the same trend of being polyextremophile, which makes it potentially versatile for use in different industrial operations, where harsh conditions are available.

The detergent industries are one of the primary consumers and users of enzymes that enhance the performance of detergents or allow the product to be more environmentally friendly [21]. Approximately 90% of commercial liquid detergents contain a mixture of enzymes that include amylases [39], since starch is considered as an attractant for various soil particles [18]. Amylases are also used in the detergent industries to remove starchy food stains, such as chocolate, custard, gravy, and potato amongst others, which are found on kitchen utensils as well as on clothes [16]. The addition of any enzyme in the detergent requires its compatibility and ability to perform in the presence of the detergents for inclusion [40]. Currently, there have been only a few reports of suitable and stable amylases that can be added into laundry detergents [41], and these need to work well at an alkaline pH [21]. Thus, it is of interest that the enriched amylase from *A. penicillioides* TISTR 3639 was found to be relatively stable in the presence of different detergents as well as alkaline conditions. Note that the compatibility test was performed under normal conditions (30°C, pH 7) that are suboptimal for this enzyme and so its activity may be improved at higher temperatures, alkalinity, and salinity levels, although of course its compatibility with these detergents would need to be evaluated at these conditions.

The rapidly changing world provides some tough challenges to humans. The limiting water resources in many parts of the world compel the use of saline or hard untreated water for daily domestic uses [42], including untreated underground hard water [43], and these decrease the cleaning efficiency of detergents [44]. In this study, the enriched α-amylase from *A. penicillioides* TISTR 3639, in comparison with three tested detergents and a commercial amylase, was found to work well in a low water activity, which means that the inclusion of this amylase could help solve the problem of a low cleaning capacity of detergents in a high saline environment. Moreover, when such underground hard water is not suitable for drinking it can be used for laundry purposes, saving the clean and drinkable water for consumption in areas where drinking water resources is limited.

5. Conclusions

Increasing demands from biotechnology, climate change, and decreasing water resources form the need to find amylases that can withstand high temperatures, salt concentrations (low water activity), and alkalinity levels. The polyextremophilic behavior of this enriched α-amylase obtained from *A. penicillioides* TISTR 3639 appears to be a promising candidate for fulfilling the current needs of many industrial processes requiring amylases, especially for laundry detergent industries. Of interest was the fact that the α-amylase from the obligate halophilic *A. penicillioides* TISTR 3639 was more extremophilic than the fungus itself. This provides an opportunity to exploit more interesting biotechnological applications from obligate halophilic fungi.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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References

[1] I. Ali, L. Kanhayuwa, S. Rachdawong, and S. K. Rakshit, "Identification, phylogenetic analysis and characterization of obligate halophilic fungi isolated from a man-made solar saltern in Phetchaburi province, Thailand," *Annals of Microbiology*, vol. 63, no. 3, pp. 887–895, 2013.

[2] N. Gunde-Cimerman, J. C. Frisvad, and P. Zalaretal, "Halotolerant and halophilic fungi," in *Biodiversity of Fungi-Their Role in Human Life*, S. K. Deshmukh and M. K. Rai, Eds., pp. 96–128, Oxford & IBH, New Delhi, India, 2005.

[3] I. Ali, N. Siwarungson, H. Punnapayak et al., "Screening of potential biotechnological applications from obligate halophilic fungi, isolated from a man-made solar saltern located in Phetchaburi province, Thailand," *Pakistan Journal of Botany*, vol. 46, pp. 983–988, 2014.

[4] I. Ali, S. K. Rakshit, A. Akbar et al., "Identification and phylogenetic analysis of halophilic fungus isolated from a man-made

solar saltern in Thailand," *Lasbella University Journal of Science and Technology*, vol. 2, pp. 47–52, 2013.

[5] G. Antranikian, "Extreme environments as a resource for microorganisms and novel biocatalysts," *Marine Biotechnology*, vol. 96, pp. 219–262, 2005.

[6] C. Gostinčar, M. Lenassi, N. Gunde-Cimerman, and A. Plemenitaš, "Fungal adaptation to extremely high salt concentrations," *Advances in Applied Microbiology*, vol. 77, pp. 71–96, 2011.

[7] A. Oren, "Industrial and environmental applications of halophilic microorganisms," *Environmental Technology*, vol. 31, no. 8-9, pp. 825–834, 2010.

[8] S. DasSarma and P. DasSarma, *Halophiles*, Encyclopedia of Life Sciences, Wiley, London, UK, 2002.

[9] M. Delgado-García, B. Valdivia-Urdiales, C. N. Aguilar-González, J. C. Contreras-Esquivel, and R. Rodríguez-Herrera, "Halophilic hydrolases as a new tool for the biotechnological industries," *Journal of the Science of Food and Agriculture*, vol. 92, no. 13, pp. 2575–2580, 2012.

[10] M. Moshfegh, A. R. Shahverdi, G. Zarrini, and M. A. Faramarzi, "Biochemical characterization of an extracellular polyextremophilic α-amylase from the halophilic archaeon *Halorubrum xinjiangense*," *Extremophiles*, vol. 17, no. 4, pp. 677–687, 2013.

[11] H. Dalbøge, "Expression cloning of fungal enzyme genes: a novel approach for efficient isolation of enzyme genes of industrial relevance," *FEMS Microbiology Reviews*, vol. 21, no. 1, pp. 29–42, 1997.

[12] I. Ali, A. Akbar, B. Yanwisetpakdee, S. Prasongsuk, P. Lotrakul, and H. Punnapayak, "Purification, characterization, and potential of saline waste water remediation of a polyextremophilic α-amylase from an obligate halophilic *Aspergillus gracilis*," *BioMed Research International*, vol. 2014, Article ID 106937, 7 pages, 2014.

[13] X. D. Liu and Y. Xu, "A novel raw starch digesting α-amylase from a newly isolated *Bacillus* sp. YX-1: purification and characterization," *Bioresource Technology*, vol. 99, no. 10, pp. 4315–4320, 2008.

[14] M. Shafiei, A.-A. Ziaee, and M. A. Amoozegar, "Purification and biochemical characterization of a novel SDS and surfactant stable, raw starch digesting, and halophilic α-amylase from a moderately halophilic bacterium, *Nesterenkonia* sp. strain F," *Process Biochemistry*, vol. 45, no. 5, pp. 694–699, 2010.

[15] K. K. Kiran and T. S. Chandra, "Production of surfactant and detergent-stable, halophilic, and alkalitolerant alpha-amylase by a moderately halophilic *Bacillus* sp. Strain TSCVKK," *Applied Microbiology and Biotechnology*, vol. 77, no. 5, pp. 1023–1031, 2008.

[16] R. Gupta, P. Gigras, H. Mohapatra, V. K. Goswami, and B. Chauhan, "Microbial α-amylases: a biotechnological perspective," *Process Biochemistry*, vol. 38, no. 11, pp. 1599–1616, 2003.

[17] S. Chakraborty, A. Khopade, C. Kokare, K. Mahadik, and B. Chopade, "Isolation and characterization of novel α-amylase from marine *Streptomyces* sp. D1," *Journal of Molecular Catalysis B: Enzymatic*, vol. 58, no. 1–4, pp. 17–23, 2009.

[18] N. Hmidet, A. Bayoudh, J. G. Berrin, S. Kanoun, N. Juge, and M. Nasri, "Purification and biochemical characterization of a novel α-amylase from *Bacillus licheniformis* NH1. Cloning, nucleotide sequence and expression of *amy* N gene in *Escherichia coli*," *Process Biochemistry*, vol. 43, no. 5, pp. 499–510, 2008.

[19] G. L. Miller, "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Analytical Chemistry*, vol. 31, no. 3, pp. 426–428, 1959.

[20] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, "Protein measurement with the Folin phenol reagent," *The Journal of Biology and Chemistry*, vol. 193, no. 1, pp. 265–275, 1951.

[21] P. M. de Souza and P. D. O. Magalhães, "Application of microbial α-amylase in industry—a review," *Brazilian Journal of Microbiology*, vol. 41, no. 4, pp. 850–861, 2010.

[22] K. Kathiresan and S. Manivannan, "α-Amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil," *African Journal of Biotechnology*, vol. 5, no. 10, pp. 829–832, 2006.

[23] F. Niknejad, M. Moshfegh, M. J. Najafzadeh et al., "Halotolerant ability and α-amylase activity of some saltwater fungal isolates," *Iranian Journal of Pharmaceutical Research*, vol. 12, pp. 113–119, 2013.

[24] R. Kammoun, B. Naili, and S. Bejar, "Application of a statistical design to the optimization of parameters and culture medium for α-amylase production by *Aspergillus oryzae* CBS 819.72 grown on gruel (wheat grinding by-product)," *Bioresource Technology*, vol. 99, no. 13, pp. 5602–5609, 2008.

[25] M. E. Setati, "Diversity and industrial potential of hydrolase-producing halophilic/halotolerant eubacteria," *African Journal of Biotechnology*, vol. 9, no. 11, pp. 1555–1560, 2010.

[26] M. F. Najafi and A. Kembhavi, "One step purification and characterization of an extracellular α-amylase from marine *Vibrio* sp.," *Enzyme and Microbial Technology*, vol. 36, no. 4, pp. 535–539, 2005.

[27] B. Prakash, M. Vidyasagar, M. S. Madhukumar, G. Muralikrishna, and K. Sreeramulu, "Production, purification, and characterization of two extremely halotolerant, thermostable, and alkali-stable α-amylases from *Chromohalobacter* sp. TVSP 101," *Process Biochemistry*, vol. 44, no. 2, pp. 210–215, 2009.

[28] K. N. Varalakshmi, B. S. Kumudini, B. N. Nandini et al., "Production and characterization of α-amylase from *Aspergillus niger* JGI 24 isolated in Bangalore," *Polish Journal of Microbiology*, vol. 58, no. 1, pp. 29–36, 2009.

[29] O. Prakash and N. Jaiswal, "α-Amylase: an ideal representative of thermostable enzymes," *Applied Biochemistry and Biotechnology*, vol. 160, no. 8, pp. 2401–2414, 2010.

[30] S. A. Fincan and B. Enez, "Production, purification, and characterization of thermostable a-amylase from thermophilic *Geobacillus stearothermophilus*," *Starch/Staerke*, vol. 66, no. 1-2, pp. 182–189, 2014.

[31] I. Gomes, J. Gomes, and W. Steiner, "Highly thermostable amylase and pullulanase of the extreme thermophilic eubacterium *Rhodothermus marinus*: production and partial characterization," *Bioresource Technology*, vol. 90, no. 2, pp. 207–214, 2003.

[32] M. A. Amoozegar, F. Malekzadeh, and K. A. Malik, "Production of amylase by newly isolated moderate halophile, *Halobacillus* sp. strain MA-2," *Journal of Microbiological Methods*, vol. 52, no. 3, pp. 353–359, 2003.

[33] K. K. Kondepudi and T. S. Chandra, "Production of surfactant and detergent-stable, halophilic, and alkalitolerant alpha-amylase by a moderately halophilic *Bacillus* sp. strain TSCVKK," *Applied Microbiology and Biotechnology*, vol. 77, no. 5, pp. 1023–1031, 2008.

[34] M. Shafiei, A.-A. Ziaee, and M. A. Amoozegar, "Purification and characterization of a halophilic α-amylase with increased activity in the presence of organic solvents from the moderately halophilic *Nesterenkonia* sp. strain F," *Extremophiles*, vol. 16, no. 4, pp. 627–635, 2012.

[35] J. Chung, S. Shin, and J. Oh, "Characterization of a microbial community capable of reducing perchlorate and nitrate in high salinity," *Biotechnology Letters*, vol. 31, no. 7, pp. 959–966, 2009.

[36] R. Margesin and F. Schinner, "Biodegradation and bioremediation of hydrocarbons in extreme environments," *Applied Microbiology and Biotechnology*, vol. 56, no. 5-6, pp. 650–663, 2001.

[37] T. Zhang, S. Datta, J. Eichler et al., "Identification of a haloalkaliphilic and thermostable cellulase with improved ionic liquid tolerance," *Green Chemistry*, vol. 13, no. 8, pp. 2083–2090, 2011.

[38] W. Asad, M. Asif, and S. A. Rasool, "Extracellular enzyme production by indigenous thermophilic bacteria: partial purification and characterization of α-amylase by *Bacillus* sp. WA21," *Pakistan Journal of Botany*, vol. 43, no. 2, pp. 1045–1052, 2011.

[39] S. Mitidieri, A. H. S. Martinelli, A. Schrank, and M. H. Vainstein, "Enzymatic detergent formulation containing amylase from *Aspergillus niger*: a comparative study with commercial detergent formulations," *Bioresource Technology*, vol. 97, no. 10, pp. 1217–1224, 2006.

[40] A. K. Mukherjee, H. Adhikari, and S. K. Rai, "Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using *Imperata cylindrica* grass and potato peel as low-cost medium: characterization and application of enzyme in detergent formulation," *Biochemical Engineering Journal*, vol. 39, no. 2, pp. 353–361, 2008.

[41] A. K. Mukherjee, M. Borah, and S. K. Rai, "To study the influence of different components of fermentable substrates on induction of extracellular α-amylase synthesis by *Bacillus subtilis* DM-03 in solid-state fermentation and exploration of feasibility for inclusion of α-amylase in laundry detergent formulations," *Biochemical Engineering Journal*, vol. 43, no. 2, pp. 149–156, 2009.

[42] I. Ali, A. Akbar, H. Punnapayak et al., "Seven big challenges for Pakistan—and the lessons they could teach," *The Futurist*, vol. 48, pp. 22–26, 2014.

[43] S. Ghizellaoui and A. Chibani, "Use of nanofiltration for partial softening of very hard water," *Desalination*, vol. 179, no. 1–3, pp. 315–322, 2005.

[44] J.-S. Park, J.-H. Song, K.-H. Yeon, and S.-H. Moon, "Removal of hardness ions from tap water using electromembrane processes," *Desalination*, vol. 202, no. 1–3, pp. 1–8, 2007.

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Research Article

Lipase-Secreting *Bacillus* Species in an Oil-Contaminated Habitat: Promising Strains to Alleviate Oil Pollution

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Lipases are of great interest for different industrial applications due to their diversity and versatility. Among different lipases, microbial lipases are preferable due to their broad substrate specificity, and higher stability with lower production costs compared to the lipases from plants and animals. In the past, a vast number of bacterial species have been reported as potential lipases producers. In this study, the lipases-producing bacterial species were isolated from an oil spillage area in the conventional night market. Isolated species were identified as *Bacillus* species by biochemical tests which indicate their predominant establishment, and further screened on the agar solid surfaces using lipid and gelatin as the substrates. Out of the ten strains tested, four potential strains were subjected to comparison analysis of the lipolytic versus proteolytic activities. Strain 10 exhibited the highest lipolytic and proteolytic activity. In all the strains, the proteolytic activity is higher than the lipolytic activity except for strain 8, suggesting the possibility for substrate-based extracellular gene induction. The simultaneous secretion of both the lipase and protease is a mean of survival. The isolated bacterial species which harbour both lipase and protease enzymes could render potential industrial-based applications and solve environmental issues.

1. Introduction

Oil pollution or spillage is a prevalent problem in developing and industrialized countries. One of the major sources of oil pollution is the dietary oil spillage from both the producers and end-users, which is a very widespread form of pollution in the environment leading to a number of other complications such as the clogging of pipes and drainages. In order to alleviate the problem associated with this, degradation of the oil can be mediated with an environment-friendly technology and cost-saving system [1]. However, this cleaning-up system is dependent on several factors such as the nature of the place of oil contamination occurrence, temperature, as well as the microbial consortium. There are different microbial species reported from the oil-polluted environments, which include bacteria, fungi, and actinomyces [2–7]. Owing to the presence of a large diversity of microorganisms, one amenable approach to assuage this oil contamination-related problem is the microbial-based degradation. To expedite microbial-based degradation, the type of bacteria and the corresponding products secreted must be identified prior to the characterization. In addition to this, identification and characterizations are able to unveil the presence of a potpourri of extracellular enzymes, which can be purified. Previous studies revealed the presence of lipolytic bacteria isolated from different habitats such as industrial wastes, vegetable oil processing factories, dairy plants, and soil contaminated with oil, where these oily environments provide good conditions for these bacteria to flourish [8, 9]. For example, a novel species of lipase producing bacteria, *Geobacillus zalihae*, was isolated from the palm oil mill effluent in Malaysia [10].

One of the most common extracellular secretions by the bacteria-inhabiting oil-rich soil is the lipase. Lipases or triacylglycerol acyl hydrolases (E.C.3.1.1.3) are enzymes that

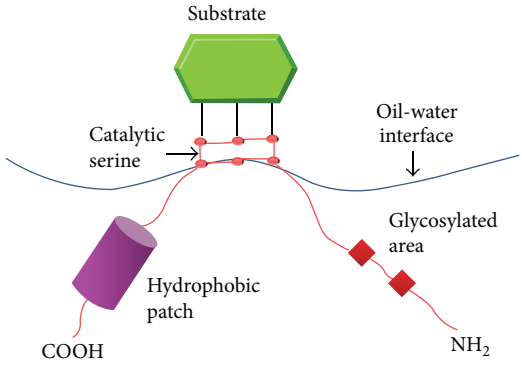


Figure 1: Hydrolysis of the triglyceride by lipase takes place at the interface of the insoluble substrate phase and the aqueous phase, the region where enzyme is dissolved.

catalyze the hydrolysis of ester bonds in fats and oils into glycerol and free fatty acids at the oil-water interface [9]. This hydrolysis takes place at the interface between the insoluble substrate phase and the aqueous phase, the region where the enzyme dissolves (Figure 1). Apart from hydrolysis, lipases also catalyze synthesis reactions, for example, esterification, amidation, alcoholysis, acidolysis, and aminolysis [11]. Yapoudjian et al. [12] have shown that the two possible binding modes of *Thermomyces lanuginosa* lipase mutants TLL (S146A) and the substrate oleic acid involve interaction with the tryptophan 89 of the lipase (protein data bank accession number-1GT6) (Figure 2(a)). Meanwhile, Zdunek et al. [13] have determined the global structure of apolipoprotein, the activator of lipoprotein lipase that forms complex with the sodium dodecyl sulfate (SDS) micelles (protein data bank accession number-1O8T) (Figure 2(b)).

The ability of lipases to catalyze reactions in a broad range of substrates without the addition of expensive cofactors and their stability in organic solvents resulted in the enzymes being listed as the third largest group of commercialized enzymes after proteases and carbohydrases [11, 14]. Lipases are commonly used in the food industry, pulp and paper processes, medical field, and as cleaning agents [14–16]. Lipases were produced naturally in several species of animals, plants, bacteria, yeasts, and fungi [17]. However, lipases isolated from bacteria have gained vast attraction due to their higher activities under optimum pH at neutral or alkaline condition. Microbial lipases also have shorter generation times, and genetic manipulations can be performed more easily on bacterial cells to increase the enzyme production [14]. Besides, bacterial cultures were more readily scaled up for production and purification with lower production costs [17, 18]. Due to the wide ranging versatility of lipases in biotechnological applications, the demands for new lipase sources continues to stimulate the screening and identification of novel lipolytic bacteria with the highest ability for the biodegradation of oils and fats [1, 19, 20]. Thus, in this study, samples from night market were chosen for screening lipase producing bacteria in the oil-contaminated soil, which can be potential microbial-based degradation approach and an "extraction pool" for the extracellular enzymes. Moreover, the differential secretion of the lipase and protease enzyme was also analyzed. Lipase and protease are the class of enzymes that perform both the degradative and synthetic function for the physiological necessity of the microorganism. The comparison can provide knowledge on the differential secretion of lipase/protease in the oil-contaminated soil. Moreover, the identification of the strains that excrete these enzymes can be also potentially manipulated by culturing them in media to expedite large scale enzyme production. These enzymes can also be useful to decontaminate the oil contaminants in the collection area of the night market that are very difficult to be cleaned.

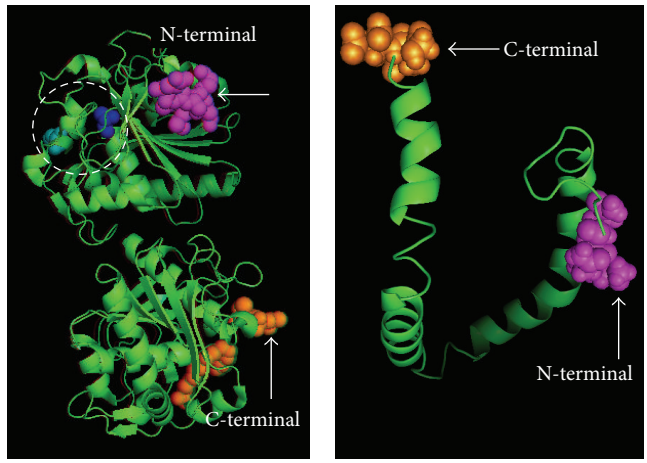
2. Materials and Methods

2.1. Isolation of Bacteria from Oil-Contaminated Soil. Oil-contaminated soil samples were collected from the night market at Bandar Putra Bertam, Penang, Malaysia, and were processed immediately on the same day of collection. The soil samples (5%) were inoculated into enrichment medium (EM1) containing 1% olive oil and incubated at 60°C for 2 days under shaking condition as described by Abd Rahman et al. [10]. Enriched cultures were then streaked onto LB agar plate (Lennox) (Laboratorios CONDA, Spain) for isolation of single colonies.

2.2. Identification of Lipase Producing Bacteria. The bacterial isolates were grown in 10 mL of Luria Broth (Laboratorios CONDA, Spain) at 37°C overnight at 200 rpm and later subjected to Gram staining by using BD Gram Stain Kit (Becton, Dickinson and Company, USA). In order to identify the bacteria, the cultures were subjected to catalase, coagulase, oxidase, motility, indole, citrase, urease, methyl red, and Triple Sugar Iron (T.S.I) test. Prior to the lipolytic activity assay, the enriched cultures of ten bacterial strains were streaked onto blood agar plates and later incubated at 37°C overnight. Blood agar plates were prepared by the addition of meat extract (1%), peptone (1%), sodium chloride (0.5%), and agar (1.5%). After autoclaving, the media is cooled to RT before the addition of 5% v/v sterile defibrinated blood prior to use.

2.3. Screening of Microbial Lipases Production on the Agar Solid Surface. The bacterial single colonies were screened for their ability to produce lipases by using solid media containing different substrates, including Tween-20 and olive oil with phenol red. The screening assays were performed using solid media due to difficulty in the determination of lipolytic activity as the water soluble lipases catalyse reaction of only the water insoluble substrates [1, 21, 22]. The relative enzymatic activity was identified based on visual observation and measuring the formation of a clearance zone on the agar surface.

2.3.1. Lipolytic Enzyme Assay Using Tween-20 Agar. Culture medium which contained peptone (1% w/v), NaCl (0.5% w/v), $CaCl_2{\cdot}2H_2O$ (0.01% w/v), agar (2% w/v), and Tween-20 (1% v/v) was prepared as described by Gopinath et al. [23]. Bacterial samples were then plated on the Tween-20 agar plates and incubated at 37°C overnight. The presence of lipolytic activity was indicated by a visible precipitate resulting from the calcium salt formed by the fatty acid from



(a) (b)

Figure 2: (a) Crystalized structure of *Thermomyces lanuginosa* lipase mutants TLL (S146A) and the substrate oleic acid (protein data bank accession number-1GT6). (b) Crystalized structure of apolipoprotein-SDS micelle complex (protein data bank accession number-1O8T).

the hydrolysis reaction or a clearance zone around the colony due to the complete degradation of the salt of the fatty acid [23].

2.3.2. Lipolytic Enzyme Assay Using Olive Oil with Phenol Red Agar. The serial diluted bacterial samples were also plated on phenol red agar and incubated at 37°C overnight. The phenol red agar plates were prepared by incorporating phenol red (0.01% w/v), olive oil (0.1% v/v), $CaCl_2$ (0.1% w/v), and agar (2% w/v) [24]. Phenol red has an end point at pH 7.3-7.4, where a slight decrease in pH will turn its color from pink to yellow. The change in color of phenol red was used as an indicator for lipase activity, where lipase producing bacteria will turn the dye into yellow color.

2.4. Proteolytic Enzyme Assay Using Gelatin Agar. The gelatin agar plates were prepared by adding 5 mL of sterile 8% solution of gelatin (Sigma) into 100 mL of nutrient agar medium (HiMedia, India) [23]. Serial diluted bacterial samples were plated on gelatin agar and incubated at 37°C overnight. The clearance zone around the colonies indicated the presence of proteolytic activity, which was due to the complete degradation of gelatin. Aqueous saturated solution of ammonium sulfate was added on the surface of the agar for clear visualization.

3. Results and Discussion

Isolation of extracellular enzyme-producing microorganism has garnered immense attention due to its application in numerous biotechnological processes such as detergents; textile; dairy industries; oil processing; surfactant production; and synthesis of chiral pharmaceuticals. Since there is a different requisite for the specific properties of the enzyme for each industrial application, there is a constant interest for the identification of new lipase for novel applications. Microorganisms such as bacteria, yeast, and fungi secrete certain enzymes for growth on insoluble organic substrates. For example, enzyme lipase is secreted and favored, attributable to its high reaction specificity, stereo specificity, less energy consumption, and having higher stability than the plant and animal enzymes. Previously, Gopinath et al. [23] have demonstrated the isolation of 34 fungal species from the oil-spill contaminated soils from several major cities in India and analyzed their seasonal-based changes of survival. The type of habitat for the bacteria, which harbour different substrates, can be a significant factor for the presence of the extracellular enzymes (Figure 3(a)). These enzymes also can be a potential source for economic isolation of enzymes and also a potential target for the microbial degradation-based control of oil spillage decontamination.

In this study, oil-contaminated soil samples were collected from a spot in a night market at Bandar Putra Bertam, Penang, Malaysia (Figure 3(b)). Night market was chosen as the location for sample collection as it is a very common oil-contaminated location in Malaysia and for the readiness to access the location. Samples were cultured in enrichment medium (EM1) to promote the growth of lipolytic bacteria [10] and olive oil was used as the sole carbon source (a cheaper alternative to triolein as lipase inducer).

3.1. Identification of Lipase Producing Bacteria. Even though 16S rDNA-based sequencing is the pragmatic approach for bacterial identification, the traditional method of Gram staining and biochemical characterization does not only aid in the identification of bacteria, but also provides information on the extracellular secretions of the bacteria. Nine of the bacteria strains (strains 2–9) were identified as Gram-positive bacilli while the other one was identified as Gram-negative bacilli (strain 1) by Gram staining studies (Table 1). Based on the differentiation via Gram stains and biochemical characterization, the Gram-positive bacteria strains were identified as *Bacillus* spp. The growth observed on the surface of the blood agar also implied the presence of Gram-positive bacteria, in this case *Bacillus* spp. (Figure 4). For the extracellular secretion analysis, the bacteria samples were subjected to lipase assays on Tween-20 with olive oil as the substrate

(a) (b)

Figure 3: (a) Common oil spillage which may harbour certain bacteria that excrete extracellular enzymes. (b) Spot in the night market at Bandar Putra Bertam, Penang, Malaysia whereby soil sample was collected.

Table 1: Gram staining and biochemical characterization of the bacterial strains.

Strain	Gram staining	Catalase test	Coagulase test	Oxidase test	Motility test	Indole test	Citrase test	Urease test	Methyl red test	T.S.I test (slant and butt)
1	–	+	–	–	–	–	–	–	–	No changes
2	+	+	–	–	–	–	–	–	–	Acid
3	+	+	–	–	–	–	–	–	–	Acid
4	+	+	–	–	–	–	–	–	–	Acid
5	+	+	–	–	–	–	–	–	–	Acid
6	+	+	–	–	–	–	–	–	+	Acid
7	+	+	–	–	–	–	–	–	–	Acid
8	+	+	–	–	–	–	–	–	+	Acid
9	+	+	–	–	–	–	–	–	+	Acid
10	+	+	–	–	–	–	–	–	+	Acid

+, positive activity; –, no activity detected.

and phenol red agar as the indicator and protease assay by using gelatin agar plates.

3.2. Analyses of the Lipase Activity. Tween-20, a detergent that has fatty acids (C_{12}) with medium chain length, has been reported as a potential substrate for the assay of soil lipase activity [23, 25, 26]. The use of Tweens has been criticized due to the possibility of Tweens being hydrolyzed by esterases, thus resulting in false-positive results in the lipase-screening assay [27, 28]. However, Tweens are still favored as lipase substrates in screening assay due to their readiness to be incorporated into culture media and their ability to promote optimal contact between cells and/or enzymes and the substrate [28]. Seven out of the 10 bacterial strains selected showed visible precipitates on the colonies (Table 2) which could be an indication of lipolytic activity due to the release of the fatty acids from Tween-20 and their precipitation as the calcium salts [23, 26].

In order to further confirm the above determined lipolytic activity, phenol red method was carried out. Phenol red, or phenolsulfonphthalein (PSP), is a pH indicator dye that has an end point at pH 7.3-7.4 where the color is pink. When the pH gradually decreases to pH 7.0-7.1, this will result in yellow coloration. Singh et al. [24] proved that the use of phenol red in lipase assay was highly reproducible with sensitivity level as low as 0.5 p-nitrophenyl palmitate (p-NPP) enzyme units within 15 min. Therefore, by using olive oil as the lipidic substrates in phenol red agar, the presence of lipolysis activity

Table 2: Screening of bacteria strains for lipolytic enzyme activity.

Strain	Tween-20 agar	Phenol red agar	Gelatin agar
1	–	+	–
2	+	+	+
3	+	+	+
4	+	+	+
5	–	+	–
6	+	+	+
7	+	+	+
8	+	+	–
9	–	+	+
10	+	+	+

+, positive activity; –, no activity detected.

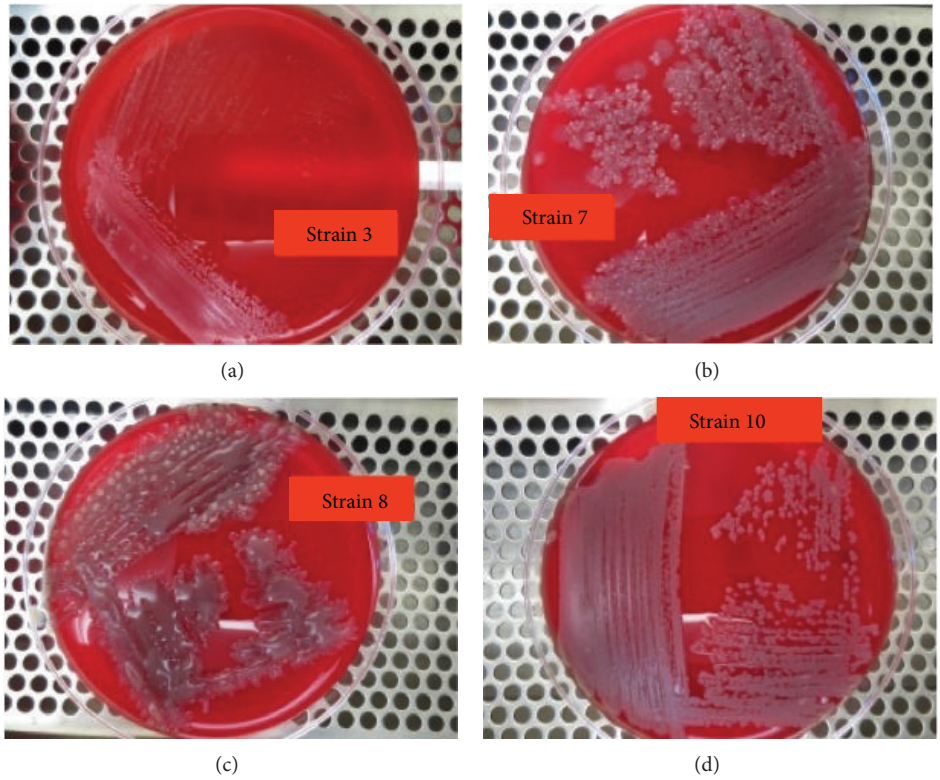


Figure 4: Blood agar culture of strains 3, 7, 8, and 10. Bacterial isolates grown overnight in the LB broth at 37°C are streaked onto blood agar plates (meat extract (1%), peptone (1%), sodium chloride (0.5%), and agar (1.5%)) and incubated further at 37°C overnight.

could be indicated by the yellow coloration. This assay is based on the principle where free fatty acids were released from the bacterial lipolysis reaction [24]. All the ten bacterial strains showed lipolytic activity based on the analysis (Table 2). Four strains, 3, 7, 8, and 10, were selected for comparative lipolytic analysis by subjecting to diameter measurement of the clear zones around the colonies. Negative control (*Escherichia coli*) showed no formation of clear zone, which implied that the zone of clearance formed is accounted for by the extracellular product excreted in the sample, which is the lipase enzyme. Positive control of the strain *Pseudomonas aeruginosa* showed a clear zone of lysis, which indicated the correct formulation of the media prepared (Figure 5).

3.3. Proteolytic Enzyme Assay Using Gelatin Agar. Besides lipases, protease, also one of the highest value commercial enzymes, has broad applications in food and pharmaceutical and detergent industries [29]. Identification of bacterial strains with both lipases and proteases producing ability could possibly meet up the industrial demand for new sources of lipases with different catalytic characteristics [9]. The zone of clearance formed around the colonies as a result from hydrolysis of gelatin indicated that the bacteria strains are positive for proteolytic activity (Figure 6). Addition of an aqueous saturated solution of ammonium sulfate on the surface of the agar results in the opaqueness of the agar with clearer zone formation around the colonies. All the strains except for strains 1, 5, and 8 manifested proteolytic activity (Table 2). Four strains, 3, 7, 8, and 10, were selected for comparative proteolytic analysis by subjecting to diameter measurement of the clear zones around the colonies. Out of the four bacterial strains, only strains 3, 7, and 10 showed proteolytic activity, substantiated by the formation of the clear zone around the colonies. No zone of clearance was observed with negative control, which indicates that the proteolytic activity of the sample is imparted by the protease enzyme. The positive control constituted by the strain *Pseudomonas aeruginosa* showed the formation of zone of clearance, validating the authenticity of the components present in the media (Figure 6).

3.4. Concomitant Secretion of Lipase and Protease by Bacillus spp. Is a Mean of Survival. As microbes are the good source of enzyme owing to their biochemical diversity, small space for cell cultivation, and the ease of the genetic manipulation of the enzymes for the production of new enzymes for application, bacteria are widely exploited for protein production. *Bacillus subtilis, B. amyloliquefaciens*, and *B. licheniformis* are widely exploited for the purpose of protein production due to the immense fermentation nature, very high product production, and the very low level of toxic by-product. *Bacillus* strains are also able to produce a large amount of the alkaline proteases which have high significant proteolysis activity and are stable at high pH and temperature [30].

This study has reported the secretion of lipase and protease by the *Bacillus* spp. From the measurement of

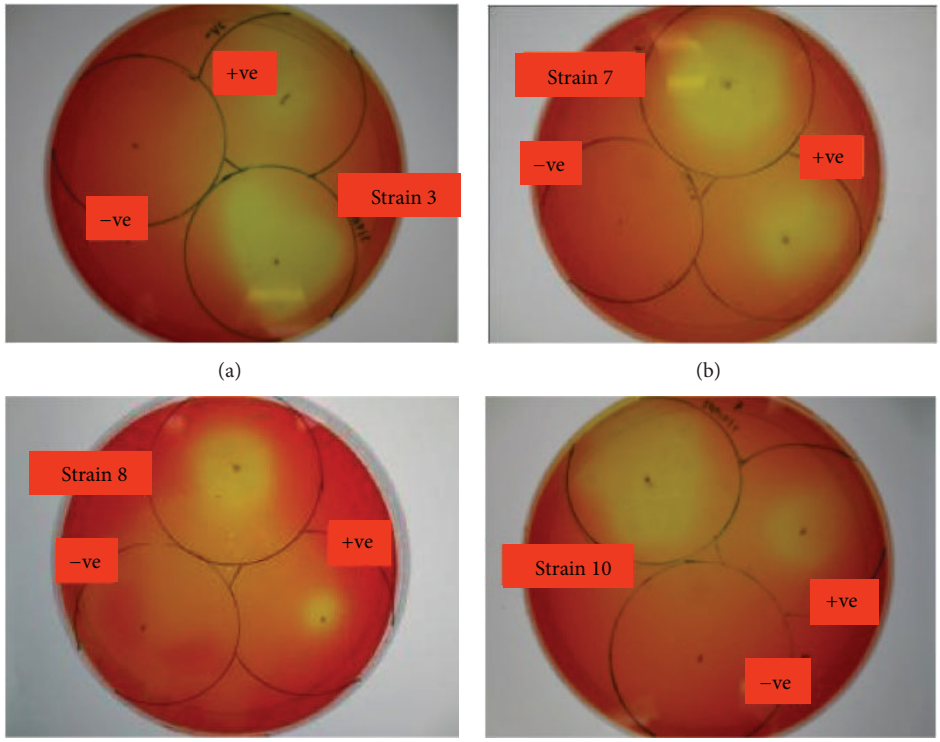


Figure 5: Screening of microbial lipases (strains 3, 7, 8, and 10) production on the agar plate containing olive oil as the substrate with phenol red as the pH indicator.

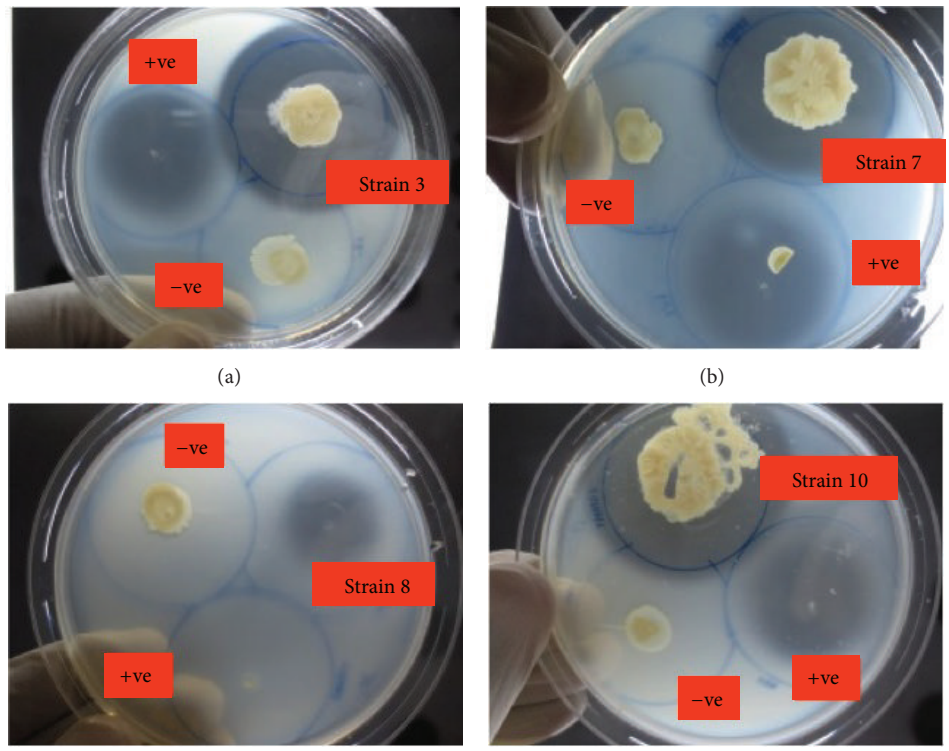


Figure 6: Proteolytic enzyme assay (strains 3, 7, 8, and 10) using gelatin agar. The overnight grown culture of the bacterial isolates is streaked onto gelatin agar (8% gelatin) and incubated at 37°C overnight.

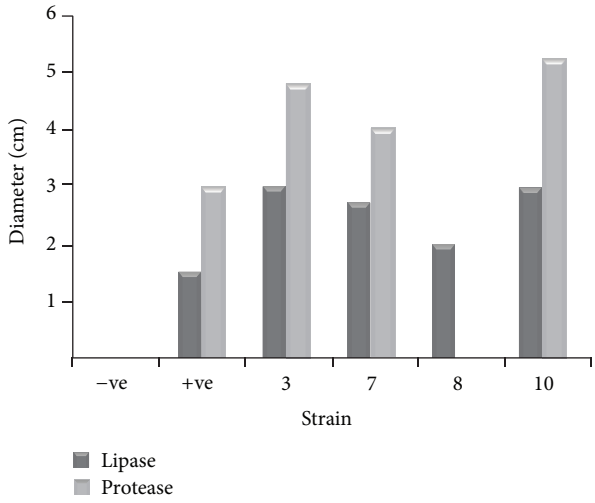


Figure 7: Measurement of zone of clearance of the lipolytic versus protease activity of strains 3, 7, 8, and 10. Serially diluted bacterial isolates grown overnight at 37°C were plated onto gelatin agar plate for proteolytic activity and phenol red agar for lipolytic activity.

the diameter of the zone of clearance, it was shown that the diameter of lipolytic activity of strain 3 has a value of 3 cm while that of protease activity has a value of 4.8 cm. On the other hand, for strain 7, the lipolytic activity exerted a zone of inhibition with the diameter value of 2.8 cm, while that of protease resulted in a value of 3.9 cm. Interestingly, strain 8 secreted no protease while the zone of clearance constituted by the lipase activity is 2 cm. Strain 10 has a value of 3 and 5.2 cm for lipolytic and protease activity, respectively. The size of the zone of inhibition is a direct indicator of the amount of enzymes excreted by the bacteria. Strain 10 has excreted the highest amount of both the lipase and protease, while strain 8 does not excrete protease (Figure 7).

The process of enzyme secretion is an energy-consuming regulatory process that is vital for the catalysis of the corresponding substrate. Moreover, the ability of the microorganism to secrete extracellular enzymes is a mean of survival, as an adaptation to the hostility of the environment. The presence of both lipid and protein in the soil has triggered the extracellular secretion of lipase and protease for the breakdown of these substrates. The accumulation of these substrates exerts pressure on the bacteria, which triggered the excretion of the enzymes. Beside substrates, other inducers for the excretion of these enzymes are the temperature, pH, sunlight, or other stress factors. These extracellular enzymes are located in the periplasmic space and are secreted depending on the sensing capacity of the microbes or quorum sensing that responds to the inducers.

3.5. Quorum Sensing Possibly Accounts for the Differential Secretions of the Extracellular Enzymes by the Bacillus spp. in the Oil-Contaminated Soil. Quorum sensing is a mechanism in bacteria whereby small molecules are excreted into the environment which aids in the adaptive response to a population [31]. The identified bacteria are Gram-positive bacteria that secrete extracellular enzymes and they are beneficial for the bacterial population [32–34]. The presence of any signalling molecules such as protein or lipid serves as the substrate or inducer for the increased secretion of both the lipase and the protease by the *Bacillus* spp. The lipid and protein molecules present in the soil initiate a mechanism that leads to the increased expression of both the lipase- and protease-encoding genes. In fact, most of the strains were reported to have secreted more protease than lipase. This could be due to the accumulated protein molecules in the soil, which are much higher than the lipid substrates. As a result, the expression of the protease-encoding genes is much more pronounced than the lipase-encoding genes. However, despite its survival, strain 8 does not secrete any protease, which is effectuated by the quorum sensing mechanism of the strain which provides less adaptive response to the presence of the substrate protein. This ignites interest to look further into the expression analysis of its protease-encoding genes, to unravel its mechanism of survival, which is unique compared to other strains.

In these findings, the excreted lipase and protease are potentially more stable than their corresponding intracellular enzymes with possible posttranslational modifications such as glycosylation and extra disulfide bond formation for increased temperature stability and higher pH resistance [35]. These enzymes also probably have enhanced stability via interaction with clay minerals, humic acid, or other compounds present in the soil [35, 36]. Hence, the oil-contaminated soil can be a promising resource of enzyme extraction, as the secreted enzymes have enhanced properties in response to the presence of the substrate in the soil. Most importantly, the isolated lipase-secreting *Bacillus* species can be a potential target strain towards the amelioration of the oil pollution.

Comprehension of the secretion and the function of the enzymes in soil are the crux of intense interest [37] which has taken an accelerating pace with the advancement of molecular and analytical techniques. As the environment is very hostile towards the stability of the enzymes, the enzymes secreted must be able to withstand the ever-present denaturation effect. Hence, this has stimulated more interest to understand the function and activity of the enzyme. A thorough insight into the function and properties of the extracellular enzymes also has many practical applications. The study shows that oil-contaminated soil is a potential location to isolate lipase producing microorganisms. With the tremendous potential of lipases in industrial applications, extensive and persistent screening for new sources of lipases with different catalytic characteristics is a matter of the utmost importance.

Environmental conditions may influence the production of the lipase and protease enzymes. Under certain conditions, the expression of the lipase- and protease-encoding genes is higher, which elevates the level of transcripts and subsequently more production of lipase/protease enzymes. Future work may focus on the identification or characterization of the regulatory elements such as transcriptional factors or small RNAs that influence the expression of the lipase-/protease-encoding genes. Identification and functional studies of these elements could render enhancement in the *in vitro* production of the recombinant lipase or protease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] S. C. B. Gopinath, P. Anbu, T. Lakshmipriya, and A. Hilda, "Strategies to characterize fungal lipases for applications in medicine and dairy industry," *BioMed Research International*, vol. 2013, Article ID 154549, 10 pages, 2013.

[2] K.-H. Baek, B.-D. Yoon, B.-H. Kim et al., "Monitoring of microbial diversity and activity during bioremediation of crude oil-contaminated soil with different treatments," *Journal of Microbiology and Biotechnology*, vol. 17, no. 1, pp. 67–73, 2007.

[3] J. P. Essien and E. D. Udosen, "Distribution of actinomycetes in oil contaminated ultisols of the Niger Delta (Nigeria)," *Journal of Environmental Sciences*, vol. 12, no. 3, pp. 296–302, 2000.

[4] V. Jensen, "Bacterial flora of soil after application of oily waste," *Oikos*, vol. 26, no. 2, pp. 152–158, 1975.

[5] C. Llanos and A. Kjoller, "Changes in the flora of soil fungi following oil waste application," *Oikos*, vol. 27, no. 3, pp. 377–382, 1976.

[6] Y. Pinholt, S. Struwe, and A. Kjøller, "Microbial changes during oil decomposition in soil," *Ecography*, vol. 2, no. 3, pp. 195–200, 1979.

[7] I. Saadoun, M. J. Mohammad, K. M. Hameed, and M. A. Shawaqfah, "Microbial populations of crude oil spill polluted soils at the Jordan-Iraq Desert (the Badia Region)," *Brazilian Journal of Microbiology*, vol. 39, no. 3, pp. 453–456, 2008.

[8] R. Sharma, Y. Chisti, and U. C. Banerjee, "Production, purification, characterization, and applications of lipases," *Biotechnology Advances*, vol. 19, no. 8, pp. 627–662, 2001.

[9] H. Treichel, D. de Oliveira, M. A. Mazutti, M. di Luccio, and J. V. Oliveira, "A review on microbial lipases production," *Food and Bioprocess Technology*, vol. 3, no. 2, pp. 182–196, 2010.

[10] R. N. Z. R. Abd Rahman, T. C. Leow, A. B. Salleh, and M. Basri, "*Geobacillus zalihae* sp. nov., a thermophilic lipolytic bacterium isolated from palm oil mill effluent in Malaysia," *BMC microbiology*, vol. 7, article 77, 2007.

[11] L. Casas-Godoy, S. Duquesne, F. Bordes, G. Sandoval, and A. Marty, "Lipases: an overview," *Methods in Molecular Biology*, vol. 861, pp. 3–30, 2012.

[12] S. Yapoudjian, M. G. Ivanova, A. M. Brzozowski et al., "Binding of Thermomyces (Humicola) lanuginosa lipase to the mixed micelles of cis-parinaric acid/NaTDC: fluorescence resonance energy transfer and crystallographic study," *European Journal of Biochemistry*, vol. 269, no. 6, pp. 1613–1621, 2002.

[13] J. Zdunek, G. V. Martinez, J. Schleucher et al., "Global structure and dynamics of human apolipoprotein CII in complex with micelles: evidence for increased mobility of the helix involved in the activation of lipoprotein lipase," *Biochemistry*, vol. 42, no. 7, pp. 1872–1889, 2003.

[14] F. Hasan, A. A. Shah, and A. Hameed, "Industrial applications of microbial lipases," *Enzyme and Microbial Technology*, vol. 39, no. 2, pp. 235–251, 2006.

[15] A. Houde, A. Kademi, and D. Leblanc, "Lipases and their industrial applications: An overview," *Applied Biochemistry and Biotechnology—Part A Enzyme Engineering and Biotechnology*, vol. 118, no. 1–3, pp. 155–170, 2004.

[16] A. Pandey, S. Benjamin, C. R. Soccol, P. Nigam, N. Krieger, and V. T. Soccol, "The realm of microbial lipases in biotechnology," *Biotechnology and Applied Biochemistry*, vol. 29, part 2, pp. 119–131, 1999.

[17] S. Nagarajan, "New tools for exploring old friends-microbial lipases," *Applied Biochemistry and Biotechnology*, vol. 168, no. 5, pp. 1163–1196, 2012.

[18] R. K. Saxena, A. Sheoran, B. Giri, and W. S. Davidson, "Purification strategies for microbial lipases," *Journal of Microbiological Methods*, vol. 52, no. 1, pp. 1–18, 2003.

[19] F. D. Gunstone, "Enzymes as biocatalysts in the modification of natural lipids," *Journal of the Science of Food and Agriculture*, vol. 79, no. 12, pp. 1535–1549, 1999.

[20] M. Sánchez, N. Prim, F. Rández-Gil, F. I. J. Pastor, and P. Diaz, "Engineering of baker's yeasts, *E. coli* and *Bacillus* hosts for the production of *Bacillus subtilis* lipase A," *Biotechnology and Bioengineering*, vol. 78, no. 3, pp. 339–345, 2002.

[21] R. Kanchana, U. D. Muraleedharan, and S. Raghukumar, "Alkaline lipase activity from the marine protists, thraustochytrids," *World Journal of Microbiology and Biotechnology*, vol. 27, no. 9, pp. 2125–2131, 2011.

[22] O. Redondo, A. Herrero, J. F. Bello et al., "Comparative kinetic study of lipases A and B from *Candida rugosa* in the hydrolysis of lipid p-nitrophenyl esters in mixed micelles with Triton X-100," *Biochimica et Biophysica Acta*, vol. 1243, no. 1, pp. 15–24, 1995.

[23] S. C. B. Gopinath, P. Anbu, and A. Hilda, "Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments," *Mycoscience*, vol. 46, no. 2, pp. 119–126, 2005.

[24] R. Singh, N. Gupta, V. K. Goswami, and R. Gupta, "A simple activity staining protocol for lipases and esterases," *Applied Microbiology and Biotechnology*, vol. 70, no. 6, pp. 679–682, 2006.

[25] Y. Sakai, M. Hayatsu, and K. Hayano, "Use of tween 20 as a substrate for assay of lipase activity in soils," *Soil Science and Plant Nutrition*, vol. 48, no. 5, pp. 729–734, 2002.

[26] R. G. von Tigerstrom and S. Stelmaschuk, "The use of Tween 20 in a sensitive turbidimetric assay of lipolytic enzymes," *Canadian Journal of Microbiology*, vol. 35, no. 4, pp. 511–514, 1989.

[27] R. G. Jensen, "Detection and determination of lipase (acylglycerol hydrolase) activity from various sources," *Lipids*, vol. 18, no. 9, pp. 650–657, 1983.

[28] C. A. Thomson, P. J. Delaquis, and G. Mazza, "Detection and measurement of microbial lipase activity: a review," *Critical Reviews in Food Science and Nutrition*, vol. 39, no. 2, pp. 165–187, 1999.

[29] A. B. Vermelho, M. N. L. Meirelles, A. Lopes, S. D. G. Petinate, A. A. Chaia, and M. H. Branquinha, "Detection of extracellular proteases from microorganisms on agar plates," *Memorias do Instituto Oswaldo Cruz*, vol. 91, no. 6, pp. 755–760, 1996.

[30] T. Kuberan, S. Sangaralingam, and V. Thirumalai Arasu, "Isolation and optimization of protease producing bacteria from Halophilic soil," *Journal of Biosciences Research*, vol. 1, no. 3, pp. 163–174, 2010.

[31] J. M. Henke and B. L. Bassler, "Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*," *Journal of Bacteriology*, vol. 186, no. 12, pp. 3794–3805, 2004.

[32] S. P. Diggle, A. S. Griffin, G. S. Campbell, and S. A. West, "Cooperation and conflict in quorum-sensing bacterial populations," *Nature*, vol. 450, no. 7168, pp. 411–414, 2007.

[33] S. A. West, A. S. Griffin, and A. Gardner, "Evolutionary explanations for cooperation," *Current Biology*, vol. 17, no. 16, pp. R661–R672, 2007.

[34] S. A. West, A. S. Griffin, A. Gardner, and S. P. Diggle, "Social evolution theory for microorganisms," *Nature Reviews Microbiology*, vol. 4, no. 8, pp. 597–607, 2006.

[35] S. D. Allison, "Soil minerals and humic acids alter enzyme stability: implications for ecosystem processes," *Biogeochemistry*, vol. 81, no. 3, pp. 361–373, 2006.

[36] H. Quiquampoix and R. G. Burns, "Interactions between proteins and soil mineral surfaces: environmental and health consequences," *Elements*, vol. 3, no. 6, pp. 401–406, 2007.

[37] M. D. Wallenstein and M. N. Weintraub, "Emerging tools for measuring and modeling the in situ activity of soil extracellular enzymes," *Soil Biology and Biochemistry*, vol. 40, no. 9, pp. 2098–2106, 2008.

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Research Article

Optimization of Culture Conditions for Production of the Anti-Leukemic Glutaminase Free L-Asparaginase by Newly Isolated *Streptomyces olivaceus* NEAE-119 Using Response Surface Methodology

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Among the antitumor drugs, bacterial enzyme L-asparaginase has been employed as the most effective chemotherapeutic agent in pediatric oncotherapy especially for acute lymphoblastic leukemia. Glutaminase free L-asparaginase producing actinomycetes were isolated from soil samples collected from Egypt. Among them, a potential culture, strain NEAE-119, was selected and identified on the basis of morphological, cultural, physiological, and biochemical properties together with 16S rRNA sequence as *Streptomyces olivaceus* NEAE-119 and sequencing product (1509 bp) was deposited in the GenBank database under accession number KJ200342. The optimization of different process parameters for L-asparaginase production by *Streptomyces olivaceus* NEAE-119 using Plackett-Burman experimental design and response surface methodology was carried out. Fifteen variables (temperature, pH, incubation time, inoculum size, inoculum age, agitation speed, dextrose, starch, L-asparagine, KNO_3, yeast extract, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, NaCl, and $FeSO_4{\cdot}7H_2O$) were screened using Plackett-Burman experimental design. The most positive significant independent variables affecting enzyme production (temperature, inoculum age, and agitation speed) were further optimized by the face-centered central composite design-response surface methodology.

1. Introduction

L-asparaginase (L-asparagine aminohydrolase, EC 3.5.1.1) is an important enzyme as therapeutic agents used in combination therapy with other drugs in the treatment of acute lymphoblastic leukemia in children, Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarcoma, and melanosarcoma [1, 2]. L-asparaginase has an antioxidant property [3]. It is also used in food industry as a food processing aid; it can effectively reduce the level of acrylamide up to 90% in a range of starchy fried foods without changing the taste and appearance of the end product [4].

Notwithstanding its high therapeutic efficacy, the therapeutic use of L-asparaginase by the patients exerts toxicity to normal cells which in turn causes the unpleasant side effects to the patients. L-asparaginase administration has been limited by a high rate of hypersensitivity in the long-term use [5] and development of anti-asparaginase antibodies, which causes an anaphylactic shock or neutralization of the drug effect. To overcome these limitations, modified versions of L-asparaginase (such as L-asparaginase from other new sources, pegylated formulations, and L-asparaginase loaded into erythrocytes) have been recently proposed [6]. The L-asparaginases of *Erwinia chrysanthemi* and *E. coli* have been employed for many years as effective drugs in the treatment

of acute lymphoblastic leukaemia and leukaemia lymphosarcoma [7], but their therapeutic response rarely occurs without some evidence of toxicity [8], suggesting the need to discover new L-asparaginases that are serologically different but have similar therapeutic effects. Therefore there is a continuing need to screen newer organisms in order to obtain strains capable of producing new and high yield of L-asparaginase with less adverse effects [9].

The toxicity of L-asparaginases is partially attributable to the glutaminase activity of these enzymes [10]. L-glutamine is required for several metabolic pathways including the formation of L-asparagine by the enzyme L-asparagine synthetase [11]. The L-glutaminase activity may cause such a reduction in glutamine in the body that it limits the tolerable therapeutic dose. Nowadays most of the research is focused on production of glutaminase free L-asparaginase by using microbial systems. L-asparaginases with high specificity for L-asparagine and low-to-negligible activity against L-glutamine are reported to be less troublesome during the course of anticancer therapy [12].

Microorganisms like bacteria, fungi, yeast, actinomycetes, and algae are very efficient producers and the better source of L-asparaginase, because they can be cultured easily and the extraction and the purification of L-asparaginase from them are also convenient, facilitating the large scale production [13].

Most of the microbial L-asparaginase is intracellular in nature except few which are secreted outside the cell [14]. Extracellular L-asparaginase is more advantageous than intracellular type because of higher accumulation of enzyme in culture broth under normal conditions, easy extraction, and downstream processing [15]; the extracellular L-asparaginase in bacteria is protease deficient and the liberated protein exported to the medium is mostly soluble and biologically active and has an authentic N-terminus, relatively free from endotoxins that lead to the minimization of adverse effects. Secretion also facilitates proper folding of proteins specially that requiring disulfide bridge formation, as it passes through a more favorable redox potential in the periplasmic space [16].

Production of L-asparaginase is greatly influenced by fermentation medium composition and culture conditions such as temperature, pH, inoculum size, agitation rate, and incubation time [17]. Statistical experimental designs have been used for many decades by several researchers in biotechnology for an optimization strategy [18–21] and can be adopted on several steps, the first step is to screen the important parameters and the second step is to optimize those parameters [22]. These have several advantages that included less experiment numbers, suitability for multiple factor experiments, search for relativity between factors, and finding of the most suitable conditions and forecast response [23]. Response surface methodology (RSM) is an efficient strategic experimental tool by which the optimal conditions of a multivariable system can be determined.

In the present study, strain NEAE-119 was identified as *Streptomyces olivaceus* strain NEAE-119. A statistical approach has been employed for which a Plackett-Burman design is used for identifying significant variables influencing glutaminase free L-asparaginase production by *Streptomyces olivaceus* NEAE-119. The levels of the positive significant variables were further optimized using face-centered central composite design.

2. Materials and Methods

2.1. Microorganisms and Cultural Conditions. Actinomycete strains used in this study were isolated from various soil samples collected from different localities in Egypt. Actinomycetes from the soils had been isolated using standard dilution plate method procedure on Petri plates containing starch nitrate agar medium of the following composition (g/L): starch, 20; KNO_3, 2; K_2HPO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.5; NaCl, 0.5; $CaCO_3$, 3; $FeSO_4{\cdot}7H_2O$, 0.01; agar, 20, and distilled water up to 1 L; then plates were incubated for a period of 7 days at 30°C. *Streptomyces* isolates were purified and maintained as spore suspensions in 20% (v/v) glycerol at −20°C for subsequent investigation.

2.2. Screening of L-Asparaginase Production by Plate Assay. It is generally observed that L-asparaginase production is accompanied by an increase in pH of the culture filtrates [24]. The plate assay was based on Gulati et al. [25] method with the incorporation of pH indicator phenol red (prepared in ethanol) in medium containing L-asparagine (sole nitrogen source). Phenol red at acidic pH is yellow and at alkaline pH turns pink; thus a pink zone is formed around microbial colonies producing L-asparaginase. Screening of potential L-asparaginase producing actinomycetes was carried out with the use of asparagine dextrose salts agar (ADS agar) (g/L: asparagine 10, dextrose 2, K_2HPO_4 1, $MgSO_4$ 0.5, and agar 20); pH was adjusted to 6.8 and supplemented with phenol red as a pH indicator (0.009% final concentration) [25] and sterilized at 1.5 atmospheric pressure for 20 min. Inoculated plates were incubated at 30°C for 7 days. Plates were examined for change in color of medium from yellowish to pink due to change of pH indicating the positive asparaginase activity. Colonies with pink zones were considered as L-asparaginase producing strains. Isolates exhibiting L-asparaginase activity were selected for further study. Control plates were prepared as inoculated medium without dye and uninoculated medium with dye.

2.3. Agar Well Diffusion Technique. L-asparaginase-producing strains were selected for subsequent screening under submerged fermentation conditions. Fifty mL of asparagine dextrose salts broth medium were dispensed in 250 mL Erlenmeyer conical flasks, sterilized, inoculated, and incubated at 30°C for 5 days in a rotatory incubating shaker at 150 rpm. After the incubation time, the mycelium of each isolate was collected by centrifugation at 6000 rpm for 20 min. 100 μL of cell free culture broth was poured into the agar well of diameter 8 mm prepared in plates containing asparagine dextrose salts agar medium supplemented with phenol red. The filtrate was allowed to diffuse into the medium for 12 hours at 4°C. The diameter of zone (mm) of L-asparaginase activity, as indicated by the formation of pink colored zone around

the well against the yellow background, was measured. For further studies, cultures showing greater enzyme production were selected.

2.4. Inoculum Preparation. 250 mL Erlenmeyer flasks containing 50 mL of asparagine dextrose salts broth (g/L: asparagine 10, dextrose 2, K_2HPO_4 1, and $MgSO_4$ 0.5) were inoculated with three disks of 8 mm diameter taken from the 7-day-old stock culture grown on starch nitrate agar medium. The flasks were incubated for 48–72 h in a rotatory incubator shaker at 30°C and 150 rpm and were used as inoculum for subsequent experiments.

2.5. Production of L-Asparaginase by Submerged Fermentation. The selected strain was cultured in fifty mL of asparagine dextrose salts broth medium (at a specified pH) dispensed in 250 mL Erlenmeyer conical flasks. The inoculated flasks were incubated on a rotatory incubator shaker at 30–37°C with shaking at 100–200 rpm. After the specified incubation time for each set of experimental trials, the mycelium of the tested isolate was collected by centrifugation at 5000 g for 20 min at 4°C.

2.6. Assay of L-Asparaginase Activity. L-asparaginase activity was determined by measuring the amount of ammonia formed by nesslerization [26]. The reaction mixture contains 1.5 mL of 0.04 M L-asparagine prepared in 0.05 M Tris-HCl buffer, pH 8.6, and 0.5 mL of an enzyme to make up the total volume to 2 mL. The tubes were incubated at 37°C for 30 minutes. The reaction was stopped by adding 0.5 mL of 1.5 M Trichloroacetic acid (TCA). The blank was prepared by adding enzyme after the addition of TCA. The precipitated protein was removed by centrifugation at 10,000 g for 5 min and the liberated ammonia in the supernatant was determined colorimetrically by direct nesslerization by adding 1 mL Nessler's reagent into tubes containing 0.5 mL of clear supernatant and 7 mL of distilled water and incubated at room temperature for 20 min. A yellow coloration indicates the presence of ammonia: at higher concentrations, a brown precipitate may form. The yellow color was read using a UV-visible spectrophotometer (Optizen Pop—UV/Vis spectrophotometer) at 480 nm. The amount of ammonia liberated was calculated using ammonium chloride standard curve. One unit (U) of L-asparaginase is defined as the amount of enzyme which catalyzed the formation of 1 μmole of ammonia from L-asparagine per minute under the standard assay conditions.

2.7. Assay of L-Glutaminase. L-Glutaminase activity was determined using L-glutamine as substrate and the product ammonia, released during the catalysis, was measured by using Nessler's reagent. L-glutaminase was assayed according to Imada et al. [27].

2.8. Morphology and Cultural Characteristics. The spore chain morphology, the spore surface ornamentation, and spore size of strain NEAE-119 were examined on starch nitrate agar medium after 14 days at 30°C. The gold-coated dehydrated specimen was examined with Analytical Scanning Electron Microscope Jeol JSM-6360 LA operating at 20 Kv at the Central Laboratory, City of Scientific Research and Technological Applications, Alexandria, Egypt. Aerial spore-mass color, substrate mycelial pigmentation, and the production of diffusible pigments were observed on ISP media (1–7) as described by Shirling and Gottlieb [28].

2.9. Chemotaxonomy and Physiological Characteristics. Sugars and diaminopimelic acid (DAP) isomers were identified by the method described by Staneck and Roberts [29]. Physiological characteristics were performed following the methods of Shirling and Gottlieb [28]. The ability of the organism to inhibit the growth of several bacterial, yeast, and fungal strains was determined: three bacterial strains (*Staphylococcus aureus* A9897, *Pseudomonas aeruginosa* T9934, and *Klebsiella pneumonia* A9898) isolated from various clinical specimens and kindly provided by Infection Control Unit, Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University, Mansoura, Egypt and two bacterial strains belonging to the Culture Collection of NRRL: Gram-positive (*Bacillus subtilis* NRRL B-543) and Gram-negative (*Escherichia coli* NRRL B-210), yeast (*Candida albicans* NRRL Y-477), and five fungal strains (*Rhizoctonia solani, Fusarium oxysporum, Alternaria solani, Bipolaris oryzae,* and *Aspergillus niger*) kindly provided by Plant Pathology Department, Faculty of Agriculture, Mansoura University, Egypt. Some additional tests can be considered to be useful in completing the description of a strain or species, even if they are not very significant or indicative on their own. The ability of strain NEAE-119 to produce uricase [30] and asparaginase activity [25] was tested.

2.10. 16S rRNA Sequencing. The preparation of genomic DNA of the strain was performed according the method described by Sambrook et al. [31]. The PCR reaction was performed according to the method of El-Naggar et al. [32].

2.11. Sequence Alignment and Phylogenetic Analysis. The complete 16S rRNA gene sequence (1509 bp) of strain NEAE-119 was aligned with the corresponding 16S rRNA sequences of the type strains of representative members of the genus *Streptomyces* retrieved from the GenBank, EMBL, DDBJ, and PDB databases by using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [33] and the software package MEGA4 version 2.1 [34] was used for multiple alignment and phylogenetic analysis. The phylogenetic tree was constructed via the bootstrap test of neighbor-joining algorithm [35] based on the 16S rRNA gene sequences of strain NEAE-119 and related organisms.

2.12. Selection of Significant Variables by Plackett-Burman Design. The Plackett-Burman statistical experimental design [36] is a two factorial design, which identifies the critical physicochemical parameters required for elevated production, and is very useful for screening the most important factors with respect to their main effects [37]. This model does not describe interaction among factors and it is used

Table 1: Experimental independent variables at two levels used for the production of L-asparaginase by *Streptomyces* sp. strain NEAE-119 using Plackett-Burman design.

Code	Variables	Levels	
		−1	+1
X_1	Temperature (°C)	30	35
X_2	pH	7	9
X_3	Incubation time (days)	5	7
X_4	Inoculum size (%, v/v)	2	4
X_5	Inoculum age (h)	24	36
X_6	Agitation speed (rpm)	100	150
X_7	Dextrose (g/L)	2	4
X_8	Starch (g/L)	10	20
X_9	L-asparagine (g/L)	5	10
X_{10}	KNO_3 (g/L)	1	3
X_{11}	Yeast extract (g/L)	0	1
X_{12}	K_2HPO_4 (g/L)	1	2
X_{13}	$MgSO_4 \cdot 7H_2O$ (g/L)	0.1	0.5
X_{14}	NaCl (g/L)	0.1	0.5
X_{15}	$FeSO_4 \cdot 7H_2O$ (g/L)	0	0.01

to screen and evaluate the important factors that influence the response. The total number of experiments to be carried out according to Plackett-Burman is $n + 1$, where n is the number of variables. Sixteen different independent variables including temperature, pH, incubation time, inoculum size, inoculum age, agitation speed, dextrose, starch, L-asparagine, KNO_3, yeast extract, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, NaCl, and $FeSO_4 \cdot 7H_2O$ were screened in Plackett-Burman experimental design. Each variable is represented at two levels, high and low, denoted by (+) and (−), respectively (Table 1). Plackett-Burman experimental design is based on the first order model:

$$Y = \beta_0 + \sum \beta_i X_i, \tag{1}$$

where Y is the response or dependent variable (L-asparaginase activity) and it will always be the variable we aim to predict, β_0 is the model intercept and β_i is the linear coefficient, and X_i is the level of the independent variables; it is the variables that will help us to explain L-asparaginase activity. All trials were performed in duplicate and the average of L-asparaginase activity was treated as responses.

2.13. Face-Centered Central Composite Design (FCCD). This step involved optimization of the levels and the interaction effects between various significant variables which exerted a positive effect on the L-asparaginase activity by using face-centered central composite design (FCCD). FCCD is an effective design that is used for sequential experimentation and provides reasonable amount of information for testing the goodness of fit and does not require large number of design points thereby reducing the overall cost associated with the experiment [38]. In this study, the experimental plan consisted of 20 trials and the independent variables were studied at three different levels, low (−1), middle (0), and high (+1). The center point was repeated six times in order to evaluate the curvature and the experiment replication facilitated the pure error estimation, so that the significant lack of fit of the models could be predicted. All the experiments were done in duplicate and the average of L-asparaginase activity obtained was taken as the dependent variable or response (Y). The experimental results of FCCD were fitted via the response surface regression procedure using the following second order polynomial equation:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j, \tag{2}$$

in which Y is the predicted response, β_0 is the regression coefficients, β_i is the linear coefficient, β_{ii} is the quadratic coefficients, β_{ij} is the interaction coefficients, and X_i is the coded levels of independent variables. However, in this study, the independent variables were coded as X_1, X_5, and X_6. Thus, the second order polynomial equation can be presented as follows:

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 x_1 + \beta_5 x_5 + \beta_6 x_6 + \beta_{15} x_1 x_5 + \beta_{16} x_1 x_6 \\ & + \beta_{56} x_5 x_6 + \beta_{11} x_1^2 + \beta_{55} x_5^2 + \beta_{66} x_6^2. \end{aligned} \tag{3}$$

2.14. Statistical Analysis. The experimental data obtained was subjected to multiple linear regressions using Microsoft Excel 2007. The P values were used as a tool to check the significance of the interaction effects, which in turn may indicate the patterns of the interactions among the variables [39]. The statistical software package, STATISTICA software (Version 8.0, StatSoft Inc., Tulsa, USA), was used to plot the three-dimensional surface plots.

3. Results and Discussion

L-asparaginase activity of *Streptomyces* sp. NEAE-119 was detected by plate assay. Production of the enzyme was indicated by color change in the medium from yellow to pink zone surrounding the colony (Figure 1). L-asparaginase activity was confirmed by agar-well diffusion technique. The potential culture, strain NEAE-119, was identified on the basis of morphological, cultural, physiological, and chemotaxonomic properties, together with 16S rRNA sequence as *Streptomyces olivaceus* strain NEAE-119.

3.1. Morphology and Cultural Characteristics of Isolate Number NEAE-119. Morphological observation of the 14-day-old culture of strain NEAE-119 grown on yeast extract-malt extract agar (ISP 2) [28] revealed that strain NEAE-119 had

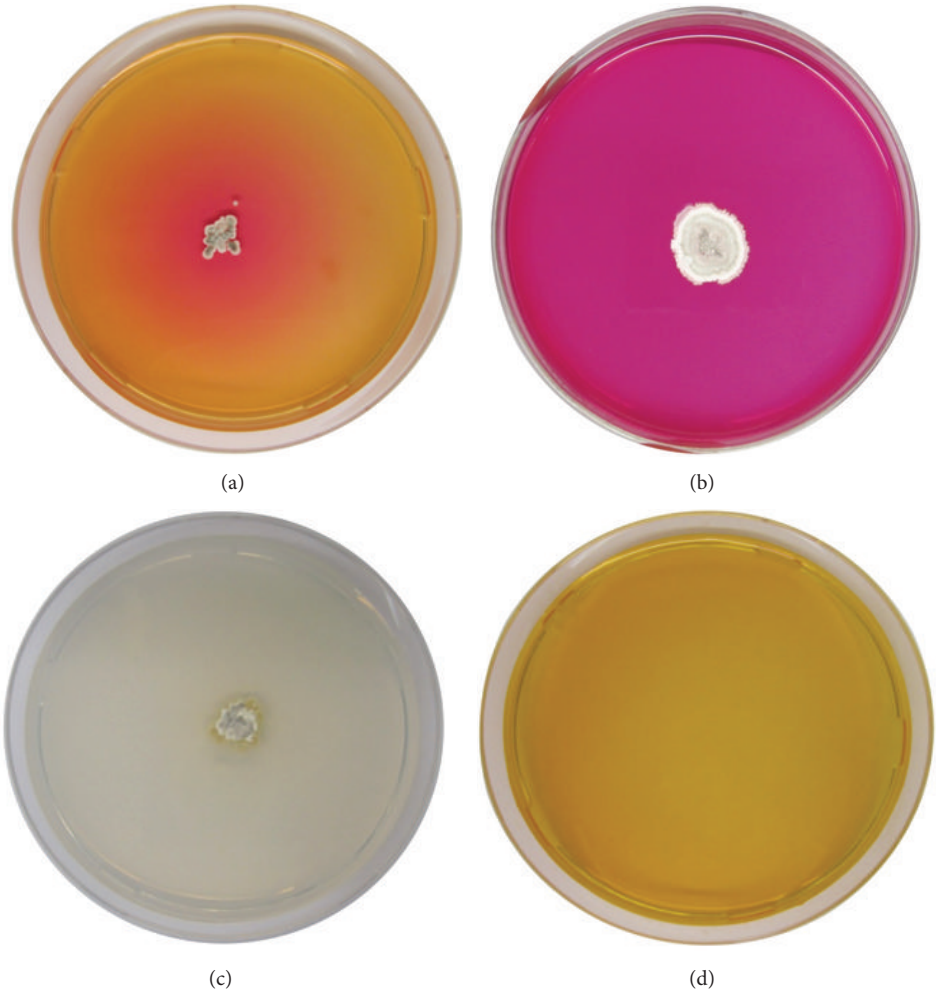
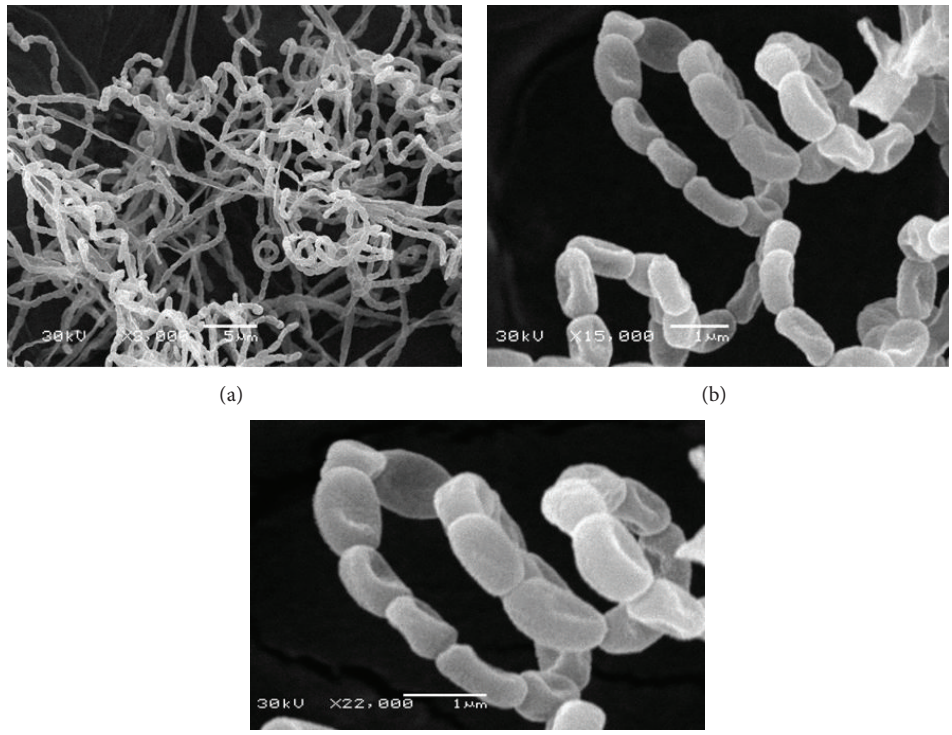


Figure 1: L-asparaginase activity of *Streptomyces* sp. NEAE-119 detected by plate assay. (a, b) Production of the enzyme indicated by color change in the medium from yellow to pink zone surrounding the colony after two and five days, respectively. (c) Control plates were prepared as inoculated medium without dye. (d) Uninoculated medium with dye.

the typical characteristics of the genus *Streptomyces* [40]; it is aerobic and mesophilic; both aerial and vegetative hyphae were abundant, well-developed, and not fragmented. Aerial mycelium color was varied from the grey color to greyish beige or whitish grey on different test media. Cultural characteristics of strain NEAE-119 are shown in the table in the Supplementary Materials available online at http://dx.doi.org/10.1155/2015/627031. Strain NEAE-119 grew well on yeast extract-malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), inorganic salt-starch agar (ISP medium 4), glycerol-asparagine agar (ISP medium 5), peptone-yeast extract iron agar (ISP medium 6), and tyrosine agar (ISP medium 7). Verticils are not present. The mycelium does not fragment. It formed an extensively branched substrate mycelium and aerial hyphae which differentiated into spore chains. Spore chains with many spores were in section *Spirals*, with open spirals intergrading through flexuous spore chains suggestive of section *Rectiflexibiles*. Mature spore chains are generally long, often with more than 50 spores per chain. This morphology is seen on starch nitrate agar medium. Spore surface is smooth (0.55–0.90 × 1.16–1.34 μm in diameter) (Figure 2).

3.2. Physiological and Biochemical Characteristics. The physiological characteristics of strain NEAE-119 are shown in Table 2. Strain NEAE-119 grew well on yeast extract-malt extract agar (ISP medium 2). The substrate hyphae are yellowish grey; substrate mycelium pigment is not a pH indicator. No pigment was found in medium in yeast extract-malt extract agar. Melanoid pigments were not formed in peptone-yeast-iron agar and tyrosine agar. Starch hydrolysis, lecithinase activity, milk coagulation and peptonization, growth on cellulose, and nitrate reduction were positive. Gelatin liquification, melanin production, and hydrogen sulphide production were negative. α-amylase, cellulase, uricase, chitosanase, and asparaginase are produced while protease is not produced. D-fructose, D-xylose, D-galactose, D-Glucose, L-arabinose, ribose, D-mannose, sucrose, maltose, rhamnose, cellulose, and trehalose are utilized for growth.



(a) (b)

(c)

Figure 2: Scanning electron micrograph showing the spore chain morphology and spore surface ornamentation of strain NEAE-119 grown on starch nitrate agar medium for 14 days at 30°C at magnification of 3000x (a), 15000x (b), and 22000x (c).

It exhibited antimicrobial activity against *Staphylococcus aureus, Alternaria solani,* and *Bipolaris oryzae*. It exhibited no antimicrobial activity against *Candida albicans, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Rhizoctonia solani, Fusarium oxysporum,* and *Aspergillus niger*. The optimal growth temperature of strain NEAE-119 was 30°C and optimal pH was 7.0. Data for reference species (*Streptomyces olivaceus*) were taken from Bergey's Manual of Systematic Bacteriology: Volume 5: the Actinobacteria [40].

Chemotaxonomic tests showed that the cell wall contained LL-diaminopimelic acid in whole-organism hydrolysates, indicating that it was of cell-wall type I. The whole-cell hydrolysates contained mainly mannose and arabinose. On the basis of morphological, cultural, and chemotaxonomic properties, together with the physiological properties of strain NEAE-119 shown in Table 2, it is evident that strain NEAE-119 belongs to the genus *Streptomyces*.

3.3. 16S rRNA Gene Sequence Comparisons and Phylogenetic Analysis. The 16S rRNA gene sequence (1509 bp) was determined for strain NEAE-119. A BLAST search [33] of the GenBank database using this sequence showed its similarity to that of many species of the genus *Streptomyces*. A phylogenetic tree (Figure 3) based on 16S rRNA gene sequences of members of the genus *Streptomyces* was constructed according to the bootstrap test of neighbor-joining algorithm method of Saitou and Nei [35] with MEGA4 [34]. This tree shows the close phylogenetic association of strain NEAE-119 with certain other *Streptomyces* species. Phylogenetic analysis indicated that the strain NEAE-119 consistently falls into a clade together with *Streptomyces enissocaesilis* strain ACCA1 (GenBank/EMBL/DDBJ accession number JX042471.1, 99% sequence similarity), *Streptomyces plicatus* strain RT-57 (GenBank/EMBL/DDBJ accession number HQ909761.1, 99% sequence similarity), and *Streptomyces olivaceus* strain RT-54 (GenBank/EMBL/DDBJ accession number HQ909759.1, 99% sequence similarity). On the basis of the collected data and in view of the comparative study of the recorded properties of isolate number NEAE-119 in relation to the closest related species of the genus *Streptomyces*, it is most closely related to the type strains of *Streptomyces olivaceus* strain RT-54 (GenBank/EMBL/DDBJ accession number HQ909759.1) (99% sequence similarity). Therefore, this strain was identified as *Streptomyces olivaceus* strain NEAE-119 and its sequencing product was deposited in the GenBank database under accession number KJ200342.

3.4. Evaluation of Variables Affecting L-Asparaginase Activity Using Plackett-Burman Design. Sixteen different independent (assigned) variables including temperature, pH, incubation time, inoculum size, inoculum age, agitation speed, dextrose, starch, L-asparagine, KNO_3, yeast extract, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, NaCl, and $FeSO_4 \cdot 7H_2O$ and four unassigned variables (commonly referred to as dummy variables) were screened in Plackett-Burman experimental design of 20 trials

Table 2: Physiological and biochemical (phenotypic) characteristics of *Streptomyces* sp. strain NEAE-119. Data for reference species (*Streptomyces olivaceus*) were taken from Bergey's Manual of Systematic Bacteriology: Volume 5: the Actinobacteria [40].

Characteristic	*Streptomyces* sp. strain NEAE-119	*Streptomyces olivaceus*
Aerial mycelium on ISP medium 2	Grey	Grey
Substrate mycelium on ISP medium 2	Yellowish grey; substrate pigment is not a pH indicator	Greyed yellow; substrate pigment is not a pH indicator
Production of diffusible pigment	No diffusible pigment	
Spore chain morphology	Spirals, with open spirals intergrading through flexuous spore chains suggestive of section Rectiflexibiles	Spirals, with open spirals intergrading through flexuous spore chains suggestive of section Rectiflexibiles.
Spore surface	Smooth	Smooth
Spore shape	Spherical or oval to ellipsoidal	
Melanin production on peptone-yeast extract iron agar (ISP 6 medium)	−	−
Melanin production on tyrosine agar (ISP 7 medium)	−	−
Melanin production on tryptone-yeast extract broth (ISP 1 medium)	−	
Max NaCl tolerance (%, w/v)	8	
Growth on sole carbon source (1%, w/v)		
D(−) Fructose	+	+
D(+) Xylose	+	+
D(+) Galactose	+	+
D(+) Glucose	+	+
L-arabinose	+	+
Ribose	+	+
D(+) Mannose	+	+
Sucrose	+	+
Maltose	+	+
Rhamnose	+	+
Cellulose	+	+
Trehalose	+	+
Enzymes		
Lecithinase activity	+	
α-amylase (starch hydrolysis)	+	
Protease	−	
Cellulase (growth on cellulose)	+	
Uricase	+	
Chitosanase	+	
Asparaginase	+	
Reduction of nitrates to nitrite	+	
Coagulation of milk	+	
Peptonization of milk	+	
Antimicrobial activities against		
Candida albicans, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Rhizoctonia solani, Fusarium oxysporum, Aspergillus niger.	−	
Staphylococcus aureus, Alternaria solani, Bipolaris oryzae.	+	

+: positive; −: negative; blank cells: no data available. Growth temperature range (°C): 25–40, growth at pH 5–9.

Table 3: Twenty-trial Plackett-Burman experimental design for evaluation of fifteen independent variables with coded values along with the observed L-asparaginase activity.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	Dummy$_1$	Dummy$_2$	Dummy$_3$	Dummy$_4$	L-asparaginase activity (U/mL)
1	1	−1	1	−1	−1	−1	−1	1	1	−1	1	1	−1	−1	1	1	1	1	−1	9.793
2	−1	1	1	−1	1	1	−1	−1	1	1	1	1	−1	1	−1	1	−1	−1	−1	11.330
3	1	−1	−1	−1	−1	1	1	−1	1	1	−1	−1	1	1	1	1	−1	1	−1	12.867
4	1	1	1	1	−1	1	−1	1	−1	−1	−1	−1	1	1	−1	1	1	−1	−1	16.852
5	1	−1	1	−1	1	−1	−1	−1	−1	1	1	−1	1	1	−1	−1	1	1	1	5.181
6	−1	−1	−1	−1	1	1	−1	1	1	−1	−1	1	1	1	1	−1	1	−1	1	20.610
7	1	1	−1	1	1	−1	−1	1	1	1	1	−1	1	−1	1	−1	−1	−1	−1	13.778
8	−1	1	1	−1	−1	1	1	1	1	−1	1	−1	1	−1	−1	−1	−1	1	1	20.708
9	1	1	−1	1	−1	1	−1	−1	−1	−1	1	1	−1	1	1	−1	−1	1	1	16.552
10	−1	−1	−1	1	1	−1	1	1	−1	−1	1	1	1	1	−1	1	−1	1	−1	11.501
11	−1	−1	1	1	−1	1	1	−1	−1	1	1	1	1	−1	1	−1	1	−1	−1	6.946
12	1	1	−1	−1	1	1	1	1	−1	1	−1	1	−1	−1	−1	−1	1	1	−1	40.479
13	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	19.016
14	−1	1	1	1	1	−1	1	−1	1	−1	−1	−1	−1	1	1	−1	1	1	−1	9.223
15	1	−1	−1	1	1	1	1	−1	1	−1	1	−1	−1	−1	−1	1	1	−1	1	49.874
16	−1	1	−1	−1	−1	−1	1	1	−1	1	1	−1	−1	1	1	1	1	−1	1	2.790
17	−1	−1	1	1	1	1	−1	1	−1	1	−1	−1	−1	−1	1	1	−1	1	1	21.293
18	1	−1	1	1	−1	−1	1	1	1	1	−1	1	−1	1	−1	−1	−1	−1	1	9.451
19	1	1	1	−1	1	−1	1	−1	−1	−1	−1	1	1	−1	1	1	−1	−1	1	13.550
20	−1	1	−1	1	−1	−1	−1	−1	1	1	−1	1	1	−1	−1	1	1	1	1	12.127

The "−1" sign corresponds to the minimum value and the "+1" sign corresponds to the maximum value of the input parameter range.

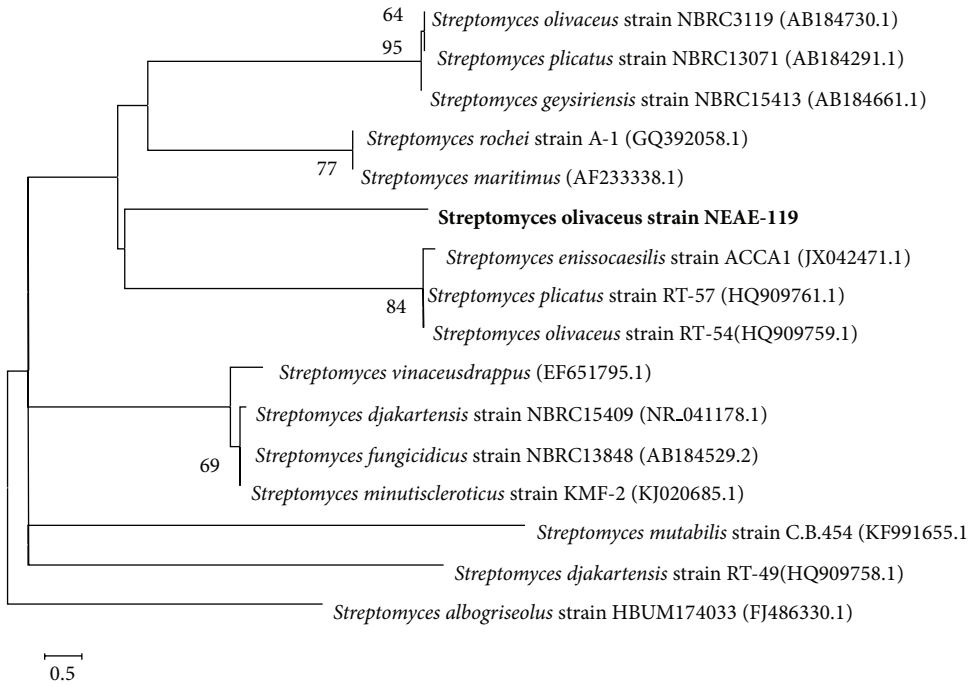


Figure 3: The phylogenetic tree was constructed via the bootstrap test of neighbor-joining algorithm based on the 16S rRNA gene sequences of strain NEAE-119 and related species of the genus *Streptomyces*. Only bootstrap values above 50%, expressed as percentages of 1000 replications, are shown at the branch points. GenBank sequence accession numbers are indicated in parentheses after the strain names. Phylogenetic analyses were conducted in the software package MEGA4. Bar: 0.5 substitution per nucleotide position.

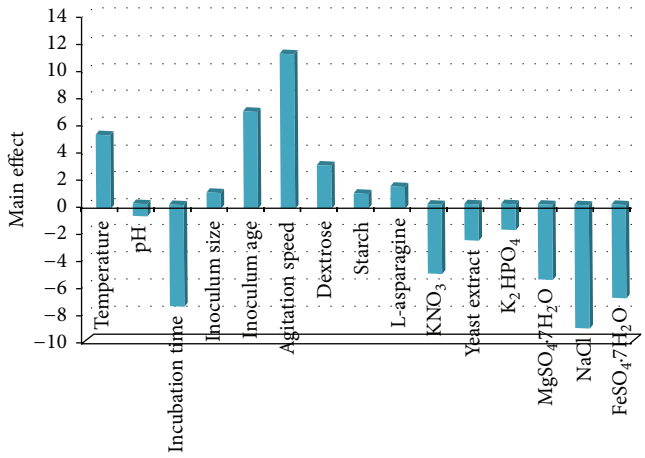


Figure 4: The main effects of the fermentation conditions on L-asparaginase production according to the Packett-Burman experimental results.

to study the effect of the selected variables on the production of L-asparaginase. Four dummy variables are used to estimate experimental errors in data analysis (Table 3). Table 3 represents the results of the screening of significant variables for L-asparaginase production and the corresponding response (Y) using Plackett-Burman design. The maximum L-asparaginase activity (49.874 U/mL) was achieved in the run number 15, while the minimum L-asparaginase activity (5.181 U/mL) was observed in the run number 5. Statistical analysis of the L-asparaginase activity was performed and represented in Table 4. With respect to the main effect of each variable (Figure 4), we can see that seven variables from the fifteen named including temperature, inoculum size, inoculum age, agitation speed, dextrose, starch and L-asparagine positively affect L-asparaginase production where the other eight variables named pH, incubation time, KNO_3, yeast extract, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, NaCl, and $FeSO_4{\cdot}7H_2O$ negatively affect L-asparaginase production. The Pareto chart illustrates the order of significance of the variables affecting L-asparaginase production in Plackett-Burman experimental design (Figure 5). Among the fifteen variables, agitation speed showed the highest positive effect by 15.87%, followed by inoculum age (9.96%), and then temperature by 7.55%. Among the 15 variables, NaCl showed the highest negative significance by 13.03%. Next to NaCl, incubation time showed negative effect by 10.75% followed by $FeSO_4{\cdot}7H_2O$ and $MgSO_4{\cdot}7H_2O$ by 9.87% and 7.95%, respectively.

The R^2 values provide a measure of how much variability in the observed response values can be explained by the experimental factors. The R^2 value is always between 0 and 1. The closer R^2 is to the 1, the stronger the model is and the better it predicts the response [41]. In this case, the value of the determination coefficient (R^2 = 0.9791) indicates that 97.91% of the variability in the response was attributed to the given independent variables and only 2.09% of the total variations are not explained by the independent variables. In addition, the value of the adjusted determination coefficient

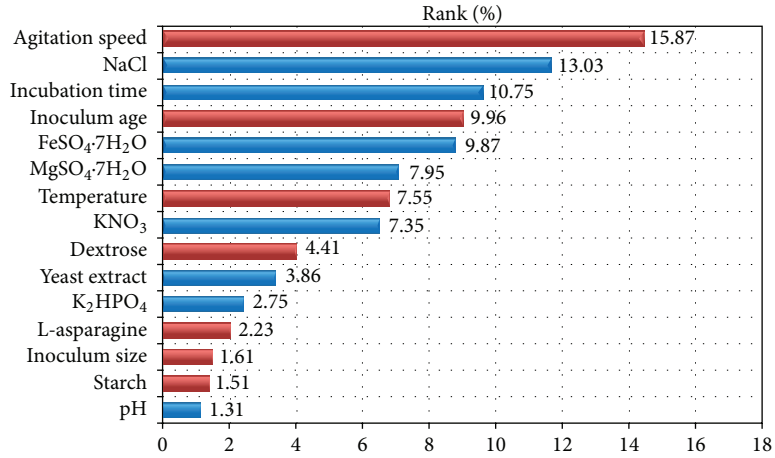


FIGURE 5: Pareto chart illustrates the order of significance of the variables affecting L-asparaginase production by *Streptomyces* sp. strain NEAE-119 (the red color represents positive effects and the blue color represents negative effects; ranks (%) values ranging from 1.31 to 15.87).

(Adj. $R^2 = 0.9010$) is also very high which indicates a high significance of the model. A higher value of the correlation coefficient ($R = 0.9895$) signifies an excellent correlation between the independent variables, this indicated a good correlation between the experimental and predicted values. Thus, the analysis of the response trend using the model was considered to be reasonable. The significance of each coefficient was determined by Student's t-test and P values, which are listed in Table 4. The larger the magnitude of the t-value is and the smaller the P value is, the more significant the corresponding coefficient is [42]. In the current experiment, variables evidencing P values of less than 0.05 (confidence levels exceeding 95%) were considered to have significant effects on the L-asparaginase activity.

Agitation speed, with a probability value of 0.0022, t-value of 7.0197, and confidence level of 99.783, was determined to be the most significant factor, followed by NaCl (P value 0.0045, t-value −5.7627, and confidence level 99.550), incubation time (P value 0.0089, t-value −4.7554, and confidence level 99.106), inoculum age (P value 0.0116, t-value 4.4049, and confidence level 98.835), $FeSO_4{\cdot}7H_2O$ (P value 0.0120, t-value −4.3669, and confidence level 98.800), $MgSO_4{\cdot}7H_2O$ (P value 0.0245, t-value −3.5180, and confidence level 97.551), temperature (P value 0.0289, t-value 3.3382, and confidence level 97.111), and then KNO_3 (P value 0.0314, t-value −3.2499, and confidence level 96.862). Screened significant variables, temperature, inoculum age, and agitation speed exerted positive effect on L-asparaginase production by *Streptomyces* sp. NEAE-119, whereas incubation time, KNO_3, $MgSO_4{\cdot}7H_2O$, NaCl, and $FeSO_4{\cdot}7H_2O$ exerted negative effect. On the basis of the calculated t-values (Table 4), temperature (X_1), inoculum age (X_5), and agitation speed (X_6) were chosen for further optimization using FCCD, since these factors had the most positive effects on L-asparaginase production. The model F value of 12.5404 (Table 4) implies that the model is significant. The values of significance F (P value) < 0.05 (0.0126) indicate that model terms are significant. By neglecting the terms that were insignificant ($P > 0.05$), the first order polynomial equation was derived representing L-asparaginase production as a function of the independent variables:

$$\begin{aligned} Y_{(\text{L-asparaginase production})} = {} & 16.196 + 2.642(X_1) - 3.763(X_3) \\ & + 3.486(X_5) + 5.555(X_6) \\ & - 2.572(X_{10}) - 2.784(X_{13}) \\ & - 4.560(X_{14}) - 3.456(X_{15}), \end{aligned} \tag{4}$$

where Y is the response (L-asparaginase production) and X_1, X_3, X_5, X_6, X_{10}, X_{13}, X_{14}, and X_{15} are temperature, incubation time, inoculum age, agitation speed, KNO_3, $MgSO_4{\cdot}7H_2O$, NaCl, and $FeSO_4{\cdot}7H_2O$, respectively.

Checking the adequacy of the model needs all of the information on lack of fit, which is contained in the residuals. The normal probability plot of the residuals is an important diagnostic tool to detect and explain the systematic departures from the normality [39]. Figure 6 shows a plot of normal probability of the experimental results. The normal probability plot of the residuals shows the points close to a diagonal line; therefore, the residuals appear to be approximately normally distributed. This indicates that the model was well fitted with the experimental results.

Khamna et al. [43] have reported 30°C for the maximum activity and the growth by *Amycolatopsis* CMV-H002. Narayana et al. [14] have also reported that *Streptomyces albidoflavus* produces high amount of L-asparaginase at 35°C. Amena et al. [15] have reported that L-asparaginase activity was maximum at 40°C by *Streptomyces gulbargensis*. Siddalingeshwara and Lingappa [44] have reported 35°C as the optimum temperature for maximum L-asparaginase production. The temperature optima for L-asparaginase production vary widely in different strains. The variation may be due to the strains employed during the fermentation for L-asparaginase production and to fermentation conditions.

Table 4: Statistical analysis of Plackett-Burman design showing coefficient values, main effect, t-test, P values, and confidence level (%) for each variable affecting L-asparaginase production and analysis of variance.

(a)

Variables	Coefficients	Main effect	t-Stat	P value	Confidence level (%)
Intercept	16.196	32.392	20.4659	0.0000	99.996
Temperature (°C)	2.642	5.283	3.3382	0.0289	97.111
pH	−0.457	−0.914	−0.5776	0.5945	40.549
Incubation time (days)	−3.763	−7.527	−4.7554	0.0089	99.106
Inoculum size (%, v/v)	0.564	1.127	0.7123	0.5156	48.436
Inoculum age (h)	3.486	6.972	4.4049	0.0116	98.835
Agitation speed (rpm)	5.555	11.110	7.0197	0.0022	99.783
Dextrose (g/L)	1.543	3.086	1.9496	0.1230	87.699
Starch (g/L)	0.529	1.059	0.6691	0.5401	45.991
L-asparagine (g/L)	0.780	1.560	0.9856	0.3801	61.989
KNO_3 (g/L)	−2.572	−5.144	−3.2499	0.0314	96.862
Yeast extract (g/L)	−1.351	−2.702	−1.7070	0.1630	83.700
K_2HPO_4 (g/L)	−0.962	−1.924	−1.2159	0.2909	70.912
$MgSO_4{\cdot}7H_2O$ (g/L)	−2.784	−5.568	−3.5180	0.0245	97.551
NaCl (g/L)	−4.560	−9.121	−5.7627	0.0045	99.550
$FeSO_4{\cdot}7H_2O$ (g/L)	−3.456	−6.912	−4.3669	0.0120	98.800

(b)

	Analysis of variance (ANOVA)				
	df	SS	MS	F-test	Significance F (P value)
Regression	15	2356.0614	157.0707	12.5404	0.01262
Residual	4	50.100594	12.52514		
Total	**19**	**2406.1620**			

t: Student's test; P: corresponding level of significance; df: degree of freedom; SS: sum of squares; MS: mean sum of squares; F: Fishers's function; Significance F: corresponding level of significance.
Multiple R 0.9895, R square 0.9791, and adjusted R square 0.9010.

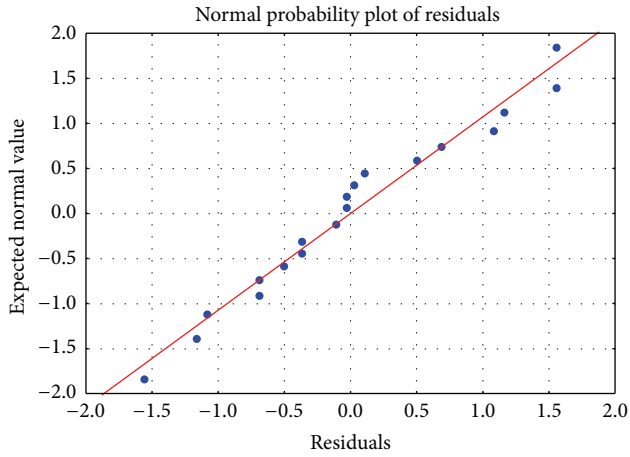


Figure 6: The normal probability plot of the residuals.

In the screening and fermentation development work which utilizes shaken cultures, it is essential that an oxygen level be provided which is sufficient to meet both the growth requirements of the organism and the yield of desired end product. Since aeration above or below an optimal level may induce conditions unsuitable for the formation of the desired end product, precise measurement and control of this variable are essential [45].

Since microorganisms growing in submerged culture utilize oxygen dissolved in the fermentation medium, the supply in vessels used for shaken cultures may become critical for microbial biosynthesis of specific end products. In addition, oxidation-reduction mechanisms existing in the fermentation mixture may exert a chemical influence on biosynthetic products [45]. Gentle aeration enables obtaining both good growth and high L-asparaginase yield [46]. Heinemann and Howard [47] observed that agitation in shaken culture was essential for optimal growth of *Serratia marcescens* and that the tumor inhibitory enzyme, asparaginase, was produced during a period of zero dissolved oxygen concentration in the fermentation medium. *Serratia marcescens* produces large amount of L-asparaginase with limited aeration than it does anaerobically [48]. Most of the organisms demand yeast extract for the growth and L-asparaginase production [49]. While using yeast extract, it was found to increase the viscosity of the medium, thereby reducing the oxygen uptake; good mixing was critical especially when fermentation medium becomes viscous [50]. In such cases, growth was limited by transfer of oxygen to the cell surface rather than by oxygen

Table 5: Face-centered central composite design, representing the response of L-asparaginase production as influenced by temperature (X_1), inoculum age (X_5), and agitation speed (X_6) along with the predicted L-asparaginase production and residuals and the levels of variables.

(a)

Trials	Variables			L-asparaginase activity (U/mL)		Residuals
	X_1	X_5	X_6	Experimental	Predicted	
1	0	0	0	68.196	66.667	1.530
2	1	1	1	34.451	38.978	−4.528
3	1	0	0	55.406	54.083	1.323
4	0	0	−1	38.765	38.104	0.662
5	1	−1	−1	15.501	21.237	−5.737
6	0	0	0	67.658	66.667	0.992
7	1	1	−1	29.266	25.752	3.514
8	0	−1	0	53.242	56.407	−3.165
9	−1	1	1	65.753	58.786	6.967
10	0	0	0	68.313	66.667	1.646
11	−1	0	0	52.860	59.104	−6.244
12	0	0	0	70.076	66.667	3.410
13	−1	−1	1	39.671	41.955	−2.284
14	0	0	0	68.196	66.667	1.530
15	0	0	0	67.403	66.667	0.736
16	−1	1	−1	29.771	33.968	−4.197
17	0	0	1	54.375	59.958	−5.583
18	−1	−1	−1	17.230	11.472	5.758
19	0	1	0	65.323	67.080	−1.756
20	1	−1	1	45.556	40.129	5.427

(b)

Level	Temperature (°C)	Inoculum age (h)	Agitation speed (rpm)
−1	30	24	100
0	35	48	150
1	37	72	200

The measured L-asparaginase activity obtained under the optimal conditions obtained from FCCD was 68.59 U/mL.

solubility which was observed in *E. coli* and *E. chrysanthemi* [51]. This suggests that lower level of aeration found to be suitable for the growth and yield of enzyme. This may be due to the fact that lower level of aeration may facilitate the suitable mycelium branching for the yield of enzyme.

Inoculum is generally transferred at the logarithmic phase of growth; the age of inoculum is important to achieve optimum yield of the metabolites. Inoculum age of 48–72 h was found to be the most suitable conditions for maximum production of L-asparaginase from *Streptomyces* sp. NEAE-119, because cells are in the logarithmic or early exponential growth phase and the cells are more active. The higher inoculum density is inhibitory to the enzyme production as too much biomass can deplete the substrate nutrients or accumulation of some nonvolatile self-inhibiting substances that inhibit the product formation [52] and lower density may give insufficient biomass causing induced product formation whereas higher inoculum may produce too much biomass which is inhibitory to the product formation [53]. Adequate inoculums can initiate fast mycelium growth and product formation, thereby reducing other organism contamination. Quantity of inoculum had a definite effect on enzyme titers.

According to Prakasham et al. [51] temperature and inoculum level are the major influential parameters and contributed to more than 50% of total L-asparaginase production. Amena et al. [15] have reported the inoculum size (1×10^8 spores/mL) for the maximum L-asparaginase production of 6.9 IU/mL by *Streptomyces gulbargensis* using ground nut extract using submerged fermentation. Kumari et al. [54] have reported optimum inoculum level of 10.36% (v/v) for L-asparaginase production by *Streptomyces griseoluteus* WS3/1 under submerged fermentation.

3.5. Optimization by Face-Centered Central Composite Design. Face-centered central composite design was employed to study the optimal levels and the interactions among the selected significant factors; those had positive effect on the L-asparaginase production. The other variables in the study

Table 6: Statistical analysis of face-centered central composite design showing coefficient values, main effect, t-test, P values and analysis of variance.

(a)

Variables	Coefficients	Main effect	t-Stat	P value
Intercept	66.667	133.33	35.0717	0.0000
X_1	−2.510	−5.02	−1.4358	0.1816
X_5	5.337	10.67	3.0520	0.0122
X_6	10.927	21.85	6.2494	0.0001
X_1X_5	−4.495	−8.99	−2.2995	0.0443
X_1X_6	−2.898	−5.80	−1.4824	0.1690
X_5X_6	−1.416	−2.83	−0.7245	0.4854
X_1X_1	−10.073	−20.15	−3.0210	0.0129
X_5X_5	−4.923	−9.85	−1.4765	0.1706
X_6X_6	−17.636	−35.27	−5.2891	0.0004

(b)

	Analysis of variance (ANOVA)				
	df	SS	MS	F-test	Significance F (P value)
Regression	9	5854.7578	650.5286	21.2771	$2.22009E-05$
Residual	10	305.7400	30.5740		
Total	**19**	**6160.4978**			

X_1: the coded value of temperature, X_5: the coded value of inoculum age, and X_6: the coded value of agitation speed.
t: Student's test; P: corresponding level of significance; df: degree of freedom; SS: sum of squares; MS: mean sum of squares; F: Fishers's function; Significance F: corresponding level of significance.
Multiple R 0.9748, R square 0.9503, and adjusted R square 0.9057.

were maintained at a constant level which gave maximal yield in the Plackett-Burman experiments. In this study, a total of 20 experiments with different combination of temperature (X_1), inoculum age (X_5), and agitation speed (X_6) were performed and the results of experiments for studying the effects of three independent variables on L-asparaginase activity are presented along with predicted response and residuals (Table 5). The results showed considerable variation in the L-asparaginase activity. Runs 1, 6, 10, 12, 14, and 15 showed a high L-asparaginase activity (≥67.403 U/mL). The minimum L-asparaginase activity (15.501 U/mL) was observed in run number 5, while the maximum L-asparaginase activity (70.076 U/mL) was achieved in run number 12.

3.6. Multiple Regression Analysis and ANOVA. Multiple regression analysis was used to analyze the data; the goodness of fit of the model was checked by the coefficient of determination (R^2), which was found to be 0.9503, indicating that the sample variation of 95.03% was attributed to the variables and only 4.97% of the total variance could not be explained by the model. Therefore, the present R^2-value reflected a very good fit between the observed and predicted responses and implied that the model is reliable for L-asparaginase production in the present study. Analysis of variance (ANOVA) which is required to test the significance and adequacy of the model is presented in Table 6. The analysis of variance (ANOVA) of the regression model demonstrates that the model is highly significant as is evident from Fisher's F-test (21.2771) and a very low probability value ($2.22009E-05$). The significance of each coefficient was determined by t-values and P values which are listed in Table 6. The P values denote the significance of the coefficients and are also important in understanding the pattern of the mutual interactions between the variables. Interpretation of the data was based on the signs (positive or negative effect on the response) and statistical significance of coefficients ($P < 0.05$). Interactions between two factors could appear as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient).

It can be seen from the degree of significance that the linear coefficients of inoculum age (X_5), agitation speed (X_6), interaction between temperature (X_1), inoculum age (X_5), and quadratic effect of temperature (X_1), and agitation speed (X_6) are significant. The probability values of the coefficient suggest that among the three variables studied, X_1, X_5 shows maximum interaction between the two variables (P value 0.0443), indicating that 95.57% of the model is affected by these variables. The linear coefficients of temperature (X_1), interaction between X_1 and X_6, and quadratic effect of X_5 are not significant (P value > 0.05). On the other hand, among the different interactions, interaction between X_1 and X_6 and that between X_5 and X_6 are not significant (P values 0.1690 and 0.4854, resp.), indicating that there is no significant correlation between each two variables and that they did not help much in increasing the production of L-asparaginase.

In order to evaluate the relationship between dependent and independent variables and to determine the maximum L-asparaginase production corresponding to the optimum levels of temperature (X_1), inoculum age (X_5), and agitation

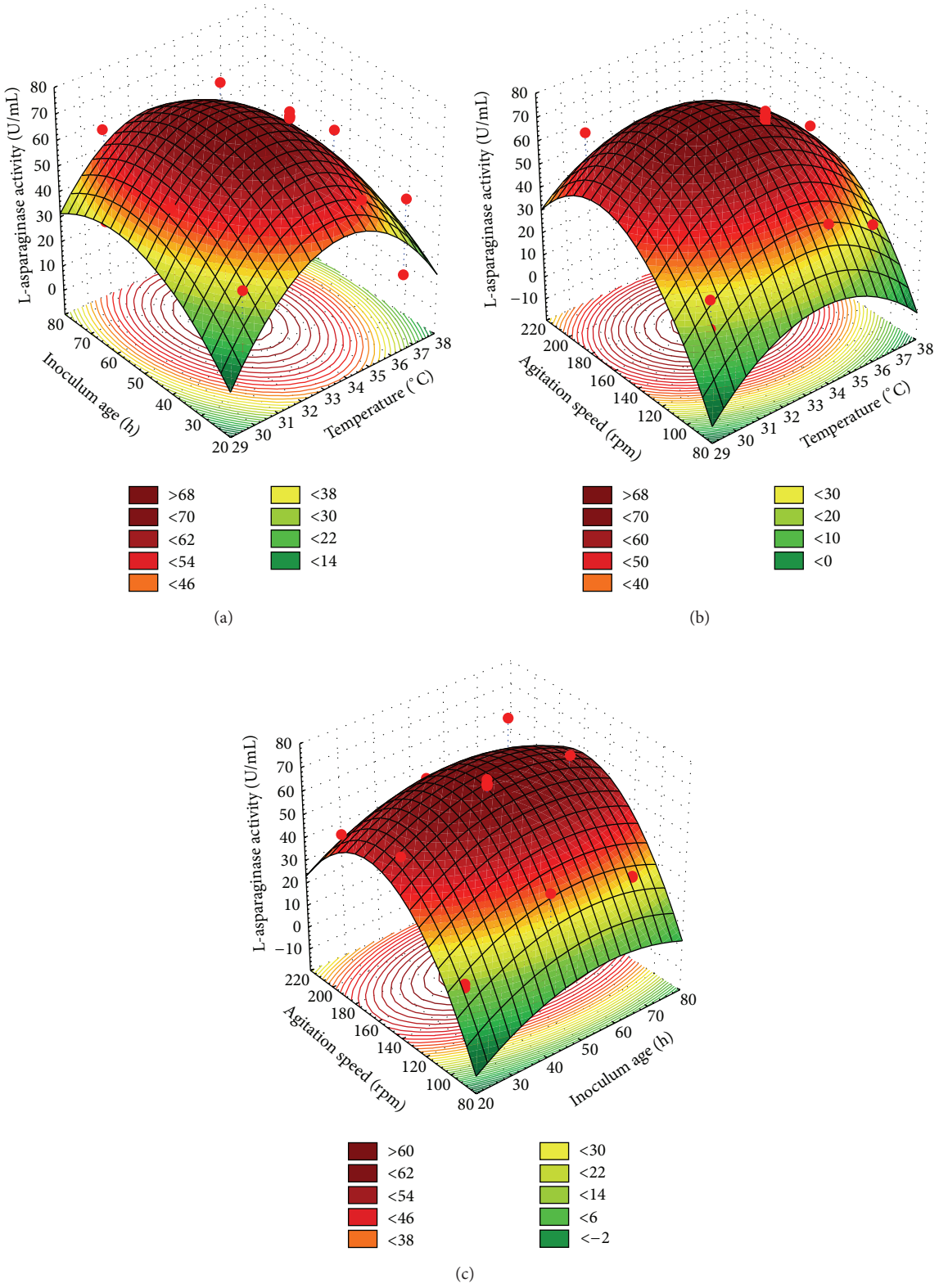


Figure 7: Three-dimensional response surface plots showing the effect of temperature (X_1), inoculum age (X_5) and agitation speed (X_6) and their mutual effect on the production of L-asparaginase.

speed (X_6), a second-order polynomial model (equation (5)) was proposed to calculate the optimum levels of these variables. By applying the multiple regression analysis on experimental data, the second-order polynomial equation that defines predicted response (Y) in terms of the independent variables (X_1, X_5, and X_6) was obtained:

$$\begin{aligned} Y_{(\text{L-asparaginase production})} = {} & 66.667 - 2.510X_1 + 5.337X_5 \\ & + 10.927X_6 - 4.495X_1X_5 \\ & - 2.898X_1X_6 - 1.416X_5X_6 \\ & - 10.073X_1^2 - 4.923X_5^2 \\ & - 17.636X_6^2, \end{aligned} \quad (5)$$

where the Y is the predicted response, X_1 the coded value of temperature, X_5 the coded value of inoculum age, and X_6 the coded value of agitation speed.

The interaction effects and optimal levels of the variables were determined by plotting the three-dimensional response surface curves (Figures 7(a)–7(c)) when one of the variables is fixed at optimum value and the other two are allowed to vary. Figure 7(a) represents the L-asparaginase activity as a function of temperature (X_1) and inoculum age (X_5) by keeping agitation speed (X_6) at optimum value. It showed that lower and higher levels of temperature support relatively low levels of L-asparaginase activity; the highest value of L-asparaginase activity was obtained with middle level of temperature and inoculum age. Further increase of inoculum age did not result in higher L-asparaginase activity. Figure 7(b) represents the L-asparaginase activity as a function of temperature (X_1) and agitation speed (X_6) by keeping inoculum age (X_5) at optimum value; the maximum L-asparaginase activity was attained at moderate to high levels of agitation speed and moderate levels of temperature and further increase in the temperature resulted in a gradual decrease in the L-asparaginase activity. Figure 7(c) showed that the maximum L-asparaginase production was attained beyond middle levels of inoculum age and lower and higher levels of inoculum age resulted in a gradual decrease in L-asparaginase production. Highest value of L-asparaginase production was obtained beyond high agitation speed.

3.7. Verification of the Model. In order to determine the accuracy of the model and to verify the result, an experiment under the optimal conditions obtained from face-centered central composite design-response surface methodology was performed and compared with the predicted data. The measured L-asparaginase activity obtained was 68.59 U/mL, close to the predicted one 70.46 U/mL, revealing a high degree of accuracy. The verification revealed a high degree of accuracy of the model of more than 97.35%, indicating the model validation under the tested conditions. The predicted optimal levels of the process variables for L-asparaginase production by *Streptomyces olivaceus* strain NEAE-119 were temperature (35°C), inoculum age (72 h), and agitation speed (200 rpm).

4. Conclusion

A statistical approach has been employed for which a Plackett-Burman design is used for identifying significant variables influencing glutaminase free L-asparaginase production by *Streptomyces olivaceus* NEAE-119. The levels of the significant variables were further optimized using face-centered central composite design. *Streptomyces olivaceus* strain NEAE-119 was identified on the basis of morphological, cultural, and physiological properties, together with 16S rRNA sequence and phylogenetic analysis. The sequencing product was deposited in the GenBank database under accession number KJ200342.

Conflict of Interests

The authors declare that there is no conflict of interests.

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References

[1] N. Verma, K. Kumar, G. Kaur, and S. Anand, "L-asparaginase: a promising chemotherapeutic agent," *Critical Reviews in Biotechnology*, vol. 27, no. 1, pp. 45–62, 2007.

[2] A. L. Stecher, P. M. de Deus, I. Polikarpov, and J. Abrahão-Neto, "Stability of L-asparaginase: an enzyme used in leukemia treatment," *Pharmaceutica Acta Helvetiae*, vol. 74, no. 1, pp. 1–9, 1999.

[3] E. M. Maysa, M. Amira, E. Gamal, T. Sanaa, and E. I. Sayed, "Production, immobilization and anti-tumor activity of L-asparaginase of *Bacillus* sp R36," *Journal of American Science*, vol. 6, no. 8, pp. 157–165, 2010.

[4] H. V. Hendriksen, B. A. Kornbrust, P. R. Ostergaard, and M. A. Stringer, "Evaluating the potential for enzymatic acrylamide mitigation in a range of food products using an asparaginase from *Aspergillus oryzae*," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 10, pp. 4168–4176, 2009.

[5] D. R. Reynolds and J. W. Taylor, *The Fungal Holomorph: A Consideration of Mitotic Meiotic and Pleomorphic Speciation*, CAB International, Wallingford, UK, 1993.

[6] X. Thomas, G. Cannas, Y. Chelghoum, and A. Gougounon, "Therapeutic alternatives to native L-asparaginase in the treatment of adult acute lymphoblastic leukemia," *Bulletin du Cancer*, vol. 97, no. 9, pp. 1105–1117, 2010.

[7] M. L. Graham, "Pegaspargase: a review of clinical studies," *Advanced Drug Delivery Reviews*, vol. 55, no. 10, pp. 1293–1302, 2003.

[8] M. Duval, S. Suciu, A. Ferster et al., "Comparison of *Escherichia coli*-asparaginase with *Erwinia*-asparaginase in the treatment of childhood lymphoid malignancies: results of a randomized European Organisation for Research and Treatment of Cancer—Children's Leukemia Group phase 3 trial," *Blood*, vol. 99, no. 8, pp. 2734–2739, 2002.

[9] N. E. El-Naggar, S. M. El-Ewasy, and N. M. El-Shweihy, "Microbial L-asparaginase as a potential therapeutic agent for

the treatment of acute lymphoblastic leukemia: the pros and cons," *International Journal of Pharmacology*, vol. 10, no. 4, pp. 182–199, 2014.

[10] J. B. Howard and F. H. Carpenter, "L-asparaginase from *Erwinia carotovora*. Substrate specificity and enzymatic properties," *The Journal of Biological Chemistry*, vol. 247, no. 4, pp. 1020–1030, 1972.

[11] M. D. Prager and N. Bachynsky, "Asparagine synthetase in L-asparaginase resistant and susceptible mouse lymphomas," *Biochemical and Biophysical Research Communications*, vol. 31, no. 1, pp. 43–47, 1968.

[12] D. S. Hawkins, J. R. Park, B. G. Thomson et al., "Asparaginase pharmacokinetics after intensive polyethylene glycol-conjugated L-asparaginase therapy for children with relapsed acute lymphoblastic leukemia," *Clinical Cancer Research*, vol. 10, no. 16, pp. 5335–5341, 2004.

[13] K. K. R. Patro, S. Satpathy, and N. Gupta, "Evaluation of some fungi for L-asparaginase production," *Indian Journal of Fundamental and Applied Life Sciences*, vol. 1, no. 4, pp. 219–221, 2011.

[14] K. J. P. Narayana, K. G. Kumar, and M. Vijayalakshmi, "L-asparaginase production by *Streptomyces albidoflavus*," *Indian Journal of Microbiology*, vol. 48, no. 3, pp. 331–336, 2008.

[15] S. Amena, N. Vishalakshi, M. Prabhakar, A. Dayanand, and K. Lingappa, "Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis*," *Brazilian Journal of Microbiology*, vol. 41, no. 1, pp. 173–178, 2010.

[16] B. Pradhan, S. K. Dash, and S. Sahoo, "Screening and characterization of extracelluar L-asparaginase producing *Bacillus subtilis strain hswx88*, isolated from Taptapani hotspring of Odisha, India," *Asian Pacific Journal of Tropical Biomedicine*, vol. 3, no. 12, pp. 936–941, 2013.

[17] M. Hymavathi, T. Sathish, C. S. Rao, and R. S. Prakasham, "Enhancement of L-asparaginase production by isolated *Bacillus circulans* (MTCC 8574) using response surface methodology," *Applied Biochemistry and Biotechnology*, vol. 159, no. 1, pp. 191–198, 2009.

[18] N. E. El-Naggar, A. A. El-Bindary, and N. S. Nour, "Production of antimicrobial agent inhibitory to some human pathogenic multidrug-resistant bacteria and *Candida albicans* by *Streptomyces* sp. NEAE-1," *International Journal of Pharmacology*, vol. 9, no. 6, pp. 335–347, 2013.

[19] N. E. El-Naggar, A. A. El-Bindary, and N. S. Nour, "Statistical optimization of process variables for antimicrobial metabolites production by *Streptomyces anulatus* NEAE-94 against some multidrug-resistant strains," *International Journal of Pharmacology*, vol. 9, no. 6, pp. 322–334, 2013.

[20] N. E. El-Naggar and N. A. M. Abdelwahed, "Application of statistical experimental design for optimization of silver nanoparticles biosynthesis by a nanofactory *Streptomyces viridochromogenes*," *Journal of Microbiology*, vol. 52, no. 1, pp. 53–63, 2014.

[21] N. E. El-Naggar, N. A. M. Abdelwahed, and O. M. M. Darwesh, "Fabrication of biogenic antimicrobial silver nanoparticles by *Streptomyces aegyptia* NEAE 102 as eco-friendly nanofactory," *Journal of Microbiology and Biotechnology*, vol. 24, no. 4, pp. 453–464, 2014.

[22] N. N. Nawani and B. P. Kapadnis, "Optimization of chitinase production using statistics based experimental designs," *Process Biochemistry*, vol. 40, no. 2, pp. 651–660, 2005.

[23] C.-Y. Chang, C.-L. Lee, and T.-M. Pan, "Statistical optimization of medium components for the production of *Antrodia cinnamomea* AC0623 in submerged cultures," *Applied Microbiology and Biotechnology*, vol. 72, no. 4, pp. 654–661, 2006.

[24] P. J. de Jong, "L-asparaginase production by *Streptomyces griseus*," *Applied Microbiology*, vol. 23, no. 6, pp. 1163–1164, 1972.

[25] R. Gulati, R. K. Saxena, and R. Gupta, "A rapid plate assay for screening L-asparaginase producing micro-organisms," *Letters in Applied Microbiology*, vol. 24, no. 1, pp. 23–26, 1997.

[26] J. C. Wriston Jr. and T. O. Yellin, "L-asparaginase: a review," *Advances in Enzymology*, vol. 39, pp. 185–248, 1973.

[27] A. Imada, S. Igarasi, K. Nakahama, and M. Isono, "Asparaginase and glutaminase activities of micro organisms," *Journal of General Microbiology*, vol. 76, no. 1, pp. 85–99, 1973.

[28] E. B. Shirling and D. Gottlieb, "Methods for characterization of *Streptomyces* species," *International Journal of Systematic Bacteriology*, vol. 16, pp. 313–340, 1966.

[29] J. L. Staneck and G. D. Roberts, "Simplified approach to identification of aerobic actinomycetes by thin layer chromatography," *Journal of Applied Microbiology*, vol. 28, no. 2, pp. 226–231, 1974.

[30] E. A. Azab, M. M. Ali, and M. F. Fareed, "Studies on uricase induction in certain bacteria," *Egyptian Journal of Biology*, vol. 7, pp. 44–54, 2005.

[31] J. Sambrook, E. F. Fritsch, and T. Maniaties, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2nd edition, 1989.

[32] N. E. El-Naggar, A. A. Sherief, and S. S. Hamza, "*Streptomyces aegyptia* NEAE 102, a novel cellulolytic streptomycete isolated from soil in Egypt," *African Journal of Microbiology Research*, vol. 5, no. 29, pp. 5308–5315, 2011.

[33] S. F. Altschul, T. L. Madden, A. A. Schäffer et al., "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," *Nucleic Acids Research*, vol. 25, no. 17, pp. 3389–3402, 1997.

[34] K. Tamura, J. Dudley, M. Nei, and S. Kumar, "MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0," *Molecular Biology and Evolution*, vol. 24, no. 8, pp. 1596–1599, 2007.

[35] N. Saitou and M. Nei, "The neighbor-joining method: a new method for reconstructing phylogenetic trees," *Molecular Biology and Evolution*, vol. 4, no. 4, pp. 406–425, 1987.

[36] R. L. Plackett and J. P. Burnam, "The design of optimum multifactorial experiments," *Biometrika*, vol. 33, pp. 305–325, 1946.

[37] S. Krishnan, S. G. Prapulla, D. Rajalakshmi, M. C. Misra, and N. G. Karanth, "Screening and selection of media components for lactic acid production using Plackett-Burman design," *Bioprocess Engineering*, vol. 19, no. 1, pp. 61–65, 1998.

[38] B. K. Körbahti, N. Aktaş, and A. Tanyolaç, "Optimization of electrochemical treatment of industrial paint wastewater with response surface methodology," *Journal of Hazardous Materials*, vol. 148, no. 1-2, pp. 83–90, 2007.

[39] D. C. Montgomery, *Design and Analysis of Experiments*, John Wiley & Sons, New York, NY, USA, 1991.

[40] M. Goodfellow, P. Kämpfer, H.-J. Busse et al., *Bergey's Manual of Systematic Bacteriology*, vol. 5 of *The Actinobacteria Part A*, Springer, New York, NY, USA, 2nd edition, 2012.

[41] R. Kaushik, S. Saran, J. Isar, and R. K. Saxena, "Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipase production by *Aspergillus carneus*," *Journal of Molecular Catalysis B: Enzymatic*, vol. 40, no. 3-4, pp. 121–126, 2006.

[42] S. Akhnazarova and V. Kafarov, *Experiment Optimization in Chemistry and Chemical Engineering*, Mir Publishers, Moscow, Russia, 1982.

[43] S. Khamna, A. Yokota, and S. Lumyong, "L-asparaginase production by actinomycetes isolated from some Thai medicinal plant rhizosphere soils," *International Journal of Integrative Biology*, vol. 6, no. 1, pp. 22–26, 2009.

[44] K. G. Siddalingeshwara and K. Lingappa, "Key fermentation factors for the synthesis of L-asparaginase—an anti tumour agent through SSF methodology," *International Journal of Pharmaceutical Sciences*, vol. 1, no. 1, pp. 103–112, 2010.

[45] B. Heinemann, A. J. Howard, and H. J. Palocz, "Influence of dissolved oxygen levels on production of L-asparaginase and prodigiosin by *Serratia marcescens*," *Applied Microbiology*, vol. 19, no. 5, pp. 800–804, 1970.

[46] M. H. Bilimoria, "Conditions for the production of L-asparaginase 2 by coliform bacteria," *Applied Microbiology*, vol. 18, no. 6, pp. 1025–1030, 1969.

[47] B. Heinemann and A. J. Howard, "Production of tumor-inhibitory L-asparaginase by submerged growth of *Serratia marcescens*," *Applied Microbiology*, vol. 18, no. 4, pp. 550–554, 1969.

[48] W. R. Barnes, G. L. Dorn, and G. R. Vela, "Effect of culture conditions on synthesis of L-asparaginase by *Escherichia coli* A-1," *Applied and Environmental Microbiology*, vol. 33, no. 2, pp. 257–261, 1977.

[49] F. S. Liu and J. E. Zajic, "Fermentation kinetics and continuous process of L-asparaginase production," *Applied Microbiology*, vol. 25, no. 1, pp. 92–96, 1973.

[50] J. Mukherjee, S. Majumdar, and T. Scheper, "Studies on nutritional and oxygen requirements for production of L-asparaginase by *Enterobacter aerogenes*," *Applied Microbiology and Biotechnology*, vol. 53, no. 2, pp. 180–184, 2000.

[51] R. S. Prakasham, C. S. Rao, R. S. Rao, G. S. Lakshmi, and P. N. Sarma, "L-asparaginase production by isolated *Staphylococcus* sp.—6A: design of experiment considering interaction effect for process parameter optimization," *Journal of Applied Microbiology*, vol. 102, no. 5, pp. 1382–1391, 2007.

[52] Z. Baysal, F. Uyar, and Ç. Aytekin, "Solid state fermentation for production of α-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water," *Process Biochemistry*, vol. 38, no. 12, pp. 1665–1668, 2003.

[53] R. E. Mudgett, "Solid state fermentations," in *Manual of Industrial Microbiology and Biotechnology*, A. L. Demain and N. A. Solomon, Eds., vol. 66, p. 83, American Society for Microbiology, Washington, DC, USA, 1986.

[54] K. P. V. Kumari, G. Girija Shankar, and T. Prabhakar, "Optimisation of L-asparaginase production by *Streptomyces griseoluteus* WS3/1 using experimental methods," *Journal of Pharmaceutical and Biochemical Sciences*, vol. 10, no. 11, pp. 1–6, 2011.

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Research Article

Statistical Optimization of Laccase Production and Delignification of Sugarcane Bagasse by *Pleurotus ostreatus* in Solid-State Fermentation

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Laccases are oxidative enzymes related to the degradation of phenolic compounds, including lignin units, with concomitant reduction of oxygen to water. Delignification is a necessary pretreatment step in the process of converting plant biomass into fermentable sugars. The objective of this work was to optimize the production of laccases and to evaluate the delignification of sugarcane bagasse by *Pleurotus ostreatus* in solid-state fermentation. Among eight variables (pH, water activity, temperature, and concentrations of $CuSO_4$, $(NH_4)_2SO_4$, KH_2PO_4, asparagine, and yeast extract), copper sulfate and ammonium sulfate concentrations were demonstrated to significantly influence laccase production. The replacement of ammonium sulfate by yeast extract and the addition of ferulic acid as inducer provided increases of 5.7- and 2.0-fold, respectively, in laccase activity. Optimization of laccase production as a function of yeast extract, copper sulfate, and ferulic acid concentrations was performed by response surface methodology and optimal concentrations were 6.4 g/L, 172.6 μM, and 1.86 mM, respectively. Experimentally, the maximum laccase activity of 151.6 U/g was produced at the 5th day of solid-state fermentation. Lignin content in sugarcane bagasse was reduced from 31.89% to 26.36% after 5 days and to 20.79% after 15 days by the biological treatment of solid-state fermentation.

1. Introduction

Laccases are blue multicopper oxidases able to oxidize a variety of phenolic compounds and, in the presence of a mediator (e.g., 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonate or ABTS), also nonphenolic compounds [1–3]. It is known that the production of laccases by fungi, especially the white-rot basidiomycetes, can be affected by the type and concentration of the carbon and nitrogen sources and also by the presence of copper and organic compounds that act as inducers of laccase activity [4–7].

Laccases are indicated for several applications in different sectors, that is, in the food and beverages industry to remove undesired phenolics which are responsible for browning, haze formation, and turbidity, in the pulp and paper industry, for biopulping and biobleaching processes, and in the textile industry, for dyes decolourization, and there are also applications in the fields of nanotechnology, bioremediation, and synthetic chemistry [8, 9].

The utilization of agroindustrial wastes as substrates for fermentative processes producing high added value products (i.e., enzymes, ethanol, single-cell protein, mushrooms, organic acids, amino acids, biologically active secondary metabolites) has been widely explored, since they are easily available and rich in carbon and often present disposal problems [10–12]. Sugarcane bagasse is an agroresidue generated in high amount (186 million tons/year) by the sugar and

alcohol industry in Brazil. It is a porous residue of cane stalks left over after the crushing and extraction of the juice from sugarcane and is composed of 19–24% lignin, 27–32% hemicellulose, 32–44% cellulose, and 4.5–9% ashes [13]. Sugar mills generate approximately 270–280 kg of bagasse (50% moisture) per metric ton of sugarcane [14].

One of the main challenges in the utilization of lignocellulosic biomass in fermentative processes is the transformation of the complex polysaccharides into simple sugars that can be assimilated by microorganisms. This can be achieved by chemical or enzymatic hydrolysis, preceded by appropriate pretreatments that enhance the efficiency of hydrolysis by lignin removal [15].

Delignification can be performed by thermochemical processes or by the biological route, using enzymes or microorganisms. The advantages of biological delignification over the thermochemical methods may include mild reaction conditions, higher product yields, fewer side reactions, less energy demand, and less reactor resistance to pressure and corrosion [16].

Lignin decomposition in nature is primarily attributed to the metabolism of microorganisms. Among all other organisms, white-rot basidiomycetes degrade lignin more rapidly and extensively than other groups [17] through the cooperative action of several ligninolytic enzymes (laccases, manganese peroxidases, and lignin peroxidases) [18].

Some white-rot fungi such as *Ceriporiopsis subvermispora*, *Phellinus pini*, *Phlebia* spp., and *Pleurotus* spp. preferentially attack lignin more readily than hemicellulose and cellulose. Many white-rot fungi, however, such as *Trametes versicolor*, *Heterobasidion annosum,* and *Irpex lacteus,* exhibit a pattern of simultaneous decay characterized by degradation of all cell wall components [3].

Solid-state fermentation is an interesting technology to be applied in the valorization of agroindustrial residues and can be economically feasible for the production of many biotechnological products [12]. It is also an interesting process to perform biological delignification because it mimics the natural environment of lignin-degrading fungi. The advantages of the solid-state fermentation process over submerged fermentation include smaller fermenter volume, lower sterilization energy costs, easier aeration, reduced or eliminated costs for stirring and effluent treatment, higher product stability, lower catabolic repression, less favorable environment for many bacteria, and lower risk of contamination [12, 16, 19].

The objective of this work was to optimize the fermentation conditions for the production of laccases and to evaluate the delignification of sugarcane bagasse, through the process of solid-state fermentation, using a selected strain of *Pleurotus ostreatus* (coded Pl 22 Em).

2. Material and Methods

2.1. Characterization of the Sugarcane Bagasse. The sugarcane bagasse, generated by the sugar and alcohol industry, was provided by the private company Ourofino Agronegócio, located in the region of Ribeirão Preto, São Paulo, Brazil. The bagasse was previously washed with water and dried at 60°C and the portion presenting particle sizes greater than 2 mm was grinded in a knife mill. Separation of the fractions presenting different particle sizes was performed by sieving. Contents of lignin, total extractives, ashes, and moisture were determined according to the TAPPI norms T222, T264, T413, and T264, respectively. Holocellulose content was calculated by difference.

2.2. Solid-State Fermentations. Erlenmeyer flasks containing 1 g of sugarcane bagasse (particle size between 0.8 and 2 mm 50% and <0.8 mm 50%) were autoclaved and humidified with a sterilized saline solution presenting the following constant composition: $MgSO_4 \cdot 7H_2O$ (0.3 g/L), $FeSO_4 \cdot 7H_2O$ (0.005 g/L), $MnSO_4 \cdot H_2O$ (0.00156 g/L), $ZnSO_4 \cdot 7H_2O$ (0.0014 g/L), $CaCl_2$ (0.3 g/L), and $CoCl_2$ (0.002 g/L) [20]. For the experiments designed according to Plackett-Burman (Table 1), the saline solution presented some differences regarding the following variables: $CuSO_4 \cdot 5H_2O$ as the inducer (0, 75 or 150 μM, concentrations defined according to previous experiments), $(NH_4)_2SO_4$ as the nitrogen source (1.5, 2.0 or 2.5 g/L), KH_2PO_4 as the source of potassium and phosphorus (1, 1.5 or 2 g/L), asparagine as the supplementary aminoacid (0, 0.3 or 0.6 g/L), yeast extract as the source of vitamins and aminoacids (0, 0.25 or 0.5 g/L), and pH (5.0, 5.5 or 6.0). The pH was adjusted with HCl 1 M or NaOH 1 M. These variables were chosen on the basis of the composition of the basidiomycetes synthetic medium. In order to evaluate the effect of water activity (A_w) and temperature, the bagasse was humidified with different volumes of saline solution (10, 15, and 20 mL/g, corresponding to initial A_w of 0.993, 0.996, and 0.999, resp.) and the cultures were incubated at 25, 29, and 33°C. For the subsequent experiments, the concentration of KH_2PO_4 and the pH were set at 1.5 g/L and 5.5, respectively; no asparagine was added; initial A_w was fixed at 0.993 or 10 mL/g and temperature at 29°C. The studied variables were nitrogen source (yeast extract, 1.96–12.04 g/L or $(NH_4)_2SO_4$, 2.5 g/L), inorganic inducer ($CuSO_4$, 24–276 μM), and organic inducer (ferulic acid, 0.32–3.68 mM, added after 48 h of fermentation).

The strain of *P. ostreatus* (Pl 22 Em), available at the culture collection of the Bioprocess Engineering and Biotechnology Department (Federal University of Paraná, Curitiba, Brazil) was reactivated in PDA dishes and after 7 days of growth, 4 disks of 7 mm diameter were transferred to Czapek liquid medium containing the antibiotic cephalexin (0.08 g/L). After 5 days of growth at 30°C and 120 rpm, the mycelium was separated from the residual medium by a sieve, homogenized with a spatula and resuspended in the residual medium to a lower final volume (10% of the initial volume). 0.2 mL of the homogenized mycelium (containing 4% of dry biomass) was transferred to the fermentation flasks, which were manually homogenized and incubated for 5 days (optimization studies) or for 3 to 7 days (kinetic study).

2.3. Experimental Designs. Fermentations were prepared as described in Section 2.2. Tables 1 and 2 present the chosen variables and levels for the Plackett-Burman Design and Central Composite Design experiments, respectively.

Table 1: Plackett-Burman design to select significant variables to be optimized in the production of laccase by solid state fermentation in sugarcane bagasse.

Levels	Variables							
	pH	A_w [a]	T °C	$CuSO_4$ μM	$(NH_4)_2SO_4$ g/L	KH_2PO_4 g/L	Asn[b] g/L	YE[c] g/L
−1	5.0	0.993	25	0	1.5	1	0	0
+1	6.0	0.999	33	150	2.5	2	0.6	0.5

[a]Water activity; [b]asparagine; [c]yeast extract.

Table 2: Central composite design for the modeling of laccase production by solid-state fermentation in sugarcane bagasse.

Levels	Variables		
	Yeast extract g/L	$CuSO_4$ μM	Ferulic acid mM
−1.68	1.96	24	0.32
−1	4	75	1
0	7	150	2
+1	10	225	3
+1.68	12.04	276	3.68

Analysis of the results and determination of the mathematical model were performed using the software Statistica 5.0 (Statsoft, USA), and determination of the optimal levels was performed through the Solver Excel tool (Microsoft, USA).

2.4. Extraction of the Enzymes. Enzymes produced by solid-state fermentation were extracted by solid-liquid extraction using sodium phosphate buffer as solvent ($NaH_2PO_4{\cdot}H_2O$, 50 mM, pH 7.0) [21]. The fermented material was manually homogenized and weighed (around 1 g) and the extraction buffer was added in the proportion of 1 : 10 (w/w). A protease inhibitor (phenylmethylsulfonyl fluoride, PMSF 1 mM) was added to the extraction mixture. The mixture was homogenized in vortex for 1 min and centrifuged for 7,500 g, 4°C, 45 min. The supernatant was separated and submitted to analyses.

2.5. Laccase Activity Assay. The enzymatic activity of laccase was assayed by the oxidation of ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid). The reaction mixture contained 100 μL of ABTS 20 mM (in sodium citrate buffer 0.1 M, pH 3.0), sample (usually 20–50 μL), and sodium citrate buffer ($C_6H_8O_7{\cdot}H_2O$ 0.1 M, pH 3.0) up to 1 mL. Oxidation of ABTS was followed by absorbance increase at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$). The enzyme activity was expressed in international units (U), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate in 1 min.

2.6. Biological Delignification. Erlenmeyer flasks containing 3 g of sugarcane bagasse (particle size between 0.8 and 2 mm 50% and <0.8 mm 50%) were autoclaved and humidified with a saline solution (sterilized by filtration, 10 mL/g bagasse) presenting the following constant composition: $MgSO_4{\cdot}7H_2O$ (0.3 g/L), $FeSO_4{\cdot}7H_2O$ (0.005 g/L), $MnSO_4{\cdot}H_2O$ (0.00156 g/L), $ZnSO_4{\cdot}7H_2O$ (0.0014 g/L), $CaCl_2$ (0.3 g/L), $CoCl_2$ (0.002 g/L), KH_2PO_4 (1.5 g/L), and pH 5.5. Yeast extract (sterilized by autoclaving at 121°C, 1 atm, 15 min) and copper sulfate (sterilized by filtration) were added to the saline solution to reach final concentrations of 6.4 g/L and 173 μM, respectively. Ferulic acid (sterilized by filtration) was added after 48 h of fermentation to a final concentration of 1.86 mM. The inoculum was prepared according to Section 2.2. and the incubation was performed at 29°C. Physicochemical analyses of the biotreated bagasse were performed as described in Section 2.1.

3. Results and Discussion

3.1. Screening of Significant Variables Affecting Laccase Production: Plackett-Burman Design. Table 3 presents the results of the Plackett-Burman experiments to select significant variables to be optimized for laccase production by the strain *P. ostreatus* Pl 22 Em.

According to the Pareto analysis, the variables that presented significant effects on laccase production at the confidence level of 90% were copper sulfate and ammonium sulfate concentrations, both presenting positive standardized effects of 11.25 and 5.003, respectively (absolute values, $R^2 = 0.96873$).

As far as the effect of copper sulfate is concerned, the addition of copper as an inducer of laccase production has been already reported in literature. Different studies have shown that laccase production is regulated by metal ions such as Cu^{2+} and Fe^{3+} by gene expression induction or through translational or posttranslational regulation [22, 23]. Palmieri et al. [4] demonstrated that the addition of copper sulfate 150 μM to a *P. ostreatus* (Jacq.:Fr.) Kummer (type Florida) liquid culture medium caused a 30-fold increase in total laccase activity, and Hou et al. [5] reported a 4.5-fold increase in laccase activity in *P. ostreatus* (strain 32, Dalian Institute of Mushroom Study) when Cu^{2+} 1 mM was added to the liquid culture medium. Baldrian and Gabriel [24] concluded that Cu^{2+} not only induces laccase by the expression of laccase genes in *P. ostreatus* CCBAS-447 (Institute of Microbiology, Academy of Sciences of the Czech Republic) but also positively affects activity and stability of the enzyme.

As far as the effect of ammonium sulfate is concerned, the production of ligninolytic enzymes has been associated with the secondary metabolism and with conditions of limited nitrogen for many white rot fungi, including the model organism for laccase production and lignin degradation

Table 3: Results of laccase activity obtained for the Plackett-Burman experiments after 5 days of solid-state fermentation on sugarcane bagasse.

	Variables and corresponding levels								Activity
	pH	A_w[a]	T[b]	$[Cu^{2+}]$[c]	[N][d]	[PK][e]	[Asn][f]	[YE][g]	U/g
1	1	−1	1	−1	−1	−1	1	1	2.091
2	1	1	−1	1	−1	−1	−1	1	7.420
3	−1	1	1	−1	1	−1	−1	−1	3.278
4	1	−1	1	1	−1	1	−1	−1	7.366
5	1	1	−1	1	1	−1	1	−1	11.45
6	1	1	1	−1	1	1	−1	1	4.294
7	−1	1	1	1	−1	1	1	−1	6.825
8	−1	−1	1	1	1	−1	1	1	10.94
9	−1	−1	−1	1	1	1	−1	1	11.18
10	1	−1	−1	−1	1	1	1	−1	3.729
11	−1	1	−1	−1	−1	1	1	1	1.914
12	−1	−1	−1	−1	−1	−1	−1	−1	2.755
C[h]	0	0	0	0	0	0	0	0	7.318
C′	0	0	0	0	0	0	0	0	6.727

Note: enzyme activities in units per gram of dry substrate.
[a]Water activity; [b]temperature; [c]$CuSO_4$ concentration; [d]$(NH_4)_2SO_4$ concentration; [e]KH_2PO_4 concentration; [f]asparagine concentration; [g]yeast extract concentration; [h]C and C′ represent the duplicates of the intermediate level.

Phanerochaete chrysosporium [25]. For *P. ostreatus* (HAI 493, Nextlab, Hawaii), however, a higher concentration of nitrogen in the medium did not repress but rather slightly stimulated mineralization of lignin, as reported by Stajić et al. [26]. Kaal et al. [25] also suggested that several white rot fungi strains, including *P. ostreatus*, produce higher ligninolytic enzyme activities in response to a nitrogen-rich medium.

3.2. Comparison between Organic and Inorganic Nitrogen Sources and Evaluation of Ferulic Acid as Inducer of Laccase Activity Production. The comparison between the laccase activities produced by the strain *P. ostreatus* Pl 22 Em when different sources of nitrogen were used and when ferulic acid was added is presented in Table 4. Using yeast extract (7.5 g/L, containing 7% total nitrogen) instead of ammonium sulfate (2.5 g/L, containing 21% nitrogen) for the same concentration of total nitrogen caused an increase of 5.7-fold in laccase production (44.23 ± 2.44 versus 9.942 ± 1.97 U/g dry substrate after 5 days of fermentation).

These results are in accordance with Hou et al. [5], who demonstrated that the most suitable nitrogen sources for laccase production by *P. ostreatus* (strain 32) were peptone and yeast extract, in comparison with urea, ammonium sulfate, and ammonium tartrate. These organic nitrogen sources increased laccase activity in 1.55, 1.99, and 1.46, respectively, (peptone) and 1.40, 1.79, and 1.32, respectively (yeast extract). Mishra and Kumar [27] also demonstrated that, regarding enhancement of laccase production in solid-state fermentation by *P. ostreatus* MTCC1804 (Institute of Microbial Technology, Chandigarh, India), yeast extract was preferred to inorganic nitrogen sources, reaching 23 U/g dry substrate against 2.2 U/g (without nitrogen supplementation), 10.11 U/g (with ammonium sulfate), and 13.0 U/g (with urea). Highest activity, however, was obtained in the presence of cyanobacterial biomass and copper sulfate 1 mM (65 U/g after 10 days). These results may be attributed to the presence of some additives (nutrients/activators) and favorable C : N ratio of the organic nitrogen sources [27].

Table 4: Effect of inorganic and organic nitrogen sources and different inducers, Cu^{2+} and ferulic acid (Fer), on the level of laccase activity produced by the strain *P. ostreatus* 22 Em after 5 days of fermentation on sugarcane bagasse.

N source (g/L)	Inducer	U/g dry substrate
Ammonium sulfate		
2.5	$CuSO_4$ 150 μM	9.942 ± 1.97
Yeast extract		
2.5	0	2.970 ± 0.651
2.5	$CuSO_4$ 150 μM	44.23 ± 2.44
2.5	$CuSO_4$ 150 μM + Fer 2 mM	89.18 ± 3.95
7.5	$CuSO_4$ 150 μM	56.25 ± 5.08

Addition of ferulic acid to the copper containing medium further enhanced laccase production (2.0-fold) by the strain *P. ostreatus* Pl 22 Em (89.18 ± 3.95 versus 44.23 ± 2.44 U/g dry substrate). Ferulic acid is a known inducer of laccase production. The structure of this organic acid is similar to that of coniferyl alcohol, the most abundant monolignol of the three lignin precursors [28]. Vanhulle et al. [29] reported a positive effect of ferulic acid 0.5 mM on laccase production by *P. ostreatus* IT01 in submerged fermentation with glucose and lactose as substrates. A peak of laccase activity (around 7,500 U/L) was observed at the 15th day of fermentation (3-fold increase when compared to control). Ferulic acid was also shown to be the best inducer of laccase activity in *Pleurotus sajor-caju* [28]. In solid-state fermentation, Meza et al. [30] reported a laccase activity of approximately 70 U/g of sugarcane bagasse in the presence of ferulic acid 10 mM, produced by *Pycnoporus cinnabarinus*

Table 5: Results of laccase activity obtained for the central composite design experiments after 5 days of solid-state fermentation on sugarcane bagasse.

	Variables			Response
	Yeast extract g/L	$CuSO_4$ μM	Ferulic acid mM	Activity U/g
1	4	75	1	61.30
2	10	75	1	51.02
3	4	225	1	98.12
4	10	225	1	58.80
5	4	75	3	50.90
6	10	75	3	46.60
7	4	225	3	68.80
8	10	225	3	65.60
9	1.96	150	2	82.19
10	12.04	150	2	52.75
11	7	24	2	62.33
12	7	276	2	35.91
13	7	150	0.32	48.03
14	7	150	3.68	89.43
15	7	150	2	158.8
16	7	150	2	149.4

Note: enzyme activities in units per gram of dry substrate.

after 10 days, in contrast with around 10 U/g without inducers. It is worth noting that the values achieved in this study in the presence of copper sulfate (44.23 ± 2.44 U/g) and ferulic acid (89.18 ± 3.95 U/g) after 5 days of fermentation were promising when compared to other results reported in literature.

3.3. Determination of the Mathematical Model of Laccase Production through the Response Surface Methodology: Central Composite Design and Kinetics of Laccase Production under Optimized Conditions. Table 5 presents the results of 16 experiments to evaluate the effect of yeast extract, copper sulfate, and ferulic acid concentrations on laccase production by solid-state fermentation in sugarcane bagasse.

According to Table 6, the linear and quadratic terms of all three variables significantly affected the response. Interaction effects (not shown) were not significant. The mathematical model of laccase production can be given by the following equation:

$$\begin{aligned}\text{Laccase activity}\,(\text{U/g}) = {} & -249.9 + 41.53\,[\text{YE}] \\ & - 3.236\,[\text{YE}]^2 + 2.071\left[\text{Cu}^{2+}\right] \\ & - 0.0060\left[\text{Cu}^{2+}\right]^2 + 106.7\,[\text{Fer}] \\ & - 28.68\,[\text{Fer}]^2,\end{aligned} \quad (1)$$

where concentrations of yeast extract (YE), copper (Cu^{2+}), and ferulic acid (Fer) are given in g/L, μM, and mM, respectively.

Table 6: Regression coefficients and identification of significant variables ($P < 0.05$) for laccase production using central composite design, $R^2 = 0.8753$.

Factor	Coefficients	Standard error	t-value	P value
Intercept	−249.9	80.11	−3.120	0.02059
Yeast extract (L^a)	41.53	12.19	3.405	0.01440
Yeast extract (Q^b)	−3.236	0.7220	−4.482	0.004182
Cu^{2+} (L)	2.071	0.4551	4.550	0.003890
Cu^{2+} (Q)	−0.0060	0.00116	−5.483	0.001539
Ferulic acid (L)	106.7	34.13	3.127	0.02040
Ferulic acid (Q)	−28.68	6.498	−4.414	0.004501

[a]Linear; [b]quadratic.

The predicted model indicated that the maximum laccase activity (161.3 U/g dry substrate) would be obtained at the following conditions: yeast extract 6.417 g/L, Cu^{2+} 172.6 μM, and ferulic acid 1.860 mM.

Experiments for the verification of the predicted model (Table 7) revealed a correlation coefficient (R^2) of 0.8963, the most significant differences being obtained at the lowest and highest levels (24% and 44%, resp.). However, when these points were not considered, the model described the laccase production as a function of yeast extract, $CuSO_4$, and ferulic acid concentrations, within the range of 5.5 to 8.5 g/L, 112.5 to 187.5 μM, and 1.5 to 2.5 mM, respectively, with a correlation coefficient of 0.9798.

Other values reported in the literature for laccase production by *P. ostreatus* in solid-state fermentation are 65.42 U/g with copper as inducer [27], 9 U/g without inducers [31], and 36 U/g without inducers [21].

Figure 1 presents the kinetics of laccase production under optimized conditions. The peak of laccase activity (151.6 U/g) was obtained between the 4th day and the 5th day of solid-state fermentation. Other values of laccase productivity reported in the literature are 80 U/mL after 12 days in liquid culture of *P. ostreatus* hybrids without exogenous inducers [6], 12.2 U/mL after 18 days of liquid fermentation by *P. ostreatus* [32], 90 U/g of sugarcane bagasse produced by *Pycnoporus cinnabarinus* after 14 days of solid-state fermentation in columns and activities near 80 U/g after 6 days [33], and 70 U/g of sugarcane bagasse, produced by *Pycnoporus cinnabarinus* after 10 days [30]. The maximum value of laccase production achieved with the strain *P. ostreatus* Pl 22 Em in this study (151.6 U/g) after 5 days of solid-state fermentation was promising in terms of enzyme activity and productivity.

Further enhancement in the production of laccase could be achieved by other strategies such as coculturing ligninolytic fungi and filamentous microfungi, as demonstrated by Cupul et al. [34]. These authors reported an increase in laccase activity from 4,881.0 to 12,382.5 U/mg protein, when the fungus *Paecilomyces carneus* was inoculated to a culture of the ligninolytic fungus *Trametes maxima*. Also, studies on gene expression could be performed since the physiological behavior of many laccase producing organisms suggests the presence of elements responsive to metals (MRE), xenobiotics

Table 7: Experiments for the verification of the predicted model of laccase production after 5 days of solid-state fermentation on sugarcane bagasse.

Yeast extract (g/L)	$CuSO_4$ (μM)	Ferulic acid (mM)	Predicted (U/g)	Experimental (U/g)
4.0	75	1.0	64.04	79.74 ± 7.94
5.5	112.5	1.5	133.2	114.7 ± 7.17
6.4	172.6	1.86	161.3	155.3 ± 5.92
7.0	150	2.0	156.6	149.2 ± 4.95
8.5	187.5	2.5	134.2	122.9 ± 5.12
10	225	3.0	66.01	95.17 ± 7.75

Note: enzyme activities in units per gram of dry substrate.

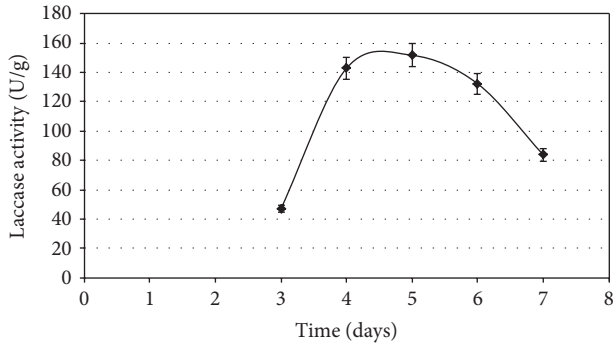

Figure 1: Kinetics of laccase production on solid-state fermentation of sugarcane bagasse by *P. ostreatus* 22 Em under optimized conditions: 6.4 g/L yeast extract, 172.6 μM $CuSO_4$, and 1.86 mM ferulic acid.

(XRE), heat shock (HSE), and oxidative stress (ARE, antioxidant response element) within the promoter region of laccase genes [35]. Regarding heterologous expression, advantages are more related to providing favorable conditions for genetic studies and enzyme characterization than to the production of high activities. Macellaro et al. [36] developed a process for heterologous production of the high redox potential *Pleurotus ostreatus* laccase POXA1b and one of its variants, 1H6C, using *Aspergillus niger* as a host, and obtained production levels of 35,000 U/L and 60,000 U/L, respectively. You et al. [37] reached a maximum laccase activity of 685.8 U/L through heterologous expression of a *Ganoderma lucidum* laccase in *Pichia pastoris*.

3.4. Characterization of the Sugarcane Bagasse and Kinetics of Biological Delignification. The culture conditions optimized for laccase production were applied in the biotreatment of sugarcane bagasse with the aim of evaluating lignin degradation. The sugarcane bagasse received from the industry presented the following particle size distribution: 21.2% between 0.8 and 2.0 mm, 22.8% < 0.8 mm, and 56% > 2.0 mm. After grinding and classification, the particle size distribution changed to 42.4% between 0.8 and 2.0 mm and 57.6% < 0.8 mm. The physicochemical composition of sugarcane bagasse before and after the biotreatment of solid-state fermentation is presented in Table 8. All values of lignin percentage were statistically different among samples. Extractives in sugarcane bagasse can be represented by waxes, pigments, alkaloids, terpenes, flavonoids, coumarins, tannins, sugars, and saponins [38]. Reduction of lignin content was of 5.53% after 5 days and of 11.1% after 15 days of solid-state fermentation. Of these percentage reductions, 3.41% were not due to fungal degradation but to the addition of nutrients for optimized laccase production.

The process developed by Pellinen et al. [39] to delignify kraft pulp and chemithermomechanical pulp (CTMP) using *Phanerochaete chrysosporium* presented delignification times of around two weeks, the kappa number (residual lignin) being reduced from 33 to less than 10 for the kraft pulp, and the lignin content decreasing from 26.5% to 21.3% for the CTMP. Delignification of sugarcane bagasse by *Ceriporiopsis subvermispora* during 30 days resulted in a pulp yield of 46–54% [40]. Meza et al. [41] presented a process for biological delignification of sugarcane bagasse and simultaneous production of laccases that yielded a laccase activity of 80 U/g and an energy economy of 50% during pulping and refining, after 28 days of fungal treatment. Knežević et al. [42] reported a reduction of 34.1% in lignin content of wheat straw after 14 days of cultivation of *Dichomitus squalens*.

The process of delignification is very complex and involves the synergistic action of laccases and peroxidases. Although laccases are directly involved in delignification through the oxidation of phenolic structures, their production level is not necessarily related to the rate of delignification. Also, lignin degradation can occur until the latest stages of fermentation, even if the peak of enzymatic activity is achieved earlier [42]. The fungus *P. ostreatus* 22 Em, which demonstrated to be a high producer of laccases on sugarcane bagasse after 5 days of solid-state fermentation, presented a moderate efficiency of delignification with the highest rate achieved between the 5th day and the 10th day. However, delignification is not the only beneficial action of laccases with respect to the use of lignocellulosic biomass. Although this action facilitates the cellulose hydrolysis and glucose release, there is also a contribution related to the degradation of phenolic compounds released during pretreatment, which reduces the toxicity of the broth for a subsequent fermentation [43, 44].

4. Conclusions

The level of laccase activity produced by *P. ostreatus* Pl 22 Em in solid-state fermentation of sugarcane bagasse was significantly affected by the concentrations of nitrogen

Table 8: Physicochemical composition of sugarcane bagasse, before and after biotreatment.

	Sugarcane bagasse	Bagasse prepared for biotreatment	Biotreated bagasse 5 days	Biotreated bagasse 10 days	Biotreated bagasse 15 days
Lignin (%)	31.89	28.48	26.36	22.37	20.79
Holocellulose (%)	63.36	63.30	64.38	67.84	69.12
Extractives (%)	2.15	5.88	5.57	6.05	6.17
Ashes (%)	2.60	2.34	3.69	3.74	3.92
Moisture (%)	7.57	91.6	88.28	87.71	85.91

Note: percentages of lignin, holocellulose, extractives, and ashes are in moisture free basis. Average standard deviations were 0.367 for lignin, 0.165 for extractives, 0.233 for ashes, and 0.0587 for moisture.

source, copper sulfate, and ferulic acid. The use of an organic nitrogen source (yeast extract) provided and increase of 5.7-fold in laccase production, in comparison with the inorganic source (ammonium sulfate). The predicted model indicated that the maximum laccase activity (161.3 U/g of sugarcane bagasse) would be obtained at the following conditions: yeast extract 6.417 g/L, Cu^{2+} 172.6 μM, and ferulic acid 1.860 mM. Experimentally, the maximum laccase activity of 151.6 U/g was produced, under optimized conditions, at the 5th day of solid-state fermentation, which is higher than that obtained in other solid-state fermentations so far reported on sugarcane bagasse. On the other hand, the process of biological delignification reduced the lignin content of sugarcane bagasse from 31.89% to 26.36% after 5 days and to 20.79% after 15 days of solid-state fermentation, which represents a moderate efficiency of delignification. The highest rate of lignin degradation was achieved between the 5th day and the 10th day.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] F. Autore, C. Del Vecchio, F. Fraternali, P. Giardina, G. Sannia, and V. Faraco, "Molecular determinants of peculiar properties of a *Pleurotus ostreatus* laccase: analysis by site-directed mutagenesis," *Enzyme and Microbial Technology*, vol. 45, no. 6-7, pp. 507–513, 2009.

[2] P. Dwivedi, V. Vivekanand, N. Pareek, A. Sharma, and R. P. Singh, "Co-cultivation of mutant *Penicillium oxalicum* SAU E-3.510 and *Pleurotus ostreatus* for simultaneous biosynthesis of xylanase and laccase under solid-state fermentation," *New Biotechnology*, vol. 28, no. 6, pp. 616–626, 2011.

[3] D. W. S. Wong, "Structure and action mechanism of ligninolytic enzymes," *Applied Biochemistry and Biotechnology*, vol. 157, no. 2, pp. 174–209, 2009.

[4] G. Palmieri, P. Giardina, C. Bianco, B. Fontanella, and G. Sannia, "Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*," *Applied and Environmental Microbiology*, vol. 66, no. 3, pp. 920–924, 2000.

[5] H. Hou, J. Zhou, J. Wang, C. Du, and B. Yan, "Enhancement of laccase production by *Pleurotus ostrea*tus and its use for the decolorization of anthraquinone dye," *Process Biochemistry*, vol. 39, no. 11, pp. 1415–1419, 2004.

[6] V. Lettera, C. del Vecchio, A. Piscitelli, and G. Sannia, "Low impact strategies to improve ligninolytic enzyme production in filamentous fungi: the case of laccase in *Pleurotus ostreatus*," *Comptes Rendus Biologies*, vol. 334, no. 11, pp. 781–788, 2011.

[7] A. Piscitelli, P. Giardina, V. Lettera, C. Pezzella, G. Sannia, and V. Faraco, "Induction and transcriptional regulation of laccases in fungi," *Current Genomics*, vol. 12, no. 2, pp. 104–112, 2011.

[8] P. Giardina, V. Faraco, C. Pezzella, A. Piscitelli, S. Vanhulle, and G. Sannia, "Laccases: a never-ending story," *Cellular and Molecular Life Sciences*, vol. 67, no. 3, pp. 369–385, 2010.

[9] S. R. Couto and J. L. T. Herrera, "Industrial and biotechnological applications of laccases: a review," *Biotechnology Advances*, vol. 24, no. 5, pp. 500–513, 2006.

[10] F. Gassara, S. K. Brar, R. D. Tyagi, M. Verma, and R. Y. Surampalli, "Screening of agro-industrial wastes to produce ligninolytic enzymes by *Phanerochaete chrysosporium*," *Biochemical Engineering Journal*, vol. 49, no. 3, pp. 388–394, 2010.

[11] A. Pandey, C. R. Soccol, P. Nigam, and V. T. Soccol, "Biotechnological potential of agro-industrial residues. I: sugarcane bagasse," *Bioresource Technology*, vol. 74, no. 1, pp. 69–80, 2000.

[12] C. R. Soccol and L. P. S. Vandenberghe, "Overview of applied solid-state fermentation in Brazil," *Biochemical Engineering Journal*, vol. 13, no. 2-3, pp. 205–218, 2003.

[13] C. R. Soccol, L. P. D. S. Vandenberghe, A. B. P. Medeiros et al., "Bioethanol from lignocelluloses: status and perspectives in Brazil," *Bioresource Technology*, vol. 101, no. 13, pp. 4820–4825, 2010.

[14] R. C. L. B. Rodrigues, M. D. G. A. Felipe, J. B. Almeida e Silva, and M. Vitolo, "Response surface methodology for xylitol production from sugarcane bagasse hemicellulosic hydrolyzate using controlled vacuum evaporation process variables," *Process Biochemistry*, vol. 38, no. 8, pp. 1231–1237, 2003.

[15] P. Binod, K. Satyanagalakshmi, R. Sindhu, K. U. Janu, R. K. Sukumaran, and A. Pandey, "Short duration microwave assisted

pretreatment enhances the enzymatic saccharification and fermentable sugar yield from sugarcane bagasse," *Renewable Energy*, vol. 37, no. 1, pp. 109–116, 2012.

[16] J. Lee, "Biological conversion of lignocellulosic biomass to ethanol," *Journal of Biotechnology*, vol. 56, no. 1, pp. 1–24, 1997.

[17] M. A. Falcón, A. Rodríguez, A. Carnicero et al., "Isolation of microorganisms with lignin transformation potential from soil of Tenerife Island," *Soil Biology and Biochemistry*, vol. 27, no. 2, pp. 121–126, 1995.

[18] P. Giardina, G. Palmieri, B. Fontanella, V. Rivieccio, and G. Sannia, "Manganese peroxidase isoenzymes produced by *Pleurotus ostreatus* grown on wood sawdust," *Archives of Biochemistry and Biophysics*, vol. 376, no. 1, pp. 171–179, 2000.

[19] R. R. Singhania, A. K. Patel, C. R. Soccol, and A. Pandey, "Recent advances in solid-state fermentation," *Biochemical Engineering Journal*, vol. 44, no. 1, pp. 13–18, 2009.

[20] C. R. Menezes, I. S. Silva, and L. R. Durrant, "Bagaço de cana: fonte para produção de enzimas ligninocelulolíticas," *Estudos Tecnológicos em Engenharia*, vol. 5, no. 1, pp. 68–78, 2009.

[21] D. Iandolo, A. Piscitelli, G. Sannia, and V. Faraco, "Enzyme production by solid substrate fermentation of *Pleurotus ostreatus* and *Trametes versicolor* on tomato pomace," *Applied Biochemistry and Biotechnology*, vol. 163, no. 1, pp. 40–51, 2011.

[22] V. Faraco, P. Giardina, G. Palmieri, and G. Sannia, "Metal-activated laccase promoters," *Progress in Biotechnology*, vol. 21, pp. 105–111, 2002.

[23] M. I. Fonseca, E. Shimizu, P. D. Zapata, and L. L. Villalba, "Copper inducing effect on laccase production of white rot fungi native from Misiones (Argentina)," *Enzyme and Microbial Technology*, vol. 46, no. 6, pp. 534–539, 2010.

[24] P. Baldrian and J. Gabriel, "Copper and cadmium increase laccase activity in *Pleurotus ostreatus*," *FEMS Microbiology Letters*, vol. 206, no. 1, pp. 69–74, 2002.

[25] E. E. J. Kaal, J. A. Field, and T. W. Joyce, "Increasing ligninolytic enzyme activities in several white-rot Basidiomycetes by nitrogen-sufficient media," *Bioresource Technology*, vol. 53, no. 2, pp. 133–139, 1995.

[26] M. Stajić, L. Persky, D. Friesem et al., "Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected *Pleurotus* species," *Enzyme and Microbial Technology*, vol. 38, no. 1-2, pp. 65–73, 2006.

[27] A. Mishra and S. Kumar, "Cyanobacterial biomass as N-supplement to agro-waste for hyper-production of laccase from *Pleurotus ostreatus* in solid state fermentation," *Process Biochemistry*, vol. 42, no. 4, pp. 681–685, 2007.

[28] P. Zucca, A. Rescigno, A. Olianas, S. MacCioni, F. A. Sollai, and E. Sanjust, "Induction, purification, and characterization of a laccase isozyme from *Pleurotus sajor-caju* and the potential in decolorization of textile dyes," *Journal of Molecular Catalysis B: Enzymatic*, vol. 68, no. 2, pp. 216–222, 2011.

[29] S. Vanhulle, R. Radman, R. Parra et al., "Effect of mannan oligosaccharide elicitor and ferulic acid on enhancement of laccases production in liquid cultures of basidiomycetes," *Enzyme and Microbial Technology*, vol. 40, no. 7, pp. 1712–1718, 2007.

[30] J. C. Meza, R. Auria, A. Lomascolo, J.-C. Sigoillot, and L. Casalot, "Role of ethanol on growth, laccase production and protease activity in *Pycnoporus cinnabarinus* ss3," *Enzyme and Microbial Technology*, vol. 41, no. 1-2, pp. 162–168, 2007.

[31] D. Iandolo, A. Amore, L. Birolo, G. Leo, G. Olivieri, and V. Faraco, "Fungal solid state fermentation on agro-industrial wastes for acid wastewater decolorization in a continuous flow packed-bed bioreactor," *Bioresource Technology*, vol. 102, no. 16, pp. 7603–7607, 2011.

[32] S. Tlecuitl-Beristain, C. Sánchez, O. Loera, G. D. Robson, and G. Díaz-Godínez, "Laccases of *Pleurotus ostreatus* observed at different phases of its growth in submerged fermentation: production of a novel laccase isoform," *Mycological Research*, vol. 112, no. 9, pp. 1080–1084, 2008.

[33] J. C. Meza, A. Lomascolo, L. Casalot, J.-C. Sigoillot, and R. Auria, "Laccase production by *Pycnoporus cinnabarinus* grown on sugar-cane bagasse: influence of ethanol vapours as inducer," *Process Biochemistry*, vol. 40, no. 10, pp. 3365–3371, 2005.

[34] W. C. Cupul, G. H. Abarca, D. M. Carrera, and R. R. Vázquez, "Enhancement of ligninolytic enzyme activities in a *Trametes maxima-Paecilomyces* carneus co-culture: key factors revealed after screening using a Plackett-Burman experimental design," *Electronic Journal of Biotechnology*, vol. 17, no. 3, pp. 114–121, 2014.

[35] G. Janusz, K. H. Kucharzyk, A. Pawlik, M. Staszczak, and A. J. Paszczynski, "Fungal laccase, manganese peroxidase and lignin peroxidase: gene expression and regulation," *Enzyme and Microbial Technology*, vol. 52, no. 1, pp. 1–12, 2013.

[36] G. Macellaro, M. C. Baratto, A. Piscitelli et al., "Effective mutations in a high redox potential laccase from *Pleurotus ostreatus*," *Applied Microbiology and Biotechnology*, vol. 98, no. 11, pp. 4949–4961, 2014.

[37] L.-F. You, Z.-M. Liu, J.-F. Lin, L.-Q. Guo, X.-L. Huang, and H.-X. Yang, "Molecular cloning of a laccase gene from *Ganoderma lucidum* and heterologous expression in *Pichia pastoris*," *Journal of Basic Microbiology*, vol. 54, no. 1, pp. 134–141, 2013.

[38] T. M. Arantes, G. B. Christofoletti, L. V. A. Gurgel, K. Marabezi, and A. A. S. Curvelo, "Estudo preliminar de extrativos do bagaço de cana-de-açúcar," in *32ªReunião Anual da Sociedade Brasileira de Química*, Fortaleza, Brasil, 2009.

[39] J. Pellinen, J. Abuhasan, T. W. Joyce, and H.-M. Chang, "Biological delignification of pulp by *Phanerochaete chrysosporium*," *Journal of Biotechnology*, vol. 10, no. 2, pp. 161–170, 1989.

[40] S. M. Costa, A. R. Gonçalves, and E. Esposito, "*Ceriporiopsis subvermispora* used in delignification of sugarcane bagasse prior to soda/anthraquinone pulping," *Applied Biochemistry and Biotechnology Part A: Enzyme Engineering and Biotechnology*, vol. 122, no. 1–3, pp. 695–706, 2005.

[41] J. C. Meza, J.-C. Sigoillot, A. Lomascolo, D. Navarro, and R. Auria, "New process for fungal delignification of sugar-cane bagasse and simultaneous production of laccase in a vapor phase bioreactor," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 11, pp. 3852–3858, 2006.

[42] A. Knežević, I. Milovanović, M. Stajić et al., "Lignin degradation by selected fungal species," *Bioresource Technology*, vol. 138, pp. 117–123, 2013.

[43] A. D. Moreno, D. Ibarra, I. Ballesteros, J. L. Fernández, and M. Ballesteros, "Ethanol from laccase-detoxified lignocellulose by the thermotolerant yeast *Kluyveromyces marxianus*—effects of steam pretreatment conditions, process configurations and substrate loadings," *Biochemical Engineering Journal*, vol. 79, pp. 94–103, 2013.

[44] A. K. Sitarz, J. D. Mikkelsen, P. Højrup, and A. S. Meyer, "Identification of a laccase from *Ganoderma lucidum* CBS 229.93 having potential for enhancing cellulase catalyzed lignocellulose degradation," *Enzyme and Microbial Technology*, vol. 53, no. 6-7, pp. 378–385, 2013.

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Review Article

Biotechnological Aspects and Perspective of Microbial Keratinase Production

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Keratinases are proteolytic enzymes predominantly active when keratin substrates are available that attack disulfide bridges in the keratin to convert them from complex to simplified forms. Keratinases are essential in preparation of animal nutrients, protein supplements, leather manufacture, textile processing, detergent formulation, feather meal processing for feed and fertilizer, the pharmaceutical and biomedical industries, and waste management. Accordingly, it is necessary to develop a method for continuous production of keratinase from reliable sources that can be easily managed. Microbial keratinase is less expensive than conventionally produced keratinase and can be obtained from fungi, bacteria, and actinomycetes. In this overview, the expansion of information about microbial keratinases and important considerations in keratinase production are discussed.

1. Introduction

Keratin is one of the most abundant biopolymers in the world [1]; it is a tough, fibrous, insoluble material that functions as an outer coat of human and animal organs, to prevent the loss of body fluids. Keratin is predominantly found in tissues of reptiles, birds, amphibians, and mammals. The structural component of feathers, hair, nails, horns, hooves, bones, furs, claws, hides, bird beaks, skin, wool, scales, and bristle is made up of keratin (Figure 1). α-keratins (alpha-helix) are usually found in the hair, wool, horns, nails, claws, and hooves of mammals, whereas the harder β-keratin (beta-sheets) is found in bird feathers, beaks, and claws. Keratin is also expressed in the epithelial cell types of digestive organs (liver, pancreas, intestine, and gallbladder), which include hepatocytes, hepatobiliary ductal cells, oval cells, acinar cells, enterocytes of the small intestine, colon, and goblet cells [2]. Keratin is rich in sulfur compounds with disulfide bridges, which imparts them with an insoluble nature. It also contains a variety of amino acids, predominantly cystine, lysine, proline, and serine. Keratin is hard, containing scleroprotein, while it is unreactive against most chemicals and is not digested by pepsin, trypsin, or papain [3]. Higher vertebrates, including humans, cannot digest keratinous materials. Keratin is a monomer that forms bundles of intermediate filaments that are expressed in epithelial cells that have been linked to human liver diseases. Structural details regarding keratin filaments 5 and 14 for heteromeric assembly and perinuclear organization have been reported ([4], Protein Data Bank Accession code: 3TNU; Figure 2(a)). Among different keratin filaments, K8 and K18 are important for the protection of hepatocytes [2]. The representation of K18 caspase-cleavage sites during apoptosis has been described in detail ([2], Figure 2(b)).

Figure 1: Sources of keratin. Different sources such as feathers, hair, nails, horns, hooves, and beak are shown. The hosts for these sources include human, bird, and animal. The hardness of these keratin materials is different in each case.

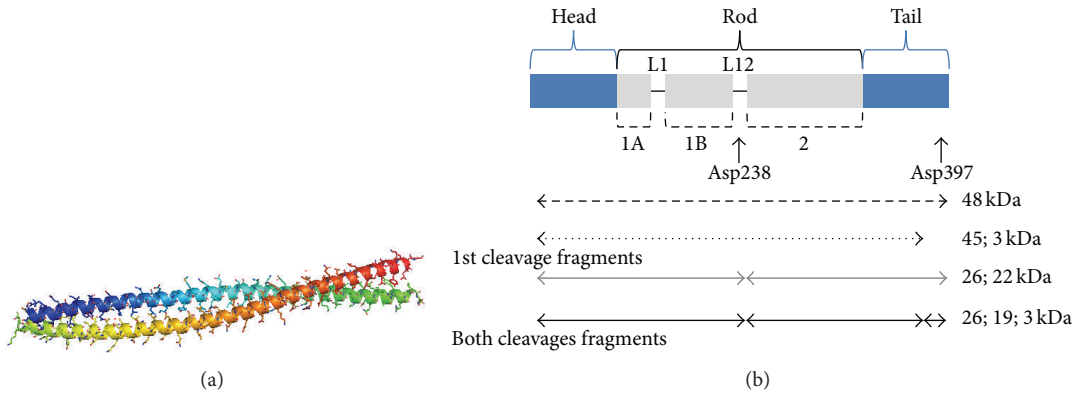

Figure 2: (a) Crystal structure of K5 and K14 coil heterocomplex (PDB accession code: 3TNU). This is a heteromeric assembly and perinuclear organization of keratin filaments. Regions from central-coiled domains of two filaments are interacting. (b) Representation of K18 caspase-cleavage sites during apoptosis. Keratin network regulates apoptotic machinery and confers a caspase-activation. The primary caspase-targets in epithelial cells are found in keratins type I family (reproduced from [2]).

The major sources of keratin accumulation, which cause environmental problems, initiate from industries that use keratin as the raw material. Poultry farms are also involved in dumping of feather wastes (barbs and rachis). Indeed, 90% of feathers are keratin, and millions of kilograms of feathers are discarded to the environment annually [6]. The disposal of feathers is also accompanied by natural falling of feathers and hairs from birds during production, so it is necessary to develop methods to reduce keratin accumulation. For environmental remediation of keratin, an immediate step that has easy processing set-up with lower cost is desired. Microbial keratinase may meet these preferences, as keratinophilic fungi, bacteria, and actinomycetes naturally reside on keratin wastes. Here, we elaborated the currently available information pertaining to microbial keratinase production.

2. Keratinophilic Fungi

Keratinophilic fungi produce the proteolytic enzymes that are capable of decomposing keratinic waste materials [7]. Several keratinophilic fungi that live as parasites on keratinous materials use keratin as their carbon and nitrogen sources, multiply in an asexual manner, and produce conidia. During the process of fungal colonization, boring hyphae are produced to drill into the keratin substrate. These keratinophilic fungi include hyphomycetes and several other taxa [8]; hyphomycetes include both dermatophytic (e.g., *Microsporum* species) and nondermatophytic (e.g., *Chrysosporium* species and other genera) keratinophilic fungi [9]. The dermatophytes are mainly from the genera *Microsporum, Epidermophyton,* and *Trichophyton*. Keratinophilic species are

usually identified by morphological features of their macro- and microconidia, molecular methods, and using DNA sequence analysis [10]. Keratinophilic fungi produce sulfide for sulphitolysis and, during this process, the disulfide bonds of cysteine, a major amino acid in keratinous materials, are broken down, after which the proteolytic enzymes released by the fungi can easily cleave the keratin. During the degradation process, the products released are cysteine, S-sulphocysteine, cysteine acid, cysteine, and inorganic sulfate, and the presence of these products in the culture media indicates the occurrence of true keratinophilic fungi. Fungi that do not show this behavior during degradation are considered nonkeratinophilic fungi. Keratinophilic fungi are predominantly anthropophilic (human loving) or zoophilic (animal loving). Many keratinophilic fungi have been isolated from soil samples due to accumulation of keratin wastes in the soils (geophilic). Soil samples from geophilic habitats including public beaches, agricultural areas, public parks, gardens, and elementary schools have been found to contain keratinophilic fungi [11–15]. Most of these studies involved an isolation technique known as keratin-baiting, in which hair or feathers are used for the isolation of keratinophilic fungi [11–15]. Keratinophilic fungi isolated in countries worldwide, including Egypt, Spain, Australia, Palestine, Kuwait, India, Iran, and Malaysia, have been described [8].

The common isolates of keratinophilic fungi from soils include *Microsporum gypseum, M. canis, M. fulvum, M. nanum, Trichophyton terrestre, T. ajelloi, T. mentagrophytes, T. interdigitale, T. verrucosum, T. equinum, T. rubrum, T. interdigitale, T. schoenleinii, T. simii, Chrysosporium keratinophilum, C. pannicola, C. tropicum,C. indicum, C. anum, C. lobatum, C. evolceanui,* and *C. indicum*. Shadzi et al. [12] have collected 330 samples from thirteen elementary schools and seven public parks and identified 214 species, among which *Chrysosporium keratinophilum* was the dominant organism, being present with a frequency of 54.2%. Anbu et al. collected 10 and 12 soil samples from poultry farms and feather dumping locations, respectively, and recovered 34 fungal species belonging to 19 genera. Among these, six species are dermatophytes belonging to five genera [13]. Kachuei et al. [15] analyzed 800 soil samples from Isfahan province of Iran and found that 588 belong to keratinophilic fungi, representing 73.5% of the total isolates. Furthermore, they recovered 16 species belonging to 11 genera. Similarly, 108 soil samples from St. Kitts and 55 samples from Nevis were shown to consist of 49 and 38 samples, respectively, positive for keratinophilic fungi. Additionally, *M. gypseum* was predominantly found in 15.7 and 40% of soils of these collections sites, respectively, followed by *Chrysosporium* species [14]. Molecular identification of keratinophilic fungi revealed 411 isolates from 22 genera in public park soils from Shiraz, Iran [9]. Another study revealed that 48 soils from Jharkhand, India, contained 10 species of keratinophilic fungi belonging to seven genera [8]. Similarly, 500 samples collected from zoos and parks of Ahvaz were found to contain keratinophilic fungi [16]. In another study, 54 soil samples from different collection sites including gardens, schools, poultry farms, rivers, hospitals, and garbage dumping sites were found to contain 23 species of keratinophilic fungi from 11 genera. The abundance of samples shown to contain keratinophilic fungi was as follows: 65% gardens, 52% schools, 43% poultry farms, 34% garbage, 30% hospitals, and 21% rivers [7]. Based on the above studies, it is clear that keratinophilic fungi are ubiquitous and present in all kinds of soils and that they are dominant in areas where humans and animals live. In addition to the above list, keratinolytic proteins from keratinophilic fungi were reported by Yu et al. [17], Asahi et al. [18], and Williams et al. [19].

3. Keratin-Degrading Bacterial Isolates

Similar to the isolates of fungi, lists of bacterial strains capable of degrading keratins have been reported. Bacteria can grow faster than fungal species and therefore have potential in industrial applications. The advantages of fungi include easier colonization of fungal hyphae into the harder keratin relative to bacteria. The isolated bacterial strains known to degrade keratin or produce the keratinase are primarily composed of *Bacillus*; it includes *B. subtilis* and *B. licheniformis* [20], although other bacteria including Gram-positive *Lysobacter, Nesterenkonia, Kocuria,* and *Microbacterium* and Gram-negative *Vibrio, Xanthomonas, Stenotrophomonas*, *Chryseobacterium, Fervidobacterium, Thermoanaerobacter,* and *Nesterenkonia* can also degrade keratin ([21] and references therein). Several other studies have investigated keratinase produced by bacterial species [22–26]. Sapna and Yamini [27] investigated the potential degradation of keratin by bacterial strains recovered from the soil samples. Four isolates from feather waste were recovered on milk agar plates and three were identified as Gram-negative bacteria (*Burkholderia, Chryseobacterium,* and *Pseudomonas* species) and one was identified as Gram-positive strain (*Microbacterium* species) [28]. Moreover, Korniłłowicz-Kowalska and Bohacz [29] reported that actinomycetes, *Streptomyces* group, namely, *S. fradiae, Streptomyces* species A11, *S. pactum, S. albidoflavus, S. thermoviolaceus* SD8, and *S. graminofaciens*, as well as *Thermoactinomyces candidus*, were capable of producing keratinase.

4. Secretion of Microbial Keratinases

Keratinolytic enzymes are proteases known as keratinases (EC 3.4.21/24/99.11) that can primarily be obtained from fungi, actinomycetes, and bacteria [29]. Fungal keratinases can be easily obtained by secretion, and their low cost makes them preferable over bacterial keratinases in some cases, even though the fungi grow slower and the recovery of keratinase from fungi has been reported for several decades. The availability of several strains that are capable of producing keratinase makes the situation to select efficient keratinase producers an important step. Screening microbial enzymes is essential in the selection process, and the chosen enzymes should be less expensive, eco-friendly, and efficient. Both keratinophilic fungi and nonkeratinophilic fungi can produce keratinases, but the difference is the rate of production, which is higher in the former case. Several methods have been proposed to screen proteolytic (including keratinolytic)

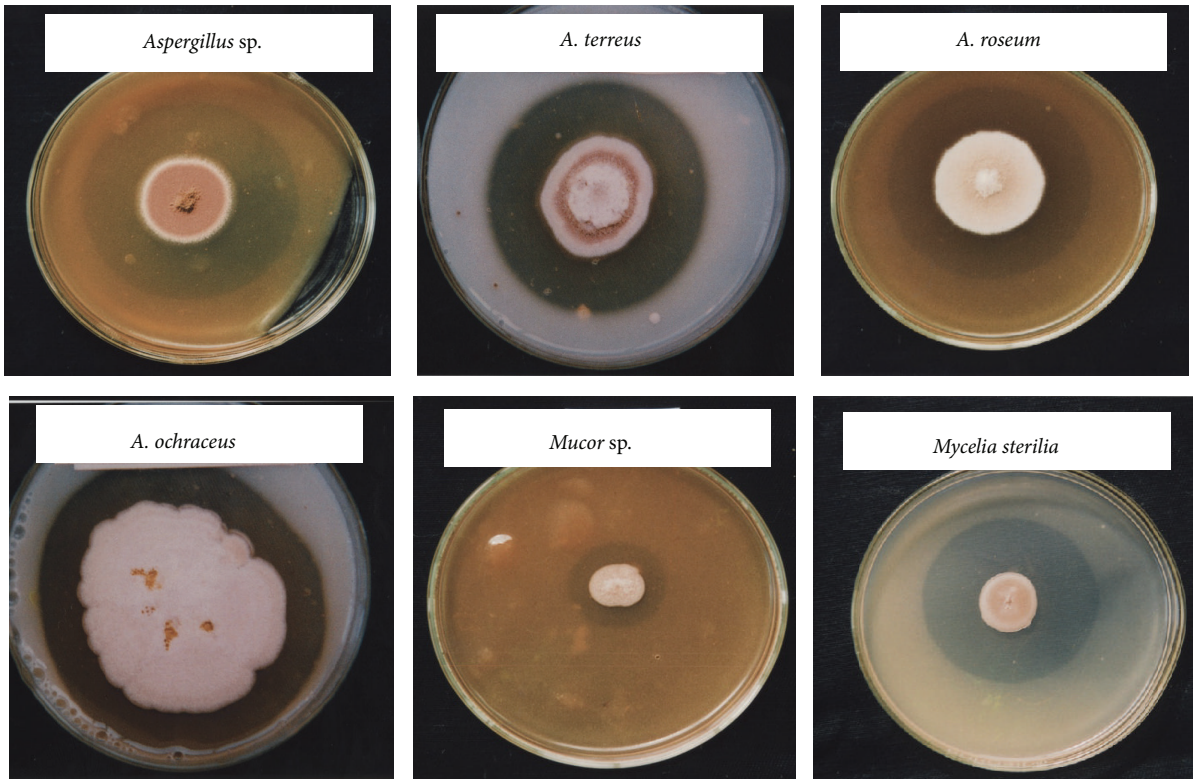

Figure 3: Plate clearance assay for proteolytic activity. Secretion of proteolytic enzymes by *Aspergillus* species, *Mucor* species, and *Mycelia sterilia* is shown as example. The 8% gelatin agar plates were prepared and a pinpoint inoculum was spotted at the center. The clear zone around the colonies indicated the presence of proteolytic activity, which was due to the complete degradation of gelatin. Aqueous saturated solution of ammonium sulfate was added on the surface of the agar for clear visualization.

enzymes, including keratin-baiting, plate screening, spectrophotometric methods, and sequence-based amplification. Jeevana Lakshmi et al. [30] identified feather-degrading bacteria using the 16S rDNA sequence. Among the aforementioned methods, the plate-clearing assay is one of the popular methods due to displaying visual results, as well as being less expensive and easier than other methods (Figure 3). The keratin-baiting method is used for the initial screening and isolation of keratinolytic species. In this method, any keratin source can be the bait; hair and feathers are routinely in use [11, 13]. Even though the pour plate method can be used to isolate the keratinophilic microbes as an alternate, keratin-baiting is also commonly applied because it enables the direct selection of keratinophilic species on the substrate.

5. Optimized Conditions for Microbial Keratinases

Once microbes are isolated, they can be further cultivated on suitable artificial growth media under optimal conditions to obtain excess production of keratinase. Sabouraud's dextrose is commonly used to grow keratinophilic fungi due to its suitability [11, 13, 16]. Usually keratinophilic fungi will take a longer time to degrade the keratin (in weeks). Using the hair-baiting technique, Gugnani et al. [14] found that 4 to 8 weeks were required to observe keratinophilic fungal growth. Kumar et al. [8] isolated keratinophilic fungi after 2 to 4 weeks of incubation, while Mahmoudabadi and Zarrin [16] found that 4 to 5 weeks are necessary to grow. In such cases, optimal growth was found to occur at room temperature. It has also been reported that keratinophilic fungi are able to degrade 40% of keratin after 8 weeks, while less than half (<20%) of that amount can be degraded in the case of nonkeratinophilic fungi [31].

It has been reported that most keratinophilic microbes thrive well under neutral and alkaline pH, the range being 6.0 to 9.0 [32]. Most keratinophilic fungi are mesophiles, although *M. gypseum* and some species of *Chrysosporium* are thermotolerant ([29] and references therein). It has been reported that temperatures of 28°C to 50°C favor keratinase production by most bacteria, actinomycetes, and fungi, while 70°C favors its production by *Thermoanaerobacter* and *Fervidobacterium* species [33–35]. Optimal keratinase production by *Chrysosporium keratinophilum* occurs at 90°C and its half-life is 30 min [36], whereas the thermophile *Fervidobacterium islandicum* AW-1 has an optimum of 100°C and a half-life of 90 min [35].

The complete optimized conditions for microbial keratinases production are described in detail elsewhere [37]. Under optimal condition, keratinophilic fungi, *Scopulariopsis brevicaulis* and *Trichophyton mentagrophytes*, result in keratinase activity to the levels 3.2 and 2.7 Keratinase

Unit (KU)/mL with the ability to degrade 79 and 72.2% of chicken feathers, respectively [38]. Matikeviciene et al. [20] have shown keratinase activity of 152 KU/mL after 24 h of incubation using *Bacillus* species with optimal media. Higher amounts of keratinase were reported by Kanchana [3] at 37°C for 72 h in medium containing feather meal and 0.025% yeast extract at a pH 7.0 under submerged culture. Laba and Rodziewicz [39] optimized the conditions for keratinolytic feather-degrading ability of *Bacillus polymyxa* and *B. cereus*. Additionally, Sivakumar et al. [40] recently optimized the culture conditions for the production of keratinase from *Bacillus cereus* TS1. Using dimeric keratinase obtained from *Bacillus licheniformis* ER-15 complete degradation was achieved within 8 h at pH 8 and 50°C. In this case, 25 g of chicken feathers was degraded with 1200 KU [41].

6. Purification of Keratinases

In addition to the higher keratinase production under optimal conditions, purification of keratinase is necessary for further industrial applications to hasten the efficiency of keratinase action. Molyneux [42] attempted to isolate keratinase from a bacterial source. In other cases, with the purified keratinases, several sizes were reported in the apparent molecular weight range of 27 to 200 kDa from different strains of bacteria and fungi ([29] and references therein). However, Kim et al. [43] reported recovery of keratinase with a molecular weight of 440 kDa. Purified enzymes including keratinases can be obtained using different methodologies. The most common strategy is to purify the enzymes by precipitation followed by column chromatography. Keratinase with a molecular mass of 35 kDa was purified from feather-degrading bacterium using ammonium sulphate precipitation followed by ion-exchange (DEAE-Sepharose) and gel-filtration (Sephadex G-75). The purified keratinase was found to have thermotolerant and showed high specific activity [44]. Using a similar strategy, Zhang et al. [45] purified the alkaline keratinase from *Bacillus* species and identified keratinase of 27 kDa using MALDI-TOF-MS. Anbu et al. [5] isolated keratinase with a molecular weight of 39 kDa from the poultry farm isolate, *Scopulariopsis brevicaulis*, and found that this keratinase had a serine residue near the active site. Keratinase with a size of 41 ± 1 kDa and activity under the optimal conditions at pH 9.0 and 50°C was isolated from *Bacillus megaterium*. This enzyme was also found to have a serine active site and to be inhibited by PMSF [46]. Based on the pH adaptation nature of the keratinase, the column matrix and method of purification can be desired while varying the elution profile (Figure 4). In addition, keratinase purification can also be accomplished with greater efficiency by immunoprecipitation when the appropriate anti-keratinase antibody is available. Similarly, immunochromatography technique can be implemented using anti-keratinase antibody for the efficient purification of keratinase. Purified keratinases from diverse species have displayed higher stability under varied condition (Table 1).

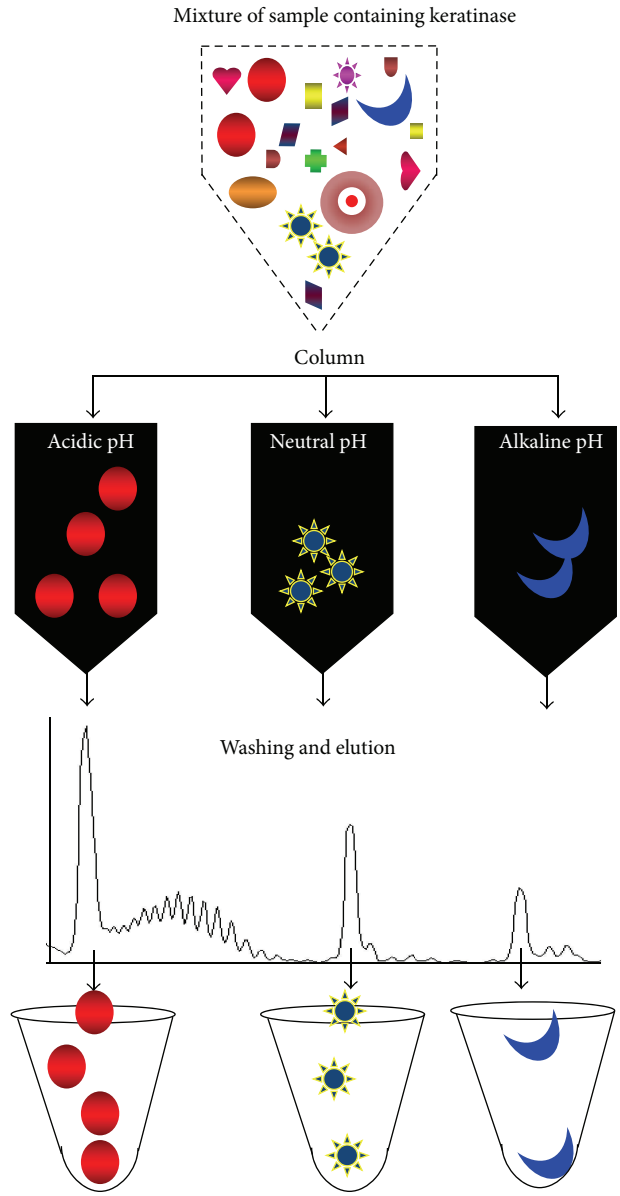

Figure 4: Purification strategy for keratinases. Options with acidic, neutral, and alkaline keratinases are shown. Peak profiles indicate the individual proteins. Conventional purification strategies include ammonium sulphate precipitation followed by ion-exchange and gel-filtration. Other methods such as immunochromatography, high-performance liquid chromatography, and fast protein liquid chromatography are also involved in the purification of keratinases.

7. Acceleration of Microbial Keratinase Production

Following the optimization of the basic conditions for keratinase production and purification, it is necessary to accelerate overproduction of keratinase. This can be accomplished by recombinant DNA technology and statistical optimization. Sequences for both the substrate-keratin and the enzyme-keratinase have been proposed. The primary sequences of the keratin involved in its recombinant production were found by

Table 1: Keratinases from different species for various applications.

Species	Optimal condition (pH)	Aim(s) of study	Reference
	Fungi		
Aspergillus oryzae	8.0	Purification and characterization	[47]
Doratomyces microsporum	8.0–9.0	Comparative analysis	[48]
Paecilomyces marquandii	8.0	Comparative analysis	[48]
Trichophyton rubrum	8.0	Purification and characterization	[18]
Microsporum gypseum	8.0	Secretion of keratinase	[49]
Scopulariopsis brevicaulis	8.0	Dehairing process	[5]
Myrothecium verrucaria	8.3	Feather degradation	[50]
Chrysosporium keratinophilum	9.0	Stable keratinase	[36]
Trichoderma atroviride	8.0–9.0	Feather degradation	[51]
	Bacteria		
Clostridium sporogenes	8.0	Novel keratinolytic activity	[52]
Microbacterium arborescens	7.0	Feather degradation	[53]
Fervidobacterium islandicum	9.0	Feather degradation	[35]
Kytococcus sedentarius	7.0–7.5	Feather degradation	[54]
Stenotrophomonas maltophilia	7.8	Purification and characterization	[55]
Kocuria rosea	7.5	Feather degradation	[56]
Xanthomonas maltophilia	8.0	Purification and characterization	[57]
Streptomyces thermoviolaceus	8.0	Feather degradation	[58]
Bacillus pumilus	10.0	Purification and characterization	[59]
Thermoanaerobacter keratinophilum	8.0	Isolation of keratinophilic species	[34]

Hanukoglu and Fuchs [60, 61] and denoted by type I and type II. Later, several amino acids sequences for keratinase were revealed. The amino acid sequence of keratinase from *Bacillus licheniformis* and other species is available in data bank ([62], e.g., accession code AAB34259). Similarly, the full length of keratin sequences from *Homo sapiens* has been reported ([63], accession code P04264). For the large-scale preparation of keratinase, recombinant DNA technology would yield a large amount of overexpressed enzyme. Recombinant or other keratinases purified using conventional methods have great potential for applications in industrial processes such as dehairing. For example, Anbu et al. [5] have accomplished dehairing using purified keratinase from the keratinophilic fungi, *Scopulariopsis brevicaulis* (Figure 5).

The production levels of any given enzyme can also be improved severalfold using statistical modeling studies. There are different formulations of statistical calculations with basic formulae that have been described. Some basic models for optimization are given in Figure 6, which shows a response surface methodology perturbation plot and mixture trace plot. One of the basic models, the Box-Behnken design, is related to experimental variables by the response equation:

$$Y = f\left(X_1, X_2, X_3, \ldots, X_k\right). \quad (1)$$

A second-degree quadratic polynomial is then used to represent the function by

$$Y = R_0 + \sum_{i=1}^{k} R_i X_i + \sum_{i=1}^{k} R_{ii} X_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} R_{ij} X_i X_j + \varepsilon. \quad (2)$$

The variables and other parameters have been described previously in detail [64]. Using a statistical optimization model, Harde et al. [65] optimized the keratinase production of *Bacillus subtilis* NCIM 2724. These authors used one-factor-at-a-time optimization and an orthogonal array design. Recently, Shankar et al. [66] used response surface methodology, for the optimization of keratinase production by *Bacillus thuringiensis*. Using this design experiment, they compared the actual experimental and predicted calculated values and found that pH 10 and 50°C with 1% mannitol were ideal for keratinase production from *B. thuringiensis*. Similarly, Ramnani and Gupta [67] optimized the medium composition for the production of keratinase from *B. licheniformis* RG1 using response surface methodology. In another study, *B. cereus* was used for the study to optimize keratinase production [68]. Using the Box-Behnken design experiments, Anbu et al. [5] optimized the activity of purified keratinase from *Scopulariopsis brevicaulis* and achieved 100% activity with 5 mM $CaCl_2$ at pH 8.0 and 40°C. Similarly, production of keratinase by *Scopulariopsis brevicaulis* and *Trichophyton mentagrophytes* has also been optimized using Box-Behnhen design experiments by Anbu et al. [38, 69].

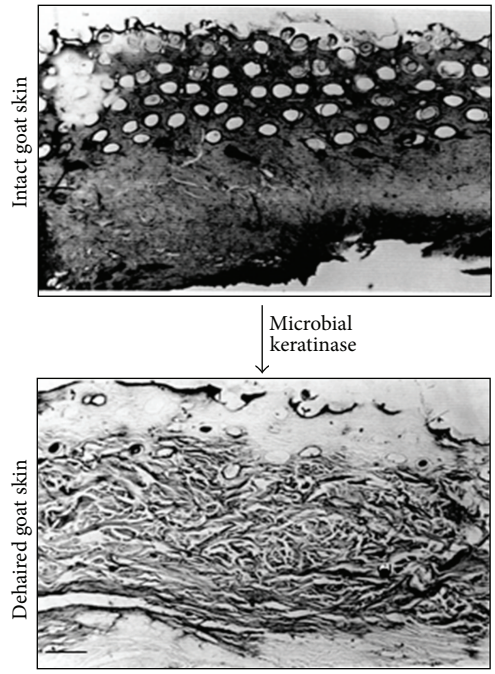

Figure 5: Dehairing using microbial keratinase (2 KU/mL) produced by *Scopulariopsis brevicaulis* (source from [5]). The purified keratinase was sprayed on the flesh side of the skin and then folded and incubated for 30 days. Every 3 days of interval, the dehairing ability was examined.

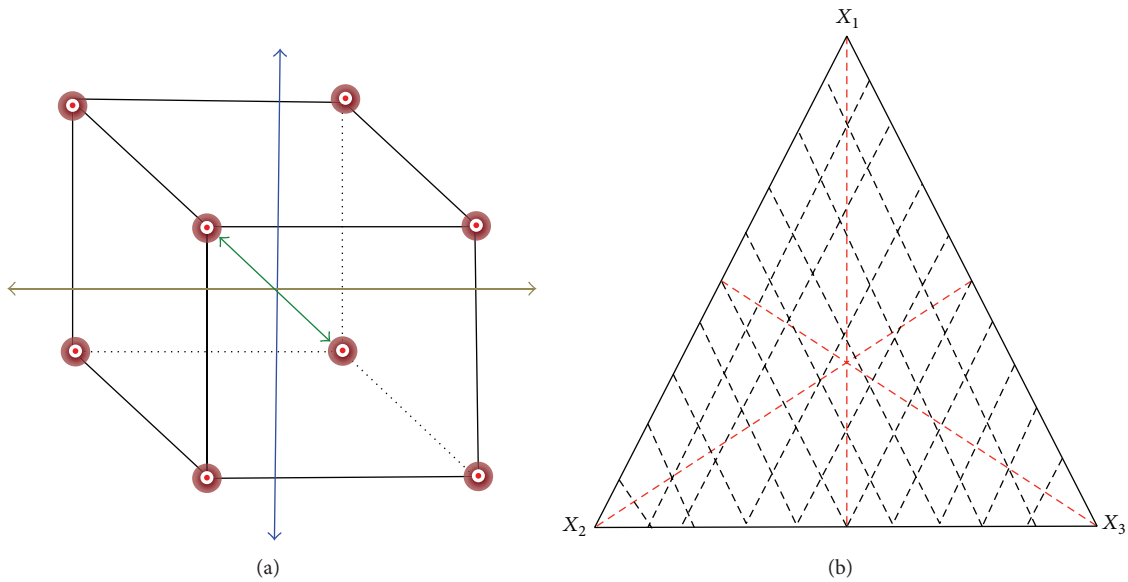

Figure 6: Basic strategy for statistical optimization of keratinase. (a) Response surface methodology perturbation plot; (b) mixture trace plot. Response surface methodology is a collection of statistical techniques for designing experiments, building models, and evaluating the effective factors. It is an efficient statistical technique for optimization of multiple variables to predict best performance conditions with minimum number of experiments.

8. Sensing Keratinases

In the above sections, various aspects regarding the conditions necessary for keratinase to degrade keratin are provided. However, detection strategies are also important for future applications of keratinase. Detection of keratinase or other biomolecules and their interactive analyses with binding partners can be accomplished using biosensors. Biosensors consist of a physicochemical detector and a biological component, enabling binding events to be transduced, thereby allowing detection of very small amounts of target biomolecules (keratinase). Sensors are broadly

classified as electrochemical, electrical, optical and mass-sensitive, chemiluminescence, fluorescence, quantum dot-based, colorimetric, and mass spectroscopic detections. Different sensing surfaces can be adopted for the detection of keratinases. Developing sensing strategies for the detection of keratinase favors the analysis of keratinase from mixtures of a given sample.

Generally, gold- or silica-based sensing surfaces have been used to analyse the biomolecules [70–77]. To capture keratinase on these surfaces, appropriate tags can chemically modify keratinase. Thiol-modification of keratinase can enable its attachment onto the surface of gold or modification of the sensing surface with the COOH-terminal for ultimate attachment to amines on keratinase. Similarly, in the case of silica, surfaces must be chemically modified using amino-coupling agent followed by suitable tags, which can couple an amino group on the keratinase. In short, both gold and silica can be modified to capture keratinase, or keratinase can be modified for specific sensing surfaces as reported in other cases [70, 77]. Diverse keratinases from different species have been reported (Table 1) and these keratinases could be active at different pH and stable, indicating the suitability for various sensing systems.

9. Perspectives

Keratin, which is one of the most abundant hard materials in soil, is difficult to degrade under natural conditions. However, microbial degradation is an easier and less expensive method for conversion of these products to useful end products. Several methods to improve keratinase production have been suggested, and keratinase has been overexpressed, successfully purified, and applied to several industrial applications. In addition, additional developments have been implemented in keratinase research recently [78, 79]. However, there is currently no highly sensitive system available for the detection of keratinases. In addition, use of recombinant keratinase chimeras has the potential to generate efficient keratinase and needs to be improved. Development of more efficient methods for the production and detection of keratinase will hasten its application to industries and environmental waste management.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] A. L. Martínez-Hernández and C. Velasco-Santos, "Keratin fibers from chicken feathers: structure and advances in polymer composites," in *Keratin: Structure, Properties and Applications*, R. Dullaart and J. Mousquès, Eds., pp. 149–211, Nova Science Publishers, 2012.

[2] A. M. Fortier and M. Cadrin, "Simple epithelial keratins k8 and k18: from structural to regulatory protein," in *Keratin: Structure, Properties and Applications*, R. Dullaart and J. Mousquès, Eds., pp. 1–35, Nova Science Publishers, 2012.

[3] R. Kanchana, "Farm waste recycling through microbial keratinases," *Journal of Applied Sciences in Environmental Sanitation*, vol. 7, no. 2, pp. 103–108, 2012.

[4] C.-H. Lee, M.-S. Kim, B. M. Chung, D. J. Leahy, and P. A. Coulombe, "Structural basis for heteromeric assembly and perinuclear organization of keratin filaments," *Nature Structural and Molecular Biology*, vol. 19, no. 7, pp. 707–715, 2012.

[5] P. Anbu, S. C. B. Gopinath, A. Hilda, T. Lakshmi Priya, and G. Annadurai, "Purification of keratinase from poultry farm isolate-*Scopulariopsis brevicaulis* and statistical optimization of enzyme activity," *Enzyme and Microbial Technology*, vol. 36, no. 5-6, pp. 639–647, 2005.

[6] M. N. Acda, "Waste chicken feather as reinforcement in cement-bonded composites," *Philippine Journal of Science*, vol. 139, no. 2, pp. 161–166, 2010.

[7] M. A. Ganaie, S. Sood, G. Rizvi, and T. A. Khan, "Isolation and identification of keratinophilic fungi from different soil samples in Jhansi City (India)," *Plant Pathology Journal*, vol. 9, no. 4, pp. 194–197, 2010.

[8] R. Kumar, R. Mishra, S. Maurya, and H. B. Sahu, "Isolation and identification of keratinophilic fungi from garbage waste soils of Jharkhand region of India," *European Journal of Experimental Biology*, vol. 3, no. 3, pp. 600–604, 2013.

[9] H. C. Gugnani, "Non-dermatophytic filamentous keratinophilic fungi and their role in human infections," in *Biology of Dermatophytes and Other Keratinophilic Fungi*, R. K. S. Kushwaha and J. Guarro, Eds., pp. 109–114, Revista Iberoamericana de Micologia Apartado, Bilbao, Spain, 2000.

[10] K. Pakshir, M. Rahimi Ghiasi, K. Zomorodian, and A. R. Gharavi, "Isolation and molecular identification of keratinophilic fungi from public parks soil in Shiraz, Iran," *BioMed Research International*, vol. 2013, Article ID 619576, 5 pages, 2013.

[11] V. M. Ramesh and A. Hilda, "Incidence of keratinophilic fungi in the soil of primary schools and public parks of Madras city, India," *Mycopathologia*, vol. 143, no. 3, pp. 139–145, 1998.

[12] S. Shadzi, M. Chadeganipour, and M. Alimoradi, "Isolation of keratinophilic fungi from elementary schools and public parks in Isfahan, Iran," *Mycoses*, vol. 45, no. 11-12, pp. 496–499, 2002.

[13] P. Anbu, A. Hilda, and S. C. B. Gopinath, "Keratinophilic fungi of poultry farm and feather dumping soil in Tamil Nadu, India," *Mycopathologia*, vol. 158, no. 3, pp. 303–309, 2004.

[14] H. C. Gugnani, S. Sharma, B. Gupta, and S. Gaddam, "Prevalence of keratinophilic fungi in soils of St. Kitts and Nevis," *Journal of Infection in Developing Countries*, vol. 6, no. 4, pp. 347–351, 2012.

[15] R. Kachuei, M. Emami, B. Naeimi, and K. Diba, "Isolation of keratinophilic fungi from soil in Isfahan province, Iran," *Journal de Mycologie Medicale*, vol. 22, no. 1, pp. 8–13, 2012.

[16] A. Z. Mahmoudabadi and M. Zarrin, "Isolation of dermatophytes and related keratinophilic fungi from the two public

parks in Ahvaz," *Jundishapur Journal of Microbiology*, vol. 1, no. 1, pp. 20–23, 2008.

[17] R. J. Yu, S. R. Harmon, and F. Blank, "Isolation and purification of an extracellular keratinase of *Trichophyton mentagrophytes*," *Journal of Bacteriology*, vol. 96, no. 4, pp. 1435–1436, 1968.

[18] M. Asahi, R. Lindquist, K. Fukuyama, G. Apodaca, W. L. Epstein, and J. H. McKerrow, "Purification and characterization of major extracellular proteinases from *Trichophyton rubrum*," *Biochemical Journal*, vol. 232, no. 1, pp. 139–144, 1985.

[19] C. M. Williams, C. S. Richter, J. M. MacKenzie Jr., and J. C. H. Shih, "Isolation, identification and characterization of a feather-degrading bacterium," *Applied and Environmental Microbiology*, vol. 56, no. 6, pp. 1509–1515, 1990.

[20] V. Matikevicienė, D. Masiliuniene, and S. Grigiskis, "Degradation of keratin containing wastes by bacteria with keratinolytic activity," in *Proceedings of the 7th International Scientific and Practical Conference*, vol. 1, pp. 284–289, 2009.

[21] R. Gupta and P. Ramnani, "Microbial keratinases and their prospective applications: an overview," *Applied Microbiology and Biotechnology*, vol. 70, no. 1, pp. 21–33, 2006.

[22] H. Korkmaz, H. Hür, and S. Dinçer, "Dincer, Characterization of alkaline keratinase of *Bacillus licheniformis* strain HK-1 from poultry waste," *Annals of Microbiology*, vol. 54, no. 2, pp. 201–211, 2004.

[23] M. M. Hoq, K. A. Z. Siddiquee, H. Kawasaki, and T. Seki, "Keratinolytic activity of some newly isolated *Bacillus* species," *Journal of Biological Sciences*, vol. 5, no. 2, pp. 193–200, 2005.

[24] S. G. Joshi, M. M. Tejashwini, N. Revati, R. Sridevi, and D. Roma, "Isolation, identification and characterization of a feather degrading bacterium," *International Journal of Poultry Science*, vol. 6, no. 9, pp. 689–693, 2007.

[25] C.-G. Cai, B.-G. Lou, and X.-D. Zheng, "Keratinase production and keratin degradation by a mutant strain of *Bacillus subtilis*," *Journal of Zhejiang University Science B*, vol. 9, no. 1, pp. 60–67, 2008.

[26] M. Cortezi, J. Contiero, C. J. B. de Lima, R. B. Lovaglio, and R. Monti, "Characterization of a feather degrading by *Bacillus amyloliquefaciens* protease: a new strain," *World Journal of Agricultural Sciences*, vol. 4, no. 5, pp. 648–656, 2008.

[27] R. Sapna and V. Yamini, "Study of keratin degradation by some potential bacterial isolates from soil," *Journal of Soil Science*, vol. 1, no. 1, pp. 1–3, 2011.

[28] A. Riffel and A. Brandelli, "Keratinolytic bacteria isolated from feather waste," *Brazilian Journal of Microbiology*, vol. 37, no. 3, pp. 395–399, 2006.

[29] T. Korniłłowicz-Kowalska and J. Bohacz, "Biodegradation of keratin waste: theory and practical aspects," *Waste Management*, vol. 31, no. 8, pp. 1689–1701, 2011.

[30] P. Jeevana Lakshmi, C. M. Kumari Chitturi, and V. V. Lakshmi, "Efficient degradation of feather by keratinase producing *Bacillus* sp.," *International Journal of Microbiology*, vol. 2013, Article ID 608321, 7 pages, 2013.

[31] J. Kunert, "Physiology of keratinophilic fungi," in *Revista Iberoamericana de Micología*, R. K. S. Kushawaha and and J. Guarro, Eds., pp. 77–85, Bilbao, Spain, 2000.

[32] N. Jain and M. Sharma, "Biodiversity of keratinophilic fungal flora in university campus, Jaipur, India," *Iranian Journal of Public Health*, vol. 41, no. 11, pp. 27–33, 2012.

[33] A. B. Friedricht and G. Antranikian, "Keratin degradation by *Feividobacterium pennavomns*, a novel thermophilic anaerobic species of the order thermotogales," *Applied and Environmental Microbiology*, vol. 62, no. 8, pp. 2875–2882, 1996.

[34] S. Riessen and G. Antranikian, "Isolation of *Thermoanaerobacter keratinophilus* sp. nov., a novel thermophilic, anaerobic bacterium with keratinolytic activity," *Extremophiles*, vol. 5, no. 6, pp. 399–408, 2001.

[35] G.-W. Nam, D.-W. Lee, H.-S. Lee et al., "Native-feather degradation by *Fervidobacterium islandicum* AW-1, a newly isolated keratinase-producing thermophilic anaerobe," *Archives of Microbiology*, vol. 178, no. 6, pp. 538–547, 2002.

[36] I. N. S. Dozie, C. N. Okeke, and N. C. Unaeze, "A thermostable, alkaline-active, keratinolytic proteinase from *Chrysosporium keratinophilum*," *World Journal of Microbiology and Biotechnology*, vol. 10, no. 5, pp. 563–567, 1994.

[37] A. Brandelli, D. J. Daroit, and A. Riffel, "Biochemical features of microbial keratinases and their production and applications," *Applied Microbiology and Biotechnology*, vol. 85, no. 6, pp. 1735–1750, 2010.

[38] P. Anbu, S. C. B. Gopinath, A. Hilda, N. Mathivanan, and G. Annadurai, "Secretion of keratinolytic enzymes and keratinolysis by *Scopulariopsis brevicaulis* and *Trichophyton mentagrophytes*: regression analysis," *Canadian Journal of Microbiology*, vol. 52, no. 11, pp. 1060–1069, 2006.

[39] W. Laba and A. Rodziewicz, "Keratinolytic potential of feather-degrading *Bacillus polymyxa* and *Bacillus cereus*," *Polish Journal of Environmental Studies*, vol. 19, no. 2, pp. 371–378, 2010.

[40] T. Sivakumar, T. Shankar, V. Thangapand, and V. Ramasubram, "Optimization of cultural condition for keratinase production using *Bacillus cereus* TS1," *Insight Microbiology*, vol. 3, no. 1, pp. 1–8, 2013.

[41] E. Tiwary and R. Gupta, "Rapid conversion of chicken feather to feather meal using dimeric keratinase from *Bacillus licheniformis* ER-15," *Journal of Bioprocessing & Biotechniques*, vol. 2, no. 4, Article ID 1000123, 2012.

[42] G. S. Molyneux, "The digestion of wool by a keratinolytic *Bacillus*," *Australian Journal of Biological Sciences*, vol. 12, no. 3, pp. 274–281, 1959.

[43] J.-S. Kim, L. D. Kluskens, W. M. de Vos, R. Huber, and J. Van Der Oost, "Crystal structure of fervidolysin from *Fervidobacterium pennivorans*, a keratinolytic enzyme related to subtilisin," *Journal of Molecular Biology*, vol. 335, no. 3, pp. 787–797, 2004.

[44] W. Suntornsuk, J. Tongjun, P. Onnim et al., "Purification and characterisation of keratinase from a thermotolerant feather-degrading bacterium," *World Journal of Microbiology and Biotechnology*, vol. 21, no. 6-7, pp. 1111–1117, 2005.

[45] B. Zhang, Z. W. Sun, D. D. Jiang, and T. G. Niu, "Isolation and purification of alkaline keratinase from *Bacillus* sp. 50-3," *African Journal of Biotechnology*, vol. 8, no. 11, pp. 2598–2603, 2009.

[46] V. Saibabu, F. N. Niyonzima, and S. S. More, "Isolation, partial purification and characterization of keratinase from Bacillus magaterium," *International Research Journal of Biological Sciences*, vol. 22, no. 2, pp. 13–20, 2013.

[47] A. M. Farag and M. A. Hassan, "Purification, characterization and immobilization of a keratinase from *Aspergillus oryzae*," *Enzyme and Microbial Technology*, vol. 34, no. 2, pp. 85–93, 2004.

[48] H. Gradišar, J. Friedrich, I. Križaj, and R. Jerala, "Similarities and specificities of fungal keratinolytic proteases: comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases," *Applied and Environmental Microbiology*, vol. 71, no. 7, pp. 3420–3426, 2005.

[49] K. C. Raju, U. Neogi, R. Saumya, and N. R. Goud, "Studies on extra cellular enzyme keratinase from dermatophyte *Microsporum gypseum*," *International Journal of Biological Chemistry*, vol. 1, no. 3, pp. 174–178, 2007.

[50] F. G. Moreira-Gasparin, C. G. M. de Souza, A. M. Costa et al., "Purification and characterization of an efficient poultry feather degrading-protease from *Myrothecium verrucaria*," *Biodegradation*, vol. 20, no. 5, pp. 727–736, 2009.

[51] L. Cao, H. Tan, Y. Liu, X. Xue, and S. Zhou, "Characterization of a new keratinolytic *Trichoderma atroviride* strain F6 that completely degrades native chicken feather," *Letters in Applied Microbiology*, vol. 46, no. 3, pp. 389–394, 2008.

[52] E. Ionata, F. Canganella, G. Bianconi et al., "A novel keratinase from *Clostridium sporogenes* bv. pennavorans bv. nov., a thermotolerant organism isolated from solfataric muds," *Microbiological Research*, vol. 163, no. 1, pp. 105–112, 2008.

[53] R. C. S. Thys, F. S. Lucas, A. Riffel, P. Heeb, and A. Brandelli, "Characterization of a protease of a feather-degrading *Microbacterium* species," *Letters in Applied Microbiology*, vol. 39, no. 2, pp. 181–186, 2004.

[54] C. M. Longshaw, J. D. Wright, A. M. Farrell, and K. T. Holland, "*Kytococcus sedentarius*, the organism associated with pitted keratolysis, produces two keratin-degrading enzymes," *Journal of Applied Microbiology*, vol. 93, no. 5, pp. 810–816, 2002.

[55] Z.-J. Cao, Q. Zhang, D.-K. Wei et al., "Characterization of a novel *Stenotrophomonas* isolate with high keratinase activity and purification of the enzyme," *Journal of Industrial Microbiology and Biotechnology*, vol. 36, no. 2, pp. 181–188, 2009.

[56] L. Vidal, P. Christen, and M. N. Coello, "Feather degradation by Kocuria rosea in submerged culture," *World Journal of Microbiology and Biotechnology*, vol. 16, no. 6, pp. 551–554, 2000.

[57] C. H. de Toni, M. F. Richter, J. R. Chagas, J. A. P. Henriques, and C. Termignoni, "Purification and characterization of an alkaline serine endopeptidase from a feather-degrading *Xanthomonas maltophilia* strain," *Canadian Journal of Microbiology*, vol. 48, no. 4, pp. 342–348, 2002.

[58] R. R. Chitte, V. K. Nalawade, and S. Dey, "Keratinolytic activity from the broth of a feather-degrading thermophilic *Streptomyces thermoviolaceus* strain SD8," *Letters in Applied Microbiology*, vol. 28, no. 2, pp. 131–136, 1999.

[59] X.-Q. Han and S. Damodaran, "Purification and characterization of Protease Q: a detergent- and urea-stable serine endopeptidase from bacillus pumilus," *Journal of Agricultural and Food Chemistry*, vol. 46, no. 9, pp. 3596–3603, 1998.

[60] I. Hanukoglu and E. Fuchs, "The cDNA sequence of a human epidermal keratin: divergence of sequence but conservation of structure among intermediate filament proteins," *Cell*, vol. 31, no. 1, pp. 243–252, 1982.

[61] I. Hanukoglu and E. Fuchs, "The cDNA sequence of a type II cytoskeletal keratin reveals constant and variable structural domains among keratins," *Cell*, vol. 33, no. 3, pp. 915–924, 1983.

[62] X. Lin, D. W. Kelemen, E. S. Miller, and J. C. H. Shih, "Nucleotide sequence and expression of kerA, the gene encoding a keratinolytic protease of *Bacillus licheniformis* PWD-1," *Applied and Environmental Microbiology*, vol. 61, no. 4, pp. 1469–1474, 1995.

[63] L. D. Johnson, W. W. Idler, X.-M. Zhou, D. R. Roop, and P. M. Steinert, "Structure of a gene for the human epidermal 67-kDa keratin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 82, no. 7, pp. 1896–1900, 1985.

[64] S. C. B. Gopinath, P. Anbu, T. Lakshmipriya, and A. Hilda, "Strategies to characterize fungal lipases for applications in medicine and dairy industry," *BioMed Research International*, vol. 2013, Article ID 154549, 10 pages, 2013.

[65] S. M. Harde, I. B. Bajaj, and R. S. Singhal, "Optimization of fermentative production of keratinase from *Bacillus subtilis* NCIM 2724," *Agriculture, Food and Analytical Bacteriology*, vol. 1, no. 1, pp. 54–65, 2011.

[66] T. Shankar, P. Thangamathi, C. Sathiya, and T. Sivakumar, "Statistical optimization of keratinase production by *Bacillus thuringiensis*," *Journal of Global Biosciences*, vol. 3, no. 2, pp. 477–483, 2014.

[67] P. Ramnani and R. Gupta, "Optimization of medium composition for keratinase production on feather by *Bacillus licheniformis* RGI using statistical methods involving response surface methodology," *Biotechnology and Applied Biochemistry*, vol. 40, no. 2, pp. 191–196, 2004.

[68] T. Sivakumar, T. Shankar, P. Vijayabaskar, and V. Ramasubramanian, "Statistical optimization of keratinase production by *Bacillus cereus*," *Global Journal of Biotechnology and Biochemistry*, vol. 6, no. 4, pp. 197–202, 2011.

[69] P. Anbu, S. C. B. Gopinath, A. Hilda, T. Lakshmipriya, and G. Annadurai, "Optimization of extracellular keratinase production by poultry farm isolate *Scopulariopsis brevicaulis*," *Bioresource Technology*, vol. 98, no. 6, pp. 1298–1303, 2007.

[70] S. C. B. Gopinath, K. Awazu, J. Tominaga, and P. K. R. Kumar, "Monitoring biomolecular interactions on a digital versatile disk: a BioDVD platform technology," *ACS Nano*, vol. 2, no. 9, pp. 1885–1895, 2008.

[71] S. C. B. Gopinath, R. Kumaresan, K. Awazu et al., "Evaluation of nucleic acid duplex formation on gold over layers in biosensor fabricated using Czochralski-grown single-crystal silicon substrate," *Analytical and Bioanalytical Chemistry*, vol. 398, no. 2, pp. 751–758, 2010.

[72] S. C. B. Gopinath, K. Awazu, and M. Fujimaki, "Waveguide-mode sensors as aptasensors," *Sensors*, vol. 12, no. 2, pp. 2136–2151, 2012.

[73] S. C. B. Gopinath, K. Awazu, M. Fujimaki, K. Shimizu, and T. Shima, "Observations of immuno-gold conjugates on influenza viruses using waveguide-mode sensors," *PLoS ONE*, vol. 8, no. 7, Article ID e69121, 2013.

[74] T. S. Dhahi, U. Hashim, N. M. Ahmed, and H. Nazma, "Fabrication and characterization of gold nano-gaps for ssDNA immobilization and hybridization detection," *Journal of New Materials for Electrochemical Systems*, vol. 14, no. 3, pp. 191–196, 2011.

[75] M. E. Ali, U. Hashim, S. Mustafa et al., "Nanoparticle sensor for label free detection of swine DNA in mixed biological samples," *Nanotechnology*, vol. 22, no. 19, Article ID 195503, 2011.

[76] M. E. Ali, U. Hashim, S. Mustafa, Y. B. Che Man, and K. N. Islam, "Gold nanoparticle sensor for the visual detection of pork adulteration in meatball formulation," *Journal of Nanomaterials*, vol. 2012, Article ID 103607, 7 pages, 2012.

[77] T. Lakshmipriya, M. Fujimaki, S. C. B. Gopinath, K. Awazu, Y. Horiguchi, and Y. Nagasaki, "A high-performance waveguide-mode biosensor for detection of factor IX using PEG-based blocking agents to suppress non-specific binding and improve sensitivity," *Analyst*, vol. 138, no. 10, pp. 2863–2870, 2013.

[78] D. J. Daroit and A. Brandelli, "A current assessment on the production of bacterial keratinases," *Critical Reviews in Biotechnology*, vol. 34, no. 4, pp. 372–384, 2014.

[79] S. Gupta and R. Singh, "Hydrolyzing proficiency of keratinases in feather degradation," *Indian Journal of Microbiology*, vol. 54, no. 4, pp. 466–470, 2014.