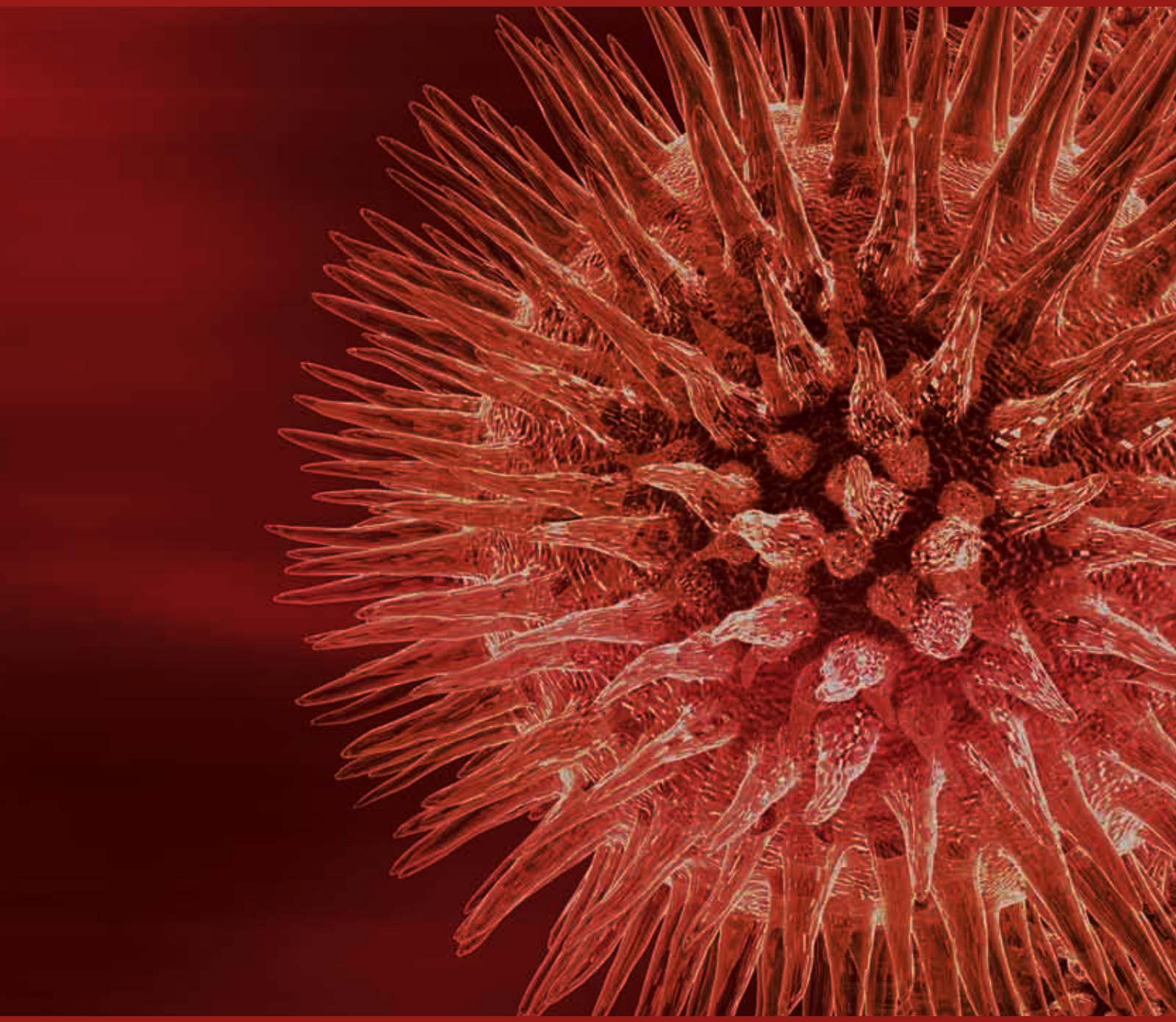
BioMed Research International

Microbial Enzymes and Their Applications in Industries and Medicine

Guest Editors: Periasamy Anbu, Subash C. B. Gopinath, Arzu Coleri Cihan, and Bidur Prasad Chaulagain



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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 204014, 2 pages
http://dx.doi.org/10.1155/2013/204014

Editorial

Microbial Enzymes and Their Applications in Industries and Medicine

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Received 23 October 2013; Accepted 23 October 2013

Enzymes are considered as a potential biocatalyst for a large number of reactions. Particularly, the microbial enzymes have widespread uses in industries and medicine. The microbial enzymes are also more active and stable than plant and animal enzymes. In addition, the microorganisms represent an alternative source of enzymes because they can be cultured in large quantities in a short time by fermentation and owing to their biochemical diversity and susceptibility to gene manipulation. Industries are looking for new microbial strains in order to produce different enzymes to fulfil the current enzyme requirements. This special issue covers ten articles including three review articles, mainly highlighting the importance and applications of biotechnologically and industrially valuable microbial enzymes.

M. Dinarvand et al. in their paper optimized the conditions for overproduction of intraextracellular inulinase and invertase from the fungus *Aspergillus niger* ATCC 20611. Optimization is one of the most important criteria in developing any new microbial process. Response surface analysis is one of the vital tools to determine the optimal process conditions. This kind of design of a limited set of variables is advantageous compared to the conventional method. The response surface methodology was used for this optimization and achieved the increment until 16 times. This study would be highly useful for the potential application in fermentation industries.

In this review, N. Gurung et al. have made an attempt to highlight the importance of different enzymes with a special focus on amylase and lipase. Enzymes generally increase the reaction rates by several million times than normal chemical reactions. Lipases play an important role in the food, detergent, chemical, and pharmaceutical industries. In the past, microbial lipases gained significant attention in the industries due to their substrate specificity and stability under varied conditions. Amylase is an enzyme that catalyses the breakdown of starch into sugars, abundant in the process of animal and human digestion. The major advantage of microbial amylases is being economical and easy to manipulate. Currently, much attention is paid to rapid development of microbial enzyme technology, and these enzymes are relatively more stable than the enzymes derived from plants and animals.

P. Mukherjee and P. Roy in their paper have purified and characterized the enzyme hydrocarbon dioxygenase from *Stenotrophomonas maltophilia* PM102, which has a broad substrate specificity. They found that the presence of copper induces the enzyme activity to be 10.3-fold higher, and NADH induces the increment to be 14.96-fold. Proposed copper enhanced monooxygenase activity and Fourier transform-infrared (FT-IR) characterization of biotransformation products from trichloroethylene satisfy the production of industrially and medically important chemicals and make bioremediation more attractive by improving the development of this technology.

C. Huynen et al. in their review paper discuss the importance of protein scaffold to develop hybrid enzymes.

The paper discusses the use of class A betalactamase as versatile scaffolds to design hybrid enzymes mentioned as betalactamase hybrid proteins (BHPs), in which an external polypeptide, peptide, protein, or their fragment is inserted at various suitable positions. The paper highlights further how BHPs can be specifically designed to develop as bifunctional proteins to produce and characterize the proteins otherwise difficult to express, to determine the epitope of specific antibodies, to generate antibodies against nonimmunogenic epitopes, and to understand the structure/function relationship of proteins. The hybrid proteins can be applied to produce difficult-to-express peptides/proteins/protein fragments, to map epitopes, to display antigens, and to study protein structure/function relationships. Among other applications, BHPs could be an important player in biosensors and in affinity chromatography, drug screening, and drug targeting.

P. Manivasagan et al. in their paper focus on purification and characterization of the protease from *Streptomyces* sp. MAB18. The authors have optimized the conditions for overproduction of protease using response surface methodology. They have also determined the molecular mass of purified enzyme and great activity and stability of enzyme in different pH and temperatures. Furthermore, the authors confirmed that the protease has an antioxidant ability. In industries, the poultry waste derived protease will be useful as a protein or as an antioxidant.

The paper titled "*β-Glucosidases from the fungus Trichoderma: an effeicient cellulose machinery in biotechnological applications*" is a detailed review on *β*-glucosidases which are members of the cellulose enzyme complex described by P. Tiwari et al. The authors especially focus on *β*-glucosidases from the fungus *Trichoderma,* mostly used for the saccharification of cellulosic biomass for biofuel production. They describe the enzyme family, their classification, structural parameters, properties, and studies at the genomics and proteomics levels. In addition, by bypassing the low enzyme production with hypersecretory strains, they give an insight on using these strains for renewable energy sources like bioethanol production. They imply the importance of fungal *β*-glucosidases which might be successful for biofuel production in order to meet the need in energy crisis.

A. Khoramnia et al. in their paper discuss yeast enzyme application for medium chain fatty acids (MCFAs) modification for industrial purpose and antibacterial applications. The paper focuses on the conceptualization, design, and assay of the enzyme produced from a Malaysian strain of *Geotrichum candidum*. With the modification on fatty acid processing using a naturally derived enzyme, a free lauric acid rich MCFAs can be obtained which can become a source of antibacterial use for both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*E. coli*) bacteria which are difficult microbes due to some of their strains becoming drug resistant. They also describe that the higher lipolysis by the strain specific enzyme is associated with the increased moisture content in the reaction environment on coconut oil hydrolysis.

M. A. Hassan et al. in their paper discuss isolation of *Bacillus amyloliquefaciencs* and *B. subtilis* from soil and production and characterization of keratinolytic protease. These bacteria were able to degrade the wool completely within 5 days and also produced the highest enzyme activity. The characterization studies confirmed that the enzyme is stable in a broad range of pH and temperatures. Furthermore, they confirmed that the keratinolytic proteases from isolated bacteria are stable in various organic solvents.

In this review article, S. C. B. Gopinath et al. put different strategies to characterize fungal lipases for their role in industry and medicine. The advantage of fungal lipases is bestowed with their extracellular nature of production thus reducing the complexities and high operation cost comparing to other bacterial enzymes. The authors provide several illustrations to show how lipolysis can be utilized and put strategies for the characterization of fungal lipases that are capable of degrading fatty substances from different sources, with an effort to highlight further applications. This review would contribute to the isolation and characterization of lipase from various fungal sources and application of lipase for medical and dairy industry and degradation of fatty substance from oil spillages.

A. Knob et al. in their paper focus on xylanses and discuss the purification and characterization of a xylanase produced by *Penicillium glabrum* using brewer's spent grain as a substrate in their paper. This study is the first report as the characterization of xylanase was carried out by using such an agroindustrial waste. Furthermore, the researchers also determined the molecular mass of the purified xylanase, the enzyme activity and stability on various pH and temperature ranges, the optimal enzyme production conditions, and the effect of some metal ions and inhibitors on xylanase activity. The authors concluded that the use of substrate brewer's spent grain for xylanase production not only decreased the amount of this waste but also reduced the xylanase production cost as desired in biotechnological processes.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 508968, 13 pages
http://dx.doi.org/10.1155/2013/508968

Research Article

Effect of C/N Ratio and Media Optimization through Response Surface Methodology on Simultaneous Productions of Intra- and Extracellular Inulinase and Invertase from *Aspergillus niger* ATCC 20611

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Received 24 May 2013; Revised 28 July 2013; Accepted 29 July 2013

Academic Editor: Subash C. B. Gopinath

The study is to identify the extraction of intracellular inulinase (exo- and endoinulinase) and invertase as well as optimization medium composition for maximum productions of intra- and extracellular enzymes from *Aspergillus niger* ATCC 20611. From two different methods for extraction of intracellular enzymes, ultrasonic method was found more effective. Response surface methodology (RSM) with a five-variable and three-level central composite design (CCD) was employed to optimize the medium composition. The effect of five main reaction parameters including sucrose, yeast extract, $NaNO_3$, Zn^{+2}, and Triton X-100 on the production of enzymes was analyzed. A modified quadratic model was fitted to the data with a coefficient of determination (R^2) more than 0.90 for all responses. The intra-extracellular inulinase and invertase productions increased in the range from 16 to 8.4 times in the optimized medium (10% (w/v) sucrose, 2.5% (w/v) yeast extract, 2% (w/v) $NaNO_3$, 1.5 mM (v/v) Zn^{+2}, and 1% (v/v) Triton X-100) by RSM and from around 1.2 to 1.3 times greater than in the medium optimized by one-factor-at-a-time, respectively. The results of bioprocesses optimization can be useful in the scale-up fermentation and food industry.

1. Introduction

In *A. niger* ATCC 20611, there are two associated intra- and extracellular glycoproteins enzymes to target the β-2, 1 linkage: inulinase (EC 3.2.1.7) and invertase (EC 3.2.1.26) [1, 2]. These enzymes exhibit corresponding hydrolytic activities towards sucrose but differ in their specificities for higher-molecular-weight oligosaccharides and fructans of the inulin type [3]. The potential of microorganisms to produce enzymes is vastly exploited for industrial purposes. *A. niger* ATCC 20611 efficiently utilizes wide a variety of inexpensive nutritional compounds to produce several intra- and extracellular enzymes. The productivity of many fermentation processes is affected by different parameters such as nutritional (medium composition) and physicochemical (agitation rates, pH value, inoculum size, and temperature) conditions to which the microorganism is exposed. The optimal design of culture media is an important aspect to be considered in the development of a fermentation process. The formulation of media containing complex nutrients is generally preferred for large-scale fermentations, since it leads to the development of cost-effective processes that

support maximum product yield. In the initial formulation of the medium in batch culture, an effort is made to understand the best source of carbon and energy, as well as the regulatory aspects of the enzyme. RSM is a mathematical modelling system which assesses the relationships between the response(s) and the independent variables [4] and defines the importance of the independent variables, alone or in combination, in the model. In addition, RSM has been furnished by ANOVA analysis, which can help to statistically analyze the whole model produced, every single parameter involved and its interactions [5, 6].

The production of enzymes through experimental design has gained much attention in biotechnology and industry studies for producing inulinase and invertase. Also, the selection of suitable microorganism is an important aspect of experimental design for producing the enzymes. *A. niger* is important in the food, beverage, and pharmaceutical industries and known as a potential producer of enzymes and organic acids [7, 8]. Furthermore, it is generally regarded as safe (GRAS) status, indicating that enzymes and other products produced by *A. niger* are deemed safe to be used for pharmaceutical and food applications [9]. Generally, the catalytic activities of inulinase (I) and invertase (S) are described in terms of I/S ratio (relative activities with inulin and sucrose) and employed to distinguish between inulinase and invertase [10]. Differences in I/S ratios and K_m value were considered by some authors to be insufficient for distinction and characterization of the enzymatic complex. When I/S ratio is higher than 10^{-2}, the inulinase production is preponderated in the culture, while for invertase production, the I/S ratio lower than 10^{-4} indicted the higher production [11]. A low I/S ratio (high activity with sucrose) is taken to indicate invertase. The naming of inulinase or invertase as β-fructosidase is based on their relative hydrolytic capacity for inulin and sucrose (I/S) [12].

Therefore, the main objective of the present study is to improve simultaneous productions of intra- and extracellular inulinase (exo- and endo) and invertase by *A. niger* ATCC 20611 through the optimization of the medium by RSM. At first, the effects of nutritional factors on intra- and extracellular inulinase and invertase productions were investigated by one-factor-at-a-time. Then, the concentration of the medium components was optimized using RSM. The main advantage of this study is the achievement of the higher productivities by using low cost carbon source and energy requirements. Based on our knowledge, optimization of two intra- and extracellular glycoproteins production by RSM has not yet been reported.

2. Materials and Methods

2.1. Microorganism, Preparation of Inoculum, and Medium Composition. *A. niger* ATCC 20611 was obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, USA, and was used in this study for intra- and extracellular inulinase and invertase productions. The strain was maintained at 4°C on potato dextrose agar (PDA) and subcultured every 3 weeks. The spores were harvested and suspended in sterile distilled water containing 0.01% (v/v) Tween 80 to obtain approximately 2.0×10^6 spores/mL. Preliminary experiments were performed by using a basal medium containing inulin 1% (w/v) and peptone 0.5% (w/v). The initial pH of the basal medium was adjusted at 6.5, prior to sterilization at 121°C for 15 min. The basal medium was inoculated with a 6% (v/v) of stock culture and incubated at 30°C with 150 rpm shaking for 96 h [13, 14]. All the fermentations procedures were carried out in triplicate in 250 mL Erlenmeyer flasks containing 50 mL of the basal medium.

2.2. Inulinase and Invertase Production Media. Six different media for extracellular inulinase and invertase productions were tested for the production of the enzymes by *A. niger* ATCC 20611. The composition of the media included the medium A (g/L): yeast extract (3 g), malt extract (3 g), peptone (5 g), and dextrose (10 g) [15]; medium B (g/L): potato dextrose broth (40 g); medium C (%): for the production of inulinase (inulin 1% (w/v), NH_4Cl 2.4% (w/v), and $Mg_2SO_4{\cdot}7H_2O$ 1.2% (w/v)) and for invertase production (inulin 5% (w/v), NH_4Cl 4.8% (w/v), and $Mg_2SO_4{\cdot}7H_2O$ 1.2% (w/v)) [2]; medium D (g/L): sucrose (20 g), yeast extract (2 g), $NaNO_3$ (2 g), $MgSO_4{\cdot}7H_2O$ (0.05 g), and K_2HPO_4 (0.5 g) [16]; medium E (g/L): inulin (10 g), yeast extract (1.5 g), NH_4NO_3 (2.3 g), $(NH_4)_2HPO_4$ (3.7 g), KH_2PO_4 (1.0 g), and $MgSO_4$ (0.5 g) [17]; medium F (g/L): inulin (2 g), peptone (2 g), $(NH_4)H_2PO_4$ (1.2 g), NaCl (0.5 g), and $MgSO_4{\cdot}7H_2O$ (0.05 g) [18].

2.3. Experimental Design. RSM offers a large amount of information from a small number of experiments because of using special designs that help the appropriate model to be fitted to the response(s). The experiments were generated by central composite design (CCD). All treatment combinations were performed in 250 mL Erlenmeyer flasks containing 50 mL of the medium which had been optimized by one-factor-at-a-time method [13, 14]. After 96 h of incubation, each flask was assayed for enzymes activity. RSM modelling was used to optimize the best range of the five effective nutrient factors (sucrose, yeast extract, $NaNO_3$, Zn^{2+}, and Triton X-100), which resulted from previous studies [13, 14], on the responses of intra- and extracellular inulinase and invertase productions, biomass, and C/N ratio. The lowest and highest concentrations of the selected ingredients in the media were sucrose (4% to 16%, w/v), yeast extract (1% to 4%, w/v), $NaNO_3$ (1% to 3%, w/v), Zn^{2+} (1 mm to 2 mm, v/v), and Triton X-100 (0.5% to 1.5%, v/v). The independent variables and their levels are shown in Table 1. The CCD design experimental data were employed using Design Expert version 6.06 (Stat-Ease Inc. Minneapolis, USA) and then interpreted. By using a five-factor and three-level CCD, 26 treatment combinations were generated as small type in experimental design. Often four or more replicates at center point are considered to estimate the experimental error (pure error variance). This offered an adequate estimate of the variation of the response and provided the number of degrees of freedom needed for an adequate statistical test of the model [5, 6, 19]. Behaviour

Table 1: Range and levels of experimental variables.

Factors	Level of factors		
	Low (−)	Medium (0)	High (+)
Sucrose (A/(w/v))	4.0	10	16
Yeast extract (B/(w/v))	1.0	2.5	4.0
$NaNO_3$ (C/(w/v))	1.0	2.0	3.0
Zn^{+2} (D/(v/v))	1.0	1.5	2.0
Triton X-100 (E/(v/v))	0.5	1.0	1.5

of the system is explained by the following second-order polynomial equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j, \quad (1)$$

where Y is the predicted intra- and extracellular inulinase and invertase productions (U/mL), biomass (mg/mL), and C/N ratio; x_i and x_j are the parameters (sucrose, yeast extract, $NaNO_3$, Zn^{+2}, and Triton X-100); β_0 is the intercept term; β_i, β_{ii}, and β_{ij} are the linear, squared and interaction coefficients, respectively. In order to test the estimation capabilities of the technique, the predicted responses obtained from RSM were compared with the actual responses. The R^2 was determined for finding the best models by measuring the amount of the reduction in the variability of response which represses variables in the model and must be close to 1.

2.4. Analytical Method for Intra- and Extracellular Inulinase and Invertase Activities. The supernatants of the cultures at 96 h of fermentation were harvested by centrifugation at 4°C (20 min, 10,000 ×g) in triplicate to determinate extracellular inulinase activity (I) and invertase activity (S). Then, cell pellet was used as a source of intracellular inulinase and invertase activities. For the extraction of intracellular enzymes, two methods were used. In the first method, the pellet was resuspended in 50 mL of sodium acetate buffer (200 mM, pH 5.0) with vigorous vortexing followed by incubation at 30°C and 150 rpm for 30 min. Then, it was sonicated on ice in glass tubes using a Branson Sonic Power Sonicator (48 Bransonic Power, 40 W, 30 s with 30 s cooling periods) for 5 min followed by centrifugation at 4°C (20 min, 10,000 ×g). In the second method, the mycelia were blotted with filter paper and grounded in a porcelain mortar with sand at 4°C and then resuspended in 50 mL of sodium acetate buffer (200 mM, pH 5.0) [20]. Cell debris was removed by centrifugation at 4°C (20 min, 10,000 ×g). The supernatant was used as the crude intracellular inulinase and invertase enzymes. Inulinase and invertase assay was carried out according to Dinarvand et al. [13, 14]. Briefly, 0.5 mL of 1% (w/v) substrate (inulin and sucrose) in sodium acetate buffer (200 mM, pH 5.0) was added to 0.5 mL of supernatant containing crude inulinase and invertase. Then, the reaction was incubated at temperature of 50°C for 20 min. The same reaction mixture without the enzyme extract was used as the control. The amount of the reducing sugars in the reaction mixture was assayed using the DNS method [21]. Inulinase and invertase activities (U/mL) were defined as 1 μmol of fructose and glucose liberated per min under the assay conditions, respectively. Specific activity was defined as a rate of total enzyme activity over protein content in milligram. Experiments were carried out in triplicate runs, and all standard deviations were lower than 10%.

Table 2: Extracellular inulinase and invertase productions by different commercial media.

Production media	Extracellular inulinase activity (U/mL)	Extracellular invertase activity (U/mL)
A	157 ± 0.94	536 ± 2.08
B	707 ± 3.14	1167 ± 7.05
C	697 ± 2.16	955 ± 5.02
D	2553 ± 9.17	1983 ± 8.03
E	1664 ± 6.16	1052 ± 6.05
F	880 ± 4.15	663 ± 3.08

A: yeast extract 3 g, malt extract 3 g, peptone 5 g, and dextrose 10 g.
B: potato dextrose broth.
C: for the production of inulinase [inulin (1%), NH_4Cl (2.4%), and $Mg_2SO_4{\cdot}7H_2O$ (1.2%)] and invertase [inulin (5%), NH_4Cl (4.8%), and $Mg_2SO_4{\cdot}7H_2O$ (1.2%)].
D: sucrose (20 g), yeast extract (2 g), $NaNO_3$ (2 g), $MgSO_4{\cdot}7H_2O$ (0.05 g), and K_2HPO_4 (0.5 g).
E: inulin (10 g), yeast extract (1.5 g), NH_4NO_3 (2.3 g), $(NH_4)_2HPO_4$ (3.7 g), KH_2PO_4 (1.0 g), and $MgSO_4$ (0.5 g).
F: inulin (2 g), peptone (2 g), $(NH_4)H_2PO_4$ (1.2 g), NaCl (0.5 g), and $MgSO_4{\cdot}7H_2O$ (0.05 g).

2.5. Determination of Protein, Biomass, Polysaccharide, and Nitrogen Content. Total protein was determined according to Bradford [22], using bovine serum albumin (BSA) as standard. The biomass was determined by dry weight measurement [13, 14]. The polysaccharide content was determined using the phenol-sulphuric acid method as described by Dubois et al. [23]. The nitrogen content in the medium was determined using the Kjeldahl method [24]. All the assays were carried out in triplicate.

3. Results and Discussion

3.1. Effect of Different Media on Inulinase and Invertase Productions. The ability of *A. niger* ATCC 20611 to simultaneously produce inulinase and invertase was studied in six different liquid media. The results (Table 2) indicated that all the media supported inulinase and invertase productions, although the levels of inulinase and invertase productions were shown to be greatly different. Maximum productions of inulinase (2553 U/mL) and invertase (1983 U/mL) in medium D were significantly higher than other production media containing sucrose as carbon source, yeast extract and $NaNO_3$ as nitrogen sources, and Mg^{+2} and K^{+2} as metal ions. The lowest production of inulinase (157 U/mL) and invertase (536 U/mL) in medium A were observed with the composition of dextrose as carbon source, yeast extract, malt extract, and peptone without any metal ions. The productions of inulinase and invertase are mostly inducer dependent. The different media have various effects on

Table 3: Determination of intracellular inulinase and invertase productions by the two different methods.

Inulinase activity (U/mL)			Invertase activity (U/mL)		
Intracellular		Extracellular	Intracellular		Extracellular
SN	PM		SN	PM	
158 ± 0.80	82 ± 0.10	356 ± 1.56	295 ± 1.20	127 ± 0.30	250 ± 1.18

Mean ± SD, $P \leq 0.05$ significant.
SN: sonication.
PM: porcelain mortar.

inulinase and invertase productions based on the physiological and biochemical pathways of the fungi. The higher inulinase and invertase productions obtained in fermentation by various microorganisms such as *A. japonicas*, *A. ficuum, Candida guilliermondii* TISTR 5844, and *A. niveus* Blochwitz 4128URM ranged from 0.5 to 810 U/mL [2, 16, 18, 19]. Based on the previous-obtained results, inulinase and invertase productions by *A. niger* ATCC 20611 were 10 fold higher than other microorganisms, suggesting that this strain could be a potential source for industrial section.

3.2. Comparative Evaluation of Extraction Methods for Intracellular Enzymes. In order to monitor the cell disruption process, two different methods of mechanical cellular breakage, sonication and porcelain mortar, were tested. Optimized conditions of sonication and porcelain mortar presented comparable yields of enzyme activity (Table 3). The maximum intracellular inulinase (158 U/mL) and invertase (295 U/mL) activities were obtained by using the sonication method and the porcelain mortar method which showed 82 and 127 U/mL activities, respectively. In order to obtain the optimal method for extracting intracellular inulinase and invertase, we aimed at establishing sonication as the best method for enzymes extraction in this study. Secreted invertase resides mainly in the cell wall as an octamer. The equal amount of invertase present in the culture fluid as well as the fraction, which is removable from the cells by treatment with sonication, was found to be composed of dimers. It has been suggested that oligomerization helped to retain the enzyme within the cell wall [3]. Similarly, the inulinase of yeast was associated with the cell wall. In contrast to the invertase, much more of the inulinase from fungi was actually secreted into the culture fluid. When *A. niger* ATCC 20611 was grown under conditions which derepress the enzyme production, around 80% of its inulinase was secreted into the culture fluid and the rest of the enzyme was gained by sonication from the cell wall. In comparison to the biochemistry of the invertase of fungi, less information has been reported about the biochemistry of inulinase.

3.3. Effect of Time on the Intra- and Extracellular Inulinase and Invertase Productions. The *A. niger* ATCC 20611 strain was grown in the basal medium; biomass, intra- and extracellular inulinase and invertase productions, and pH were measured during 168 h incubation at different time points. The maximum intra- and extracellular inulinase and invertase productions and biomass were obtained after 96 h of cultivation at 30°C in a shaker incubator with agitation speed of 150 rpm. Intra- and extracellular inulinase and invertase productions were shown to coincide with the exponential growth phase. There was a reduction in pH from 6.5 to 3.0 during intra- and extracellular inulinase and invertase productions, and maximum enzymes production was observed at pH 4.6 (data not shown). The maximum productions of inulinase and invertase were obtained at the end of the logarithmic growth phase [20, 25]. It was an interest to determine inulinase production together with invertase; many microbial preparations of inulinase possess remarkable invertase activity (S) accompanying the inulinase activity (I), where their catalytic activity is described in terms of I/S (Inulin/Sucrose) ratio [25, 26]. There was a significant positive correlation (r) coefficient at $P \leq 0.01$ between growth on one side and the production of inulinase ($r = 0.98$) and invertase ($r = 0.99$) on the other side. In addition, inulinase and invertase were also positively correlated ($r = 1$). The enzymes production by *A. niger* ATCC 20611 was growth associated, because the maximum activities were obtained at the stationary phase [26]. Higher inulinase and invertase productions in a very short fermentation time could be an advantage of using *A. niger* ATCC 20611. Increasing incubation time led to reduction in enzymes production. Reduced enzymes activities after 120 h of fermentation could be either due to the decrease in nutrient availability in the medium, proteolytic activity, or catabolic repression of enzymes [26, 27]. Reduction in the culture pH may have also made an impact on enzymes production [26]. The decrease in pH was probably due to the deamination of some amino acids or formation of organic acids [13, 14].

3.4. Response Surface Methodology Study on Optimization of Medium Ingredients for Intra- and Extracellular Inulinase and Invertase Productions and Biomass

3.4.1. Model Fitting and Analysis of Variance (ANOVA). The average intra- and extracellular inulinase and invertase-productions, biomass, and C/N ratio were obtained after 4 days of fermentation in 26 experiments of the chosen experimental design. The best fitting models were determined through quadratic regressions. Finally, the modified quadratic model was highly significant ($P < 0.001$) to represent the actual relationships between the responses and the significant variables. Analysis of variance (ANONA) was used to evaluate the significance of the coefficients of the modified quadratic model. In the model for all responses, very small "model P values" (<0.0002) and large "lack of fit P values" (<0.1701) with a suitable R^2 (≥0.90) and adjusted

coefficient of determination was absorbed (Table 4). The second-order regression equation provided the levels of extracellular inulinase and invertase productions, biomass, and C/N ratio as the functions of sucrose, yeast extract, $NaNO_3$, Zn^{+2}, and Triton X-100. They can be presented in terms of coded factors as in the following equations:

$$\begin{aligned}
&\text{extracellular inulinase activity (U/mL)} \\
&\quad = +2681.56 + 88.31A - 198.16B - 66.73C \\
&\qquad + 490.15D + 268.81E - 1057.79A^2 \\
&\qquad - 462.17B^2 - 434.03C^2 + 472.26D^2 \\
&\qquad - 162.67E^2 + 350.71AB + 425.54\,AC \\
&\qquad + 290.77BC - 330.10DE, \\
&\text{extracellular invertase activity (U/mL)} \\
&\quad = +2887.82 + 226.53A + 16.96B \\
&\qquad + 60.23C + 440.70D + 36.42E \\
&\qquad - 1057.62A^2 - 399.88B^2 - 403.29C^2 \\
&\qquad + 392.21D^2 - 180.19E^2 + 239.56\,AC \\
&\qquad + 249.02AE + 121.82BE + 203.35CE, \\
&\text{Biomass (mg/mL)} \\
&\quad = +48.30 + 2.08A + 3.93B + 0.36C \\
&\qquad + 11.94D + 0.38E - 12.79A^2 - 7.72B^2 \\
&\qquad - 7.90C^2 + 10.43D^2 - 3.51E^2 + 10.46\,AC \\
&\qquad + 9.88AE - 8.71BD + 9.63CE, \\
&\frac{C}{N} = +0.61 - 0.13A + 0.021B + 0.016C \\
&\qquad - 0.37D - 0.027E + 0.87A^2 \\
&\qquad + 0.42B^2 + 0.42C^2 - 0.23D^2 \\
&\qquad + 0.015E^2 - 0.30\,AC - 0.26AE \\
&\qquad + 0.17BD - 0.26CE,
\end{aligned} \tag{2}$$

where A, B, C, D, and E are sucrose, yeast extract, $NaNO_3$, Zn^{+2}, and Triton X-100, respectively. ANOVA for the response surface is shown in Table 4. The R^2 for the quadratic regression model of extracellular inulinase and invertase productions, biomass, and C/N ratio is more than 0.90, and the model was highly significant ($P < 0.001$) for all responses.

3.4.2. Main Effects and Interactions between Parameters. In this research, we tried to analyze, model, and interpret the experimental data using RSM as a mathematical modelling system. The concentration of parameters (sucrose, yeast extract, $NaNO_3$, Zn^{+2}, and Triton X-100) and the effect of their interactions on intra- and extracellular inulinase and invertaseproductions, biomass, and C/N ratio were determined by CCD of RSM. The interaction effects of the extracellular inulinase and invertase productions, biomass, C/N ratio, and optimal value of variables are clearly represented in the three dimensional response surface plots. Figures 1, 2, and 3 represent the three dimensional plots as functions of extracellular inulinase and invertase productions and biomass, respectively, when other parameters were kept constant at the center point. The results were analyzed via ANOVA. Twenty-six experiments were performed at the different combinations of the factors shown in Table 5. The maximum intra- and extracellular inulinase and invertase productions were obtained with sucrose (10%, w/v) as the sole carbon source (Figures 1 and 2). The composition of the culture medium is the most important factor with influence on enzyme production, growth, and physiology of the cell, and it enhances formation of bioproducts. Readily metabolized or utilized carbon sources in the medium could increase or inhibit the enzyme synthesis. It has been reported that most fungal and yeast strains produce inulinase and invertase in the presence of sucrose, while presence of polysaccharide sugars had an inhibitory effect on both enzymes production [13, 14]. Inulinase and invertase were retained by the cell wall and secreted from cells, residing mainly in the cell wall, where the diffused sucrose can be easily hydrolyzed. Such specific localization of inulinase and invertase may be ecologically beneficial for the efficient scavenging of hydrolyzed products. However, this may not be the case for the other carbon sources because other sugars molecules can hardly penetrate the cell wall and must therefore be hydrolyzed outside the cell wall [13, 14]. Among the carbon sources tested, only sucrose possessed a structure consisting of β-1,2 linkage which can be cleared by inulinase and invertase. In addition, the localization of an enzyme, its mode of action, and yield

Table 4: Analysis of variance (ANOVA).

Responses	Model	Lack of fit
Extracellular inulinase (U/mL)		
P value	0.0002	0.1120
F value	10.21	3.70
R^2	0.93	—
Extracellular invertase (U/mL)		
P value	<0.0001	0.1536
F value	19.22	2.99
R^2	0.96	—
Biomass (mg/mL)		
P value	<0.0001	0.0601
F value	17.54	5.46
R^2	0.96	—
C/N%		
P value	<0.0001	0.1704
F value	18.84	2.77
R^2	0.96	—

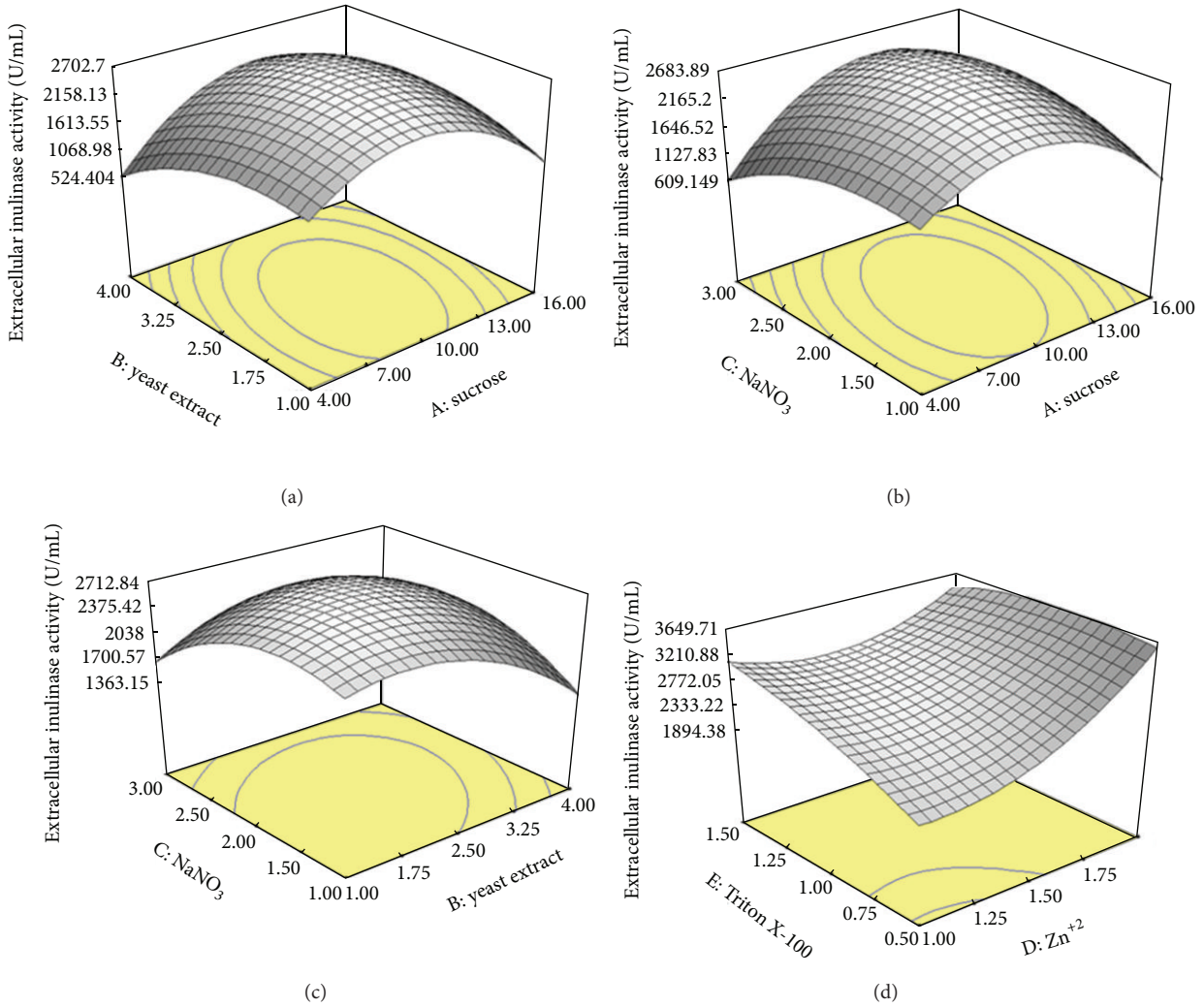

Figure 1: Response surface showing the interaction between five parameters and extracellular inulinase production (U/mL); (a) sucrose and yeast extract, (b) sucrose and $NaNO_3$, (c) yeast extract and $NaNO_3$, and (d) Zn^{+2} and Triton X-100. Other variables are constant at their center points. The numbers inside the contour plots indicate conversion yield (U/mL) of the extracellular inulinase.

depend upon the kind of microorganism and the substrate used during fermentation [28]. The localization of inulinase and invertase in *A. niger* ATCC 20611 may be different or altered by cultivation conditions. Inulinase and invertase secreted from *A. niger* ATCC 20611 cells resides mainly in the cell wall to perform their physiological function, the cleavage of sucrose molecule diffusible into the cell wall [15, 29]. Such specific localization of inulinase and invertase may be ecologically beneficial for efficient scavenging of hydrolyzed products. The cell wall retention of inulinase and invertase may be advantageous for sucrose utilization in *A. niger* ATCC 20611. The polysaccharides such as inulin are too large to enter the cell wall. Generally, those sugar molecules hardly penetrate into the cell wall, and thus, their hydrolysis occurs outside the cell wall [29]. Therefore, it was suggested that sucrose might act as an inducer for intra- and extracellular inulinase and invertase productions. As lower cost of raw materials for the industrial fermentation processes is mostly preferable, sucrose was selected as the best and most economic of carbon source for intra- and extracellular inulinase and invertase productions by *A. niger* ATCC 20611 [10, 12].

Nitrogen sources that produced maximum intracellular inulinase and invertase (data not shown) and extracellular inulinase and invertase (Figures 1 and 2) were obtained by using a mixture of yeast extract (2.5%, w/v) and $NaNO_3$ (2%, w/v). Thus, yeast extract and $NaNO_3$ were selected as the best sources of nitrogen. In addition, the total specific activity of the enzymes by using the mixture of nitrogen sources (organic and inorganic) was higher compared to using optimum concentrations of organic and inorganic nitrogen sources individually (data not shown). The enzyme productions in fermentation by using a mixture of yeast extract and $NaNO_3$ were improved by about 2.5 to 1.6 times, respectively, compared to using yeast extract and $NaNO_3$ alone. Enhancement of inulinase and invertase productions in fermentation by using a mixture of yeast extract or $NaNO_3$ has also been reported by Skowronek and Fiedurek [31]

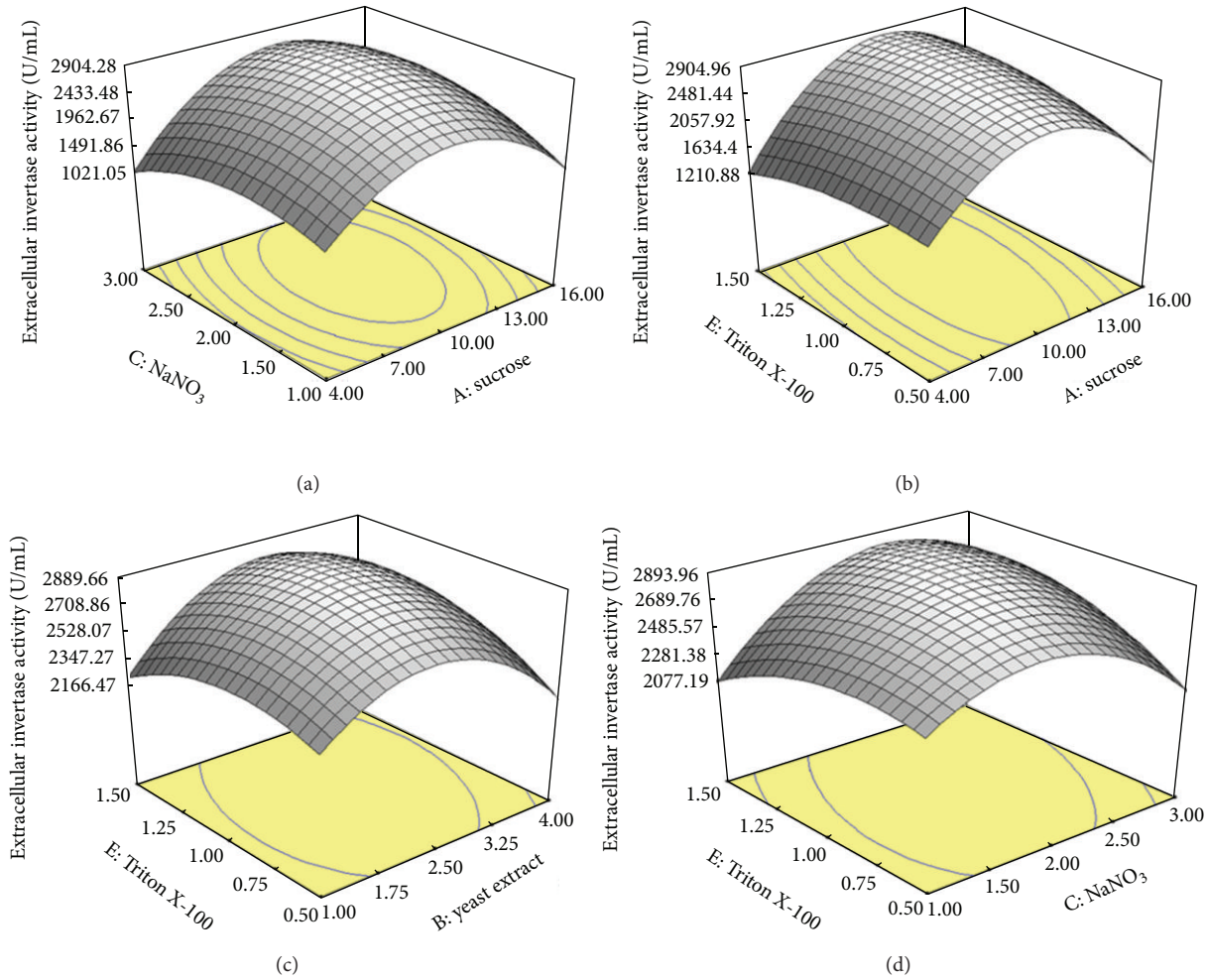

Figure 2: Response surface showing the interaction between five parameters and extracellular invertase production (U/mL); (a) sucrose and $NaNO_3$, (b) sucrose and Triton X-100, (c) yeast extract and Triton X-100, and (d) $NaNO_3$ and Triton X-100. Other variables are constant at their center points. The numbers inside the contour plots indicate conversion yield (U/mL) of the extracellular invertase.

and Kaur and Sharma [30]. Furthermore, complex nitrogen sources (yeast extract and $NaNO_3$) at higher concentrations might have a toxic effect on enzymes production [13, 14].

As shown in Figures 1 and 2, maximum extracellular enzymes productions were observed in the production medium containing Zn^{2+} (1.5 mM) as a trace element followed by Ca^{2+} which enhanced the productions to 77% and 97%, respectively. The same trend was observed for intracellular enzymes (data not shown). Trace elements have significant effects on growth, metabolism, and enzyme synthesis by many microorganisms [15]. Trace metals function as cofactors in enzymatic reactions and also play an important role in enzyme structure stabilization. Metal ions are important in maintaining cell wall rigidity, stabilizing oligomeric proteins, and covalently bounding protein peptidoglycan complexes in the outer membrane [32]. In addition, transition metal ions might change the enzyme activity. This phenomenon was due to the interaction between ions and the enzyme surface charge which could markedly affect the ionization of some amino acid residues, then change the enzyme conformation, and alter the enzyme activity. Zn^{2+} is a multifunctional element found in almost 300 enzymes. It plays essential roles in enzyme production in many microbial species [15, 33].

Among the various surfactants supplemented in the production medium, only Triton X-100 (1%, v/v) enhanced intracellular enzymes production (data not shown) and extracellular inulinase and invertase productions (Figures 1 and 2). Triton X-100 in high concentrations decreased the intra- and extracellular enzymes production and biomass of *A. niger* ATCC 20611 (data not shown). The studies claim that surfactants affect the cell membrane permeability [25], might affect cell bound enzymes, and enhance the release of enzymes into the medium [34]. The solubilization of membrane bound proteins and phospholipids by Triton X-100 at higher concentrations causes lethal effects on yeast cells [25]. The previous results showed that intra- and extracellular inulinase and invertase productions and biomass were increased with increase in sucrose, yeast extract, $NaNO_3$, Zn^{+2}, and Triton X-100 up to 10% (w/v), 2.5% (w/v), 2% (w/v), 1.5 mM, and 1% (v/v), respectively. Thereafter, intra-

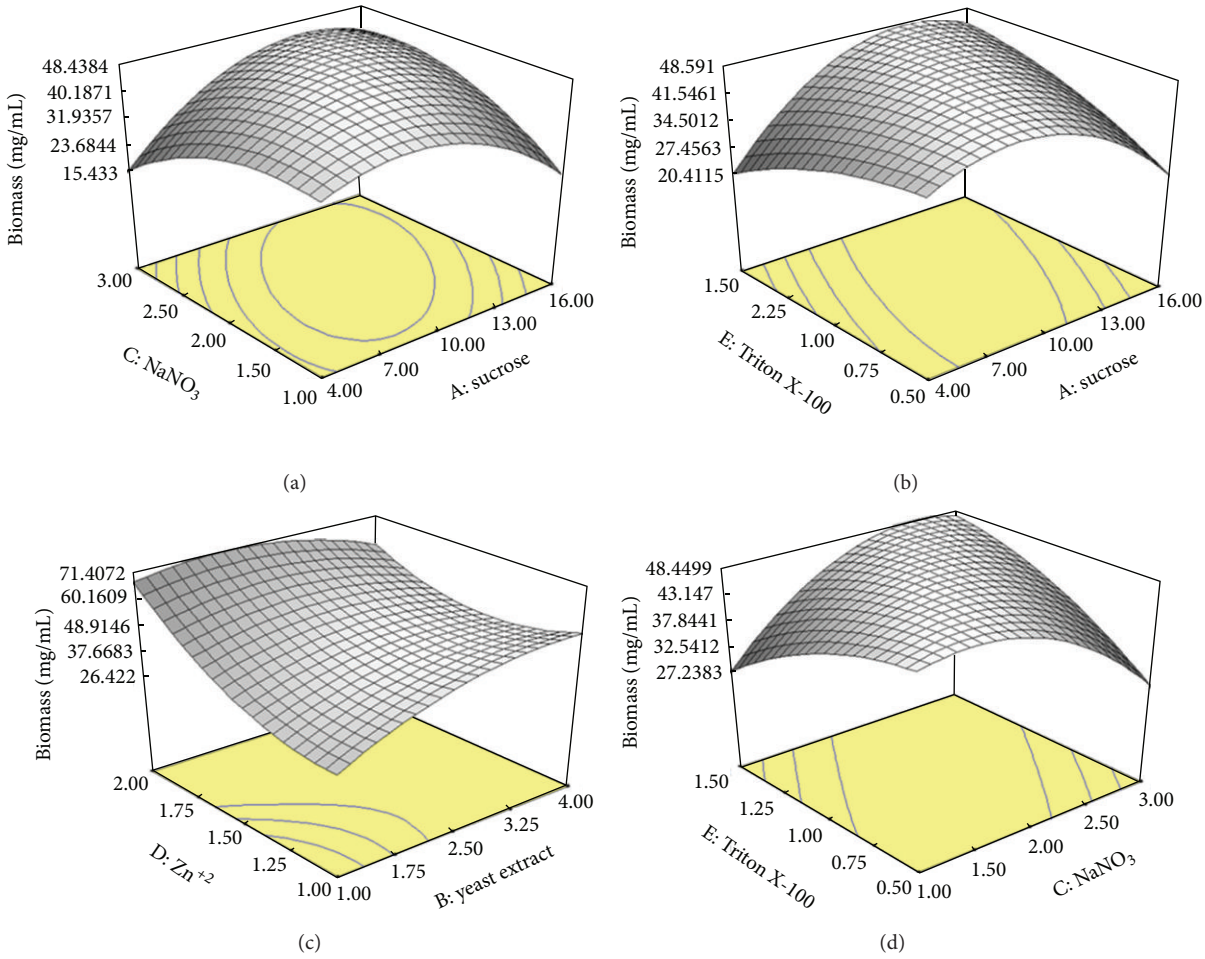

Figure 3: Response surface showing the interaction between five parameters and biomass (mg/mL); (a) sucrose and $NaNO_3$, (b) sucrose and Triton X-100, (c) yeast extract and Zn^{+2}, (d) $NaNO_3$ and Triton X-100. Other variables are constant at their center points. The numbers inside the contour plots indicate conversion yield (mg/mL) of the biomass.

and extracellular inulinase and invertase productions and biomass were decreased with further increase in the parameters. The same trend was observed similar to the other variables (data not shown). The previous model can be used to predict the enzymes production within the limits of the experimental factors.

3.4.3. Model Validation and Optimum Conditions. From the previous results, the economically optimized composition of fermentation medium for the maximum productions of intra- and extracellular inulinase and invertaseand biomass was as follows: sucrose 10% (w/v), yeast extract 2.5% (w/v), $NaNO_3$ 2% (w/v), Zn^{+2} 1.5 mM, and Triton X-100 1% (v/v). Validation of the experimental model was tested by carrying out the batch experiment under optimal operation conditions. The intra- and extracellular inulinase and invertase productions and biomass obtained from experiments were very close to the actual responses predicted by the regression model, which proved the validity of the model. The maximum values for the three responses (extracellular inulinase, extracellular invertase, and biomass) predicted from the model were 3681.21 U/mL, 3854.48 U/mL, and 73.39 mg/mL, respectively. Repeated experiments were performed to verify the predicted optimum conditions. At these optimized conditions, the maximum extracellular inulinase, extracellular invertase, and biomass were found to be 3687.93 U/mL, 3876.72 U/mL responds, and 70.82 mg/mL, respectively (Table 5). The average results from the three replications were coincident with the predicted values and the model was proven to be adequate. Table 6 presents the optimal combination of parameters that can be used to obtain high enzymes production and biomass. The optimum conditions can be used for future scale-up productions of the intra- and extracellular inulinase and invertase.

3.5. Effect of C/N Ratio on Variables. The effect of independent variables on C/N was shown in Figure 4. The results showed the desired C/N ratio obtained in the presence of 10% (w/v) sucrose, 2.5% (w/v) yeast extract, and 2% (w/v) $NaNO_3$. Relatively high specific activities of intracellular inulinase (33.71 U/mg), intracellular invertase (43.78 U/mg), extracellular inulinase (73.48 U/mg) and extracellular invertase

Table 5: Composition of various experiments of the central composite design (CCD) for independent variables and predicted and experimental values of responses.

Run no.	Sucrose (w/v)	Yeast extract (w/v)	$NaNO_3$ (w/v)	Zn^{+2} (v/v)	Triton X-100 (v/v)	Extracellular inulinase (U/mL)		Extracellular invertase (U/mL)		Biomass mg/mL		C/N	
						Actual	Predicted	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	16.00	4.00	1.00	2.00	0.50	1032.23	1140.22	1318.67	1311.74	24.25	24.51	2.1	2.09
2	16.00	1.00	3.00	2.00	0.50	1430.86	1367.56	1764.30	1698.08	36.29	37.83	1.5	1.56
3	4.00	4.00	3.00	1.00	1.50	219.53	123.44	458.12	461.95	19.00	16.24	2.6	2.65
4	16.00	4.00	3.00	1.00	0.50	862.46	867.01	903.05	833.45	21.18	19.06	2.5	2.51
5	16.00	4.00	1.00	1.00	1.50	884.57	748.36	1128.66	1059.05	20.21	20.19	2.4	2.45
6	16.00	1.00	1.00	2.00	1.50	1332.80	1297.23	1395.53	1416.14	34.61	34.69	1.7	1.63
7	4.00	1.00	3.00	2.00	1.50	1235.41	1172.11	1342.99	1293.56	29.04	30.75	2.0	2.02
8	4.00	4.00	1.00	2.00	1.50	1030.99	1038.33	1293.56	1376.84	23.20	23.00	2.2	2.19
9	16.00	1.00	3.00	1.00	1.50	1402.18	1366.60	1621.43	1625.26	35.40	34.74	1.6	1.61
10	4.00	4.00	3.00	2.00	0.50	930.16	970.30	1202.20	1198.66	22.44	21.60	2.3	2.38
11	4.00	1.00	1.00	1.00	0.50	1259.39	1163.32	1377.56	1321.19	29.76	29.64	1.9	1.96
12	4.00	2.50	2.00	1.50	1.00	1282.62	1490.61	1382.73	1404.97	30.81	33.02	1.8	1.60
13	16.00	2.50	2.00	1.50	1.00	1598.38	1756.51	2068.23	2256.15	37.11	38.01	1.4	1.35
14	10.00	1.00	2.00	1.50	1.00	2167.92	2461.75	2413.59	2561.17	39.37	36.80	1.1	1.02
15	10.00	4.00	2.00	1.50	1.00	1904.32	1976.61	2352.84	2415.42	38.69	44.36	1.2	1.03
16	10.00	2.50	1.00	1.50	1.00	2247.08	2399.61	2452.98	2481.99	39.62	39.62	1.0	0.98
17	10.00	2.50	4.00	1.50	1.00	1881.45	2095.04	2306.64	2487.78	38.07	41.18	1.3	1.07
18	10.00	2.50	2.00	1.00	1.00	2259.90	2619.30	2496.12	2684.04	40.96	46.63	0.9	0.73
19	10.00	2.50	2.00	2.00	1.00	3681.21	3687.93	3854.48	3876.72	73.39	70.82	0.1	0.02
20	10.00	2.50	2.00	1.50	0.50	2287.32	2294.04	2528.36	2731.03	43.17	44.44	0.8	0.60
21	10.00	2.50	2.00	1.50	1.50	2383.92	2743.32	2677.46	2684.94	43.29	45.13	0.7	0.64
22	10.00	2.50	2.00	1.50	1.00	2726.29	2681.88	2854.61	2887.25	49.57	48.29	0.6	0.61
23	10.00	2.50	2.00	1.50	1.00	2959.09	2681.88	3079.77	2887.25	48.69	48.29	0.4	0.61
24	10.00	2.50	2.00	1.50	1.00	3166.54	2681.88	3105.58	2887.25	52.16	48.29	0.3	0.61
25	10.00	2.50	2.00	1.50	1.00	2807.25	2681.88	2939.38	2887.25	49.92	48.29	0.5	0.61
26	10.00	2.50	2.00	1.50	1.00	3214.72	2681.88	3297.57	2887.25	53.55	48.29	0.2	0.61

Table 6: Optimum conditions for independent variables and predicted and experimental values of responses.

Run no.	Sucrose (w/v)	Yeast extract (w/v)	$NaNO_3$ (w/v)	Zn^{+2} (v/v)	Triton X-100 (v/v)	Extracellular inulinase (U/mL)		Extracellular invertase (U/mL)		Biomass mg/mL		C/N	
						Actual	Predicted	Actual	Predicted	Actual	Predicted	Actual	Predicted
2	7.17	1.47	1.28	2.00	0.50	3802.43	3733.14	3858.98	3862.30	72.54	73.49	0.02	0.00
3	7.35	1.73	1.09	1.99	0.50	3754.98	3812.80	3930.42	3917.98	72.98	73.39	0.15	0.10
4	7.13	1.76	1.42	2.00	0.50	3793.65	3765.34	3906.97	3885.80	73.03	73.48	0.06	0.08
5	8.95	2.16	1.00	2.00	0.50	3698.96	3768.61	3865.38	3866.43	74.07	73.47	0.02	0.03

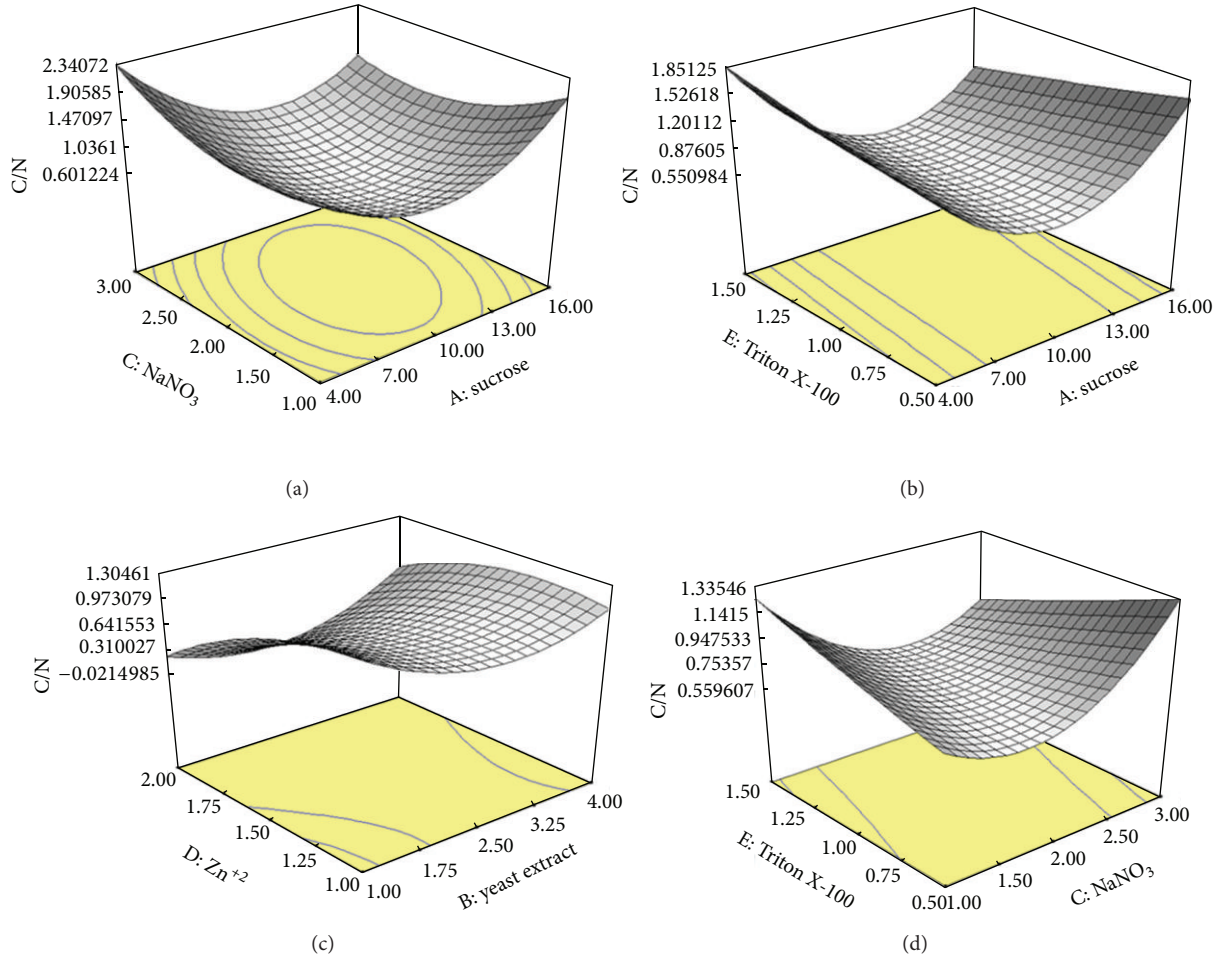

Figure 4: Response surface showing the interaction between five parameters and C/N; (a) sucrose and $NaNO_3$, (b) sucrose and Triton X-100, (c) yeast extract and Zn^{+2}, and (d) $NaNO_3$ and Triton X-100. Other variables are constant at their center points. The numbers inside the contour plots indicate the C/N.

(76.94 U/mL) were observed in optimized culture with high biomass (73.39 mg/mL), and lower C/N ratios (0.1) (data not shown). In the lowest intra- and extracellular enzymes production, C/N ratio was 2.0. Similar compositions of medium were used to attain high intra- and extracellular enzymes production and growth by *A. niger* ATCC 20611. During media optimization, with increase in the productions of intra- and extracellular inulinase and invertase, the C/N ratio contrarily decreased from 2.6 to 0.1 (Table 5). Similarly, the biomass progressively increased simultaneously with decreasing C/N ratio from 2.6 to 0.1. The highest I/S ratio (1.03) was observed in the medium with a C/N ratio of 0.3. The ratio of I/S sharply increased for the media with C/N ratios ranging from 2.6 to 2.5 and was progressively decreased for the media with C/N ratios from 2.5 to 2.3. I/S ratio proximately remained stable with C/N ratios from 2.3 to 0.1 which was an optimum point of C/N ratio (data not shown). The productions rate of extracellular inulinase and invertase per gram dry mycelium was about 12 and 30 times higher in optimum medium compared to the basal medium, respectively (data not shown). The effect of C/N ratio on enzyme production was strain dependent [35]. Carbon and nitrogen were critical nutritional parameters in the productions of intra- and extracellular inulinase and invertase and biomass built-up by *A. niger* ATCC 20611. Upon the selection of the preferred carbon and nitrogen sources, the effect of them on C/N ratios during intra- and extracellular inulinase and invertase productions was studied. So, to investigate the change of the C/N ratios, the quantity of nitrogen content (mixture of yeast extract and $NaNO_3$) in the medium was varied and the quantity of carbon content (sucrose) was fixed. Based on our previous study, organic and inorganic nitrogen sources have been claimed to promote cell growth and synthesis of enzymes [13, 14]. Maximum enzymes were obtained under carbon limitation, while enough nitrogen was available for the cells. This result might be the consequence of channelling more nitrogen for cell growth and intra- and extracellular enzymes production.

In the formulation of medium, natural and relative concentrations of carbon and nitrogen sources are very important [36]. The significant relation between C/N ratio, enzymes production, biomass, and I/S ratio was observed. Clearly, the enzymes production and growth were strongly

related to a low C/N ratio that resulted in better production of enzymes and biomass. Productions of intra- and extracellular enzymes were generally associated with the stationary phase of carbon, when an excess of nitrogen was channelled into secondary metabolism [37]. The imbalanced ratio between carbon and nitrogen content in the medium led to a decrease in the enzymes production. Results from this study suggested that the production of intra- and extracellular enzymes and growth were highly dependent on C/N ratios. A high productivity of enzymes was generally obtained by using a slowly metabolized nitrogen source by limiting the carbon content conditions. However, the presence of excess nitrogen under consumption of all carbon greatly enhanced the rate of production of enzymes. Apparently, the metabolic pathways for the synthesis of intra- and extracellular inulinase and invertase from nitrogen were much slower than the pathways that convert nitrogen to biomass. Therefore, carbon limitation enhanced enzymes production by diverting the extra nitrogen to enzymes production. This ability had made *A. niger* ATCC 20611 an important microorganism in industrial production of secreted glycoproteins.

4. Conclusions

Results from this study have demonstrated that *A. niger* ATCC 20611 is an efficient intra- extracellular inulinase and invertase producer. Also, the ultrasonic method was found more effective than grinding by porcelain mortar to extract the intracellular enzymes. The results showed that optimization of the reaction parameters by RSM design was an effective method for attaining high intra- and extracellular inulinase and invertase productions and biomass. The C/N ratio of the culture could indicate the intra- and extracellular inulinase and invertase productions and growth. The high yields of enzymes and growth were generally obtained by using an excess nitrogen source under conditions of carbon limitation. The developed model and optimum conditions could be used for future process scale-up.

Conflict of Interests

The authors report no conflict of interests in this work.

Acknowledgment

This research was supported by the Universiti Putra Malaysia.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 329121, 18 pages
http://dx.doi.org/10.1155/2013/329121

Review Article

A Broader View: Microbial Enzymes and Their Relevance in Industries, Medicine, and Beyond

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Received 3 May 2013; Revised 7 July 2013; Accepted 9 July 2013

Academic Editor: Subash C. B. Gopinath

Enzymes are the large biomolecules that are required for the numerous chemical interconversions that sustain life. They accelerate all the metabolic processes in the body and carry out a specific task. Enzymes are highly efficient, which can increase reaction rates by 100 million to 10 billion times faster than any normal chemical reaction. Due to development in recombinant technology and protein engineering, enzymes have evolved as an important molecule that has been widely used in different industrial and therapeutical purposes. Microbial enzymes are currently acquiring much attention with rapid development of enzyme technology. Microbial enzymes are preferred due to their economic feasibility, high yields, consistency, ease of product modification and optimization, regular supply due to absence of seasonal fluctuations, rapid growth of microbes on inexpensive media, stability, and greater catalytic activity. Microbial enzymes play a major role in the diagnosis, treatment, biochemical investigation, and monitoring of various dreaded diseases. Amylase and lipase are two very important enzymes that have been vastly studied and have great importance in different industries and therapeutic industry. In this review, an approach has been made to highlight the importance of different enzymes with special emphasis on amylase and lipase in the different industrial and medical fields.

1. Introduction

Enzymes are the biological substance or biological macromolecules that are produced by a living organism which acts as a catalyst to bring about a specific biochemical reaction. These are like the chemical catalysts in a chemical reaction which helps to accelerate the biological/biochemical reactions inside as well as outside the cell. These are generally known as "Biocatalyst." In 1877, Wilhelm Friedrich Kühne a professor of physiology at the University of Heidelberg first used the term enzyme, which comes from a Greek word *ενζυμον* meaning "in leaven" [1]. Even many centuries ago enzyme and its use were well known to the mankind but Wilhelm Friedrich Kühne was the first person to give a scientific terminology to this biomolecule. Use of enzyme has been seen in ancient Egyptians where they were used for the preservation of food and beverages. Cheese making has always involved the use of enzymes, and it goes as far as back in about 400 BC, when Homer's Iliad mentioned the use of a kid's stomach for making cheese. In 1783, the famous Italian catholic priest Lazzaro Spallanzani first mentioned the importance of this biomolecule in his work of biogenesis (spontaneous generation of microbes) where he mentioned that there is a life-generating force inherent to certain kinds of inorganic matter that causes living microbes to create themselves given sufficient time [2]. In the year 1812 Gottlieb Sigismund Kirchhoff was investigating the procedure of converting starch into glucose. In his experiment he also enlightens the application of these biomolecules as catalyst [3]. In 1833, French chemist Anselme Payen discovered the first enzyme, diastase [4]. In 1835, the hydrolysis of starch by diastase was acknowledged as a catalytic reaction by another Swedish scientist Jöns Jacob Berzelius. In 1839, he also interpreted fermentation as being caused by a catalytic force and postulated that a body—by its mere presence—could, by affinity to the fermentable substance, cause its

rearrangement to the products [5]. In 1846 the activity of invertase was demonstrated by Dubonfout. A few decades later in 1862, when studying the fermentation of sugar to alcohol by yeast, Louis Pasteur along with Ferdinand Cohn and Robert Koch came to the conclusion that this fermentation was catalysed by a vital force contained within the yeast cells called "ferments," which were thought to function only within living organisms [6]. In 1894, Jokichi Takamine discovered takadiastase which is the form of diastase obtained from *Aspergillus oryzae*. In 1897, Eduard Buchner demonstrated the conversion of glucose to ethanol by a cell-free extract from the yeast. Later in 1908, Otto Rohm, German scientist, introduced application of pancreatic enzymes with inorganic salts to meet the requirement in tanneries for bating of hides. In 1916, J. M. Nelson and E. G. Griffin showed the adsorption of invertase on charcoal and alumina demonstrating that immobilised enzymes can be retained. At present, immobilized cells have been used for production of organic acids, amino acids, antibiotics, enzymes, alcohol, and other compounds. Immobilized cell techniques have several advantages as compared to the free cell system, such as higher production rate and easier product separation [7]. It was not until 1926, however, that the first enzyme was obtained in pure form, a feat accomplished by James B. Sumner of Cornell University. Sumner in 1947 was able to isolate and crystallize the enzyme urease from the jack bean. His work was to earn him the Nobel Prize. John H. Northrop and Wendell M. Stanley shared the 1947 Nobel Prize with Sumner. They discovered a complex procedure for isolating pepsin. This precipitation technique devised by Northrop and Stanley has been used to crystallize several enzymes [8]. In 1960, NOVO started producing protease using Bacillus lichnformis on a commercial scale. After 1980, many scientists started application of genetic engineering techniques in order to improve the production of enzymes and also to alter the properties of enzymes by protein engineering.

Naturally found enzymes have been used widely since ancient times and in the manufacture of products such as linen, leather, and indigo. All of these processes dependent on either enzymes produced by microorganisms or enzymes present in added preparations such as calves' rumen or papaya fruit. The development of fermentation processes was aimed specifically at the production of enzymes by use of particularly selected strains, due to which it is possible to produce purified, well-characterized enzymes on a large scale. This development allowed the introduction of enzymes into true industrial products and processes, for example, within the detergent, textile, and starch industries. The recombinant DNA technology has further improved production processes and helped to produce enzymes commercially that could not be produced previously. Furthermore, the developments in biotechnology, such as protein engineering and directed evolution, further revolutionized the commercialization of industrial important enzymes. This advance in biotechnology is providing different kinds of enzymes displaying new activities, adaptability to new conditions leading to their increase use in industrial purposes. Since 1940s, the intensive research biochemistry confronted the use of enzymes as diagnostic tool and also provided basis in clinical chemistry. It is, however, only within the recent past few decades that interest in diagnostic enzymology has multiplied. Many methods currently on record in the literature are not in wide use, and there are still large areas of medical research in which the diagnostic potential of enzyme reactions has not been explored at all.

The majority of currently used industrial enzymes are hydrolytic in action, being used for the degradation of various natural substances. Proteases remain the dominant enzyme type, because of their extensive use in the detergent and dairy industries. Various carbohydrases, primarily amylases and cellulases, used in industries such as the starch, textile, detergent, and baking industries, represent the second largest group [9]. The global market for industrial enzymes is estimated at 3.3 billion dollars in 2010. This market is expected to reach more than 4 billion dollars by 2015. Enzymes play key roles in numerous biotechnology products and processes that are commonly encountered in the production of food and beverages, cleaning supplies, clothing, paper products, transportation fuels, pharmaceuticals, and monitoring devices. At present, the most frequently used enzymes in biotechnology are hydrolases, which catalyse the breakdown of molecules. Enzymes can display regional stereospecificity, properties that have been exploited for asymmetric synthesis and racemic resolution. Chiral selectivity of enzymes has been employed to prepare enantiomerically pure pharmaceuticals, agrochemicals, chemical feedstock, and food additives.

Thus, enzymes do show us a wide range of applications in different industries whether it may be food, textile, medicine, dairy, or any other. With the advancement of modern biotechnology and protein engineering we have the capability to introduce or modify the capability of the genes that are important for us to produce these novel enzymes. Our objective in writing this review is to emphasize the current role of the microbial enzymes and the current status of their use in different industries along with the biotechnological perspectives of its future development.

2. Enzymes, Classification, and Their Use

Enzymes are large biological molecules responsible for all those important chemical interconversions that are required to sustain life [10]. They are highly selective catalysts which can greatly accelerate both the rate and specificity of metabolic reactions, which range from the digestion of food to the synthesis of DNA. Almost all chemical reactions in a biological cell need enzymes in order to occur at rates sufficient for life. Since enzymes are selective for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell. Enzymes are known to catalyse about 4,000 biochemical reactions [11]. Enzymes are very specific, and it was suggested by the Nobel laureate Emil Fischer in 1894 that this was because both the enzyme and the substrate possess specific complementary geometric shapes that fit exactly into one another [12]. This is often referred to as "the lock and key" model. However, while this model explains enzyme specificity, it fails to explain the stabilization of the transition state that enzymes achieve.

Most enzymes are much larger than the substrates they act on, and only a small portion of the enzyme (around 2–4 amino acids) is directly involved in catalysis [13]. The region that contains these catalytic residues, binds the substrate, and then carries out the reaction is known as the active site. Enzymes can also contain sites that bind cofactors, which are needed for catalysis. Some enzymes also have binding sites for small molecules, which are often direct or indirect products or substrates of the reaction catalyzed. This binding can serve to increase or decrease the enzyme's activity, providing a means for feedback regulation. Like all proteins, enzymes are long, linear chains of amino acids that fold to produce a three-dimensional product. Each unique amino acid sequence produces a specific structure, which has unique properties. Individual protein chains may sometimes group together to form a protein complex. Most enzymes can be denatured that is, unfolded and inactivated by heating or chemical denaturants, which disrupt the three-dimensional structure of the protein. Depending on the enzyme, denaturation may be reversible or irreversible.

Due to their wide range of activities based on their nature of reaction enzymes are being classified according to their enzyme catalysing reaction as shown in Table 1. The Enzyme Commission number (EC number) is a numerical classification scheme for enzymes, based on the chemical reactions they catalyze [14]. As a system of enzyme nomenclature, every EC number is associated with a recommended name for the respective enzyme. Except for some of the originally studied enzymes such as pepsin, rennin, and trypsin, most enzyme names end in "ase." The enzyme nomenclature scheme was developed starting in 1955, when the International Congress of Biochemistry in Brussels sets up an Enzyme Commission. The first version was published in 1961. The current sixth edition, published by the International Union of Biochemistry and Molecular Biology in 1992, contains 3196 different enzymes. The International Union of Biochemistry (I.U.B.) initiated standards of enzyme nomenclature which recommend that enzyme names indicate both the substrate acted upon and the type of reaction catalyzed. According to the enzyme commission the enzymes are divide into 6 parts:

(i) oxidoreductase (EC 1),

(ii) transferase (EC 2),

(iii) hydrolase (EC 3),

(iv) lyase (EC 4),

(v) isomerase (EC 5),

(vi) ligase (EC 6).

In Table 2 examples of few classes of industrially important enzymes are given.

3. Application of Enzyme

3.1. Amylase. Amylase is an enzyme that catalyses the breakdown of starch into sugars. Amylase is abundantly present in human saliva as shown in Figure 1, where it begins the mechanical process of digestion. Foods that contain much starch but little sugar, such as rice and potato, taste slightly sweet as they are chewed because amylase turns some of their starch into sugar in the mouth. The pancreas also makes amylase (alpha amylase) to hydrolyse dietary starch into disaccharides and trisaccharides which are converted by other enzymes to glucose to supply the body with energy. Plants and some bacteria also produce amylase. As diastase, amylase was the first enzyme to be discovered and isolated by Anselme Payen in 1833. All amylases are glycoside hydrolases and act on α-1,4-glycosidic bonds. It is widely used in industries and have nearly 25% of the enzyme market [15, 16]. Today amylase has almost replaced chemical hydrolysis of starch in starch processing industry. The amylases obtained from microorganisms have a broad spectrum of industrial uses as they are more stable than plant and animal amylases. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity, and also microbes are easy to manipulate to derive enzymes of desired nature. Starch-degrading amylolytic enzymes are of great importance in biotechnological sector ranging from food, fermentation, textile to paper industries [17, 18]. Although amylases can be obtained from several sources, like plants and animals, the enzymes from microbial sources generally satisfy industrial demands and had made significant contribution to the foods and beverages industry in the last three decades.

3.2. Types. Amylase has been divided into three sub classes—α- β- γ-amylase. The classification is based on the bonding type.

3.3. α-Amylase. α-Amylase are enzymes that helps in the hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight products, such glucose, maltose, and maltotriose units [19, 20]. It is the major form of amylase found in humans and other mammals [21]. It is also present in seeds containing starch as a food reserve and is secreted by many fungi. Although found in many tissues, amylase is most prominent in pancreatic juice and saliva, each of which has its own isoform of human α-amylase. In humans, all amylase isoforms link to chromosome 1p21 (AMY1A).

3.4. Use. α-Amylase is used in ethanol production to break starches in grains into fermentable sugars. The first step in the production of high-fructose corn syrup is the treatment of cornstarch with α-amylase, producing shorter chains of sugars called oligosaccharides. An α-amylase called "Termamyl," sourced from *Bacillus licheniformis*, is also used in some detergents, especially dishwashing and starch-removing detergents.

3.5. β-Amylase. Another form of amylase, β-amylase (EC 3.2.1.2) (alternative names: 1,4-α-D-glucan maltohydrolase; glycogenase; saccharogen amylase) is also synthesized by bacteria, fungi, and plants and shown in Figure 2. Working from the nonreducing end, β-amylase catalyzes the hydrolysis of the second α-1, 4 glycosidic bond, cleaving off two glucose units (maltose) at a time. During the ripening of fruit, β-amylase breaks starch into maltose, resulting in the sweet flavor of ripe fruit. Both α-amylase and β-amylase are present

Table 1: Enzyme classes and types of reactions [14].

Enzyme commission number	Class of enzyme	Reaction profile
EC 1	Oxidoreductases	Oxidation reactions involve the transfer of electrons from one molecule to another. In biological systems we usually see the removal of hydrogen from the substrate. Typical enzymes in this class are called dehydrogenases. For example, alcohol dehydrogenase catalyzes reactions of the type $R–CH_2OH + A \; R–CHO + H_2A$, where A is an acceptor molecule. If A is oxygen, the relevant enzymes are called oxidases or laccases; if A is hydrogen peroxide, the relevant enzymes are called peroxidases.
EC 2	Transferases	This class of enzymes catalyzes the transfer of groups of atoms from one molecule to another. Aminotransferases or transaminases promote the transfer of an amino group from an amino acid to an alpha-oxoacid.
EC 3	Hydrolases	Hydrolases catalyze hydrolysis, the cleavage of substrates by water. The reactions include the cleavage of peptide bonds in proteins, glycosidic bonds in carbohydrates, and ester bonds in lipids. In general, larger molecules are broken down to smaller fragments by hydrolases.
EC 4	Lyases	Lyases catalyze the addition of groups to double bonds or the formation of double bonds through the removal of groups. Thus bonds are cleaved using a principle different from hydrolysis. Pectate lyases, for example, split the glycosidic linkages by beta-elimination.
EC 5	Isomerases	Isomerases catalyze the transfer of groups from one position to another in the same molecule. In other words, these enzymes change the structure of a substrate by rearranging its atoms.
EC 6	Ligases	Ligases join molecules together with covalent bonds. These enzymes participate in biosynthetic reactions where new groups of bonds are formed. Such reactions require the input of energy in the form of cofactors such as ATP.

Table 2: A selection of enzymes used in industrial processes.

Sl no.	Class	Industrial enzymes
1	Oxidoreductases	Catalases Glucose oxidases Laccases
2	Transferases	Fructosyltransferases Glucosyltransferases
3	Hydrolases	Amylases Cellulases Lipases Mannanases Pectinases Phytases Proteases Pullulanases Xylanases
4	Lyases	Pectate lyases Alpha-acetolactate decarboxylases
5	Isomerases	Glucose isomerases Epimerases Mutases Lyases Topoisomerases
6	Ligases	Argininosuccinate Glutathione synthase

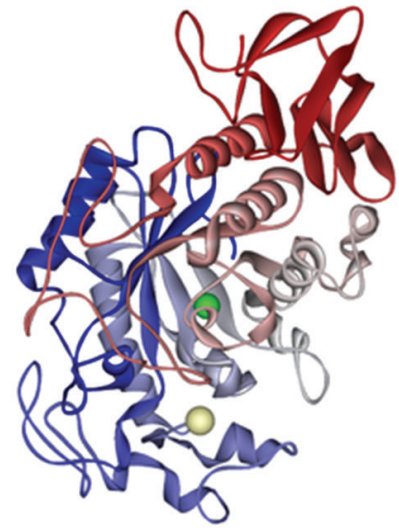

Figure 1: Computer simulated 3D image of human salivary amylase [22].

in seeds; β-amylase is present in an inactive form prior to germination, whereas α-amylase and proteases appear once germination has begun. Cereal grain amylase is key to the production of malt. Many microbes also produce amylase to degrade extracellular starches. Animal tissues do not contain β-amylase, although it may be present in microorganisms contained within the digestive tract. The optimum pH for β-amylase is 4-5.

3.6. Use. α and β amylases are important in brewing beer and liquor made from sugars derived from starch. In fermentation, yeast ingest sugars and excrete alcohol. In beer and some liquors, the sugars present at the beginning of fermentation

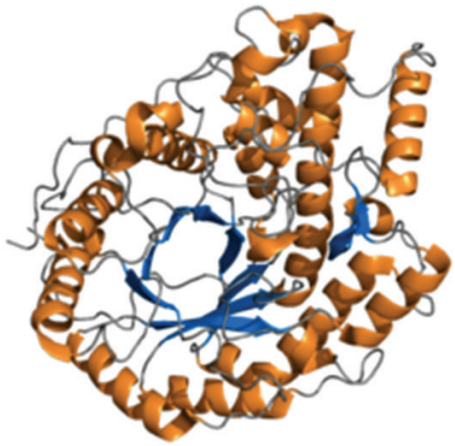

Figure 2: Computer simulated 3D image of barley beta-amylase [23].

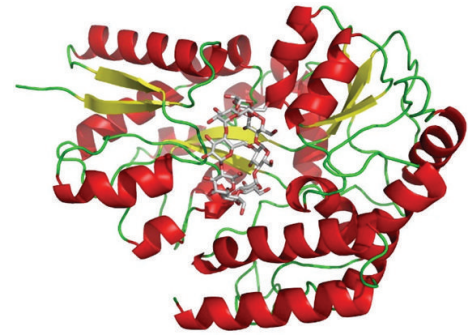

Figure 3: Computer simulated 3D image of gamma amylase from *Thermoactinomyces vulgaris* R-47 cyclodextrin binding protein (2DFZ) [24].

have been produced by "mashing" grains or other starch sources (such as potatoes). In traditional beer brewing, malted barley is mixed with hot water to create a "mash," which is held at a given temperature to allow the amylases in the malted grain to convert the barley's starch into sugars. Different temperatures optimize the activity of α and β amylase, resulting in different mixtures of fermentable and unfermentable sugars. In selecting mash temperature and grain-to-water ratio, a brewer can change the alcohol content, mouthfeel, aroma, and flavor of the finished beer.

3.7. γ-Amylase. γ-Amylase (EC3.2.1.3) (alternative names: Glucan 1,4-α-glucosidase; amyloglucosidase; Exo-1,4-α-glucosidase; glucoamylase; lysosomal α-glucosidase; 1,4-α-D-glucan glucohydrolase) will cleave α(1-6) glycosidic linkages, as well as the last α(1-4) glycosidic linkages at the nonreducing end of amylose and amylopectin, yielding glucose. The image of γ-amylase is shown in Figure 3. The γ-amylase has most acidic pH optimum because it is most active around pH 3.

3.8. Use. They are used in food, pharmaceutical, drug delivery, and chemical industries, as well as agriculture and environmental engineering. Hydroxypropyl beta cyclodextrin (HPβCD) is the chief active compound found in Procter and Gamble's deodorizing product "Febreze" under the brand name "Clenzaire."

3.9. Bacterial Amylases. Amylase can be obtained from different species of microorganisms, but for commercial use, α-amylase derived from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* has number of application in different industries such as in food, fermentation, textiles and paper industries [25, 26]. Thermostability is a desirable characteristic of a major group of industrial enzymes. Thermostable enzymes have found a large number of commercial applications due to their stability. Thermostable amylolytic enzymes have been currently in research to improve industrial method of starch degradation and also in the production of valuable products like crystalline dextrose, glucose, maltose, dextrose syrup, and maltodextrins [27, 28]. *Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis,* and *Bacillus amyloliquefaciens* are found to be good producers of thermostable α-amylase and are widely in use for commercial production of the enzyme for numerous applications. Today, thermostable amylases of *Bacillus stearothermophilus* or *Bacillus licheniformis* are used in starch processing industries [28]. Some halophilic microorganisms have optimal activity at high salinity and enzymes produced by them could be used in many harsh industrial processes where highly concentrated salt solutions are used which would otherwise inhibit many enzymatic reactions [29]. Also, most of the halobacterial enzymes are thermotolerant and remain stable at room temperature for a long period [30]. Halophilic amylases have been derived from bacteria such as *Chromohalobacter* sp. [29], *Halobacillus* sp. [31], *Haloarcula hispanica* [32], *Halomonas meridiana* [33], and *Bacillus dipsosauri* [34].

3.10. Fungal Amylase. Most of the mesophilic fungi are reported to produce α-amylase, and many researches have been done for specific cultural conditions and to choose the best strains to produce commercially. Fungal enzymes are limited to terrestrial isolates, mostly to *Aspergillus and Penicillium* [35]. The *Aspergillus* species usually produces a variety of extracellular enzymes, and amylases are the ones with the most significant industrial value. Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce large quantities of enzymes that can be used extensively in the industry. *A. oryzae* is considered to be the favourable host for the production of heterologous proteins as it has ability to secrete a vast amount of high value proteins and industrial enzymes, for example, α-amylase [28]. *Aspergillus oryzae* has been extensively used in the production of food such as soy sauce and organic acid such as citric and acetic and commercial enzymes including α-amylase [36]. *Aspergillus niger* is acid tolerant (pH < 3) and hence has important hydrolytic capacities in the α-amylase production, and it also avoids bacterial contamination [37]. The fungal α-amylase is usually preferred over other microbial sources because of their more accepted (GRAS) Generally Recognized as Safe

status [19]. The thermophilic fungus *Thermomyces lanuginosus* is an excellent producer of amylase. Jensen et al. [38] and Kunamneni et al. [39] purified the α-amylase, proving its thermostability.

3.11. Application of Amylase. Amylases have a wide range of application in various industries such as in the food, bread making, paper industries, textiles, sweeteners, glucose and fructose syrups, fruit juices, detergents, fuel ethanol from starches, alcoholic beverages, digestive aid, and spot remover in dry cleaning. Bacterial α-amylases are also being used in clinical, medicinal, and analytical chemistry [26]. The widely used thermostable enzymes in the starch industry are the amylases [38].

3.12. Use in Starch Industry. The starch industry has the most widespread applications of amylases, which are used during starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups [40]. The enzymatic conversion of all starch includes gelatinization, which involves the dissolution of starch granules, thereby forming a viscous suspension; liquefaction, which involves partial hydrolysis and loss in viscosity; and saccharification, involving the production of glucose and maltose via further hydrolysis [28].

3.13. Use in Detergent Industry. Both in terms of volume and value detergent industry are the primary consumers of enzymes. The application of enzymes in detergents making enhances the detergents ability to remove tough stains and also makes detergent ecofriendly. Amylases are the second type of enzymes used in the detergent formulation, and 90% of all liquid detergents contain these enzymes [41]. These enzymes are used for laundry and automatic dishwashing to clean up residues of starchy foods such as custard, gravies, potato, and chocolate. and other smaller oligosaccharides.

3.14. Use in Food Industry. There is an extensive use of amylase in processed food industry such as baking, brewing, production of cakes, preparation of digestive aids, fruit juices, and starch syrups. The α-amylases have been used in the baking industry widely [42]. These enzymes are generally added to the dough of bread in order to degrade the starch into smaller dextrins, which are further fermented by the yeast. The α-amylase enhances the fermentation rate and the reduction of the viscosity of dough, which results in improvements in the volume and texture of the product.

3.15. Use in Textile Industry. Amylases are utilized for desizing process in textile industry. Sizing agents like starch are added to yarn before fabric production for fast and secure weaving process. Starch is a very attractive size, because it is cheap, easily available all over, and it can be easily removed. Desizing is the process where removal of starch from the fabric takes place and acts as the strengthening agent to prevent breaking of the warp thread during the weaving process. The α-amylases selectively remove, the size and do not affect the fibres [43]. For a long time amylase from *Bacillus* strain was employed in textile industry.

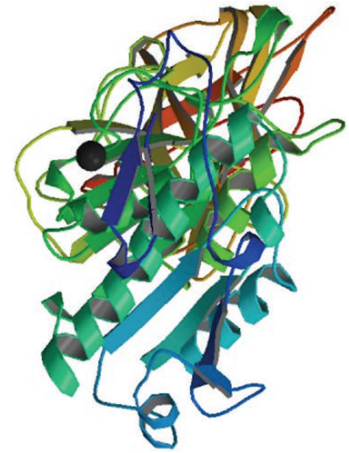

Figure 4: Computer simulated 3D image of pancreatic lipase [48].

3.16. Use in Paper Industry. The main use of α-amylases in the pulp and paper industry is the modification of starch of coated paper, that is, for the production of low-viscosity, high-molecular weight starch [42]. The coating treatment makes the surface of paper smooth and strong to improve the writing quality of the paper. For paper sizing the viscosity of natural enzyme is too high, and this can be changed by partially degrading the polymer with α-amylases in a batch or continuous processes. Starch is considered to be the good sizing agent for the finishing of paper, improving the quality and reusability, besides being a good coating for the paper.

3.17. Use in Medicine. A higher than normal concentration of amylases may predict one of several medical conditions, including acute inflammation of the pancreas, perforated peptic ulcer, strangulation ileus, torsion of an ovarian cyst, macroamylasemia, and mumps. In other body fluids also amylase can be measured, including urine and peritoneal fluid. In various human body fluids the level α- amylase activity is of clinical importance, for example, in diabetes, pancreatitis, and cancer research [44].

3.18. Lipase. It is an enzyme that catalyzes the breakdown or hydrolysis of fats [45]. Lipases are a subclass of the esterases. Lipases perform essential roles in the digestion, transport, and processing of dietary lipids (e.g., triglycerides, fats, and oils) in most, if not all, living organisms. Genes encoding lipases are even present in certain viruses [46]. Most lipases act at a specific position on the glycerol backbone of lipid substrate especially in small intestine. For example, human pancreatic lipase as shown in Figure 4, which is the main enzyme that breaks down dietary fats in the human digestive system, converts triglyceride substrates found in ingested oils to monoglycerides and two fatty acids. Several other types of lipase activities exist in nature, such as phospholipases and sphingomyelinases; however, these are usually

treated separately from "conventional" lipases. Some lipases are expressed secreted by pathogenic organisms during the infection. In particular, *Candida albicans* has a large number of different lipases, possibly reflecting broad lipolytic activity, which may contribute to the persistence and virulence of *C. albicans* in human tissue [47]. Lipases are considered as major group of biotechnologically valuable enzymes, mainly due to the versatility of their applied properties and easy mass production. Microbial lipases are largely diversified in their enzymatic properties and substrate specificity, which make them potential source for industrial applications. The interest in this enzyme is primarily due to investigations of their role in pathogenesis and their wide application in biotechnology. Bacterial lipases are more stable than animal or plant lipases. The energy expenditure required to conduct reactions at elevated temperatures and pressures is eliminated as lipases are active under ambient temperature, and it also reduces the denaturation of labile reactants and products.

3.19. Types. There are no such distinguished types of lipase, but mainly it is categorized according to its use, namely, human digestive system in human pancreatic lipase (HPL) and pancreatic lipase. Others include hepatic lipase (HL), endothelial lipase, and lipoprotein lipase.

3.20. Application of Lipase. Lipases are involved in diverse biological processes ranging from routine metabolism of dietary triglycerides to cell signaling and inflammation [53, 54]. Thus, some lipase activities are confined to specific compartments within cells, while others work in extracellular spaces.

(i) In the example of lysosomal lipase, the enzyme is confined within an organelle called the lysosome.

(ii) Other lipase enzymes, such as pancreatic lipases, are secreted into extracellular spaces where they serve to process dietary lipids into more simple forms that can be more easily absorbed and transported throughout the body.

(iii) Fungi and bacteria may secrete lipases to facilitate nutrient absorption from the external medium (or in examples of pathogenic microbes to promote invasion of a new host).

(iv) Certain wasp and bee venoms contain phospholipases that enhance the "biological payload" of injury and inflammation delivered by a sting.

(v) As biological membranes are integral to living cells and are largely composed of phospholipids, lipases play important roles in cell biology.

(vi) *Malassezia globosa*, a fungus that is thought to be the cause of human dandruff, uses lipase to break down sebum into oleic acid and increase skin cell production, causing dandruff.

Lipases serve important roles in human practices as ancient as yogurt and cheese fermentation. However, lipases are also being exploited as cheap and versatile catalysts to degrade lipids in more modern applications. For instance, a biotechnology company has brought recombinant lipase enzymes to market for use in applications such as baking, laundry detergents, and even as biocatalysts [55] in alternative energy strategies to convert vegetable oil into fuel [56]. High enzyme activity lipase can replace traditional catalyst in processing biodiesel; this enzyme is more environmental and safe.

3.21. Bacterial Lipase. Some of the lipase-producing bacterial genera include *Bacillus*, *Pseudomonas, and Burkholderia*. The commercially important bacterial lipases are usually extracellular, and also their bulk production is much easier. There are a number of lipase-producing bacteria, but only a few are commercially exploited as wild or recombinant strains [57]. Of these, the important ones are *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium*, *Enterococcus*, *Corynebacterium,* and *Pseudomonas*. In a variety of biotechnological applications lipases from *Pseudomonas* are widely used [58, 59]. Various products based on bacterial lipases have been launched in the market in the past few years, such as Lumafast and Lipomax from *Pseudomonas* with their major application in detergent enzymes, while Chiro CLEC-PC, Chirazyme L-1, and Amano P, P-30 and PS have numerous applications in organic synthesis.

3.22. Fungal Lipase. Fungi capable of synthesizing lipases are found in several habitats, including soils contaminated with wastes of vegetable oils, dairy byproduct, seeds, and deteriorated food [60, 61]. *Candida rugosa* lipases have been known for their diverse biotechnological potential [62]. The presence of *C. rugosa* lipase isoforms has been reported by many researchers [62, 63]. *Rhizopus oryzae* lipase, *Rhizopus delemar* lipase and *Rhizopus javanicus* lipase have a substitution in the His 134 and the Leu 234 was referred by Minning et al. [64]. The LIP2 lipase from the *Yarrowia lipolytica* (YLLIP2) have a high potential for enzyme replacement therapy due to its unique biochemical properties: It shows highest activity at low pH values and is not repressed by bile salts. It was also reported that *Thermomyces lanuginosus* lipase has variety of applications in the field of detergents and biotechnological processes and it was also reported that YLLIP2 belongs to same family [65]. Other major lipase-producing fungi are *Mucor, Candida, Penicillium, Rhizopus, Geotrichum, Rhizomucor*, *Aspergillus, Humicola,* and *Rhizopus* [66]. The extracellular thermostable lipase is produced by thermophilic *Mucor pusillus*, *Rhizopus homothallicus*, and *Aspergillus terreus*. *Mucor* sp. produces an extracellular, thermostable, inducible, and alkaliphilic lipase. There are few reports that have been made so far with molds with alkaliphilic and thermostable lipase [67, 68].

3.23. Use in Textile Industry. In the textile industry lipases are used for the removal of size lubricants, which increases fabrics absorbance ability for improved levelness in dyeing. In the denim abrasion systems, it is used to lessen the frequency of cracks and streaks. Commercial preparations used for the desizing of denim and other cotton fabrics contain both alpha

amylases, and lipase enzymes are used for the desizing of cotton fabrics and denim during its commercial preparation [69].

3.24. Use in Detergent Industry. The hydrolytic lipases are commercially very important, and their addition to detergents is mainly used in laundries and household dishwashers. Enzymes reduce the environmental load of detergent products, as they save energy by enabling a lower wash temperature to be used, and use of chemicals in detergents is reduced, mostly biodegradable, leaving no harmful residues has no negative impact on sewage treatment processes; and does not possess any kind of risk to aquatic life [70].

3.25. Use in Food Industry. To modify the food flavour by synthesis of esters of short-chain fatty acids and alcohols (flavour and fragrance) lipases have been frequently used. Lipases play a major role in the fermentative steps during manufacturing of sausage and also to measure changes in long-chain fatty acid liberated during ripening. Previously, lipases of different microbial sources were used for refining rice flavour, modifying soybean milk, and for enhancing the aroma and speed up the fermentation of apple wine [71]. By adding lipases the fat is removed while processing meat and fish, and this process is called biolipolysis.

3.26. Use in Diagnosis. Lipases are considered as important drug targets or marker enzymes in the medical field. The presence or high levels of lipases can indicate certain infection or disease and can be used as diagnostic tool. They are used in the determination of serum triglycerides to liberate glycerol which is determined by enzyme-linked colorimetric reactions. Acute pancreatitis and pancreatic injury can be determined by the level of lipases in blood [72]. Few new developments have been made by using lipases for the diagnosis of pancreatitis. The development of a test for measurement of canine pancreatic lipase has been developed using pancreatic lipases as they are fixed markers for the pancreas. A serum feline pancreatic lipase immunoreactivity (fPLI) test was currently developed, and findings suggest that this test is more accurate than other diagnostic tools used for the diagnosis of feline pancreatitis [73]. *P. aeruginosa* strains are isolated from patients with cancer, and 69% of the strains showed higher level of lipase (20–150 U/mL); these elevated levels of lipases were involved mainly with nontypable strains. The nontypable strains clearly show the most frequent group with elevated level of lipase, proteinase, elastase, hydrophobicity, and motility [74].

3.27. Use in Medical Applications. Lipases isolated from *Galleria mellonella* (wax moth) were found to have a bactericidal action on *Mycobacterium tuberculosis* (MBT) H37Rv. This preliminary research may be considered as part of global unselected screening of biological and other samples for detecting new promising sources of drugs [75]. Lipases can be used as digestive aids. Lipases can be used in the treatment of malignant tumors as they are the activators of tumor necrosis factor. Human gastric lipase (HGL) is the most stable acid lipase and considered to be a good tool for enzyme substitution therapy. Earlier lipases have been used in the treatment of gastrointestinal disturbances, dyspepsias, cutaneous manifestations of digestive allergies, and so forth. Lipase from *Candida rugosa* synthesizes lovastatin, a drug that lowers serum cholesterol level. The asymmetric hydrolysis of 3-phenylglycidic acid ester which is a key intermediate in the synthesis of diltiazem hydrochloride is a widely used coronary vasodilator and is synthesized using *S. marcescens* lipase [76].

3.28. Use in Cosmetics. Retinoids (vitamin A and derivatives) are commercially very important in cosmetics and pharmaceuticals such as skin care products. Immobilized lipases are used for the preparation of water-soluble retinol derivatives. Lipases are used in hair waving preparation and have also been used as an ingredients of topical antiobese creams or as oral administration [77].

3.29. Use as Biosensor. The enzyme-catalysed dissolution of biodegradable polymer films based on biosensor has been developed. The polymer enzyme system. poly(trimethylene) succinate, Which was investigated is degraded by a lipase and can be used as biosensor. Within the last few years, different processes have been designed using enzyme-labelled probes in order to avoid unstable and harmful isotopes. While screening various hydrolytic enzymes to fulfil the special demands, fungal lipases turned out to be the most relevant one. Immobilization of lipases can be done on pH/oxygen electrodes along with glucose oxidase, and these serve as lipid biosensors and can be used in triglycerides and blood cholesterol determinations [78].

3.30. Use in Biodegradation. Margesin et al. [79] have concluded that soil microbial lipase activity can be an important indicator of diesel oil biodegradation in freshly contaminated, unfertilized, and fertilized soils. In the coastal environment fungal strains are used to degrade oil spills, which in turn increase ecorestoration and enzymatic oil processing in industries. Lipase produced by *Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens, Serratia marcescens, Pseudomonas aeruginosa,* and *Staphylococcus aureus* was reported to degrade palm oil mill, diary, slaughter house, soap industry, and domestic waste water [80]. *Pseudomonas aeruginosa* lipases were recommended for castor oil degradation [81].

4. General Therapeutic Application of Other Enzymes

Therapeutic enzymes have a wide variety of specific uses such as oncolytics, thrombolytics, or anticoagulants and as replacements for metabolic deficiencies. Proteolytic enzymes serve as good anti-inflammatory agents. The list of enzymes which have the potential to become important therapeutic agents and its microbial sources are shown in Table 3 and Table 4 respectively. A number of factors severely decrease the potential utility of microbial enzymes once we enter

Table 3: Some important enzymes and their therapeutic importance.

Enzyme	Reaction	Use	Sources	References
Asparaginase	L-Asparagine H_2O→L-aspartate + NH_3	Leukaemia	*E. coli*	[82, 83]
Collagenase	Collagen hydrolysis	Skin ulcers	*C. perfringens*	[84]
Glutaminase	L-Glutamine H_2O→L-glutamate + NH_3	Leukaemia	*E. coli* SFL-1	[85, 86]
Lysozyme	Bacterial cell wall hydrolysis	Antibiotic	*Homo sapiens*	[87, 88]
Ribonuclease	RNA hydrolysis	Antiviral	Yeast and bacteriophages	[89, 90]
Streptokinase	Plasminogen→plasmin	Blood clots	*Streptococci* sp.	[91]
Trypsin	Protein hydrolysis	Inflammation	*Homosapiens* and *other* vertebrates	[92]
Uricase	Urate + O_2 → allantoin	Gout	*A. flavus*	[93, 94]
Urokinase	Plasminogen→plasmin	Blood clots	*Bacillus subtilis*	[95, 96]
β-Lactamase	β-Lactam ring hydrolysis	Antibiotic resistance	*Citrobacter freundii, Serratia marcescens*, and *Klebsiella pneumonia*	[97]
Penicillin acylase	Binding the rings of benzylpenicillin (penicillin G) and phenoxymethylpenicillin (penicillin V)	Penicillin production/broad spectrum antibiotic production	*Penicillium* sp.	[98]

Table 4: List of some common enzymes found from different species.

Source	Enzyme	Microorganism	References
Fungal	Amylase	*Aspergillus oryzae*	[107–110]
	Glucosidases	*Aspergillus flavus*	
	Proteases	*Aspergillus niger*	
	Pectinases	*Aspergillus niger*	
	Glucose oxidase	*Penicillium notatum*	
	Catalase	*Aspergillus niger*	
Bacterial	Amylases Proteases Penicillinase	*Bacillus subtilis*	[111]
Yeast	Invertase	*Saccharomyces cerevisiae*	[112]
	Lactase	*Saccharomyces fragilis*	

the medical field due to large molecular size of biological catalyst which prevents their distribution within somatic cells, and another reason is the response of immune system of the host cell after injecting the foreign enzyme protein.

As compared to the industrial use of enzymes, therapeutically useful enzymes are required in relatively less amounts, but the degree of purity and specificity should be generally high. The kinetics of these enzymes are low K_m and high $V_{\max}$ so that it is maximally efficient even at low concentrations of enzymes and substrates. The sources of such enzymes should be selected with great care to prevent any possibility of undesirable contamination by incompatible material and also to enable ready purification. Therapeutic enzymes are usually marketed as lyophilised pure preparations with biocompatible buffering salts and mannitol diluent. The cost of these enzymes is high but comparable to those of therapeutic agents or treatments. As an example, urokinase is derived from human urine and used to dissolve blood clots. One of the major applications of therapeutic enzymes is in the treatment of cancer and various other diseases as shown in the Figure 5. For the treatment of acute lymphocytic leukaemia asparaginase enzyme has proved to be promising. Its activity depends upon the fact that tumour cells lack aspartate-ammonia ligase activity, which stops the synthesise of nonessential amino acid L-asparagine. Hence, they are extracted from body fluids. The asparaginase does not affect the normal cells which are capable of synthesizing enough for their own requirements, but they decrease the free exogenous concentration, so it causes a state of fatal starvation in the susceptible tumour cells. The enzyme can be administered intravenously and is only effective in reducing asparagine levels within the bloodstream, showing a half-life of about a day (in a dog). This half-life can be increased by 20-fold with use of polyethylene glycol-modified asparaginase.

4.1. Treatment of Damaged Tissue. A large number of proteolytic enzymes of plant and bacterial origin have been studied for the removal of dead skin of burns. Various enzymes of higher quality and purity are now in clinical trials. Debrase gel dressing, containing a mixture of several enzymes

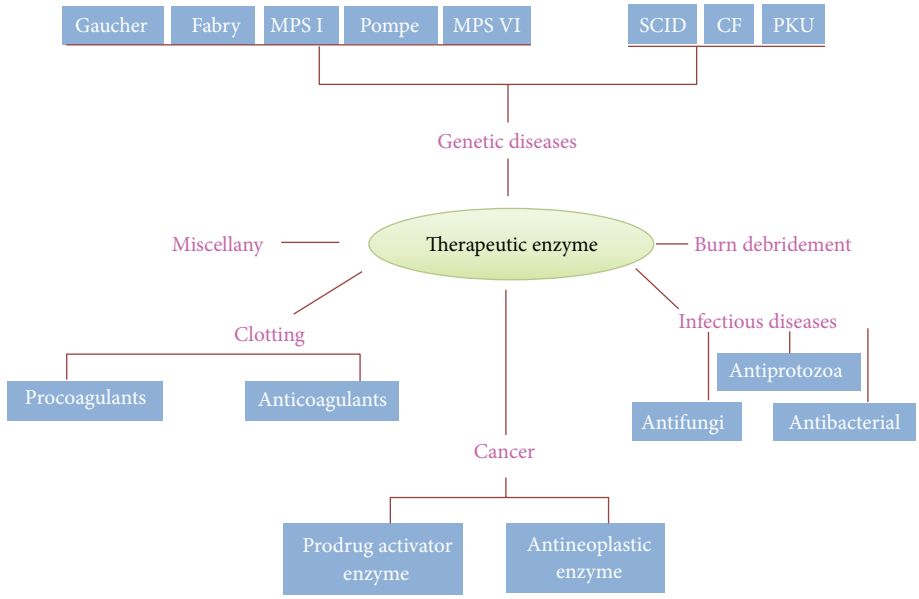

Figure 5: Application of therapeutic enzymes in different disorders and diseases [49–52].

extracted from pineapple, received clearance in 2002 from the US FDA for a Phase II clinical trial for the treatment of partial-thickness and full-thickness burns. A proteolytic enzyme (VibrilaseTM) obtained from *Vibrio proteolyticus* is found to be effective against denatured proteins such as those found in burned skin. The regeneration of injured spinal cord have been demonstrated using chondroitinases, where this enzyme acts by removing the glial scar and thereby accumulating chondroitin sulfate that stops axon growth [99]. Hyaluronidase has also been found to be a similar hydrolytic activity on chondroitin sulphate and may help in the regeneration of damaged nerve tissue [100].

4.2. Treatment of Infectious Diseases. Lysozyme is a naturally occurring antibacterial agent and used in many foods and consumer products, as it is able to breakdown carbohydrate chains in bacterial cell wall. Lysozyme has also been found to have activity against HIV, as RNase A and urinary RNase U present selectively degrade viral RNA [101] showing possibilities for the treatment of HIV infection. Chitinases is another naturally occurring antimicrobial agent. The cell wall of various pathogenic organisms, including fungi, protozoa, and helminths is made up of chitin and is a good target for antimicrobials [102]. The lytic enzyme derived from bacteriophage is used to target the cell walls of *Streptococcus pneumonia*, *Bacillus anthracis,* and *Clostridium perfringens* [103]. The application of lytic bacteriophages can be used for the treatment of several infections and could be useful against new drug-resistant bacterial strains.

4.3. Treatment of Cancer. The cancer research has some good instances of the use of enzyme therapeutics. Recent studies have proved that arginine-degrading enzyme (PEGylated arginine deaminase) can inhibit human melanoma and hepatocellular carcinomas [104]. Currently, another PEGylated enzyme, Oncaspar1 (pegaspargase), has shown good results for the treatment of children newly diagnosed with acute lymphoblastic leukemia and are already in use in the clinic. The normal cells are able to synthesize asparagine but the cancerous cells cannot and thus, die in the presence of asparagine degrading enzyme. Asparaginase and PEG-asparaginase are effective adjuncts for standard chemotherapy. Another important feature of oncogenesis is proliferation. It has been proved that the removal of chondroitin sulfate proteoglycans by chondroitinase AC and, to a lesser extent, by chondroitinase B, stops tumor growth, metastasis, and neovascularization [105]. The further application of enzymes as therapeutic agents in cancer is described by antibody-directed enzyme prodrug therapy (ADEPT). A monoclonal antibody carries an enzyme specific to cancer cells where the enzyme activates a prodrug and destroys cancer cells but not normal cells. This approach is being utilized for the discovery and development of cancer therapeutics based on tumor-targeted enzymes that activate prodrugs. The targeted enzyme prodrug therapy (TEPT) platform, involving enzymes with antibody-like targeting domains, will also be used in this effort [106].

5. Modern Application of Enzyme: A Biotechnology View

Enzymes are one of the most important biomolecules which has a wide range of applications in industrial as well as biomedical field as describe in Table 5. Today it is one of the most important molecules which are widely used since the ancient human civilization. With the growing population and raising need enzymes seem to be one of the most vital molecules that have a great impact in every sector that may be dairy, industrial, agriculture, or medicine.

Table 5: A broad spectrum idea about using the application of enzymes in different areas.

Types of industries	Enzymes	Use	References
Alcohol/beverage	Amylase, glucanases, proteases, beta-glucanases, arabinoxylans, amyloglucosidase, pullulanases, and acetolactate decarboxylase	Degradation of starch and polycarbonated into simple sugar. Also for degrading complex proteins into sugars thus to increase the fermentation efficiency. Production of low calorie beer	[23, 116–123]
Fruit drinks	Cellulases, pectinases	Clarify fruit juice	[124, 125]
Baby food	Trypsin	Predigest baby foods	[126]
Food processing	Amylase, protease, and papain	Degradation of starch and complex proteins, softening of meat	[127–129]
Dairy	Rennin, lipases, and lactases	Hydrolysing protein, cheese production (Roquefort cheese), and glucose production from lactose	[130–132]
Detergent	Protease, amylase, lipase, cellulases, and mannanase	To remove protein after staining, remove insoluble starch in dish washing, removing oils and fats, and to increase the effectiveness of detergents	[23, 124, 128, 131, 133]
Textile	Amylase, pectinase, cellulases, catalase, and protease	To remove starch size, glue between the fiber core and the waxes, fabric finishing in denims, degrading residual hydrogen peroxide after the bleaching of cotton, wool treatment, and the degumming of raw silk also known as biopolishing	[23, 124, 125, 128, 134]
Paper and pulp	Amylases, xylanases, cellulases, hemicellulose, ligninases, and esterase	Degrade starch to lower viscosity, aiding sizing, deinking, and coating paper. Xylanases reduce bleach required for decolorizing; cellulases and hemicellulase smooth fibers, enhance water drainage, and promote ink removal; lipases reduce pitch and lignin-degrading enzymes remove lignin to soften paper, for esterification	[23, 124, 135]
Animal feedstock	Phytase	Increase total phosphorous content for growth, increase in phytic acid need	[136]
Rubber	Catalase	Generate oxygen from peroxide to convert latex into foam rubber	[128]
Oil and petroleum	Cellulases, ligninases, and mannanase	Formation of ethanol, forming gel breaker in oil drilling	[133, 135]
Biopolymer/plastic	Laccases, peroxidases, lipases, and transglutaminases	Forming cross-links in biopolymers to produce materials in situ by means of polymerization processes	[131, 137]
Pharmaceutical	Nitrile hydratase, D-amino acid oxidase, glutaric acid acylase, penicillin acylase, penicillin G acylase, ammonia lyase, and humulin	Producing water soluble intermediates, semisynthetic antibiotics, intermediate for aspartame, and biosynthetic human insulin	[138, 139]
Molecular biology	Restriction enzymes, DNA ligase, and polymerases	Used to manipulate DNA in genetic engineering, essential for restriction of digestion and the polymerase chain reaction, also important in forensic science	[140]

Previously in the 19th up till mid-20th century, the world has seen great industrial expansions which we all know as industrial revolution which has created a steep raise in population and its demand for survival thus creating a great impact in the agricultural, industrial, dairy, and medicinal fields. To meet the raising demand, many scientists had put their great effort to develop many chemical processes to meet the demand, but in later years, the harmful effects of using chemical catalysts to fast up the process have come in front of the mankind. Many chemical transformation processes used in various industries have inherent drawbacks from a commercial and environmental point of view. Nonspecific reactions may result in poor product yields. High temperatures and/or high pressures needed to drive reactions lead to high energy costs and may require large volumes of cooling water downstream. Harsh and hazardous processes involving high temperatures, pressures, acidity, or alkalinity need high capital investment and specially designed equipment and control systems. Unwanted by-products may prove difficult or very costly to produce. High chemicals and energy consumption as well as harmful by-products have a negative impact on the environment. Thus, a need for environment friendly process/biocatalyst came in light which again created a great research innovation in different scientific communities thus leading to a new field called "Biotechnology" where different live organisms were utilized to obtain desirable products in an ecofriendly way. In a number of cases, some or all of these drawbacks can be

virtually eliminated by using enzymes. Interestingly enzyme reactions may often be carried out under mild conditions; they are highly specific and involve high reaction rates. Industrial enzymes originate from biological systems which can effectively contribute to sustainable development through being isolated from microorganisms which are fermented using primarily renewable resources. In addition, as only small amounts of enzymes are needed in order to carry out chemical reactions even on an industrial scale, both solid and liquid enzyme preparations take up very little storage space. Mild operating conditions enable uncomplicated and widely available equipment to be used, and enzyme reactions are generally easily controlled. Enzymes also reduce the impact of manufacturing on the environment by reducing the consumption of chemicals, water, and energy and the subsequent generation of waste. Developments in genetic and protein engineering have led to improvements in the stability, economy, specificity, and overall application potential of industrial enzymes. When all the benefits of using enzymes are taken into consideration, it is not surprising that the number of commercial applications of enzymes is increasing every year.

Biotechnology offers an increasing potential for the production of goods to meet various human needs. Enzyme technology are a subfield of biotechnology where new processes had been developed and are still developing to manufacture both bulk and high added value products utilizing enzymes as biocatalysts, in order to meet needs such as food (e.g., bread, cheese, beer, and vinegar), fine chemicals (e.g., amino acids, vitamins), agricultural (growth hormones), and pharmaceuticals (insulin). Enzymes are also used to provide services, as in washing and environmental processes (especially clean-up processes) or for analytical and diagnostic purposes. The driving force in the development of enzyme technology, both in academic research and industry, has been and will continue to the development of new and better products, processes, and services to meet these needs along with the improvement of the processes to produce existing products from new raw materials as biomass. The goal of these approaches is to design innovative products and processes that are not only competitive but also meet criteria of sustainability and economic viability. The concept of sustainability was introduced in the World Commission on Environment and Development (WCED, 1987) with the aim to promote the necessary development that meets the needs of the present and future demand without compromising the ability of future generations to meet their own needs. To determine the sustainability of a process, criteria that evaluate its economic, environmental and social impact must be used [113–115]. A positive effect in all these three fields is required for a sustainable process. Criteria for the quantitative evaluation of the economic and environmental impact are in contrast with the criteria for the social impact, easy to formulate. In order to be economically and environmentally more sustainable than the existing processes, a new process must be designed to reduce not only the consumption of resources (e.g., raw materials, energy, air, and water), waste production, and environmental impact but also to increase the recycling of waste per kilogram of product. Because enzymes are highly specific in the reactions they catalyse, an abundant supply of enzymes must be present in cells to carry out all the different chemical transformations required. Most enzymes help break down large molecules into smaller ones and release energy from their substrates. To date, scientists have identified over 10,000 different enzymes. Because there are so many, a logical method of nomenclature has been developed to ensure that each one can be clearly defined and identified.

Thus, from this table we can get a clear idea that the use of enzymes and bioengineering of them is nowadays very much practised in almost every industry. These biomolecules which are also known as biocatalyst too are now playing a very major role in the modern industrial development that is mainly aimed in economical, high efficiency, and ecofriendly production of different products and by-products. Enzymes are now an important area of studies of different human diseases.

Like other proteins, enzymes are produced inside cells by ribosomes, which link up amino acids into chains. Although the majority of industrial enzymes are produced by microorganisms, the enzymes are formed in exactly the same way as in human cells. The structure and properties of the enzymes produced by a particular cell are determined by the genetic instructions encoded in the deoxyribonucleic acid (DNA) found in chromosomes of the cell. DNA enables the production of specific enzymes through a code consisting of four bases: adenine (A), guanine (G), cytosine (C), and thymine (T). DNA's characteristic double helix consists of two complementary strands of these bases held together by hydrogen bonds. A always pairs with T, while C always pairs with G. The order in which these bases are assembled in the DNA double helix determines the sequence of amino acids in the enzyme protein molecule. Each fully functional segment of DNA—or gene—determines the structure of a particular protein, with each of the 20 different amino acids being specified by a particular set of three bases. Enzyme engineering sometimes also known as protein engineering is a modern term that is used in the application of modifying an enzyme's structure and thus altering/improving its function by modifying the catalytic activity of isolated enzymes to produce new metabolites [141, 142] or to convert from some certain compounds into others which is called biotransformation. These products will be useful as chemicals, pharmaceuticals, fuel, food, or agricultural additives. Since the tight control of enzyme activity is essential for homeostasis, any malfunction (mutation, overproduction, underproduction, or deletion) of a single critical enzyme can lead to a genetic disease. The importance of enzymes is shown by the fact that a lethal illness can be caused by the malfunction of just one type of enzyme out of the thousands of types present in our bodies. For example, the most common type of enzyme deficiency disorder is phenylketonuria, and a mutation of a single amino acid in the enzyme phenylalanine hydroxylase (which catalyzes the amino acid phenylalanine) results in buildup of phenylalanine and related products. This can lead to mental retardation if the disease is untreated in early childhood [143]. Another example of enzyme deficiency is germline mutations in genes coding for DNA repair

enzymes which cause hereditary cancer syndromes such as xeroderma pigmentosum [144]. Defects in these enzymes cause cancer since the body is less able to repair mutations in the genome. This causes a slow accumulation of mutations and results in the development of many types of cancer in the sufferer. Some enzymes are produced in increasing amounts for therapeutic purposes; this applies especially to recombinant enzymes such as factor VIII, tPA, and urokinase that cannot be produced in sufficient amounts from natural sources (blood serum or urine). Another advantage of the recombinant production of these enzymes is that possible contamination with pathogenic human viruses (HIV, herpes) can be avoided. Enzymes are now orally administrated to treat several diseases (e.g., pancreatic insufficiency and lactose intolerance). Since enzymes are proteins themselves, they are potentially subject to inactivation and digestion in the gastrointestinal environment. Therefore a noninvasive imaging assay had been developed to monitor gastrointestinal activity of exogenous enzymes like prolyl endopeptidase as potential adjuvant therapy for celiac disease [145].

6. Problems, Safety Concerns, and Possible Future Strategy to Outcome This Problem

Proteins are abundant in nature. Many proteins can cause allergies like pollen, house dust mites, animal dander, and baking flour. Like many other proteins foreign to the human body, enzymes are potential inhalation allergens. The inhalation of even small amounts of foreign protein in the form of dust or aerosols can stimulate the body's immune system to produce specific antibodies. In some individuals, the presence of these specific antibodies can trigger the release of histamine when reexposed to the allergen. This compound can cause symptoms well known to hay fever sufferers such as watery eyes, a runny nose, and a sore throat. When exposure ceases, these symptoms also cease. Enzymes must be inhaled for there to be a risk of causing sensitization that may lead to an allergic reaction. It may be necessary to monitor the working environment in facilities where enzymes are used, especially if large quantities are handled on a daily basis. Monitoring is used to confirm that threshold limit values (TLVs) for airborne enzymes are not being exceeded. In many countries, the TLVs for enzymes are based on the proteolytic enzyme subtilisin and are stated as 0.00006 mg/m^3 of pure crystalline subtilisin in air [146]. But one industry that has come a long way in the safe handling of enzymes is the detergent industry. The use of encapsulated enzymes, combined with improved industrial hygiene and operating practices, has brought levels of airborne enzyme dust down dramatically in developed countries since the occupational problem of enzyme allergies first came to light in the late 1960s. The trade association AISE has generated a guide to safe handling of enzymes in the detergent industry [147]. It should be emphasized that allergy to enzymes is solely an occupational hazard, and no effects on end consumers using products containing enzymes have ever been reported during more than 35 years of use. In one of the most important reports on the subjects, the National Research Council (NRC), USA, concluded that consumers of enzymatic laundry products did not develop respiratory allergies [148]. Further studies of enzyme allergy over the years have confirmed that enzymatic laundry and dishwashing detergents are safe for consumers to use. The HERA Risk Assessment document gives a comprehensive overview of consumer safety in regards to enzyme application within the household cleaning sector. The safe use of enzymes in food processing has been documented in a recent study by Novozymes and the University Hospital of Odense, Denmark [149].

The application of enzymes in food processing is governed by food laws. Within the EU, large parts of the food laws of individual member states have been harmonized by directives and regulations. For general purposes, the FAO/WHO Joint Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC) have made guidelines available for the application of enzymes as food additives. AMFEP in Europe and the Enzyme Technical Association (ETA) in the USA work nationally and internationally to harmonize enzyme regulations. AMFEP members ensure that the enzymes used in food processing are obtained from nonpathogenic and nontoxigenic microorganisms, that is, microorganisms that have clean safety records without reported cases of pathogenicity or toxicosis attributed to the species in question. When the production strain contains recombinant DNA, the characteristics and safety record of each of the donor organisms contributing genetic information to the production strain are assessed. The majority of food enzymes are used as processing aids and have no function in the final food. In this case, they do not need to be declared on the label because they are not present in the final food in any significant quantity. A few enzymes are used both as processing aids and as food additives. When used as additives, they must be declared on the food label. Good Manufacturing Practice is used for industrial enzymes for the food industry. The key issues in GMP are microbial control of the microorganism selected for enzyme production, the control and monitoring systems ensuring pure cultures and optimum conditions for enzyme yield during fermentation, and the maintenance of hygienic conditions throughout the recovery and finishing stages.

Commercial enzyme products are usually formulated in aqueous solutions and sold as liquids or processed into nondusting, dry products known as granulates or microgranulates. Both liquid and dry preparations must be formulated with the final application in mind. It is important for both the producer and customer to take into account storage stability requirements such as stability of enzyme activity, microbial stability, physical stability, and the formulation of the enzyme product itself. Enzyme molecules are far too complex to synthesize by purely chemical means, with a very instability of its pure form, and so the only way of making them is to use living organisms. The problem is that the useful enzymes produced by microorganisms in the wild are often expressed in tiny amounts and mixed up with many other enzymes. These microorganisms can also be very difficult to cultivate under industrial conditions, and they may create

undesirable by-products. Even there is a possibility of cross-contamination and production of toxins which may be lethal if used.

Genetic engineering is a far more efficient option because the changes are completely controlled. This process basically involves taking the relevant gene from the microorganism that naturally produces a particular enzyme (donor) and inserting it into another microorganism that will produce the enzyme more efficiently (host). The first step is to cleave the DNA of the donor cell into fragments using restriction enzymes. The DNA fragments with the code for the desired enzyme are then placed, with the help of ligases, in a natural vector called a plasmid that can be transferred to the host bacterium or fungus. The DNA added to the host in this way will then divide as the cell divides, leading to a growing colony of cloned cells each containing exact replicas of the gene coding for the enzyme in question. Since the catalytic properties of any enzyme are determined by its three-dimensional structure, which in turn is determined by the linear combination of the constituent amino acids, we can also alter an enzyme's properties by replacing individual amino acids. For example, detergent enzymes can be made more bleach-stable using this type of protein engineering. Bleach-stable protein engineered enzymes have been on the market for a number of years, for example, Novozymes' Everlase. Furthermore, enzymes can be given other useful properties using this technique, for example, improved heat stability, higher activity at low temperatures, and reduced dependency on cofactors such as calcium. New and exciting enzyme applications are likely to bring benefits in other areas like less harm to the environment, greater efficiency, lower cost, lower energy consumption, and the enhancement of a product properties. New enzyme molecules capable of achieving this will no doubt be developed through protein engineering and recombinant DNA techniques. Industrial biotechnology has an important role to play in the way modern foods are processed. New ingredients and alternative solutions to current chemical processes will be the challenge for the enzyme industry. When compared with chemical reactions, the more specific and cleaner technologies made possible by enzyme-catalyzed processes will promote the continued trend towards natural processes in the production of food.

7. Conclusion

Enzymes are being known to mankind since the ancient human civilization. The use of enzymes had been done intensively in different fields especially, in ancient brewing and other uses. But since the 18th century it has been technically known to us as enzymes. Many scientists had tried to study the use of enzymes, and from their pioneer work, we have come to know about its power and utility in our daily life. Today different types of enzymes are being manufactured by many big companies and being sold for their important role in different industries like food, dairy, detergent, and chemical as well as for their important lifesaving therapeutically application. Due to advancement of modern biotechnology and protein engineering a new area of enzyme engineering, has evolved which mainly deals with the purification and stability of these important enzymes. Different microbes as well as other model systems are extensively used for the production of these important biomolecules. Since then many microorganisms and their enzymes with unique function have also been discovered by means of extensive screening, and now they are commonly used in different industrial and medical fields. Development of these medically important enzymes has been at least as extensive as those for industrial applications thus reflecting the magnitude of the potential rewards of this sector in the near

future. Enzymes industry is one among the major industries of the world, and there exists a great market for further improvement in this field. Amylase and lipase are few of these mentionable enzymes that have a wide spectrum role in this sector. Its use is almost done in every industry whether it may be detergent, dairy, food, or medicineThis review especially emphasizes the important wide spectrum role of amylase and lipase in various sectors of industries and also discussed the role of other enzymes in therapeutic field. There is an indeed need of future research in these biomolecules which will later be beneficial for the mankind in their relevance.

Acknowledgments

The authors are thankful to the Indian Institute of Science Education and Research Kolkata (IISER-K) for providing all the necessary facilities to gather relevant data for this topic. Sutapa Bose thanks the Department of Science and Technology (DST) and Vivek Rai thanks the Department of Biotechnology (DBT) for the funding.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 723680, 9 pages
http://dx.doi.org/10.1155/2013/723680

Research Article

Copper Enhanced Monooxygenase Activity and FT-IR Spectroscopic Characterisation of Biotransformation Products in Trichloroethylene Degrading Bacterium: *Stenotrophomonas maltophilia* PM102

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Received 30 April 2013; Revised 13 July 2013; Accepted 15 July 2013

Academic Editor: Subash C. B. Gopinath

Stenotrophomonas maltophilia PM102 (NCBI GenBank Acc. no. JQ797560) is capable of growth on trichloroethylene as the sole carbon source. In this paper, we report the purification and characterisation of oxygenase present in the PM102 isolate. Enzyme activity was found to be induced 10.3-fold in presence of 0.7 mM copper with a further increment to 14.96-fold in presence of 0.05 mM NADH. Optimum temperature for oxygenase activity was recorded at 36°C. The reported enzyme was found to have enhanced activity at pH 5 and pH 8, indicating presence of two isoforms. Maximum activity was seen on incubation with benzene compared to other substrates like TCE, chloroform, toluene, hexane, and petroleum benzene. K_m and V_{max} for benzene were 3.8 mM and 340 U/mg/min and those for TCE were 2.1 mM and 170 U/mg/min. The crude enzyme was partially purified by ammonium sulphate precipitation followed by dialysis. Zymogram analysis revealed two isoforms in the 70% purified enzyme fraction. The activity stain was more prominent when the native gel was incubated in benzene as substrate in comparison to TCE. Crude enzyme and purified enzyme fractions were assayed for TCE degradation by the Fujiwara test. TCE biotransformation products were analysed by FT-IR spectroscopy.

1. Introduction

Hydrocarbon dioxygenases have broad substrate specificity which makes them attractive candidates for production of industrially and medically important chemicals and development of bioremediation technology. Bacterial aromatic oxygenases are multicomponent enzymes that add oxygen molecules to the aromatic nucleus to form arene cis-diols. For example, toluene dioxygenase that catalyses the following reaction (Figure 1) [1].

Toluene 4-monooxygenase (T4MO) of *Pseudomonas mendocina* KR1, toluene 3-monooxygenase (T3MO) of *Ralstonia pickettii* PKO1, and toluene ortho-monooxygenase (TOM) of *Burkholderia cepacia* G4 convert benzene to phenol, catechol, and 1,2,3-trihydroxybenzene by successive hydroxylations [2].

Current interests in structure and function of dioxygenases are due to their role in bioremediation of contaminants from our environment and in the green synthesis of cis-diols that have significant industrial and medical importance such as development of polyphenylene and prostaglandin E2α [2–5]. Crixivan (indinavir) is an orally active HIV protease inhibitor. A key intermediate in its synthesis is (-)-*cis*-(1*S*,2*R*)-1-aminoindan-2-ol. This compound can be synthesized directly from enantiopure *cis*-(1*S*,2*R*)-dihydroxyindan. Toluene dioxygenase oxidizes indene to produce the desired enantiomer [6].

All members of the dioxygenase family have one or two electron transport proteins preceding their oxygenase components, as shown in Figure 2 for toluene dioxygenase system [7].

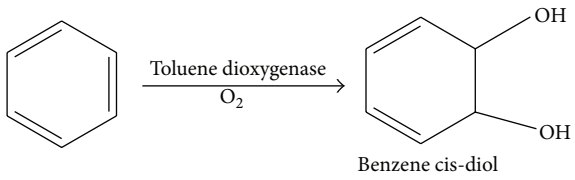

Figure 1: Oxidation of benzene to benzene *cis*-diol by toluene dioxygenase.

Pseudomonas cepacia G4 was the first organism to degrade TCE, reported to contain toluene monooxygenase. Toluene monooxygenase and dioxygenase [8, 9]; methane monooxygenase [10]; propane monooxygenase [11]; ammonia monooxygenase [12]; and also mammalian liver cytochrome P450 monooxygenase have been implicated in TCE degradation. In 1996, Werlen et al. [13] identified four dioxygenase families (naphthalene, toluene/benzene, biphenyl, and benzoate/toluate) based on amino acid sequence comparisons of the catalytic oxygenase α subunits. A novel tce350 gene product from *Stenotrophomonas maltophilia* PM102 was shown to belong to the dioxygenase family of proteins due to the presence of 71.6% each of alternating α helix and β strand [14]. Monooxygenases can be classified according to the cofactors required for catalytic activity. Heme-dependent monooxygenases are cytochrome P450 monooxygenases, for example, CYP102 from *Bacillus megaterium* BM3 that hydroxylates a variety of alkanes, fatty acids, and aromatic compounds [15]. In all CYPs, a strictly conserved cysteine is found in the active site that acts as the fifth ligand of the heme-iron center, thereby activating the metal complex [16]. CYPs catalyze a wide variety of oxidations. Besides epoxidations and hydroxylations, these monooxygenases are also able to perform heteroatom dealkylations and oxidations, oxidative deaminations, dehalogenations, dehydrogenations, dehydrations, and reductions [17]. Flavin-dependent monooxygenases require FAD/FMN, for example, luciferases and Type II Baeyer-Villiger monooxygenases [18]. Copper-dependent monooxygenases constitute a relatively small family of enzymes that require copper ions for hydroxylation of their substrates. An example of these monooxygenases is the membrane-associated methane monooxygenase (pMMO) from *Methylococcus capsulatus*. It has been reported that this enzyme contains up to 20 copper ions per heterotrimer, in which the ions are involved in either activation of molecular oxygen or electron transfer [19]. Nonheme iron-dependent monooxygenases utilize two iron atoms as cofactor for their oxidative activity. The best-characterized member of this family is the soluble methane monooxygenase (sMMO) from *M. capsulatus* [20]. Other members of this family are alkene monooxygenases, phenol hydroxylase, and toluene-4-monooxygenase. Another family of monooxygenases is the pterin-dependent monooxygenases, mainly of eukaryotic origin, that hydroxylate the amino acids phenylalanine, tyrosine, and tryptophan at their aromatic ring.

In this communication, we present the biochemical characterisation of a novel oxygenase induced by copper and benzene from PM102 isolate that utilises trichloroethylene (TCE) as the sole carbon source. A simple spectrophotometric method was used to measure enzyme activity [21]. Purification of the enzyme and investigation of its probable role in TCE degradation have been documented. Optimisation of protocol for activity staining is also reported. Fourier transform infrared (FT-IR) spectroscopy has become an important tool for rapid analysis of complex biological samples. The infrared absorbance spectrum could be regarded as a "fingerprint," which is a characteristic of biochemical substances. The ability to use FT-IR to rapidly distinguish between biotransformation product mixtures suggests this approach might be a valuable tool for screening large biotransformation assays for novel products [22]. Determination of enzymatic breakdown products by the PM102 isolate through FT-IR spectroscopy has been attempted in this communication.

2. Materials and Methods

2.1. Strain and Culture Conditions. *Stenotrophomonas maltophilia* PM102 was isolated in our laboratory from soil samples obtained from Asansol and Dhanbad industrial belt, India. It was identified by 16S rDNA sequencing (GenBank Acc. No. JQ797560). The PM102 isolate could grow on TCE as the sole carbon source and degraded TCE efficiently [23]. TCE-induced proteins from the PM102 isolate were previously identified [24], and TCE degradation in presence of other organic pollutants was also documented [25]. The PM102 isolate was grown in minimal medium: Na_2HPO_4—1 g/L, K_2HPO_4—3 g/L, NH_4Cl—1 g/L, and $MgSO_4{\cdot}7H_2O$—0.4 g/L with 0.1% peptone and 0.2% of different carbon sources: TCE, toluene, chloroform, benzene, hexane, and petroleum benzene.

2.2. Crude Enzyme Extract Preparation. PM102 cells were grown in a shaker incubator (150 rpm), at 34°C, for 24 hours in 200 mL minimal medium with 0.2% of the different carbon sources and 0.1% peptone. Cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C, and cell pellet was suspended in 1.5 mL solution I (10 mM EDTA pH 8, 50 mM glucose, and 25 mM tris HCl pH 8) with 100 μL 10 mg/mL lysozyme. The suspension was vortexed and kept at 4°C for 15 minutes followed by temperature shock at 37°C for 1 hour and further incubated at 4°C for 30 minutes. The suspension was centrifuged at 5000 rpm for 5 minutes at 4°C and the supernatant was stored as crude enzyme extract at −20°C for further studies. The supernatant of the cell cultured in media with different carbon sources was also used as the crude enzyme for mono-oxygenase assay.

2.3. Oxygenase Assay. 10 mL reaction mixture contained 5 mL reactant A (25 mg o-Dianisidine + 1 mL methanol + 49 mL phosphate buffer), 1.5 mL phosphate buffer (0.1 M KH_2PO_4, pH 5), 3.5 mL substrate—TCE and other organic compounds, 100 μL (90 μg crude enzyme extract intracellular), and 0.05 mM NADH. For extracellular assay, 1 mL of the cell-free medium supernatant (16 μg protein) was added as crude enzyme extract. Protein content in cell lysate and sup was measured by Bradford assay [26]. A dark red colour was

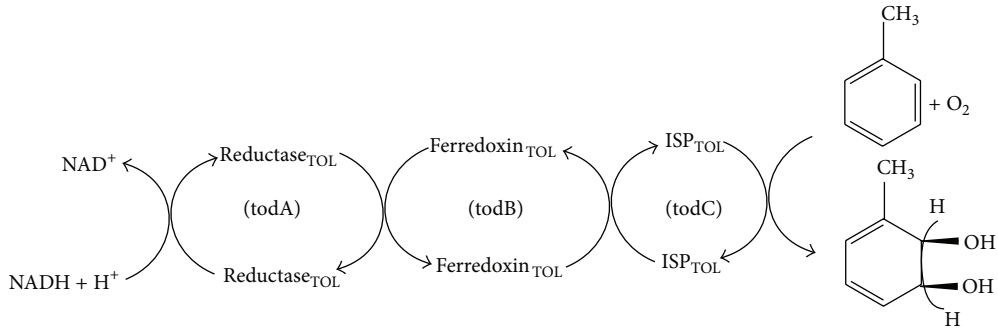

Figure 2: Oxidation of toluene to cis-toluene dihydrol by toluene dioxygenase. Electrons are transferred from NADH through a flavoprotein reductase (reductase$_{TOL}$) to a Rieske (2Fe-2S) protein (ferredoxin$_{TOL}$). The latter reduces the oxygen component, an iron-sulfur protein (ISP$_{TOL}$), which, in the presence of exogenous ferrous ion, catalyses the stereospecific addition of dioxygen to the aromatic nucleus.

formed in the substrate phase only which was pipetted out, and absorbance was noted at 480 nm. The basic principle is based on the property of oxygenases to use molecular oxygen to oxidize the hydrocarbon-substrate molecules by means of an electron transport system. Ortho-dianisidine (ortho-DNS) acts as a chromogenic acceptor of oxygen.

2.4. Characterisation of the Oxygenase. Effect of various metal cofactors was studied by adding 1 mM of the following metal salts in 10 mL reaction mixture: copper sulphate, ferric chloride, cobalt bromide, manganese sulfate, zinc chloride, aluminium chloride, lead nitrate, silver nitrate, calcium chloride, magnesium sulphate, sodium chloride, and potassium dihydrogen phosphate. Optimum copper concentration for enzymatic activity was determined by varying the concentration of copper sulfate added to 10 mL reaction mixture from 0.05 mM to 5 mM.

Optimum NADH concentration was determined by varying NADH concentration from 0.01 mM to 2 mM in presence of 0.7 mM Cu. Substrate specificity was analysed with TCE, benzene, toluene, chloroform, hexane and petroleum benzine separately to 10 mL reaction mixture setup each. K_m and V_{max} were calculated by varying concentrations of benzene and TCE under fixed enzyme concentration. Effect of temperature was studied by setting up the reaction condition at 4, 20, 30, 36, and 42°C, respectively. Optimisation of pH for oxygenase activity was performed by varying the pH of the phosphate buffer used in the reaction mixture from pH 3 to pH 8.5, varying at intervals of 0.5. For pH 8.5 to pH 10, glycine-NaOH buffer was used. Activity in lysate (intracellular) and supernatant (extracellular) was also studied.

2.5. Native PAGE for Zymogram Analysis. For zymogram studies, PM102 cells were grown in minimal medium with 0.2% TCE for 24 hours followed by induction with 0.2% of various carbon sources: TCE, toluene, chloroform, benzene, hexane, and petroleum for another 24 hours. Intracellular proteins were extracted by lysozyme, and protein concentration was measured by the Bradford assay. About 100 μg of proteins was resolved by 8% native gel that was incubated in 10 mL reactant A, 9 mL 0.1 M phosphate buffer (pH 5), 1 mL substrate (benzene/TCE), and 0.7 mM copper.

2.6. Enzyme Purification. To understand which particular protein band gave colour in activity staining, purification of the crude enzyme extract was done by ammonium sulphate precipitation followed by dialysis. As maximum enzyme activity was recorded in the supernatant, salt precipitation was carried out with 200 mL culture supernatant of PM102 grown in 0.2% TCE with 0.2% benzene induction. The online program ammonium sulphate calculator from EnCor Biotechnology, Inc, Gainesville, Florida [28], was used. Precipitation was done at 10% and 30% for 2-3 hours and at 50%, 70%, and 90% of ammonium sulphate for overnight incubation, with magnetic stirrer at 4°C. After each precipitation step, the fraction was centrifuged at 12,000 rpm for 15 minutes. Precipitate obtained as pellet (if any) was dissolved in 500 μL of 10 mM PBS for removal of Ammonium sulphate by dialysis against 200 mL 10 mM PBS for 2 hours at 25°C, followed by another 2 hours after buffer change. A third buffer change was given for overnight dialysis at 4°C. The fractions after dialysis were resolved through 8% native gel. One-half of the gel was stained with Coomassie blue, and the other half was subjected to activity staining.

2.7. Investigating TCE Degradation Activity of the Purified Enzyme Extract. TCE degradation studies with the unpurified crude protein extract and with the purified enzyme fractions (extracellular) were carried out by the Fujiwara test. In this reaction, trichloroethylene in presence of pyridine and caustic alkali gives a red colour compound [29]. In 20 mL of 10 mM PBS, 100 μL of enzyme extract was added and incubated with 0.2% TCE added initially. 2 mL aliquots were drawn after respective time intervals, that is, 1, 2, 3, and 24 hours, and Fujiwara assay was done. Absorbance of the red upper aqueous phase was measured at 470 nm. As TCE gets degraded with time, the intensity of the red colour decreases with a corresponding decline in O.D. The experiment was carried out in four sets with crude unpurified enzyme, 30% Ammonium sulphate fraction, 50% fraction and 70% fraction of the extract.

2.8. FT-IR Analysis to Determine Biodegradation Products. Biomass obtained with TCE or benzene induction and biomass grown in peptone without induction (control) were

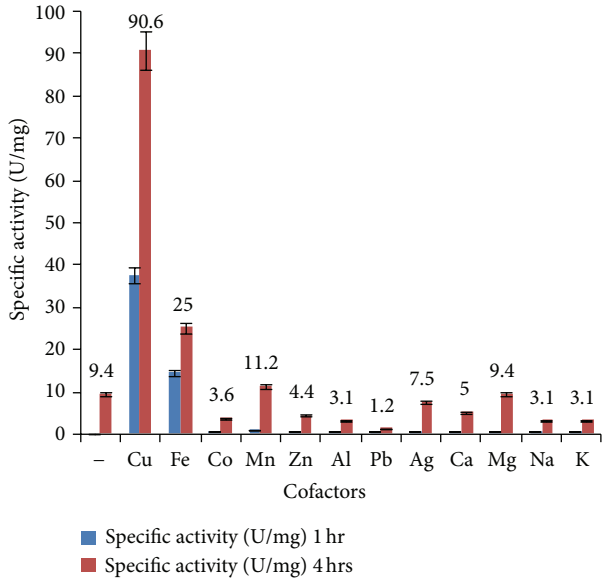

Figure 3: Effect of metal cofactors (1 mM) on enzyme activity in cell supernatant. Copper was found to considerably enhance enzymatic activity in comparison to other metal cofactors. Iron and manganese also have some inducing effect. Error bars with 5% SEM are displayed.

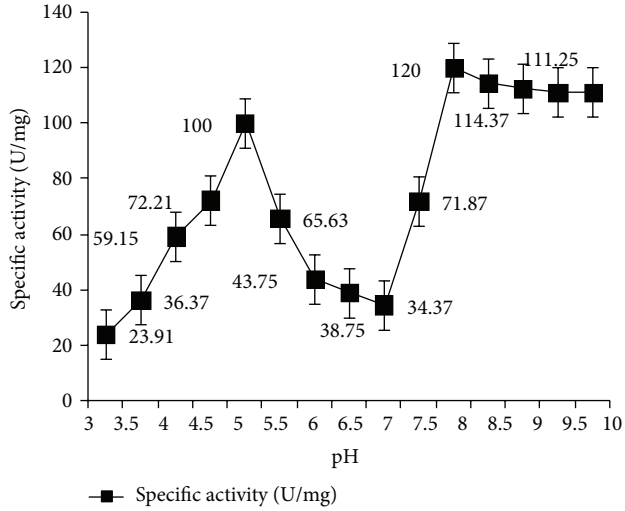

Figure 4: Oxygenase activity under varying pH with 0.7 mM Cu at 36°C. Optimum activity could be detected at pH 5 and pH 8, respectively. Error bars with 5% SEM are displayed.

used for FT-IR spectroscopy. Before analysis, the samples were dried in hot air oven at 50°C for 2 hours. Pellets formed by milling samples with KBr were subjected to FT-IR analysis and performed with a Nicolet impact 400 FT-IR spectrophotometer fitted with a high-linearity room temperature mid-infrared detector (MIR) cooled with liquid N_2 at the Instrumentation facility of Indian Association for Cultivation of Science, Kolkata, India. The spectra were recorded in the scan range 6000–500 cm^{-1} with a resolution of 4 cm^{-1}. The IR spectra obtained were analysed with Origin Pro 8 software (OriginLab, Northampton, MA).

Table 1: Characterisation of monooxygenase activity from *Stenotrophomonas maltophilia* PM102.

Conditions for enzyme activity	Enzyme parameters	Specific activity (U/mg) (4 hrs)
Metal cofactors (1 mM)	Cu, Fe, and Mn	90.6, 25, and 11.2
Optimum metal cofactor	Cu	
Range of copper concentration tested	0.05 mM–5 Mm	
Optimum Cu concentration	0.7 mM	96.87
Range of NADH concentration	0.01 mM–0.2 mM	
Optimum NADH concentration	0.05 mM	140.625
Range of temperature	4°C–42°C	
Optimum temperature	36°C	103.13
Range of pH	3–10	
Optimum pH	5 and 8	100 and 120
Substrates	TCE, benzene, toluene, chloroform, hexane, and petroleum benzine	87.5, 103.75, 31.87, 40.63, 13.75, and 11.25
Optimum substrate	Benzene	103.75
Rate when benzene is the substrate	V_{max} Benzene	340 U/mg/min
Rate when TCE is the substrate	V_{max} TCE	170 U/mg/min
K_m when benzene is the substrate	K_m Benzene	3.8 mM
K_m when TCE is the substrate	K_m TCE	2.1 mM

3. Results and Discussion

3.1. Optimisation of Conditions for Enzymatic Activity. 1000 μL supernatant (0.016 mg extracellular proteins) was used for characterisation of enzyme activity. Figure 3 shows the effect of 1 mM of different metal cofactors on enzymatic activity plotted with respect to specific activity (U/mg). Copper was found to enhance oxygenase activity by 10.3-fold in comparison to other metals. Iron and manganese also increased enzyme activity to some extent. Optimum copper concentration was found to be 0.7 mM. NADH concentration as a coenzyme in enzymatic reaction was also optimized, as shown in Table 1. A further increment of 14.96-fold in enzymatic activity was seen in presence of 0.05 mM NADH in addition to 0.7 mM Cu. A methane monooxygenase from

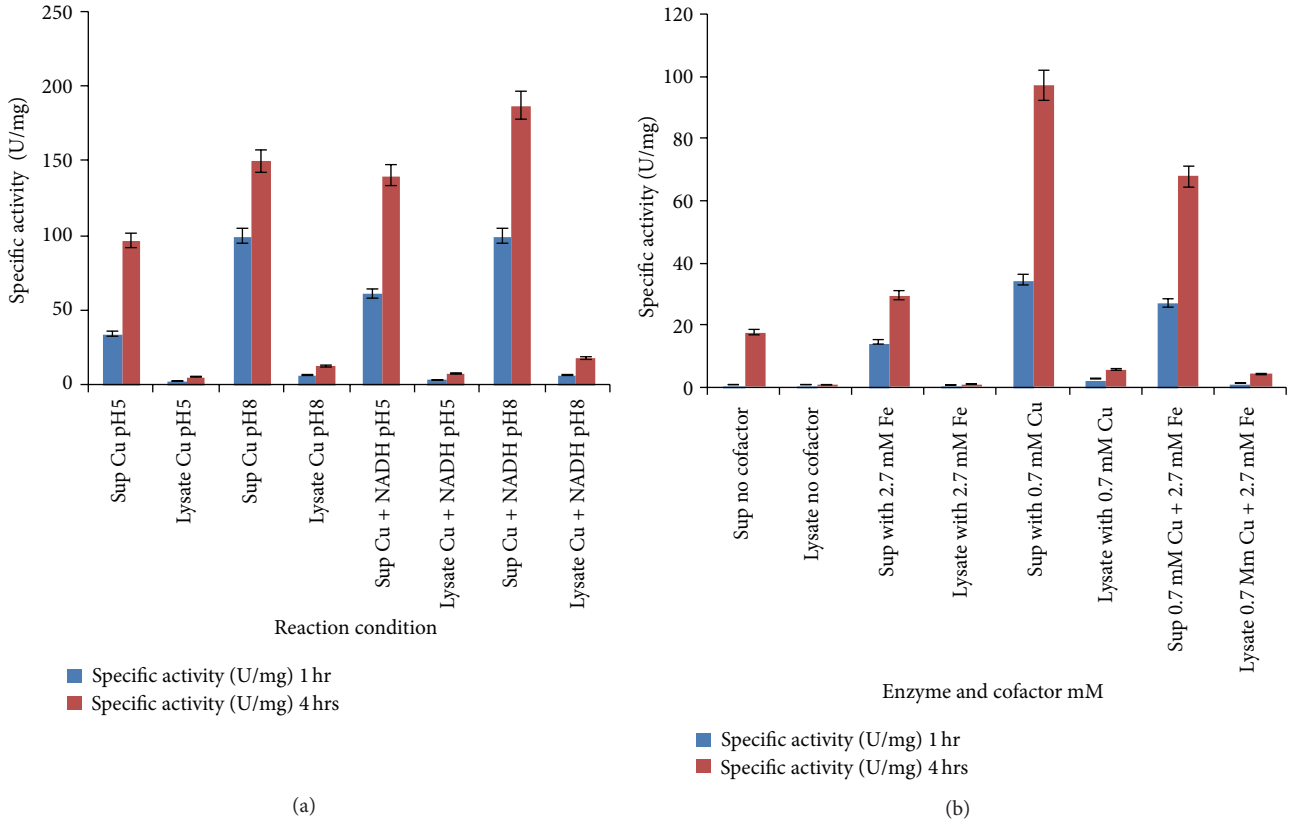

FIGURE 5: (a) Enzyme activity is maximum in culture supernatant. Comparatively, less activity is seen in cell lysate at pH 5 and pH 8. Error bars with standard error are shown. (b) At constant pH 5, enzyme activity is enhanced in presence of 0.7 mM Cu and 2.7 mM Fe. Maximum activity is seen in extracellular form. Activity in presence of both Cu and Fe is lower than that in presence of only Cu. Error bars with standard error are displayed.

Methylocystis sp. strain M has been reported to have a copper-active site that turned it into a powerful copper oxidase [30]. Manganese and iron were also found to enhance the PM102 oxygenase activity to some extent, indicating that Mn/Fe may play a role as an enzyme cofactor in absence of Cu. There are reports on polysaccharide oxygenases using Mn and Cu as cofactors [31, 32].

Table 1 is listed with the effect of substrates, temperature, pH, and optimum cofactor and coenzyme concentrations of the enzyme taken from culture supernatant. Benzene was found to enhance enzymatic activity greater than TCE or any other substrate as evident from the enzymatic rate calculations: $V_{\max}$ for benzene was 340 U/mg/min whereas $V_{\max}$ for TCE was 170 U/mg/min. K_m (benzene) was 3.8 mM, and K_m (TCE) was 2.1 mM, respectively. Two optimum pH for enzymatic activity were noted at pH 5 (acidic) and pH 8 (alkaline), respectively. Figure 4 clearly shows that first, there is a sharp increase in enzyme activity at pH 5 which drops around neutral pH followed by a peak at pH 8 that gradually falls into a plateau. The most probable explanation was the presence of two isoforms that was further confirmed by zymogram assay with the purified enzyme extract. Two brown bands in close proximity were detected in the zymogram that corresponded to two bands in the 70% purified enzyme fraction.

3.2. Comparison of Enzyme Activity in Extracellular versus Intracellular Extracts. All the optimisations in enzyme activity were done with 1 mL of culture supernatant (obtained from a total of 200 mL cell culture) that contained approximately 0.016 mg protein as measured by Bradford assay. To compare the enzyme activity in the intracellular fraction obtained by lysozyme extraction of the 200 mL cell pellet, the reaction was carried out under optimised conditions, that is, 0.7 mM Cu, 0.05 mM NADH, and 36°C, and at pH 5 and pH 8, respectively, as shown in Figure 5(a). Figure 5(b) shows enzyme activity in supernatant and cell lysate at pH 5 with Cu and Fe as cofactors and without any cofactor. Oxygenase activity was found to mainly lie in the extracellular fraction. Percentage of total activity in 1 mL supernatant (0.016 mg crude protein) with 0.7 mM Cu after 4 hours was 94.5%, while activity in 100 μL cell lysate (0.3 mg crude protein) with 0.7 mM Cu after 4 hours was 5.5%. Thus, percentage of total activity was maximum in the extracellular supernatant with comparatively less activity in intracellular lysate, at pH 5 and pH 8.

3.3. Zymogram Analysis. Three 8% native gels were electrophoresed under same conditions. One gel was subjected to the usual staining (Coomassie blue R250) and destaining

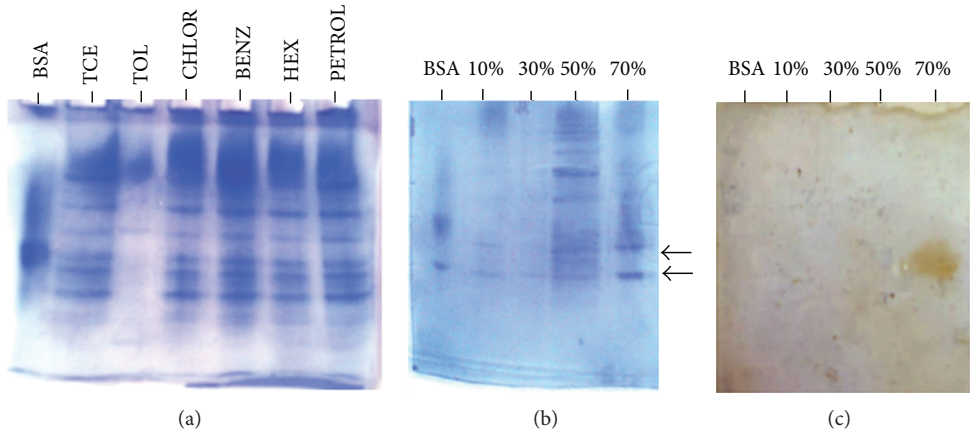

Figure 6: (a) The 8% native PAGE with proteins obtained by lysozyme extraction from PM102 cells grown in minimal medium with 0.2% TCE for 24 hours followed by induction with 0.2% of various carbon sources: TCE, toluene, chloroform, benzene, hexane, and petroleum for another 24 hours. (b) The 8% native gel with protein fractions purified from culture supernatant. As enzyme activity was higher in the supernatant, extracellular proteins were purified by ammonium sulphate precipitation. At 70% ammonium sulphate, the two protein bands that gave activity bands are shown with arrows. (c) The 8% native gel zymogram showing two activity bands lying in close proximity indicating two isoforms of the enzyme.

(30% methanol with 7.5% acetic acid) process (Figure 6(a)), while the other two parts of identical gels were subjected to activity staining. One of the zymogram gels was incubated in benzene as substrate while the other zymogram was incubated in TCE as substrate. A broad brown activity band appeared only in the benzene lane that was much prominent when the gel was incubated in benzene as substrate. A faint activity response was seen when the gel was incubated in TCE as the substrate. This observation led us to believe that the PM102 oxygenase may be a cometabolic enzyme lying in the common pathway of benzene and TCE degradation. To locate the particular protein band that showed up in the activity stain, purification of the crude enzyme extract was done. As enzyme activity was considerably higher in the extracellular form, the culture supernatant was subjected to ammonium sulphate precipitation and dialysis.

3.4. Activity Staining with Partially Purified Enzyme Extract. Figure 6(b) shows the 8% native gel with the purified enzyme fractions. At 10% and 30%, negligible amount of protein was precipitated. Most of proteins were salted out at 50%, while at 70%, moderate amount of protein was precipitated. Specific activity with the unpurified culture supernatant was 34.37 U/mg after 1 hour, while 98.69 U/mg activity was recorded with the 70% purified enzyme fraction (2.8-fold of purification could be achieved). One U of enzyme activity is defined as the amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute.

Figure 6(c) depicts the zymogram with the purified enzyme fractions from the culture supernatant. It was clearly visible that two brown bands in close proximity developed in the activity stain corresponding to the two closely lying protein bands in the 70% purified enzyme fraction. Further purification of the enzyme for subsequent crystal structure prediction needs to be investigated.

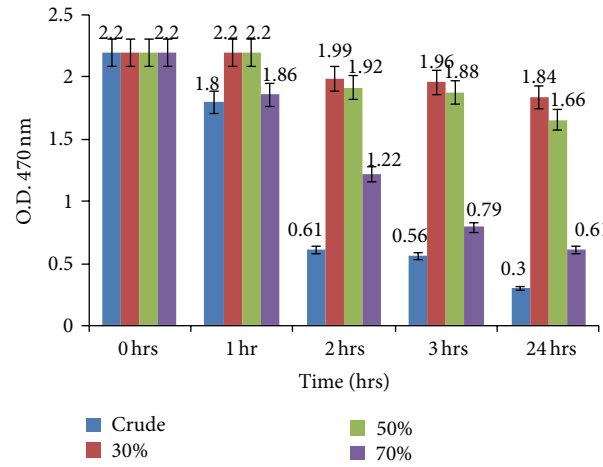

Figure 7: Fujiwara test showing TCE degradation by crude enzyme (culture supernatant obtained from PM102 isolate grown in 0.2% TCE with 0.2% benzene induction) and by ammonium sulphate precipitated fractions at 30%, 50%, and 70%. The 70% enzyme fraction showed comparatively higher rate of TCE degradation than the other fractions. Error bars with 5% SEM are displayed.

3.5. Fujiwara Test to Determine TCE Degradation Role of the Purified Enzyme Extracts. Figure 7 shows TCE degradation by the crude protein extract from PM102 isolate in comparison to the individual enzyme fractions obtained by salt precipitation. Higher rate of TCE degradation was seen in the 70% enzyme fraction that also reacted in activity staining. This further validates the assumption that common enzymes are involved in the degradation of TCE and benzene.

3.6. FT-IR Analysis. The FT-IR spectra for treated (TCE-induced and benzene-induced biomasses) and control (peptone grown biomass) are shown in Figures 8(a), 8(b), and 8(c).

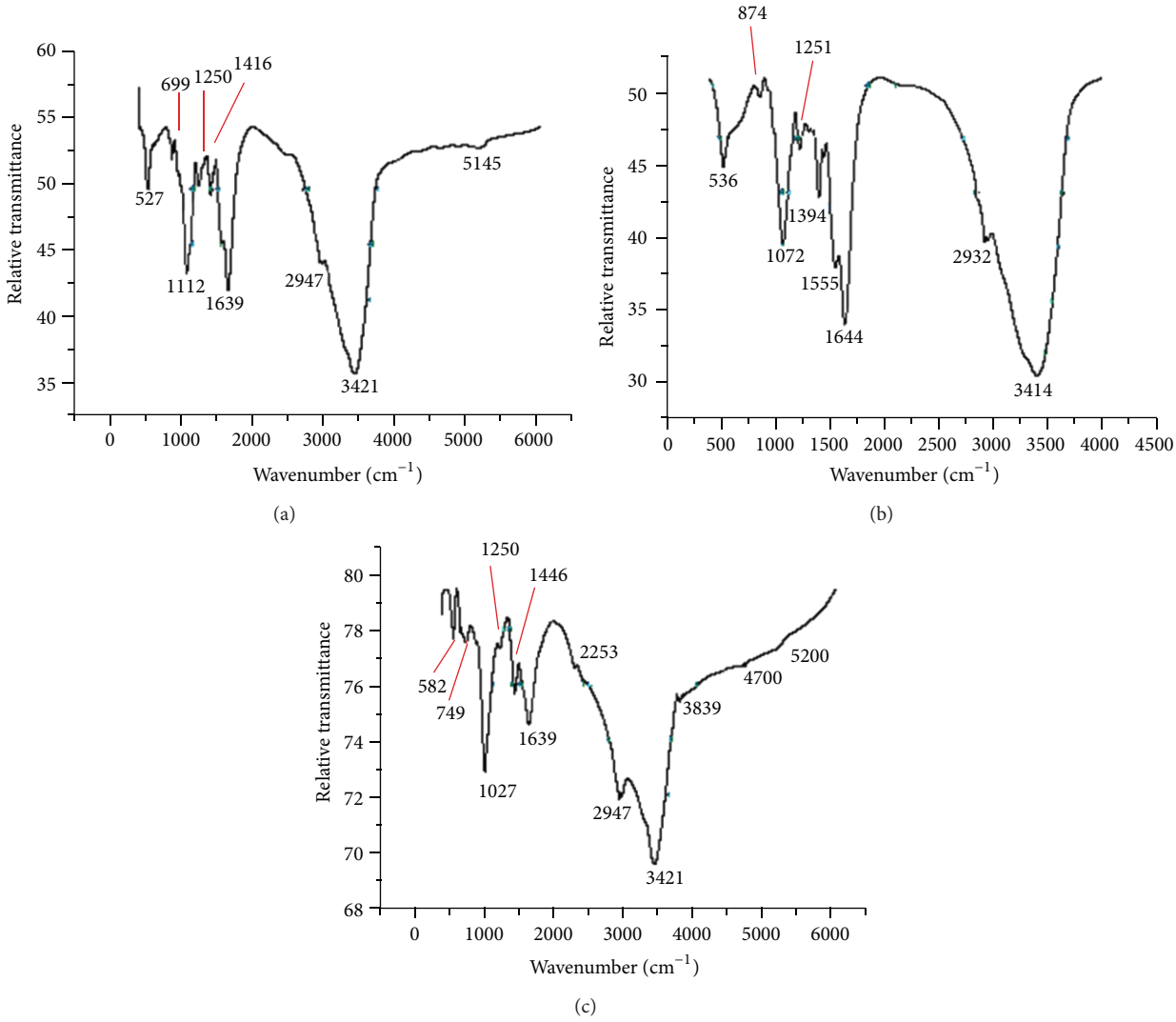

FIGURE 8: (a) FT-IR spectrum of peptone-grown PM102 biomass (control). (b) FT-IR spectra of TCE-induced PM102 biomass (treated). (c) FT-IR spectra of benzene-induced PM102 biomass (treated).

Table 2 shows the identified functional groups present in the biotransformed product of TCE-treated and benzene-treated biomasses against peptone-grown biomass. Peaks at 874, 1394, and 1555 cm^{-1} corresponding to epoxy ring, tertiary alcohol and carboxylic acid were present in TCE-treated samples only. In control, peaks at 699 and 1416 cm^{-1} correspond to C–Cl stretch and C–H in plane bend. This evidence points towards the transformation of TCE by the PM102 isolate to TCE epoxide. Previous reports show a soluble methane monooxygenase from *Methylosinus trichosporium* OB3b converts TCE to chloral hydrate, trichloroacetate, trichloroethanol, and CO_2. Toluene monooxygenase from *Burkholderia cepacia* G4 has been known to convert TCE to TCE epoxide [33]. Thus, the oxygenase involved in TCE degradation characterised in this paper is a monooxygenase as evidence of epoxide formation has been documented. Benzene-treated biomass gave peak at 749 cm^{-1} that corresponds to 1,2-disubstitution (ortho) in aromatic ring which is indicative of benzene-cis-diol or di-hydroxy benzene. Peaks at 1027, 1446, and 2253 cm^{-1} for benzene-treated cells could be designated as cyclohexane ring, aromatic ring stretch, and isocyanate, respectively.

Isocyanates are known to have specific industrial importance in the manufacture of polyurethanes which have wide known applications in preparation of dental materials, contact lenses, and medical adsorbents and as an ingredient in automobile paints. Medical and industrial importance of cis-diols has been stated previously. Industrial importance of ethylene oxide (epoxide) can be determined from the fact that it is one of the most important raw materials used in the large-scale chemical production, mainly of ethylene glycol that accounts for up to 75% of global consumption. Ethylene glycol is used as antifreeze, in the production of polyester and polyethylene terephthalate (PET—raw material for plastic bottles), liquid coolants, and solvents. Polyethylene glycols are used in perfumes, cosmetics, pharmaceuticals, lubricants,

Table 2: Interpretation of FT-IR data of TCE-treated, benzene-treated, and peptone-grown (control) PM102 biomasses. Functional groups were assigned to respective wavenumbers with reference to Coates (2000) [27].

Group frequency (cm^{-1})	Functional group
Treated (TCE-induced biomass)	
536	Aliphatic bromo C–Br stretch
874	**Epoxy ring**
1072	Cyclic ethers C–O stretch
1251	Skeletal C–C vibrations
1394	**Tertiary alcohol OH bend**
1555	**Carboxylic acid**
1644	Amide or secondary amine NH bend
2932	Methylene stretch
3414	Hydroxy group H bonded OH stretch
Control (peptone-grown biomass)	
527	Aliphatic bromo C–Br stretch
699	**Aliphatic chloro C–Cl stretch**
1112	Cyclic ethers C–O stretch
1250	Skeletal C–C vibrations
1416	**Vinyl C–H in plane bend**
1639	Amide or secondary amine NH bend
2947	Methyl C–H asym./sym. stretch
3421	Hydroxy group H bonded OH stretch
Treated (benzene-treated biomass)	
582	Aliphatic bromo C–Br stretch
749	**1,2-Disubstitution (ortho) aromatic ring**
1027	**Cyclohexane ring vibrations**
1250	Skeletal C–C vibrations
1446	**Aromatic ring stretch**
1639	Amide or secondary amine NH bend
2253	**Isocyanate (–N=C=O asym. stretch)**
2947	Methyl C–H asym./sym. stretch
3421	Hydroxy group H bonded OH stretch

paint thinners, and plasticizers. Ethylene glycol ethers are part of brake fluids, soaps, detergents, solvents, lacquers and paints [34]. Ethylene oxide is one of the most commonly used sterilization methods in the healthcare industry and for processing of storage facilities (tobacco, packages of grain, sacks of rice, etc.), clothing, furs, and valuable documents [35]. Thus, TCE and benzene degradation products by the PM102 isolate are of considerable significance in medicine and industrial applications.

Conflict of Interests

There is no conflict of interests between the authors or with any other person regarding any of the works reported or software used in this paper.

Acknowledgments

The authors thank the Department of Science and Technology (DST), New Delhi, India, for the grant of INSPIRE Fellowship (Innovation in Science Pursuit for Inspired Research) and supporting this research work. They also thank Indian Association for the Cultivation of Science(IACS), Kolkata, for allowing them to use their FT-IR spectroscopy facility.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 827621, 16 pages
http://dx.doi.org/10.1155/2013/827621

Review Article

Class A β-Lactamases as Versatile Scaffolds to Create Hybrid Enzymes: Applications from Basic Research to Medicine

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Received 31 May 2013; Accepted 4 July 2013

Academic Editor: Bidur Prasad Chaulagain

Designing hybrid proteins is a major aspect of protein engineering and covers a very wide range of applications from basic research to medical applications. This review focuses on the use of class A β-lactamases as versatile scaffolds to design hybrid enzymes (referred to as β-lactamase hybrid proteins, BHPs) in which an exogenous peptide, protein or fragment thereof is inserted at various permissive positions. We discuss how BHPs can be specifically designed to create bifunctional proteins, to produce and to characterize proteins that are otherwise difficult to express, to determine the epitope of specific antibodies, to generate antibodies against nonimmunogenic epitopes, and to better understand the structure/function relationship of proteins.

1. Introduction

Engineering proteins with improved properties or new functions is an important goal in biotechnology. In this context, numerous bi- or multifunctional proteins have been created for applications in a broad range of fields including biochemical analysis, protein purification, immunodetection, protein therapies, vaccine development, functional genomics, analysis of protein trafficking and analysis of protein interaction network [1]. The most common strategy to create such proteins involves fusing polypeptides into an end-to-end configuration. For example, the fusion of proteins with specific tagging domains is nowadays a standard tool for protein engineering. Indeed, numerous commercial kits allow the fusion of a protein of interest to an affinity polypeptide such as 6-Histidine tag, maltose-binding protein, and glutathione transferase [1]. Moreover, the fusion of a protein of interest to the green fluorescent protein (GFP) has become one of the most important tools used in contemporary bioscience, and O. Shimomura, M. Chalfie, and R. Y. Tsien were rewarded the Chemistry Nobel prize in 2008 for the initial discovery of GFP and development of important GFP-based applications.

Another strategy to create hybrid proteins, although much less commonly used, is to insert (or graft) a peptide/protein/fragment thereof at a permissive site of a carrier protein. For example, Collinet et al. [2] have successfully created bi- or trifunctional hybrid enzymes by inserting the dihydrofolate reductase (159 aa) and/or the TEM-1 β-lactamase (263 aa) into four different positions of phosphoglycerate kinase (415 aa). Betton et al. [3] have created hybrid proteins by inserting the TEM-1 β-lactamase at various sites into the MalE maltodextrin-binding protein, that is, positions 120, 133, and 303, or by fusing it to the carboxy terminus of MalE. Insertion at positions 133 and 303 or fusion at the C-terminus allows the production in the periplasm of hybrid proteins exhibiting both parental activities; indeed, the maltose binding and the penicillinase activity of these hybrid proteins is indistinguishable from that of, respectively, MalE and TEM-1 [3]. Interestingly, the two proteins with insertions displayed two additional properties compared to

their C-terminal fusion counterpart: (i) they were more resistant to degradation by endogenous proteases and, (ii) even more remarkably, the TEM-1 moiety was stabilized against urea denaturation through the binding to MalE of maltose, that is, its natural ligand. This latter observation clearly demonstrates that TEM-1 is structurally dependent on MalE or in other words that an allosteric interaction occurs between the two proteins [1, 3]. Thus, insertion of a polypeptide inside a scaffold protein can present some advantages compared to the more common end-to-end fusion. This strategy actually mimics that used by nature to generate protein diversity. Indeed, genetic rearrangements, such as the introduction of a sequence into an unrelated coding sequence, naturally occur in the genome [2]. The resulting proteins are composed of two or more domains, and the linear sequence of one domain is interrupted by the insertion, forming discontinuous domains. This process has been shown for large natural proteins such as disulfide bond isomerase A (dsbA) [4], DNA polymerase [5], and pyruvate kinase [6]. A systematic survey of structural domains showed that ~28% of them are actually not continuous, clearly indicating that the sequence continuity of a domain is not required for correct folding and function [2, 7].

In this review, we report the use of two class A β-lactamases to create hybrid proteins (β-lactamase hybrid proteins, BHPs) in which exogenous peptides/proteins/fragments thereof are inserted within β-lactamase sequences. Such hybrid proteins can be designed for a range of applications such as (i) the creation of bifunctional hybrid proteins [8–11], (ii) the production, purification, and characterization of proteins otherwise difficult to express [10, 12, 13], (iii) the determination of the epitope of a specific antibody at the surface of an antigen [8, 11], (iv) the generation of antibodies against specific antigens or fragments thereof [9, 13–15], and (v) the investigation of fundamental aspects of structure, stability, and function of proteins [10, 12, 16].

2. Class A β-Lactamases as Scaffolds to Create Hybrid Enzymes

2.1. Why the Use of Class A β-Lactamases? The two class A β-lactamases that have been extensively used as model proteins to insert exogenous polypeptides are BlaP from *Bacillus licheniformis* 749/C and TEM-1, the latter being the most encountered β-lactamase in gram-negative bacteria (Figure 1). These two enzymes are, respectively, secreted by bacteria in the external environment and expressed in the periplasmic space to inactivate β-lactam antibiotics. About 40% and 20% of the amino acids are, respectively, identical and homologous between these two serine-active β-lactamases (Figure 1). Note that the residue numbering used in this review is that specific to class A β-lactamases [17]. These enzymes, especially TEM-1, have been extensively used as reporters to study gene expression [21–23] and for immunoassays [24]. They have been selected as scaffolds to create hybrid proteins based on a series of observations. Firstly, they are fairly small (30 kDa) and stable proteins that are easily overexpressed in *E. coli* and subsequently purified [9–11]. Their three-dimensional structure has been determined by X-ray crystallography (Figure 1) [18, 19, 25, 26]. They exhibit the typical class A β-lactamase 3D structure which is composed of two domains: an α/β domain and an all-α domain, with the catalytic site at the interface between the two domains [19, 25, 26]. Finally, their catalytic properties have been thoroughly characterized and can be easily monitored using fluorescent [22, 23, 27] or chromogenic substrates [28]. This latter point is crucial as exemplified in the following sections. The enzymatic activity of the BHPs serves as a reporter to select clones resistant to β-lactam antibiotics, such as ampicillin, expressing well-folded and active BHP. It is assumed that the folding of the exogenous polypeptide and the carrier enzyme are interdependent to some degree. Indeed, the correct folding of the carrier β-lactamase requires the exogenous polypeptide to be exposed to the solvent; otherwise, the β-lactamase cannot adopt its native structure. Moreover, the correct folding of the carrier protein imposes constraints at the N- and C-terminal extremities of the inserted polypeptide, maintaining its two extremities in close proximity of the insertion site. These constraints limit therefore the conformational flexibility of the insert and could assist the insert in adopting its native structure. When a greater flexibility is required for the correct folding of the insert, small peptides, that is, linkers, can be added at both of its extremities. Note that, in some applications, these linkers have an additional functionality allowing the excision of the insert thereafter, as discussed in detail below.

2.2. A Brief History of Class A β-Lactamase Hybrid Proteins. The design of class A hybrid β-lactamases was initiated with the work of Hallet et al. in 1997 [20]. A random pentapeptide cassette was inserted at 23 different positions of TEM-1 using pentapeptide-scanning mutagenesis (Figure 1(a)) [20]. This method consists of transposing the transposon Tn*4430* into the TEM-1 gene which duplicates 5 base pairs (bp) of host sequence at the insertion point. This transposon contains *Kpn*I restriction enzyme sites 5 bp from the outer ends of its terminal inverted repeats. Digestion with *Kpn*I followed by ligation results therefore in the insertion of 10 pb derived from Tn*4430* and 5 bp from the 5-nucleotide duplication of the target gene. Each transposition event leads to a 15 bp insertion into the TEM-1 gene encoding for a random pentapeptide cassette. The 23 insertion sites have been classified into 3 groups depending on the level of resistance that the hybrid protein confers to *E. coli* cells against ampicillin, a β-lactam antibiotic (Figure 1(a)): (i) 5 permissive sites (197, 198, 265, 270, and 272) with high level of resistance. These sites are located into two loops distant from the catalytic site, one loop between α helices 8 and 9 and the other between β-strand 5 and the C-terminal helix. (ii) Eight non-permissive sites (63, 68, 163, 164, 208, 232, 233 and 246) associated with the loss of the β-lactamase activity and clustered around the catalytic site. (iii) Ten semi-permissive sites (37, 97, 99, 119, 206, 216, 218, 260, 264, and 276) are conferring an intermediate level of ampicillin resistance.

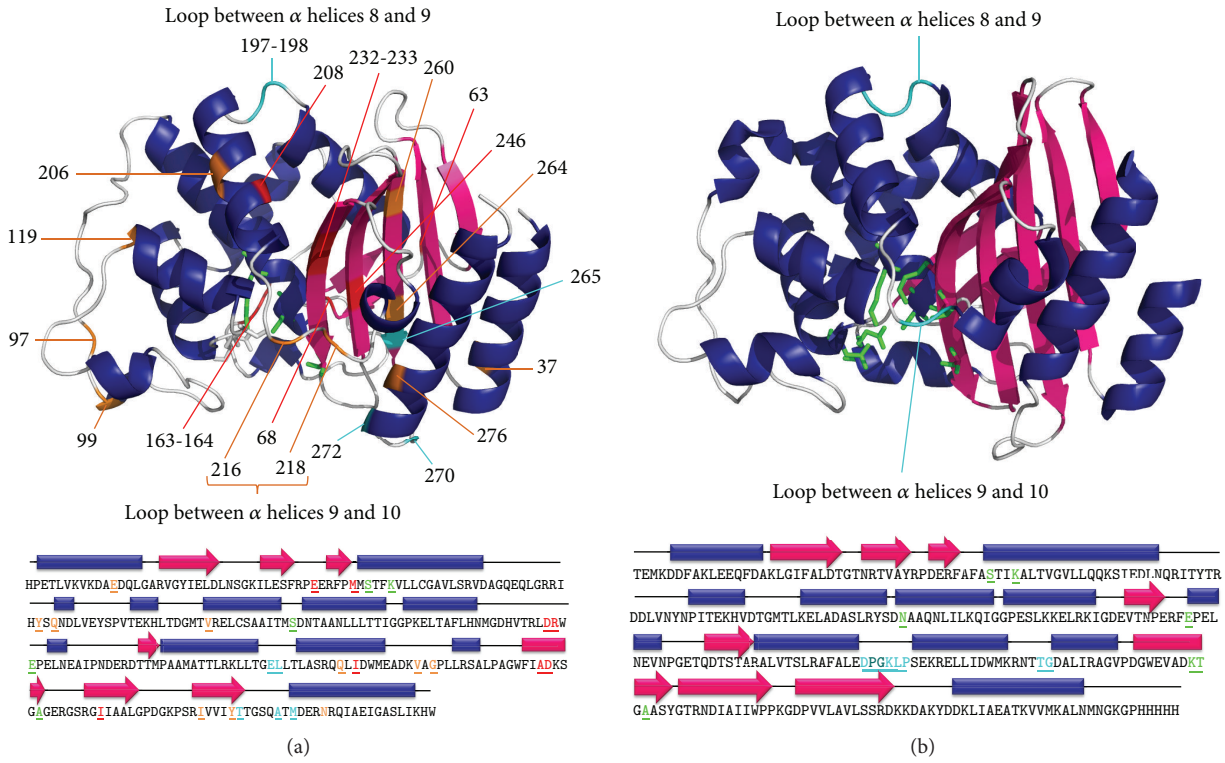

Figure 1: X-ray crystal structure and sequence of TEM-1 (a) and BlaP (b). The residue numbering is based on homology to class A β-lactamases [17]. The structures were produced using PyMOL (DeLano Scientific LLC, South San Francisco, CA, USA) and the PDB ID is 4BLM for BlaP [18] and 1XPB for TEM-1 [19]. (a) TEM-1. The residues of the active site are represented in green on the structure and are coloured and underlined in the sequence. The different insertion sites of pentapeptides reported in Hallet et al. [20] are coloured on the structure and are coloured and underlined in the sequence. Light blue, orange and red are associated with permissive, semi-permissive, and non-permissive insertion sites, respectively. (b) BlaP from *Bacillus licheniformis* 749/C. The residues of the active site are represented in green on the structure, and are coloured and underlined in the sequence. The two insertion sites most commonly used to design BHPs, located in the loop between α helices 8 and 9 (197-198) and in the loop between α helices 9 and 10 (216-217), are indicated in light blue on the structure and are coloured and underlined in the sequence. The PG dipeptide between residues 197 and 198 indicated in bold in the sequence corresponds to the SmaI restriction site inserted into the gene of BlaP for the cloning of exogenous polypeptides at this position.

Later, Ruth et al. [9] have inserted longer and more structured polypeptides in eight of the TEM-1 positions investigated by pentapeptide-scanning mutagenesis (Table 1). The thermostable 18-residues STa enterotoxin from enterotoxic *Escherichia coli* has been introduced, for example, into one of the two permissive loops (at position 195 and 198, between α helices 8 and 9), in five semi-permissive sites (37, 206, 216, 218 and 260) and into one non-permissive site (232). Insertions between α helices 8 and 9 (positions 195–200) and between α helices 9 and 10 (positions 213–220) allow the production of a soluble hybrid protein which retains both activities of parental proteins (i.e., ampicillin resistance and enterotoxicity). The level of activities and the amount of soluble hybrid enzymes produced depend however on the position of insertion.

TEM-1 is a β-lactamase sensitive to many proteases [11, 29]. Some BHPs that do not confer resistance to ampicillin to *E. coli* (i.e., BHPs containing insertions at sites identified as non-permissive) could however be successfully expressed in protease-deficient *E. coli* strains. This observation suggests that insertions at these positions into TEM-1 actually increase its sensitivity to proteolysis [11]. This increased susceptibility to proteolysis could be due to local conformational changes or to a destabilisation of the enzyme upon the insertion of the exogenous polypeptide. In contrast, BlaP presents an advantage over TEM-1 in being much less sensitive to proteases. Indeed, this enzyme evolved to withstand high levels of various and numerous proteases, which are secreted by the gram-positive *Bacillus licheniformis* bacterium [11, 30]. Based on this observation, it was anticipated that BlaP could constitute an alternative scaffold to TEM-1 to generate hybrid proteins. Since, as mentioned above, BlaP and TEM-1 share a high sequence identity and a similar three-dimensional structure, the permissive sites described for TEM-1 served as a basis to engineer hybrid BlaP proteins with the insertion of heterologous polypeptides (Figure 1(b)) [11].

2.3. How Were BHPs Designed? TEM-1 hybrid proteins were produced in the periplasm of *E. coli* via pFHX plasmids derived from the pBR332 plasmid, the X corresponding to the position of insertion into TEM-1. These plasmids carry a *KpnI* restriction site at the position of insertion

Table 1: Properties of the different BHPs discussed in this review.

Hybrid proteins [ref]	Insert		Effect on stability ($\Delta\Delta G^{\circ}_{H_2O}$—kJ·mol^{-1})	Effects on the β-lactamase activity	Function of the insert
	Name + size	Insertion site			
TEM-1-STa [14, 15]	STa 18 AA	37, 195, 197, 198, 206, 216, 218, 232 and 260	ND	Insertion at positions 37, 198, and 206: ND Insertion at position 195: no loss of activity Insertion at positions 197, 216, 232, and 260: partial loss of activity	Enterotoxicity
BlaP-HA [11]	HA 9 AA	197	ND	No loss of activity	Binding to anti-HA mab: K_D = 0.68 nM
BlaP-ChBD [10]	ChBD 72 AA	197	~3.2	No loss or partial loss of activity depending on the substrate	Binding to chitin: $K_r = 5.4 \pm 0.5$ L g^{-1}
	Thb-ChBD-Thb	197	ND	Partial loss of activity	Binding to chitin
BlaP-CD22 [12]	CD22/C2C3 202 AA	197	ND	Activity detected but not quantified	Binding to mab 8015: $K_D = 26.2 \pm 1.4$ nM
$BlaP_{197}(Gln)_x$ [16]	PolyQ tracts 23Q-30Q-55Q and 79Q	197	~7.6–8.8	No loss of activity	PolyQ tracts longer than a threshold trigger the aggregation of BHPs into amyloid fibrils

The name of the BHPs, the name of the insert, its size, and function are indicated. The effects of the insertion on the stability and on the enzymatic activity of the β-lactamase are also reported. ND: not determined.

allowing the insertion of the exogenous sequence. They allow a constitutive expression of the BHPs which are purified by ion-exchange chromatography [9].

Most BlaP hybrid proteins, on the other hand, have so far been created by inserting the exogenous polypeptide at position 197 within the permissive loop located between the α helices 8 and 9 (Figure 1(b)) [10]. A constitutive expression vector in *E. coli* (pNYBlaP), which allows the insertion of heterologous polypeptide sequences into the *SmaI* restriction site at position 197 (Figure 1(b)), has been developed [10, 11]. This vector allows the expression, in the periplasm of *E. coli*, of the BHP in fusion with a C-terminal His-tag to facilitate their purification using affinity chromatography.

3. BHPs as Tools to Produce and Study Difficult to Express Peptides/Proteins

Some proteins are difficult to produce recombinantly because of their intrinsic properties such as relative insolubility, instability and/or large size. One strategy to study their properties (i.e., structure, function, ...) consists of inserting these proteins or their structural subdomains into a carrier protein.

Vandevenne et al. [10] have, for example, designed, produced, and characterized a hybrid protein in which the chitin binding domain (ChBD) of the human macrophage chitotriosidase has been inserted in BlaP at position 197; this hybrid protein is referred to as BlaP-ChBD (Table 1). The study of the chitin binding domain of this chitinase is of interest since (i) the physiological function of mammalian chitinases remains unknown [31] and, more importantly, (ii) the concentration of chitotriosidase, a chitinase expressed in lipid-laden macrophages, is highly elevated in Gaucher disease [31–33]. Some evidence suggests that mammalian chitinases confer a defensive function against chitin-containing pathogens, such as fungals, which have cell walls consisting mainly of chitin; indeed, high levels of chitinases are present in the serum and tissues of guinea pigs after infection by *Aspergillus fumigates* [31, 34, 35]. It is also proposed that the enzyme could modulate the extracellular matrix in the vessel wall affecting the downstream tissue-remodelling processes, associated with atherogenesis. This hypothesis is based on the fact that macrophages expressing chitinases are abundant in atherosclerotic plaques [31, 33].

ChBD is a small domain of 8 kDa composed of 72 amino acids which binds insoluble chitin. It contains 3 disulfide bridges that are essential for both its structural stability and its binding to chitin [10, 13, 31]. This protein domain is extremely difficult to produce, and its three-dimensional structure has not yet been solved [10, 13, 31].

The BlaP-ChBD hybrid protein has been successfully produced in the periplasm of *E. coli* and purified in a two-step procedure, that is, affinity chromatography via a His-tag designed at the C-terminus of BlaP, followed by anion-exchange chromatography. Remarkably, the hybrid protein exhibits both parental activities (Table 1). The β-lactamase moiety hydrolyzes its β-lactam substrates, in the presence and/or absence of chitin, and the ChBD domain binds to insoluble chitin with similar K_r value (relative equilibrium association constant), 5.4 ± 0.5 L g^{-1}, of other carbohydrate-binding domains [10, 13]. It is important to note that the bifunctionality of the hybrid protein allowed the K_r value between BlaP-ChBD and insoluble chitin to be easily measured, using the β-lactamase activity as a reporter. Moreover,

the hybrid protein allowed the detection of chitin in fungal cell walls [24]. The fact that ChBD conserves its binding properties within BlaP suggests that its N- and C-terminal extremities are proximal in the three-dimensional structure. In the presence of reducing agents, BlaP-ChBD still exhibited β-lactamase activity but failed to bind to chitin, confirming that the 3 disulfide bridges of ChDB are essential to maintain its functionality.

The effects of the insertion of ChBD on the thermodynamic stability of BlaP were extensively characterized. Despite the multidomain character of BlaP-ChBD, urea unfolding occurs according to a simple two-state mechanism and is fully reversible [10]. The insertion of ChBD in position 197, however, slightly destabilizes BlaP by 3.2 kJ·mol^{-1} (Table 1). Moreover, the thermal unfolding of BlaP-ChBD was found to be less reversible and less cooperative than that of BlaP. Indeed, the thermal transition observed for BlaP-ChBD by differential scanning calorimetry (DSC) and far UV circular dichroism is characteristic of a three-state transition while the one observed for BlaP is characteristic of a two-state transition. This suggests that, within the hybrid protein, ChBD and BlaP unfold individually upon temperature increase [10].

The ChBD domain has also been inserted in position 197 of BlaP between two thrombin (Thb) cleavage sites; this hybrid protein is referred to as BlaP-*Thb/CHBD/Thb* (Table 1) [13]. This construction allowed easy separation of the ChBD domain from the BlaP carrier protein, following the action of the thrombin and subsequent purification. Upon proteolytic cleavage, the N- and C-terminal parts of BlaP remain strongly linked together and are separated from the ChBD domain by depletion using affinity chromatography via the C-terminal His-tag (Figure 2). Remarkably, the thrombin cleavage did not significantly alter either the activity of BlaP or that of of ChBD (Table 1), and ~18 mg of highly soluble and active ChBD was obtained per litre of culture [13, 24].

These studies clearly demonstrate that BlaP is an efficient scaffold to create bifunctional enzyme and to produce, purify, and characterize protein fragments that are otherwise difficult to express. This approach has been further used to produce various polypeptides of up to 40 kDa in size (unpublished results). Moreover, these results show that the BHPs can be used (i) to easily investigate the biological function of the polypeptide/protein inserted such as its interaction with a series of ligands using the β-lactamase activity as a reporter, (ii) to screen therapeutic molecules, that is, molecules neutralizing the biological properties of the polypeptide inserted, as further exemplified in the section of this review describing the use of BlaP for vaccine development, (iii) to screen molecules stabilizing and preventing protein aggregation (see section "BHPs as model proteins to investigate structure/function relationships"), and (iv) to facilitate the determination of the three-dimensional structure of polypeptides that are otherwise unnameable to X-ray crystallography due to their insolubility, since BHPs allow an increase in the solubility of the insert [10, 12, 13].

4. BHPs: an Original Approach to Map and Functionalize Epitopes

The determination of the epitope (i.e., the region of the antigen that is recognized by an antibody or an antibody fragment, which can be linear or conformational) is of crucial importance to understand, at the molecular level, the relationship between the structure and function at the antigen-antibody interface [8]. For example, the determination of the epitope recognized by two heavy-chain antibody fragments specific to human lysozyme has allowed a better understanding of how they prevent *in vitro* amyloid fibril formation by the amyloidogenic variants of human lysozyme [36, 37]. Moreover, the identification of the epitope enables the design and isolation of new antibody analogues, having higher affinity for their antigen or exhibiting different biological activities, or smaller antibody mimetics (i.e., peptides and small molecules interacting with the same epitope region) [8]. Epitope mapping is also essential in diagnostic, immunotherapy, and vaccine development.

Different techniques have been developed to identify epitopes. The gold standard for epitope identification is the determination of the three-dimensional structure of the antigen-antibody complex by X-ray crystallography. This technique is, however, not always easy to perform due to the difficulty of obtaining high concentrations of a good quality complex and finding the proper conditions of crystallisation [8, 38]. Alternative biophysical techniques include (i) H/D exchange experiments on the antigen-antibody complex coupled to proteolysis with pepsin and subsequent analysis of the digested fragments by mass spectrometry (deuterium exchange mass spectrometry) [39] and (ii) NMR spectroscopy by comparing, for example, *heteronuclear single quantum coherence* (HSQC) spectra, of small target proteins in the presence or absence of the antibodies [40]. For all these structural approaches, high quantities of pure and stable antigens are however required, and some of them are limited by the size of the complex [8]. Another set of approaches is based on the exposition of numerous small peptides (overlapping sequences from the natural antigen or peptide mixtures derived from a combinatorial library if the antigen sequence is unknown) on a chip (peptide arrays) [41], or at the surface of phages (phage display) [42–46]. Phage display is based on the expression of a peptide/protein/protein fragment of interest at the surface of phage particles, fused to one of their coat proteins [42, 47, 48]. The power of this technique is the physical link between the phenotype (the expression of the peptide/protein/protein fragment of interest that can interact with its ligand) and the genotype (the nucleotide sequence of the peptide/protein/protein fragment cloned into the phagemid genome) [42, 48]. Phage display allows high-throughput screening of libraries of variant nucleotide sequences with diversity up to 10^6 to 10^{10} [11, 42, 49].

Innovative phage display strategies are continuously implemented [50]. One of them is based on the use of the β-lactamase hybrid protein to expose random peptides/protein fragments at the surface of the phages and therefore to enzymatically functionalize the epitope (Figure 3) [8, 11,

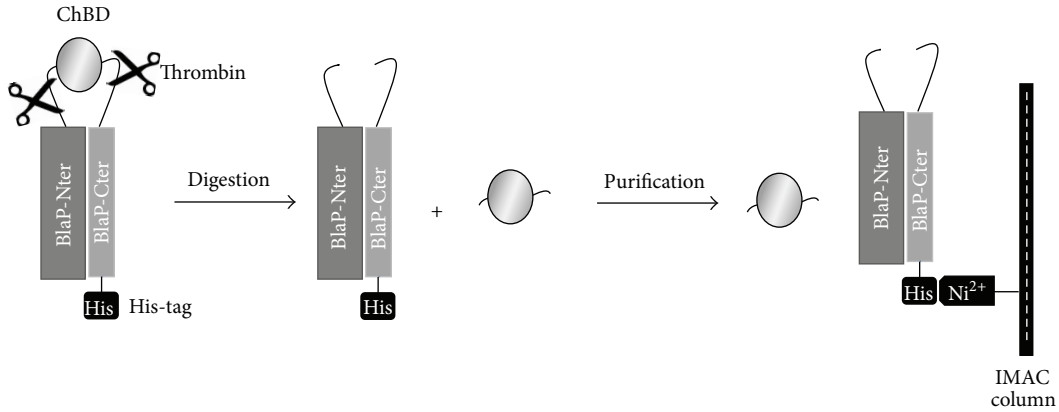

Figure 2: Schematic representation of the protocol used to cleave the ChBD domain from BlaP and to purify it. Purified ChBD is collected in the flow through after loading the hybrid protein digested by thrombin on an immobilized metal ion affinity chromatography (IMAC). Figure adapted from Vandevenne et al., 2008 [13]. BlaP-Cter: C-terminal sequence of BlaP, starting at the insertion site. BlaP-Nter: N-terminal sequence of BlaP ending at the insertion site.

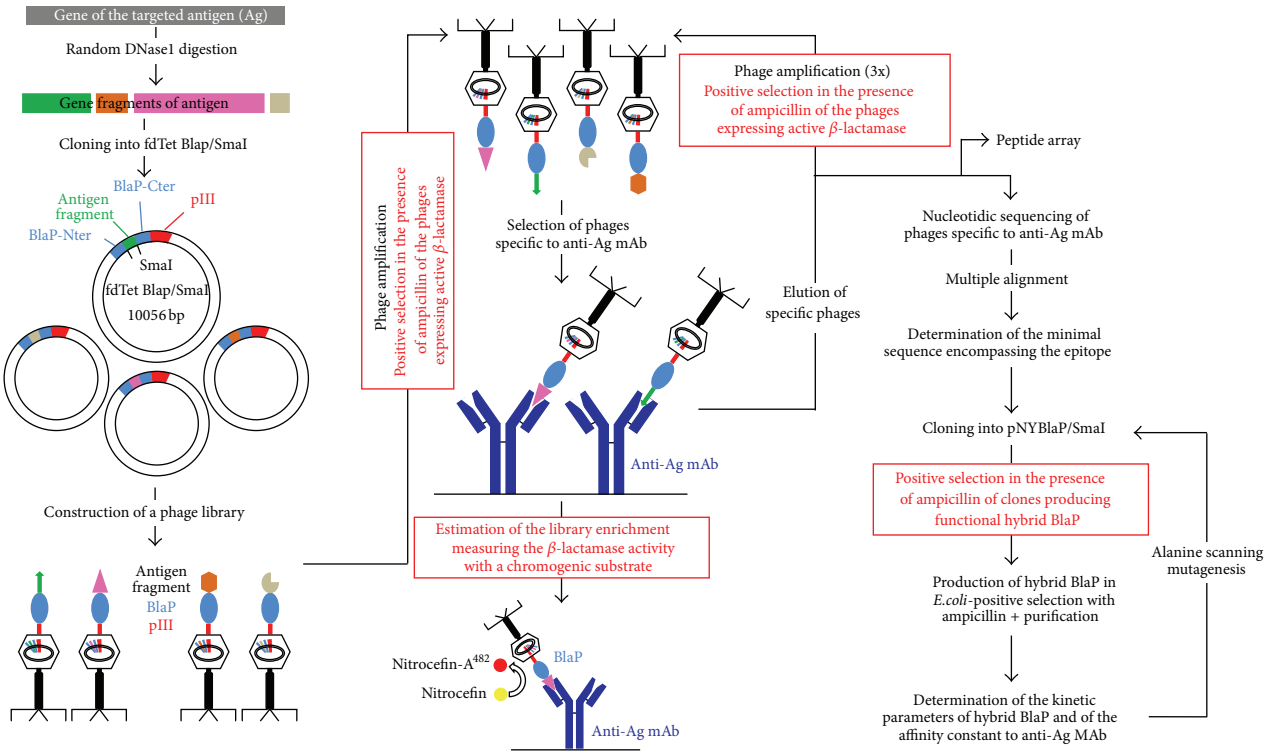

Figure 3: BHPs as unique tools for epitope mapping. Schematic representation of the epitope mapping procedure developed by Bannister et al. and Chevigné et al. [8, 11]. The steps for which the enzymatic activity of BlaP, as a reporter or as factor for selection, is critical are highlighted in red. BlaP-Cter: C-terminal sequence of BlaP, starting at the insertion site. BlaP-Nter: N-terminal sequence of BlaP ending at the insertion site. Anti-Ag mab: monoclonal antibody specific to the targeted antigen. Following the elution of phages after the last round of panning, a peptide array can be carried out to verify that the entire antigen sequence is well represented in the phage library. Alanine scanning mutagenesis can be carried out to determine which residues of the minimal sequence encompassing the epitope is in direct contact with the antigen. Figure adapted from Chevigné et al., 2007 [11].

51]. The advantages of this strategy are numerous [11, 51]. First, the polypeptide exposed within the β-lactamase could have a restricted conformational freedom and be somehow protected from proteolytic cleavage [3]. As illustrated in Figure 3, the main implementation is, however, associated with the use of β-lactamase activity which can be easily detected with chromogenic substrates such as nitrocefin [11]. The β-lactamase activity is used (i) as a reporter to directly assess the interactions between the inserted peptides and the target antibodies, and to easily monitor the enrichment of

the phage library during the successive rounds of selection, and (ii) as an agent of selection of phages expressing active BHPs. Indeed, in order to amplify phages, phage-infected cells are grown in a liquid medium in the presence of ampicillin. Under these conditions, the bacterial growth is directly associated with antibiotic resistance, and thus only cells expressing active BlaP at the surface of phages are able to grow [11].

This approach was first introduced by Legendre et al., who inserted random peptides within TEM-1, which was displayed on phage fd in fusion with the coat protein pIII [51]. The peptides were displayed on different loops surrounding the active site of TEM-1: in one library, peptides replaced residues A103 to 106; in a second library, they replaced residues Thr271 and/or Met272, and these two libraries were combined in a third one [51]. By selection from the different libraries using three unrelated conformational monoclonal antibodies (mabs) recognizing distinct epitopes on the prostate-specific antigen (PSA), several hybrid proteins expressing small peptides with an affinity for these mabs in the micro- and nanomolar range were isolated. The sequence of these small peptides presents no similarity with that of PSA, suggesting that mimotopes (i.e., peptides which bind the antibody without having any identity to the antigen sequence) were selected. These results indicate that BHPs could effectively be used to display peptides at the surface of phages.

An original epitope mapping strategy, inspired by the work of Legendre et al., was developed by Bannister et al. and Chevigné et al. [8, 11]. It involves constructing a phage display library in which random fragments of different lengths of the gene coding for the antigen of interest are exposed within the β-lactamase BlaP (at position 197) in fusion with the pIII protein at the surface of phages (Figure 3). A given region of the target protein is displayed within peptides of different lengths and could therefore adopt different conformations. Sufficiently long peptides are likely to adopt a fold similar to what they adopt in the full target protein [11]. As exemplified below, this technology allows the minimal region of an antigen interacting with an antibody to be identified with high accuracy (Figure 3) [11]. As the peptides exposed within the β-lactamase are directly related to the nucleotide sequence of the antigen, they do not correspond to mimotopes. Moreover, the key amino acids within this minimal region that are directly in contact with the antibody can be further determined by point mutations as discussed below (Figure 3) [8].

4.1. Mapping of Linear Epitopes. The BHP-implemented phage display approach, developed by Chevigné et al. [11], has allowed the determination of the linear epitope (i.e., an epitope composed of amino acids that are in close proximity in the sequence) of a high-affinity monoclonal antibody (anti-*HA* mab) specific to the virus influenza hemagglutinin (HA1). Overlapping peptides of different sizes (from gene fragments of 50 to 300 bp) of HA1 were displayed on the solvent exposed loop between α helices 8 and 9 of BlaP, which was fused to pIII coat protein of phages (Figure 3). The phages expressing peptides containing the epitope region of HA1 were selected by panning against anti-*HA* mab. After three rounds of selection and amplification in the presence of ampicillin, a series of phages expressing BHPs specific to anti-*HA* mab was selected. The sequencing of these phages shows a consensus 9-residues linear epitope of anti-*HA* mab (Figure 4). This epitope is present in fragments of various sizes (from 13 to 70 residues) and can thus in principle adopt different folds and solvent accessibilities. The gene coding for the BHP displaying the consensus 9-residues epitope sequence was cloned into the constitutive expression vector pNY. The hybrid protein, referred to as BlaP-HA, was subsequently expressed in the periplasm of *E. coli* and purified as a bifunctional hybrid protein. The kinetic parameters (i.e., K_m, k_{cat}, and k_{cat}/K_m) of BlaP-HA are similar to that of wild-type BlaP, and it binds anti-*HA* mab with an affinity (K_D) of ~0.68 nM (Table 1) [11].

4.2. Mapping of Conformational Epitopes. A similar approach was used by Bannister et al. to identify the conformational epitope (i.e., epitope composed of amino acids located far apart in the sequence but brought together by protein folding) of the anti-CD22 immunotoxin CAT-8015 (mab 8015) [8]. CD22 (cluster of differentiation-22) is a cell surface glycoprotein composed of a N-terminal Ig-like V-type domain and various Ig-like C-type domains (Figure 5) [52–55]. This protein is of particular therapeutic interest because it is a specific marker present on the surface of malignant B-cells and it is rapidly internalized upon binding; it constitutes therefore a relevant target for an antibody drug conjugate or immunotoxin approach. Despite the therapeutic interest of CD22, very little is known concerning its structure or that of the other related family members due to their high level of glycosylation [8]. CAT-8015 is an immunotoxin which combines a CD22-specific antibody variable fragment (Fv, derived from the antibody mab 8015) with a *Pseudomonas* exotoxin A (PE38); it exhibits a noteworthy clinical activity in three leukaemia (i.e., chronic lymphocyte leukaemia, hairy cell leukaemia, and acute lymphoblastic leukaemia) [8, 56, 57]. Antibody competition-binding studies have revealed that the epitope of CAT-8015 is localized in the C-like domain 2 of CD22, but there is no information on its precise location [8, 58].

In order to identify which residues of CD22 bind to CAT-8015, Bannister et al. introduced random CD22 extracellular domain gene fragments of 50 to 1000 bp into the permissive exposed loop of BlaP (position 197) fused to the phage pIII coat protein (Figure 3) [8]. A library containing 6×10^5 transformants was obtained and screened against CAT-8015 using the same strategy as that explained above. A peptide-array analysis (Figure 3) confirmed that the entire CD22 gene was well represented in the β-lactamase-positive infectious phages, except two small regions of 7 residues each. The minimal region of CD22 which binds to mab 8015 was identified. This region, referred to as CD22/C2C3, is composed of 202 residues, from V234 to G435, located in the C-terminal of the C-type domain 1, in the C-type domains

2 and 3, and in the N-terminal of the C-type domain 4 (Figure 5).

The gene coding for the BHP displaying the minimal region of CD22 was cloned into the constitutive expression vector pNY. The hybrid BlaP-CD22/C2C3, produced as a bifunctional hybrid protein in *E. coli*, conserves the β-lactamase activity and binds to mab 8015 with a high affinity (K_D ~26.2 nM) (Table 1). To identify the key amino acids involved in the antibody binding, an alanine-scanning mutagenesis was performed (Figure 3) [11]. Thirty-four single-point mutations to alanine were performed in three clusters (referred to as clusters 1, 2, and 3) of CD22 encompassing the epitope, and the resulting hybrid proteins were produced and purified. Cluster 1 is located in the junction of C-type domains 1 and 2, cluster 2 is located in C-type domain 3 and cluster 3 encompasses the junction between C-type domain 3 and 4 and the N-terminal of C-type domain 4 (Figure 5). The binding of each mutant to mab 8015 was then measured by enzyme-linked immunosorbent assay (ELISA) and compared to that of the parent hybrid protein BlaP-CD22/C2C3. This technique has allowed the identification of the amino acids of high or intermediate importance in the CD22-mab 8015 complex. The most important residues encompass three discontinuous regions and they are mainly located in C-type domains 2 and 3 (clusters 1 and 2), and few are located in cluster 3. Modelling of different orientations of these domains strongly suggests that they adopt a U-shaped arrangement which allows the three clusters to be in close enough proximity to form the epitope (Figure 5) [8].

These two examples show that alternating successive affinity selections of phages and growth of phage-infected cells in the presence of antibiotic permits the selection of BHPs exhibiting both a high affinity for the target antigen and an efficient enzymatic activity. They clearly demonstrate that BHP technology using BlaP is a unique tool that allows the identification of the antigen region involved in the antigen-antibody interactions. It allows (i) the identification of linear epitopes (i.e., the linear epitope of HA for anti-*HA* mab) and (ii) the characterisation of complex epitopes (i.e., the U-shaped discontinuous CD22 epitope of mab 8015). Moreover, BlaP has enough conformational flexibility to allow inserted antigen fragments of various lengths to fold and interact with the antibody. BHPs allow therefore the identification of non-linear epitopes (i.e., conformational epitopes) that are directly linked to the nucleotide sequence of the antigen (i.e., not a mimotope) [8, 11]. This approach can be further used to identify the protein region involved in any protein-ligand interactions.

4.3. BHPs as a Unique Tool for Immunoassay Development. The β-lactamase implemented phage-display technique described above allows the selection of a particular epitope as a bifunctional hybrid protein which associates the epitope recognizing its specific target with an efficient enzymatic activity. This functionalization of the epitope allows the rapid characterisation of the antigen-antibody interaction. For example, the BlaP-HA hybrid protein was successfully coated on solid surface to perform an ELISA, suggesting that the solvent accessibility of the inserted polypeptide (i.e., the epitope) was not altered when the hybrid protein is coated [11]. Moreover, the enzymatic activity of BlaP can be used to check the coating efficiency and to quantify the amount of coated protein.

5. BHPs as Immunogenic Carriers for Vaccine Development

Antibodies are specialized fighter proteins that are effective in preventing infectious diseases. This property is based on the recognition of specific epitopes on the surface of the antigen that promotes the neutralization of the biological activity of the antigen or the opsonisation of the pathological agent. It is assumed that immunization with a precise epitope, corresponding to an effective neutralizing antibody, would elicit the generation of similarly potent antibodies in the vaccine [38]. Thus, the insertion of these particular epitopes into a carrier protein is often the starting point for the development of a new generation of safe vaccines. Within the carrier protein, the properties of the polypeptides, including their antigenicity and immunogenicity, can be further modified [59]. In this context, the BHP technology, allowing the insertion of a domain, subdomain, or a short polypeptide from the native targeted antigen into a carrier β-lactamase, is of particular interest to design such *de novo* antigens (Figure 6). This approach is particularly relevant to antibody raised against cryptic epitopes and non-immunodominant antigens.

5.1. Generation of Vaccines against Poorly Immunogenic Polypeptides/Proteins. TEM-1 hybrid proteins were created in an attempt to develop a vaccine against the poorly antigenic and non-immunogenic STa enterotoxin from enterotoxic *E. coli* strains (ETEC). This toxin, which is associated with enteric colibacillosis (characterized by severe diarrhoea) in calves and piglets, leads to significant losses in agriculture due to the death of newborns [60, 61]. STa is composed of 18 amino acids including 6 cysteins involved in three disulfide bridges which are necessary for the toxic activity [62, 63]. STa is poorly antigenic and is not immunogenic due to its small size. No vaccine is yet available against this thermostable toxin despite many attempts to design a safe vaccine (i.e., a non-toxic form of STa). The latter includes the chemical coupling of the toxin to bovine serum albumin [64, 65], to the β-subunit of cholera toxin [66], and to the heat-labile enterotoxin (LT) [65, 67], and its fusion to the major protein subunit ClpG of *E. coli* CS31A surface antigen [68], to several subunits of cholera toxin [66, 69], to LT [70, 71], to OmpC [70], and to flagellin [72]. These constructions either failed to induce the production of neutralizing antibodies against STa or retained a certain degree of toxicity. However, Wu and Chung have managed to elicit the production of protective antibodies against the antigen STLT (i.e., a fusion of the thermostable ST and thermolabile LT enterotoxins) in mice using a protein fusion between GFP and STLT. These immunized mice presented a subsequent full protection against ETEC [73].

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Cl 1  FVERSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEV
Cl 2   VERSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEVTQNGGS
Cl 3   VERSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEVTQNGGS
Cl 4   VERSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEV
Cl 5   VERSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEV
Cl 6   VERSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEVTQNGGSNACKRGPGSGFFS
Cl 7     RSKAFSNCYPYDVPDYASL
Cl 8     RSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEVTQNGGSNACKRGPGSGFFSRLNWLTKSEV
Cl 9           NCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEVTQN
Cl 10           CYPYDVPDYASLRSLVASSGTLEFITEGFTWTEVTQNGGSNACKRGPGSGFFS
Cl 11          NCYPYDVPDYASLRSL
Consensus       CYPYDVPDYASL
```

FIGURE 4: Consensus sequence recognized by anti-HA mab. Multiple sequence alignment from eleven individual clones selected after three rounds of selection of phages on anti-HA mab. The residues corresponding to the epitope recognized by the anti-HA mab are underlined. Figure from Chevigné et al. [11].

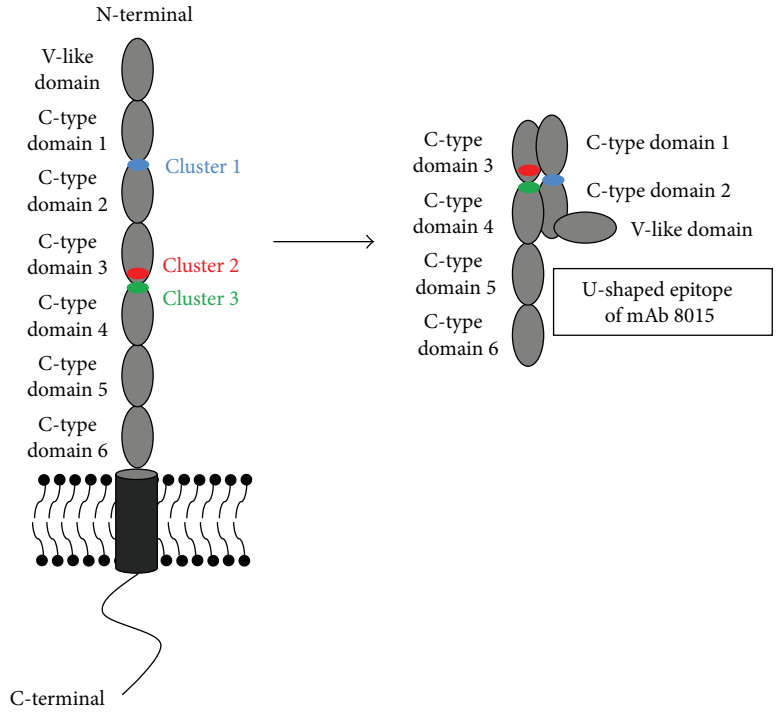

FIGURE 5: Schematic representations of the glycoprotein CD22 in a linear and U-shaped arrangement. In the linear arrangement, the three clusters forming the epitope are far away from each other whereas they are close in a U-shaped arrangement [8]. The residues involved in the complex with mab 8015 are clustered in cluster 1, 2, and 3 and are shown in light blue, red, and green, respectively. Figure adapted from Bannister et al. [8].

Different approaches based on the use of TEM-1 hybrid proteins have been used in order to design a vaccine against STa [9, 14, 15]. First, Ruth et al. have designed DNA vaccination to induce the production of neutralizing antibodies against STa [14]. STa and three variant forms, in which one (STaC6A, STaC17A) or two (STaC6A-C17A) of the six cysteines have been mutated (in order to disrupt one or two disulphide bridges and cause a complete loss of the toxicity of the toxin), have been inserted either in position 195 (loop A) or 216 (loop B) of TEM-1. Plasmid DNA encoding these hybrid proteins has been used to immunize mice. Following immunizations, different levels of anti TEM-1 antibodies were generated. The level of antibody production against TEM-1 was higher for mice immunized with plasmid DNA coding for BHPs displaying STa and its variants in position 216 (loop B) than in position 195 (loop A). Since insertions in loop B interfere less with anti TEM-1 antibody production than insertion in loop A, this suggests that the immunodominant epitope of TEM-1 is located in the region of loop A. In contrast, no antibody specific to STa could be detected, even after 3 injections of the different plasmids. Two subsequent boosts with a STa toxin obtained by peptide

synthesis, which is not immunogenic, did however induce the production of STa-specific antibodies in mice initially primed with plasmids encoding hybrid proteins, but not when primed with plasmid encoding TEM-1. This clearly indicates that hybrid proteins were expressed in mice and have primed the production of antibodies specific to STa. The ability of sera containing STa specific antibodies to neutralize the toxin was determined by suckling mouse assays. Only the sera from mice immunized with the plasmid coding for the double disulfide bridge mutated variant (STaC6A-C17A) and boosted with STa injections show neutralizing activity. These results indicate that the use of the toxic form of STa is therefore not needed to induce the production of neutralizing antibodies. These results also indicate that TEM-1 is an appropriate carrier to present nonimmunogenic peptide to the immune system. Indeed, the carrier is required for the induction of helper T cells allowing the production of antibodies against the carrier, TEM-1, and the hapten, STa. Moreover, the best location for the insertion seems to be located at position 195, the immunodominant epitope of TEM-1. Indeed, exposing the insert at this position reduces the production of anti TEM-1 antibodies and should therefore decrease the competition between the hapten and the carrier for the B-cell immune response [14].

Subsequently, recombinant hybrid TEM-1 proteins in which STa has been inserted at different positions, including positions 195 and 216, were designed, produced, and used for mice immunization (Figure 6) [9]. This study revealed that the hybrid proteins elicit the production of antibodies mainly against TEM-1, and in much lower quantity against STa. TEM-1 with the insertion at position 195 induces the highest production of anti-STa antibody and most importantly was the only hybrid protein that leads to the production of antibodies neutralizing the toxicity of STa. These results are in good agreement with results obtained with DNA vaccination and confirm that B-cell epitopes of the carrier are immunodominant. Moreover, the carrier, TEM-1, contains the functional helper T cell epitopes necessary for the immune response against the hapten, STa [9].

Following these encouraging results, Zervosen et al. immunized cattle with a TEM-1 hybrid protein containing the STa in position 197 in the presence of 3 different adjuvants (Table 1) (Montanide ISA70 - ISA206 and IMS1313) [15]. High levels of different IgG types specific to TEM-1 were detected, and *in vitro* neutralization of the β-lactamase activity was observed by mixing purified TEM-1 with sera. In contrast, specific anti-STa IgG and IgG1 antibodies were only detected at non-significant levels, and no IgG2 were detected in the sera of the immunized cattle. These results suggest that the response of the bovine immune system is different from that observed in mice [9, 15]. The use of a series of other adjuvants to immunize cattle needs to be investigated.

5.2. Production of Neutralizing Antibodies against Difficult-to-Express Antigens. As discussed above, BlaP can be used as a scaffold to produce and to purify the chitin binding domain from chitotriosidase (ChBD) [13]. The purified ChBD has been used to immunize a rabbit (Figure 6). The resulting serum contains antibodies that are able to bind to the free ChBD, the hybrid BlaP-*Thb/ChBD/Thb*, and the native human macrophage chitotriosidase. Moreover, these anti-ChBD antibodies are able to prevent the interaction of ChBD with chitin [13].

Taken all together, the results of these studies indicate that both BlaP and TEM-1 are appropriate scaffolds for producing and presenting antigens and inducing the production of antibodies neutralizing biological properties of the inserted fragment (Figure 6).

6. BHPs as Model Proteins to Investigate Structure/Function Relationships

6.1. BlaP: A Scaffold to Create Protein Models to Study the Mechanism of Amyloid Fibril Formation by Polyglutamine Proteins. Ten progressive neurodegenerative disorders, referred to as polyglutamine (polyQ) diseases, and, including Huntington's disease and several spinocerebellar ataxias, are associated with ten unrelated proteins containing an expanded polyglutamine (polyQ) tract (i.e., a tract that is longer than a pathological polyglutamine threshold). PolyQ tracts are encoded by a repetition of an unstable CAG trinucleotide repeat in the corresponding genes [74–77]. The ten disease-associated proteins show no sequence or structural similarity apart from the expanded polyQ tract, which is located at a different positions in each protein. The polyQ tract appears therefore to be a critical determinant in polyQ diseases, and several lines of evidence suggest that it confers a gain of toxic function to the mutant proteins by triggering the aggregation of the proteins into amyloid fibrils [74, 75, 78–80]. Indeed, all polyQ diseases share a number of features which suggest a common physiopathological mechanism [75, 79–81]: (i) the existence of a polyQ threshold for the aggregation of polyQ proteins and the disease development, generally comprised between 35 and 45Q [82], (ii) the so-called anticipation phenomenon, which indicates that the longer the polyQ, the earlier and more severe the disease [76, 83–85], and (iii) the presence of intranuclear inclusion bodies, containing amyloid fibrils made of polyQ proteins, in neuronal cells [75, 76, 85, 86].

So far, there is no preventive or curative treatment for these pathologies and existing therapies only treat the symptoms (i.e., they alleviate the symptoms without modifying the course of the disease). In order to design curative and/or preventive treatments, it is crucial to better understand how polyQ tracts trigger protein aggregation and by which mechanism some of the aggregates formed are cytotoxic.

Although the presence of the expanded polyQ tract is the critical trigger factor of the aggregation phenomenon, a growing number of studies suggest that the non-polyQ regions can, however, modulate both the kinetics and the aggregation pathway of polyQ proteins [74, 78, 87–90]. The non-polyQ regions can protect from aggregation by (i) sterically hindering polyQ intermolecular interactions [91], (ii) restricting polyQ conformational changes which are required for fibril formation [89] and (iii) increasing the protein solubility [87, 91]. On the other hand, non-polyQ regions

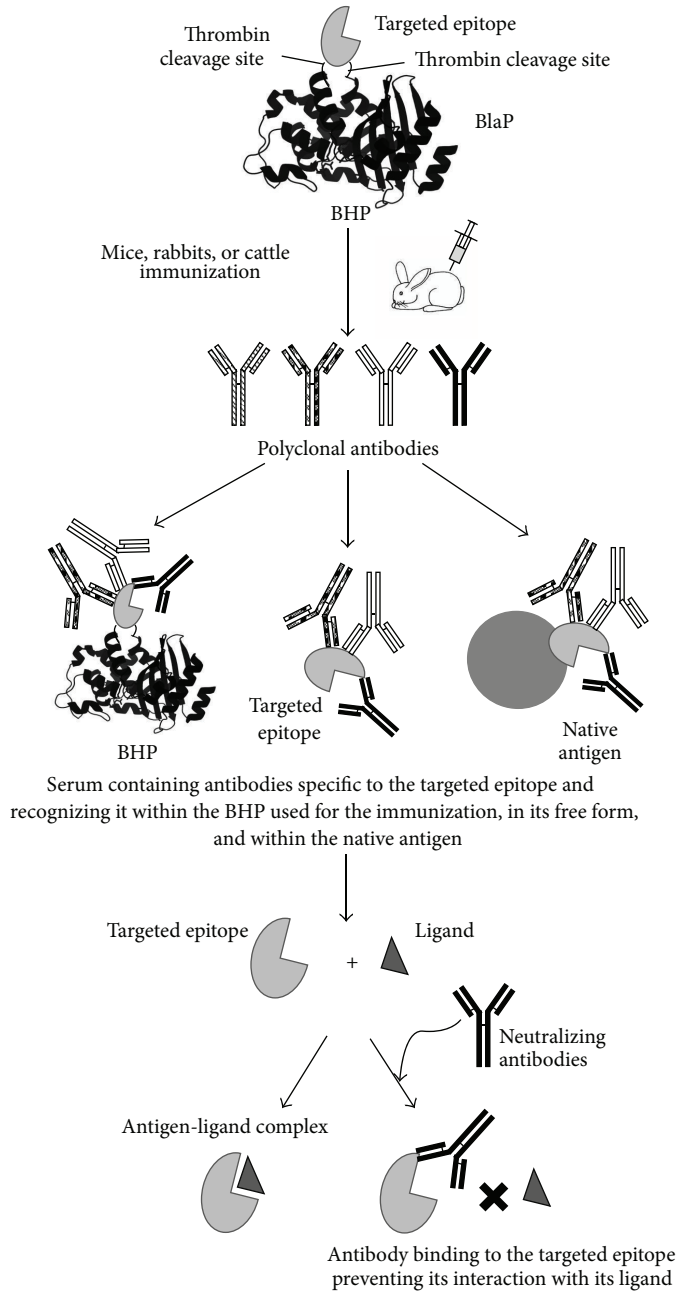

Figure 6: Schematic representation of the BHP technology used to design *de novo* antigens in order to induce the production of neutralizing antibodies. A specific region of a large antigen, that is known to promote the neutralization of the biological activity of the antigen or promote its opsonization, is inserted into BlaP. This approach can also be used to elicit the production of specific, neutralizing antibodies against polypeptides that are insoluble, difficult to express (i.e., ChBD, [13]), and poorly immunogenic (such as STa, [9]).

could assist aggregation by providing additional aggregation-prone domains [74, 92]. There is, therefore, a complex interplay between the tendency of the polyQ tract to trigger aggregation and the modulating effect of non-polyQ regions [16]. To better understand the general principles governing this complex phenomenon, it is crucial to investigate in detail which properties of the host protein (sequence, size, structure, and stability) influence the ability of polyQ tracts to mediate aggregation. The ten disease-associated proteins are difficult to produce recombinantly due to their large size and/or relative insolubility [74, 92–94]. Scarafone et al. tackled this problem by creating and characterizing model polyQ proteins consisting of the β-lactamase BlaP and polyQ tracts inserted at position 197 [16]. Significant amounts (i.e., 10–20 mg per liter of culture) of hybrid enzymes containing 23, 30, 55, and 79Q were successfully produced and purified. It is important to note that the longest polyQ tract that has been inserted in other model proteins is made of 55 residues [95].

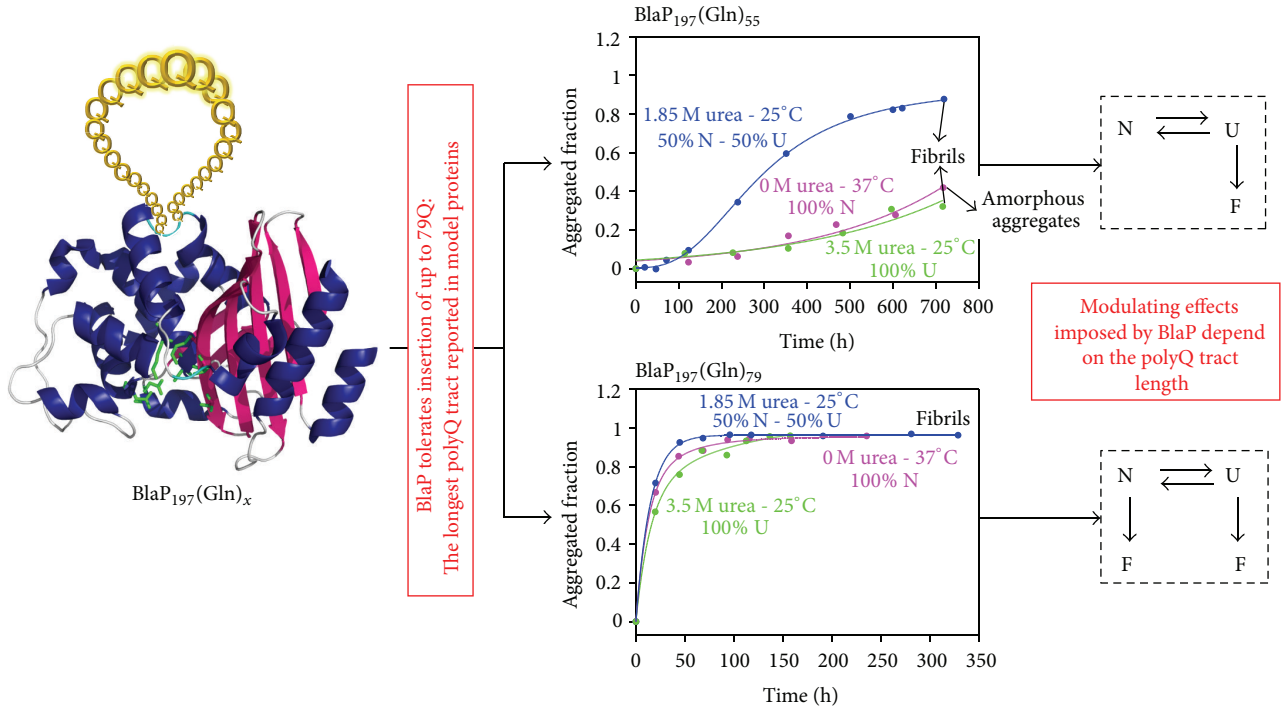

FIGURE 7: Model polyQ proteins using BlaP to investigate the amyloid fibril formation. The advantage of using BlaP as a scaffold to create polyQ proteins are highlighted in red. Left side panel: schematic representation of the polyQ-BlaP hybrid protein. Middle panel: kinetics of aggregation of BlaP$_{197}$(Gln)$_{55}$ and BlaP$_{197}$(Gln)$_{79}$ at 110 μM under the following conditions of incubation: (i) PBS, pH 7.5 and 0 M urea at 37°C (pink), (ii) PBS, pH 7.5, and 1.85 M urea at 25°C (blue) and (iii) PBS, pH 7.5, and 3.5 M urea at 25°C (green). BlaP$_{197}$(Gln)$_{55}$ does not form amyloid fibrils under native conditions, in contrast to BlaP$_{197}$(Gln)$_{79}$. Under native conditions, the threshold value is therefore comprised between 55 and 79Q. Under denaturing conditions, it is comprised between 30 and 55Q. Moreover, the kinetics of aggregation are faster with longer polyQ tracts. Right side panel: schematic pathway of aggregation for BlaP$_{197}$(Gln)$_{55}$ and BlaP$_{197}$(Gln)$_{79}$. N, native state; U, unfolded state; F, amyloid fibril. The native conformation of BlaP imposes constraints to the 55Q tract that prevent it to trigger the formation of amyloid fibrils. Figure adapted from Scarafone et al. [16].

BlaP is therefore a unique scaffold to investigate the effects of long inserted polyQ sequences (Figure 7).

The effects of the polyQ insertions on the activity, structure, and aggregation of BlaP were investigated using a range of biophysical techniques. The activity and the secondary and tertiary structures of BlaP are essentially not affected by the insertion of the polyQ tract as long as 79Q. The polyQ tract adopts a disordered structure at the surface of the protein irrespective of the number of glutamines. BlaP is significantly destabilized, however, by the insertion of the polyQ tract. Remarkably, the extent of destabilization is largely independent of the polyQ length (7.6–8.8 kJ·mol^{-1}; Table 1). This behaviour therefore makes it possible to investigate independently the role of (i) the length of the polyQ sequence and (ii) the conformational state of the β-lactamase moiety on the aggregating properties of the hybrid proteins. Accordingly, the aggregating properties of BlaP and of the different hybrid proteins were investigated under both native and denaturing conditions. The aggregation behaviour of BlaP-polyQ hybrid proteins recapitulates that of disease-associated polyQ proteins. Only hybrid proteins with 55Q and 79Q readily form amyloid fibrils; therefore, analogous to the proteins associated with diseases, there is a polyglutamine threshold required for the formation of amyloid fibrils. Moreover, above this threshold, the longer the polyQ, the faster the aggregation rate (Figure 7). Most importantly, the threshold-value critically depends on the structural integrity of BlaP. BlaP with 55Q forms fibrils under denaturing but not under native conditions (Figure 7). This means that the native conformation of BlaP imposes some conformational and/or steric constraints to the 55Q tract that inhibit fibril formation. On the other hand, the hybrid protein containing 79Q forms amyloid fibrils at similar rates whether BlaP is folded or not (Figure 7). These results therefore suggest that the influence of the protein context on the aggregation properties of polyQ disease-associated proteins could be negligible when the latter contain particularly long polyQ tracts [16].

Taken all together, these results indicate that BlaP is an appropriate host to study the aggregation of polyQ proteins. Its utility could be extended to the study of how other amyloidogenic peptides trigger protein aggregation.

6.2. Effect of the Structure of the Insert on BHP Stabilities. Vandevenne et al. [10] extensively investigated the effects of the insertion of the ChBD domain on the thermodynamic stability of BlaP, as indicated above. They observed that the insertion of this 72-residue folded domain at position 197 slightly destabilizes BlaP by 3.2 kJ·mol^{-1} (Table 1) [10].

This destabilization is significantly lower than that induced by the insertion of unstructured polyQ tracts of 23 to 79 residues, inserted into the same position (7.6–8.8 kJ·mol^{-1}, Table 1) [16]. This observation suggests that the insertion of unstructured polypeptides is more destabilizing than the insertion of folded polypeptides.

6.3. BHPs as Models to Screen Molecules Stabilizing Polypeptides. A growing number of peptide and protein drugs are utilized in therapy [96]. Unfortunately, because of unfavourable solubility, stability, and aggregation, their applications are sometimes difficult [96]. To circumvent this problem, low molecular weight additives, called cosolutes, have been developed, including cyclodextrins [96, 97]. Cyclodextrins are circular oligosaccharides containing a central cavity forming the resting site for hydrophobic molecules of an appropriate dimension [98]. They are generally used to prevent protein aggregation during the renaturation process [12, 96]. BCD07056 is a modified β-cyclodextrin, one of the most abundant classes of cyclodextrin [96].

Vandevenne et al. used the hybrid protein BlaP-ChBD, incubated under drastic conditions, to investigate the effects of BCD07056 on the stability of proteins [12]. They observed that BCD07056 does not affect the chitin binding of BlaP-ChBD, yet increases its β-lactamase activity. More interestingly, this β-cyclodextrin minimizes the inactivation of BlaP-ChBD upon storage at room temperature; its addition cannot, however, reverse the inactivation process. The β-cyclodextrin has a moderate effect on the thermal stability of BlaP-ChBD. However, its presence restores a cooperative reversible thermal unfolding, with a simple two-state transition, characteristic of BlaP without insert. This suggests that BCD07056 prevents the aggregation of BlaP-ChBD by interacting with the protein during the unfolding process. BCD07056 is therefore an effective additive to stabilize proteins during their storage and prevent their aggregation, without interfering with their activity [12]. It could be used to facilitate the application of a growing number of peptide and protein drugs in therapy [96].

7. Conclusion

Class A β-lactamase hybrid proteins, in which exogenous polypeptides of various sizes are inserted (up to 40 kDa), can be readily designed and used for multiple purposes, notably to produce difficult-to-express peptides/proteins/protein fragments, to map epitopes, to display antigens, and to study protein structure/function relationships. The wide ranging impact of the BHP approach essentially originates from (i) the efficient enzymatic activity that can be easily measured and that can serve as a reporter or factor for selection and (ii) the facility with which they can be recombinantly produced and purified. In addition to the numerous applications summarized in this review, many other applications can be envisaged: BHPs could be used as biosensors and in affinity chromatography, drug screening, and drug targeting. They are also of special interest to better understand more fundamental aspects of protein evolution and structure/function relationships.

Abbreviations

Ag: Antigen
BHPs: β-lactamase hybrid proteins
BlaP: β-lactamase from *Bacillus licheniformis* 749/C
CD22: Cluster of differentiation-22
ChBD: Chitin binding domain
dsbA: Disulfide bond isomerase A
ELISA: Enzyme-linked immunosorbent assay
ETEC: Enterotoxic *E. coli*
GFP: Green fluorescent protein
HA1: Virus influenza hemagglutinin
H/D: Hydrogen/deuterium
LT: Thermolabile enterotoxin
mab: Monoclonal antibody
MalE: Maltodextrin-binding protein
NMR: Nuclear magnetic resonance
polyQ: Polyglutamine
PSA: Prostate specific antigen
Thb: thrombin.

Acknowledgments

The authors would like to acknowledge the Fonds de la Recherche Scientifique (1.C039.09, FRFC 24.58.112.F and MIS-F.4505.11), the Belgian program of Interuniversity Attraction Poles administered by the Federal Office for Scientific Technical and Cultural Affairs (PAI nos. P6/19 and P7/44), the University of Liege (FSRC-11/108), the Walloon region and the funds from DGO6 for their support of those parts of their own research that are described in this review. We would like to thank Dr. David Thorn and Coralie Pain for their critical reading of the paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 496586, 12 pages
http://dx.doi.org/10.1155/2013/496586

Research Article

Production, Characterization and Antioxidant Potential of Protease from *Streptomyces* sp. MAB18 Using Poultry Wastes

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Received 29 April 2013; Revised 20 June 2013; Accepted 8 July 2013

Academic Editor: Periasamy Anbu

Poultry waste is an abundant renewable source for the recovery of several value-added metabolites with potential industrial applications. This study describes the production of protease on poultry waste, with the subsequent use of the same poultry waste for the extraction of antioxidants. An extracellular protease-producing strain was isolated from Cuddalore coast, India, and identified as *Streptomyces* sp. MAB18. Its protease was purified 17.13-fold with 21.62% yield with a specific activity of 2398.36 U/mg and the molecular weight was estimated as 43 kDa. The enzyme was optimally active at pH 8–10 and temperature 50–60°C and it was most stable up to pH 12 and 6–12% of NaCl concentration. The enzyme activity was reduced when treated with Hg^{2+}, Pb^{2+}, and SDS and stimulated by Fe^{2+}, Mg^{2+}, Triton X-100, DMSO (dimethyl sulfoxide), sodium sulphite, and β-mercaptoethanol. Furthermore, the antioxidant activities of protease were evaluated using *in vitro* antioxidant assays, such as DPPH radical-scavenging activity, O_2 scavenging activity, NO scavenging activity, Fe^{2+} chelating activity, and reducing power. The enzyme showed important antioxidant potential with an IC_{50} value of 78 ± 0.28 mg/mL. Results of the present study indicate that the poultry waste-derived protease may be useful as supplementary protein and antioxidant in the animal feed formulations.

1. Introduction

Feather is composed of over 90% protein, the main component being keratin, a fibrous and insoluble protein highly cross-linked with disulphide and other bonds. In mature chicken, feather accounts up to 5–7% of the live weight. Worldwide, several million tons of feather is generated annually as waste by poultry-processing industries. Considering its high protein content, this waste could serve as a good source of protein and amino acids for animal feed and for many other applications. However, because of the insoluble nature of keratin and its resistance to enzymatic digestion by animal, plant, and many known microbial proteases, use of feather as a source of value-added products has been very limited.

Thermophilic actinobacteria produce many degradative enzymes [1] and can play a major role in the biodegradation of keratinaceous waste materials [2]. Biodegradation of feathers by microorganisms represents a method for improving the utilization of feathers as a feed protein [3] and amino acids as pure chemicals [4]. Feather may also find an important application in the fermentation industry for the production of commercial enzymes.

Several studies have been made on the proteolytic enzymes of mesophilic actinobacteria [5]. In contrast, relatively little work of a similar nature has been published on alkaline protease-producing actinobacteria. In the present study, an attempt has been made to optimize the culture conditions of *Streptomyces* sp. MAB18 for protease production

using poultry wastes. In addition, protease from *Streptomyces* sp. MAB18 was purified and characterized, and the antioxidant activity of the culture supernatant was analyzed.

2. Material and Methods

2.1. Materials. Chicken feathers (whole feather) were collected immediately after slaughtering of the chickens and extensively washed with tap water until the effluent became very clear and finally with distilled water. The washed feathers were dried under sunlight and then further dried at 60°C for 48 h. After drying, the large feather stocks were cut by hand into smaller pieces to fit to the culture flask. They were stored at 4°C until used [6]. Standard proteins and tyrosine were purchased from Sigma-Aldrich, India. Other reagents were from Merck (Germany). All other chemicals and bacteriological media were from standard sources.

2.2. Isolation and Screening of Marine Actinobacteria. A marine actinobacterium *Streptomyces* sp. MAB18 was isolated from the marine sediments of Cuddalore coast (lat 11°42′ N, long 79°52′ E), India, and screened for protease production on gelatin agar medium (gelatin, 10 g; peptone, 5 g; beef extract, 5.0 g; agar, 20.0 g; and pH 8.0), and incubated at 50°C. After incubation, clear zones developed around the colony were considered positive for protease activity. The selected strain was grown in liquid medium prepared as above but in which gelatin was substituted by 10 g/L chicken feather. The cultures were incubated at 50°C with rotary shaking and solubilisation of the feather was observed visually. The level of protease production was checked from the culture supernatant obtained after centrifugation [7].

2.3. Taxonomic Studies and 16S rDNA Sequencing. The selected strain was identified according to Bergey's Manual of Determinative Bacteriology (1974) and the keys proposed [8].

A molecular identification of the isolate was achieved by 16S rDNA sequencing. DNA extraction was performed by the CTAB method [9]. The primer sequences were chosen from the conserved regions previously reported for the bacterial 16S rDNA [10]. Sequencing was done using forward primer (F 5′-CAGGCCTAACACATGCAAGTC-3′) and reverse primer (R 5′-GGGCGGTGTGTACAAGGC-3′). PCR reactions were performed with the following program for the 16S rRNA gene: 30 cycles consisting of 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, followed by a final extension step of 5 min at 72°C. The 16S rDNA sequence was analyzed by an automated DNA sequencer (Applied Biosystems). The sequence was analyzed for homology using CLUSTAL X package [11].

2.4. Protease Production. Whole-feather medium (WFM), which contained whole-chicken feather (WCF) 10 g, peptone 5 g, beef extract 5 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2$ 0.5 g, Na_2CO_3 5 g, NaCl 5 g, and pH 8.0 was used for protease production. Medium (100 mL) was dispensed in 500 mL Erlenmeyer flask and sterilized at 110°C for 20 min. Each flask was inoculated with 1 mL of 48-hours-old seed culture (8×10^9 CFU/mL), prepared in the same medium, and incubated at 55°C, 200 rpm for 7 day. Culture broth was centrifuged at 10000 ×g for 10 min and the supernatant was used as an enzyme [12].

2.5. Enzyme Assays

2.5.1. Protease Activity. Protease activity was determined with a modification of the method described by Cheng et al. [13]. Reaction mixture (2 mL) containing 1 mL of casein 1% (w/v) (dissolved in 25 mM glycine NaOH buffer pH (10.0)) and 0.95 mL of glycine NaOH buffer was preincubated at 50°C. The reaction was initiated by the addition of 0.05 mL of suitably diluted enzyme solution and kept at 50°C for 20 min reaction; 2 mL of trichloroacetic acid 10% (w/v) was added to terminate the reaction and the mixture was allowed to stand at room temperature for 1 h. The reaction mixture was centrifuged at 10000 ×g for 10 min and the absorbance of the supernatant was determined at 280 nm. Protease (1 U) activity was defined on the amount of enzyme required to liberate 1 μg of tyrosine per minute under experimental conditions [14].

2.5.2. Keratinase Activity. Keratinase activity was assayed by the modified method of Plackett and Burman [15]. The mixture of 10 mg of feather powder suspended in 1 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM $CaCl_2$ and 1 mL of culture filtrate was incubated at 35°C with shaking at 125 rpm for 15 min in a water bath shaker. This elevated temperature was used for the enzyme incubation to accelerate substrate hydrolysis. The enzyme reaction was terminated by adding 2 mL of trichloroacetic acid (TCA) solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid) into the reaction mixture. The mixture was then centrifuged at 10 000 ×g, 4°C for 30 min and the absorbance of the supernatant was spectrophotometrically measured at the wavelength of 275 nm (UV-1800, Shimadzu scientific instruments, USA). The enzyme inactivated by TCA solution was used as a control. One unit (U) of keratinase activity has been expressed as 1 μmol of tyrosine released per minute under the specific conditions [13].

2.6. Optimization of Medium Components. Medium optimization was carried out by statistical approaches. Physical parameters such as pH, agitation, and nutritional parameters, that is, carbon and nitrogen sources, were first standardized by one-variable-at-a-time method. Following this, the Plackett-Burman (PB) design and response surface methodology (RSM) were used to optimize the medium composition for maximum protease production.

2.6.1. Selection of the Most Suitable Carbon and Nitrogen Sources by One-Variable-at-a-Time Approach. Initial screening of the most significant carbon and nitrogen sources allowing the maximum protease production was performed by the one-variable at-a-time approach. To check the effect of various carbon and nitrogen sources on protease production,

media were supplemented with 1% (w/v) of different carbon and nitrogen sources. The flasks were inoculated with 2% inoculum and incubated in a shaking incubator with a shaking speed of 180 rpm at 55°C for 7 days. Samples were collected every 12 h and assayed for growth as well as enzyme production.

2.6.2. Plackett-Burman Design (PB Design). Important medium components with respect to their main effects were screened by the Plackett-Burman design with a two-factorial design. It identifies the main physico-chemical parameters required for maximal protease production by screening n variables in $n + 1$ experiments; each variable was examined at two levels [15]. Table S1A see (Supplementary Matrial available online at http://dx.doi.org/10.1155/2013/496586) lists the factors under investigation as well as the levels of each factor used in the experimental design with the symbol code and actual level of the variables. "Design expert software" (Minitab package version 16.0) was used to analyse the experimental Plackett-Burman design.

2.6.3. Optimization of Key Ingredients by Central Composite Design (CCD). Levels of four significant factors and the interaction effects between various medium constituents which influence the protease production significantly were analysed and optimized by the response surface methodology, using a CCD design. The significant factors utilized were whole-chicken feather (WCF), peptone, NaCl, and Na_2CO_3. In this study, the experimental plan consisted of 27 trials and the independent variables were studied at three different levels: low, middle, and high as shown in Table S2A. All the experiments were done in duplicate and the average protease production obtained was taken as the dependent variable or response (Y).

2.6.4. Statistical Analysis and Modeling. Data obtained from RSM on protease production were subjected to analysis of variance (ANOVA). The experimental results of RSM were fitted via the response surface regression procedure, using the following second-order polynomial equation:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j, \quad (1)$$

where Y_i is the predicted response, $X_i X_j$ are independent variables, β_0 is the offset term, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient, and β_{ij} is the ijth interaction coefficient. However, in this study, the independent variables were coded as X_1, X_2, X_3, and X_4. Thus, the second-order polynomial equation can be presented as follows:

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 \\ & + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \\ & + \beta_{44} X_4^2 + \beta_{12}\ X_1 X_2 + \beta_{13} X_1 X_3 \\ & + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4. \end{aligned} \quad (2)$$

The statistical software package Minitab package version 16.0 was used for the regression analysis of the experimental data and also to plot the response surface graphs. The statistical significance of the model equation and the model terms was evaluated via Fisher's test. The quality of fit the second-order polynomial model equation was expressed via the coefficient of determination (R^2) and the adjusted R^2. The fitted polynomial equation was then expressed in the form of three-dimensional surface plots, in order to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study. The point optimization method was employed in order to optimize the level of each variable for maximum response. The combination of different optimized variables, which yielded the maximum response, was determined in an attempt to verify the validity of the model.

2.7. Time Course of Protease Production. The kinetics of protease production were followed in batch cultures at optimum conditions. The experiment was designed for 7 days starting from the log phase to stationary phase under submerged culture conditions. The resultant cell free supernatant was removed by filtration followed by cold centrifugation at 10 000 ×g at 4°C for 20 min. The supernatant was analyzed for protease production.

2.8. Purification of Protease Enzyme. For purification of protease, ammonium sulphate was added to the culture supernatant to obtain 60% saturation (w/v) and allowed to stand overnight at 4°C. The precipitate collected through centrifugation at 10 000 ×g for 15 min was dissolved in 50 mM Tris-HCl buffer (pH-8) and dialyzed against the same buffer (4°C). The dialysate was loaded on DEAE-Cellulose column (5 × 25 cm) and eluted with linear gradient of NaCl (0–1.0 M) at a flow rate of 0.5 mL/min. Fractions were collected and assayed for enzyme activity and fractions which exhibited enzyme activity were pooled together and concentrated by ammonium sulphate precipitation. The resultant precipitate was collected by centrifugation and dissolved in 50 mM Tris-HCl buffer (pH-8.0). Concentrated fractions were loaded onto a Sephadex G-50 column (2.5 × 25 cm) equilibrated with 50 mM Tris-HCl buffer (pH-8) and eluted with the same buffer at a flow rate of 15 mL/h. Fractions exhibiting protease activity were pooled together and used as a purified enzyme for further characterization study.

2.9. Determination of Protein. Protein concentration of the protease in supernatant was determined by the method, using bovine serum albumin as the standard [16].

2.10. Molecular Weight Determination in SDS-PAGE. SDS-PAGE (10%) was performed as described, under reducing conditions. The molecular weight was determined by interpolation from a linear semilogarithmic plot of relative molecular weight versus the relative mobility, using broad-range standard molecular weight markers (29, 43, 66, 97, and 200 kDa) [17].

2.11. Effect of pH on Activity and Stability of Protease. The optimum pH of alkaline protease was determined with casein 1% (w/v) as substrate dissolved in different buffers (citrate phosphate, pH 5-6, sodium phosphate, pH 7.0, Tris-HCl, pH 8.0, and glycine NaOH, pH 9–13). The pH stability of alkaline protease was determined by preincubating enzyme in different buffers for 10 h at 50°C.

2.12. Effect of Temperature and NaCl on the Enzyme Activity and Stability. Effect of temperature on the enzyme activity was determined by incubating the reaction mixture (enzyme + substrate) at different temperatures (30–75°C). To determine the temperature stability, the purified enzyme was preincubated at different temperatures (30–70°C), for 1 h and then residual activity (%) was assayed under standard assay conditions. Effect of NaCl on the enzyme activity was studied by varying the concentrations of NaCl% (w/v). Enzyme activity has been expressed as percentage relative activity.

2.13. Effect of Inhibitors and Surfactants. The effect of inhibitors and surfactants on enzyme activity took place under standard enzyme assay conditions where the assay cocktail was supplemented with phenylmethylsulfonyl fluoride (PMSF), (10 mM), EDTA (1 mM), cystine (1 mM), SDS (0.1%), Tween-80 (0.1%), and Triton X-100 (0.1%). The effect was assessed by comparing with the control.

2.14. Effect of Metallic Salts and Inhibitors on Protease Activity. The enzyme was preincubated for 1 h in concentrations (1, 3, 5, and 10 mM) of different metallic salts ($FeCl_2$, $HgCl_2$, $MgCl_2$, $AgNO_3$, $CuCl_2$, $PbCl_2$, $CaCl_2$, $NiCl_2$, and $MnCl_2$) and then the residual activity (%) was measured under optimum conditions. From this, the inhibitory effect of various compounds on the enzyme and the nature of the alkaline protease were determined.

2.15. Detergent Stability. Stability of the protease in commercial detergents were tested by incubating measured quantity of the enzyme (500 μL) with the solutions of the different commercial detergents at a detergent concentration of 7 mg/mL (to simulate washing conditions) [18] for 1 h. The detergents tested were Ariel, Tide (Procter and Gamble Ltd.), Rin, Surf excel (Hindustan Lever Ltd.), and Henko (SPIC India Ltd.), which are widely used in India. Suitable aliquots were withdrawn at different time intervals (at 15, 30 and 60 min), for 1 h, and the residual activity was measured by standard assay procedure and compared with the control (incubated under similar conditions, without any detergent) and the relative activity has been expressed in % taking the value given by control as 100%. Also the detergent solutions in the same concentration used for this study, but without incubating with the enzyme, were assayed for protease activity to rule out the possibility of any protease (if at all present) contained as ingredient of the detergent [19, 20].

2.16. Antioxidant Activity

2.16.1. DPPH Radical-Scavenging Assay. DPPH radical-scavenging activity of the hydrolysates was determined as described [21]. A volume of 500 μL of each sample at different concentrations was mixed with 500 μL of 99.5% ethanol and 125 μL of 0.02% DPPH in 99.5% ethanol. The mixture was then kept at room temperature in the dark for 60 min, and the reduction of DPPH radical was measured at 517 nm using a UV-visible spectrophotometer. The percentage inhibition of the DPPH radical (scavenging activity) was calculated according to the following formula:

$$\text{Scavenging effect } (\%) = \frac{A_c - A_s}{A_c} \times 100, \quad (3)$$

where A_c is the absorbance of the control reaction and A_s is the absorbance of the sample extract. Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against protease concentration. A lower absorbance of the reaction mixture indicated a higher DPPH radical-scavenging activity. Butylated hydroxyanisole (BHA) was used as a standard. The test was carried out in triplicate.

2.16.2. Assay of Superoxide Radical-(O_2^-)-Scavenging Activity. The assay was based on the capacity of the antioxidant to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system [22]. The method used by Martinez et al. [23] for determination of superoxide dismutase was followed after modifications [23]. Each 3 mL of reaction mixture contained 50 mM sodium phosphate buffer, pH 8.0, 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, NBT (75 μM), and 1 mL of the protease of different concentrations. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after a 10 min illumination from a fluorescent lamp. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture were kept in the dark and served as blanks. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$(\%)\ \text{Inhibition} = \frac{A_c - A_s}{A_c} \times 100, \quad (4)$$

where A_c is the absorbance of the control and A_s is the absorbance of the protease.

2.16.3. Assay of Nitric Oxide-Scavenging Activity. The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using the Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of protease dissolved in water and incubated at room temperature for 150 min.

The same reaction mixture, without the protease but with an equivalent amount of water, served as a control. After the incubation period, 0.5 mL of the Griess reagent (1% sulfanilamide, 2% H_3PO_4, and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Ascorbic acid was used as a positive control [24].

2.16.4. Reducing Power Determination. The reducing power of protease was determined according to the method [25]. Different amounts of protease in water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 8.0) and potassium ferricyanide ($K_3Fe(CN)_6$) (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.16.5. Metal Chelating Activity. Chelating property of ferrous ions by the protease was estimated using the standard method [26]. Briefly, the protease was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL); the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. Percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $((A_c - A_s)/A_c) \times 100$, where A_c was the absorbance of the control and A_s was the absorbance of the protease/standard.

2.17. Data Handling. Results have been expressed as means ± standard deviations of four replicated determinations. Minitab software (Minitab package version 16.0, Inc., USA) was used for data analysis.

3. Results and Discussion

3.1. Isolation, Identification, and Enzyme Production of a Marine Actinobacterium. A marine actinobacterial strain MAB18 strain was isolated from the marine sediments of Cuddalore coast, India. It formed a clear zone on gelatin agar plates around the colony. Figure 1(a) indicates that the MAB18 strain has produced the most pronounced clearing zone. This isolate was pursued for identification, protease enzyme production, and the ability to degrade feathers. The degradation of whole-chicken feather by *Streptomyces* sp. MAB18 is shown in Figure 1(b). Degradation was observed after incubation at 50°C for 5 days. Mohamedin [7] reported that *Streptomyces* degraded whole intact chicken feather at 50°C [7].

The strain MAB18 showed the presence of LL-diaminopimelic acid and glycine in the cell wall and there was no characteristic sugar pattern and hence the strain belonged to the cell wall type I. Its aerial mycelium was grey. Sporophores were spiral; hooks and loops were also formed (Figure 1(d)). Conidia were oblong, the surface of which was smooth (Figure 1(e)). This strain was identified as *Streptomyces* sp. MAB18, based on its morphological, physiological, and biochemical characteristics and it was confirmed by the 16S rDNA sequencing (Figure 1(c)). The sequence was submitted to Gene Bank in NCBI (http://www.ncbi.nlm.nih.gov/nuccore/JQ068140.1) with the accession number JQ068140.

3.2. Optimization of Protease Production

3.2.1. Selection of Physicochemical Parameters, Carbon, and Nitrogen Sources by One-Variable-at-a-Time Approach. Effect of carbon and nitrogen sources on enzyme yield is shown in Table S3A. *Streptomyces* sp. MAB18 produced higher amount of protease in the presence of glucose, maltose, and sucrose. However, production of protease was higher (165 U/mL) in the medium which contained glucose than that with maltose (145.03 U/mL) and sucrose (123.57 U/mL). Nitrogen sources including casein, yeast extract, soybean meal, and peptone significantly influenced the enzyme production. Among these, casein proved to be a good nitrogen source for stimulating protease production (156.80 U/mL). Among the various carbon and nitrogen sources tested, glucose and casein were found to be the most suitable substrates for protease production. Thus, these substrates were selected for further optimization steps. The highest protease production was observed in glucose and casein, which is in conformity with an earlier study [12].

3.2.2. Screening of Parameters Using the Plackett-Burman Design. Experiment was conducted in 12 runs to study the effect of the selected variables. Table S1A represents the results of the screening experiments using the Plackett-Burman design. Statistical analysis of the responses was performed which is represented in Table S1B. The model *F* value of 3790.63 implies that the model is significant. Values of *Prob* < 0.05 indicate that the model terms are significant.

Magnitude of the effects indicates the level of the significance of the variables on protease production. Among the variables of screened whole-chicken feather (WCF), peptone, beef extract, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, Na_2CO_3, and NaCl were identified as the most significant variables, influencing protease production (Table S1C).

3.2.3. Optimization of Significant Variables Using Response Surface Methodology (RSM). Experiments conducted in the present study were targeted towards the construction of a quadratic model consisting of twenty-seven trials. The design matrix and the corresponding results of RSM experiments to determine the effects of four independent variables (whole-chicken feather (WCF), peptone, NaCl, and Na_2CO_3) are shown in Table S2A, along with the mean predicted values. The regression analysis of the optimization study indicated that the model terms, X_1, X_2, X_3, X_4, X_1^2, X_2^2, X_3^2, X_4^2, X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4 and X_3X_4, were significant ($P < 0.05$). These results indicate that the concentration of the whole-chicken feather (WCF), peptone, NaCl, and Na_2CO_3

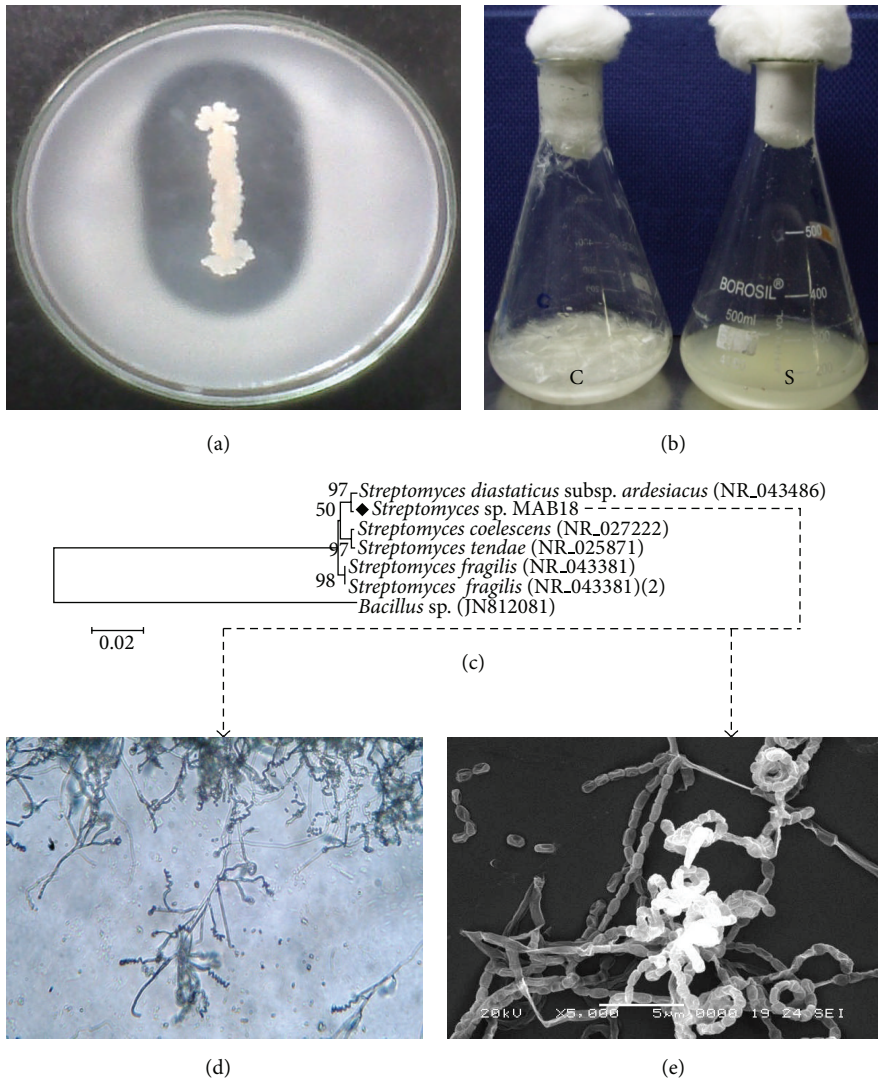

Figure 1: (a) Screening of protease enzyme on gelatin agar medium. (b) Feather degradation by *Streptomyces* sp. MAB18 in liquid media with the whole intact feather as sole carbon and nitrogen source. C: uninoculated medium (control), clear with intact feathers. S: complete hydrolysis of feathers inoculated with MAB18. (c) Phylogenetic tree of the 16S rDNA sequence of strain MAB18 and related strains. (d) Micromorphology of spore chains of the *Streptomyces* sp. MAB18 (×400). (e) Scanning electron micrograph of spores of the *Streptomyces* sp. MAB18 (×5000).

bears a direct relationship with protease production. The interactions between whole-chicken feather (WCF), peptone, NaCl and Na_2CO_3 were significant, as shown by the low P values $P < 0.0001$ and $P < 0.0002$, respectively. Analysis of variance (ANOVA) (Table S2B) depicts the P values for the model ($P < 0.0001$) and for lack of fit also suggested that the obtained experimental data are a good fit with the model.

Regression equation coefficients were calculated and the data were fitted to a second-order polynomial equation. The response, protease (Y) by *Streptomyces* sp. MAB18, can be expressed in terms of the following regression equation:

$$\begin{aligned} Y = {} & 25.6972 + 3.7770X_1 + 10.2709X_2 \\ & + 10.8862X_3 + 11.3939X_4 - 0.3154X_1^2 \\ & - 1.6146X_2^2 - 1.6599X_3^2 - 1.5509X_4^2 \\ & + 0.1756X_1X_2 + 0.0527X_1X_3 - 0.1130X_1X_4 \\ & - 0.0051X_2X_3 - 0.0940X_2X_4 + 0.0233X_3X_4, \end{aligned} \quad (5)$$

where whole chicken feather (WCF) (X_1), peptone (X_2), NaCl (X_3), Na_2CO_3 (X_4), whole chicken feather∗whole chicken feather (X_1^2), peptone∗peptone (X_2^2), NaCl∗NaCl (X_3^2), Na_2CO_3∗Na_2CO_3 (X_4^2), whole chicken feather*peptone (X_1X_2), whole chicken feather∗NaCl (X_1X_3), whole chicken feather∗Na_2CO_3 (X_1X_4), peptone∗NaCl (X_2X_3), peptone∗Na_2CO_3 (X_2X_4), and NaCl∗Na_2CO_3 (X_3X_4).

Regression equation obtained from the ANOVA showed that the R^2 (multiple correlation coefficient) was 0.999 5 (a value >0.80 indicates fitness of the model). This is an estimate of the fraction of overall variation in the data accounted by the model, and thus the model is capable of explaining 99.95% of the variation in response. The "adjusted R^2" was 0.998 8 and the "predicted R^2" was 0.996 8, and this indicates that the model is good.

In order to determine the optimal levels of each variable for maximum protease production, three-dimensional response surface plots were constructed by plotting the response (protease production) on the z-axis against any two independent variables, while maintaining other variables at their central levels (Figure S1). Maximum protease production was obtained at the middle level of each pair of factors at a constant middle level of the other factor. Further increase in these factors above the middle level showed a decrease in protease production. In order to determine the maximum protease production corresponding to the optimum levels of whole-chicken feather, peptone, NaCl, and Na_2CO_3, a second-order polynomial model was used to calculate the values of these variables. Fitting of the experimental data to equation allowed determination of the levels of whole-chicken feather (X_1 = 6.565 6 g), peptone (X_2 = 3.364 3 g), NaCl (X_3 = 3.400 7 g), and Na_2CO_3 (X_4 = 3.437 2 g), giving a maximum protease concentration of 92.37 U/mL in shake flask culture.

Fermentation was performed using 100 mL synthetic medium containing the optimized level of whole chicken feather (6.565 6 g), peptone (3.364 3 g), NaCl (3.400 7 g) and Na_2CO_3 (3.437 2). Maximum protease production (94.84 U/mL) was obtained, which was slightly higher than the value given by the model.

3.3. Time Course of Protease Production. The time course was studied up to 180 hrs in optimized medium and maximum enzyme production of 765 U/mL was achieved in 120 hrs of incubation (Figure 2(a)). It was observed that the enzyme production was built up slowly during the exponential phase and it attained the maximum at the onset of the stationary phase, thus lending support to earlier study [27].

3.4. Purification of Protease. Protease from the culture broth of *Streptomyces* sp. MAB18 was purified through multistep purification and summary of the purification profile is presented in Table S3B. The overall purification fold of protease was about 17.13 with the specific activity of 2398.36 U/mg and 21.62% yield. Homogeneity of the purified enzyme was analyzed and confirmed by the single band obtained in SDS-PAGE. Molecular weight of the purified protease was estimated as 43 kDa (Figure 2(b)) and it is worth mentioning here that different molecular masses of protease ranging from 23 to 24 kDa have been reported for *Nesterenkonia* sp. AL-20 and *Bacillus pseudofirmus* Al-89 [12].

3.5. Effect of Temperature, pH, and NaCl on Purified Enzyme. Optimum temperature and pH for the protease activity of *Streptomyces* sp. MAB18 were 50–60°C and 8–10, respectively (Figure 3(a)). Thermostability study showed that the enzyme was 100% stable up to 60°C for 1 h, 89% at 65°C for 1 h and 76% at 70°C for 1 h, and it appears that the enzyme of *Streptomyces* sp. MAB18 is considerably more stable than the other *Streptomyces* sp. protease reported to date [7, 28], as most of the other enzymes were stable only up to 30–60°C. Likewise, the enzyme from *Streptomyces* sp. MAB18 was stable up to pH 12.0; it retained 100% activity at pH 9–11 and 75% activity even after 1 h incubation at 70°C and pH 12.0 (Figure 3(a)). Previously, researchers have reported that protease was stable only between pH 4 and 11.0 [7, 28].

Protease from *Streptomyces* sp. MAB18 showed activity over a broad range of NaCl concentration (0–10%) and the optimal concentration was 6% (Figure 3(a)). The enzyme retained a good 89% activity in 6% NaCl concentration, but the enzyme activity decreased when the NaCl concentration increased from 8 to 10%. At 10% NaCl concentration, the enzyme retained 30% of its original activity after 12 h incubation (Figure 3(a)). Even recent reports reveal that the protease activity with an optimal 5% NaCl tolerance was shown by *Streptomyces clavuligerus* strain Mit-1 and *Nocardiopsis kunsanensis* sp. [29, 30]. Thus, the high salt tolerance shown by the MAB18 enzyme will have a number of applications in the biotechnological processes that depend on higher salinity or osmotic pressure for long periods of incubation including the potential applications in treating the agricultural wastes, bioremediation of keratin materials for sustainable bio-based production, and bioenergy production.

3.6. Effect of Metal Ions and Chemicals on Enzyme Activity. Effect of various metal ions on the protease activity of the purified protease enzyme is shown in Table S4. Activity of the protease was strongly inhibited by Hg^{2+} and Pb^{2+} in 1–10 mM concentrations. When treated with Fe^{2+} and Mg^{2+}, the enzyme activity was stimulated, as reported earlier [31]. Ag^{2+}, Ca^{2+}, and Mn^{2+} slightly inhibited the enzyme activity. The stimulatory effect of Fe^{2+} and Mg^{2+} at 1–10 mM concentrations on protease suggests that these metals can act as cofactors in increasing the enzyme activity.

Presence of Triton X-100, DMSO, sodium sulphite, and β-mercaptoethanol also stimulated the protease activity. SDS, 1,10-phenanthroline, and EDTA strongly inhibited the enzyme activity and Tween-80 and isopropanol slightly inhibited the activity (Table S4). Jain et al. [19] also reported that β-mercaptoethanol enhanced the enzyme activity and EDTA strongly inhibited the activity in *Bacillus* sp. [19].

3.7. Compatibility of Protease with Commercial Laundry Detergents. *Streptomyces* sp. MAB18 protease showed good stability with the commercial detergents tested (Figure 3(b)). It retained 64.2% residual activity after 1 h of incubation in Ariel, 76.2% in Tide, 120.4% in Rin, 131.3% in Surf excel and 54.4% in Henko. After 15 min, it retained 83.9%, 89.6%, 125.9%, 145.3% and 67.3%, respectively. The maximum stability was observed with Surf excel and Rin. Similar trend has been reported by Mukherjee et al. [20].

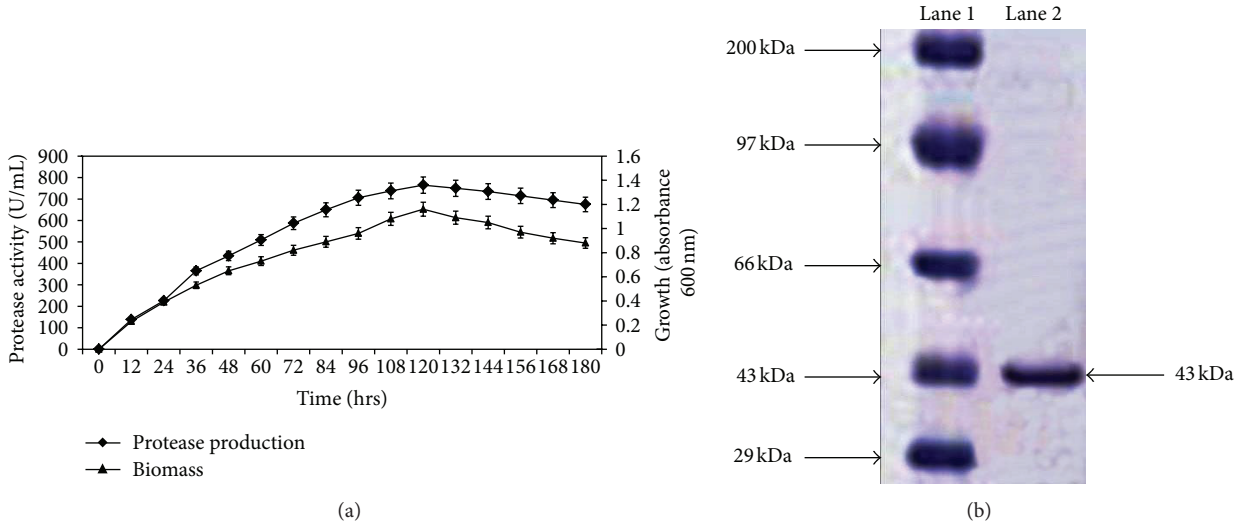

FIGURE 2: (a) Time course of protease production from *Streptomyces* sp. MAB18. (b) SDS-PAGE analysis of protease from *Streptomyces* sp. MAB18. Lane 1, molecular markers (29–200 kDa); lane 2, purified enzyme.

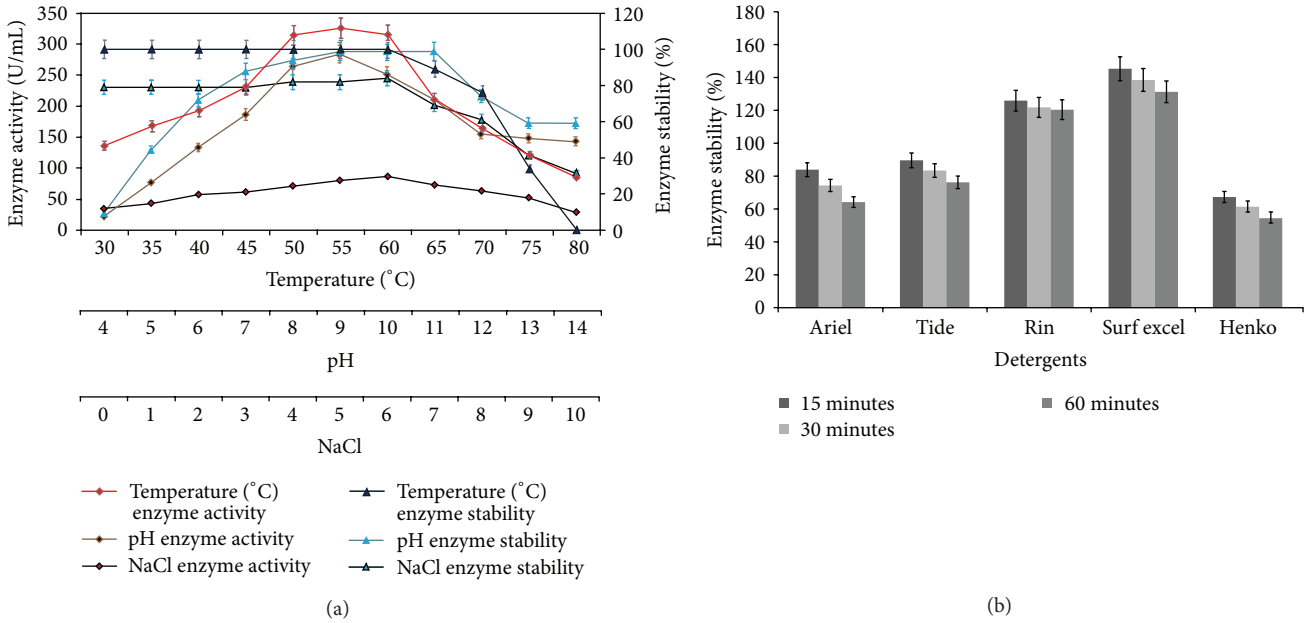

FIGURE 3: (a) Effect of temperature, pH and sodium chloride concentrations on enzyme activity and stability of purified protease from *Streptomyces* sp. MAB18. The values are mean ± SD, $n = 3$. Absence of bars indicates that errors were smaller than symbols. (b) Detergents stability and compatibility of the protease *Streptomyces* sp. MAB18 in commercial detergents. The values are mean ± SD, $n = 3$.

3.8. Antioxidant Activity of Protease. Protease was assayed for its antioxidant activity using DPPH radical-scavenging activity, O_2-scavenging activity, NO-scavenging activity, Fe^{2+} chelating activity, and reducing power.

3.8.1. DPPH Free Radical-Scavenging Activity. DPPH is a stable free radical that shows maximum absorbance at 517 nm. When DPPH radicals encounter a proton-donating substrate such as an antioxidant, the radicals would be scavenged and the absorbance would be reduced [32]. The decrease in absorbance is taken as a measure for radical-scavenging activity. The DPPH radical-scavenging activity was investigated at different concentrations (0–3.5 mg/mL) of the protease. The results presented in Figure 4(a) clearly show that the protease exhibited an interesting radicals scavenging activity with an IC_{50} value of 78 ± 0.28 mg/mL.

3.8.2. Assay of Superoxide Radical (O_2^-)-Scavenging Activity. Figure 4(b) shows the superoxide radical-(O_2^-)-scavenging

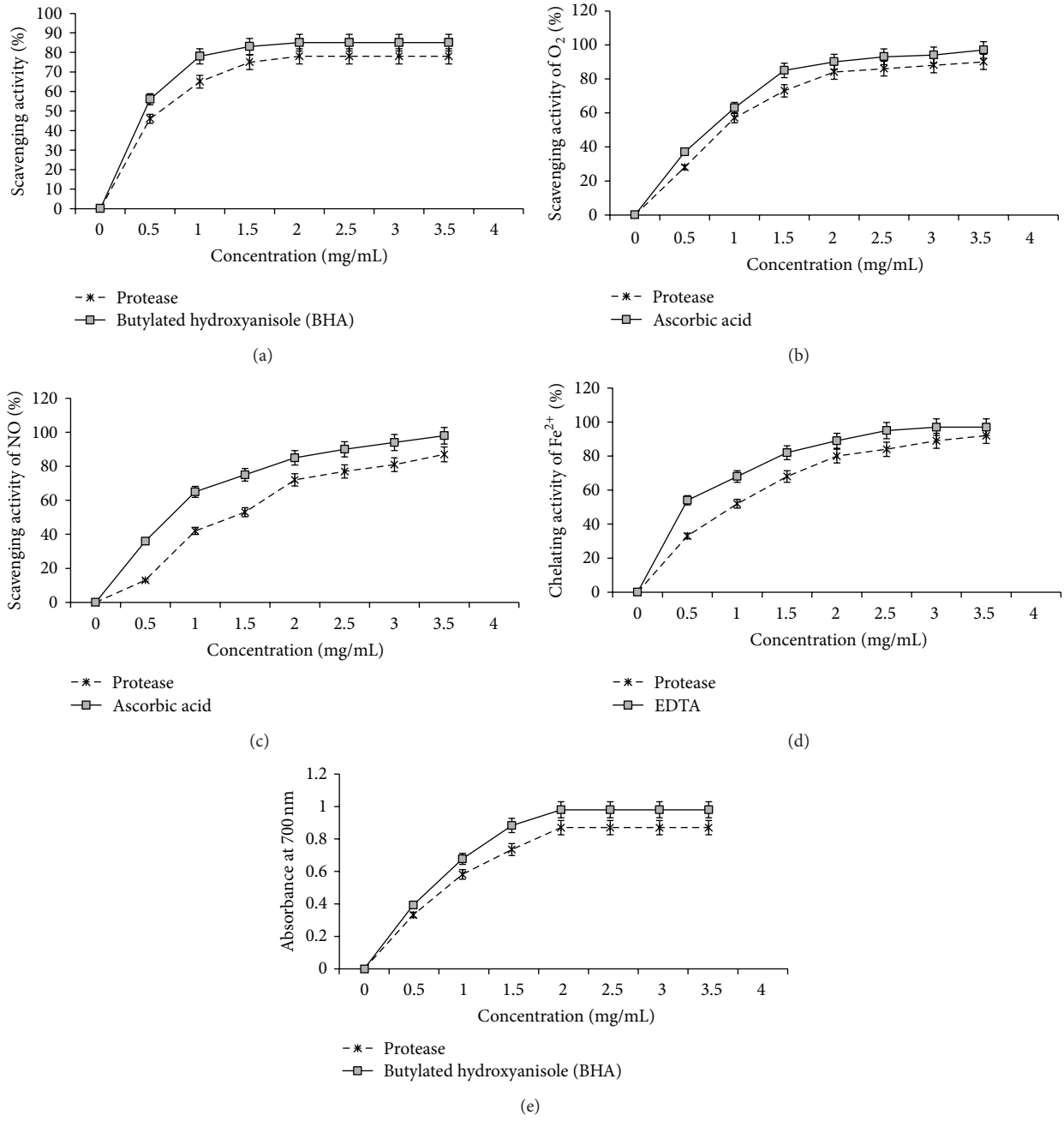

Figure 4: Antioxidant activity of protease, (a) DPPH-scavenging activity; (b) O_2-scavenging activity; (c) NO-scavenging activity; (d) Fe^{2+} chelating activity; and (e) reducing power.

activity of the protease, as measured by the riboflavin-NBT-light system *in vitro*. Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species [33]. Photochemical reduction of flavins generates O_2^-, which reduces NBT, resulting in the formation of blue formazan [22]. The protease was found to be a moderate scavenger of superoxide radical generated in riboflavin-NBT-light system *in vitro*. The protease inhibited the formation of the blue formazan and the % inhibition was proportional to the concentration with an IC_{50} value of 84 mg/mL. These results indicated that the tested protease had a notable effect on scavenging of superoxide when compared with ascorbic acid, which was used as a positive control.

3.8.3. Assay of Nitric Oxide-Scavenging Activity. Protease also showed a moderate nitric oxide-scavenging activity at different concentrations, 0.5–3.5 mg/mL, in a dose dependent manner (IC_{50} = 72 mg/mL) (Figure 4(c)). Nitric oxide is an essential bioregulatory molecule required for several physiological processes like regulation of blood pressure, prevention of aggregation and adhesion of platelets, assisting

the immune system to kill a wide variety of pathogens and block viral replication, promotion of certain types of cancer, promotion of penile erection and spermatogenesis, and facilitating childbirth [34]. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions [35]. Present results suggest that *Streptomyces* sp. MAB18 is a potent and novel source of therapeutic agents for scavenging NO and regulating the pathological conditions caused by excessive generation of NO as its protease showed a moderate nitric oxide-scavenging activity. Percent inhibition was increased with the increasing concentration of the protease.

3.8.4. Reducing Power Determination. Figure 4(e) shows the reductive capabilities of protease compared to BHA. For the measurements of the reductive ability, we investigated the Fe^{3+}-Fe^{2+} transformation in the presence of protease, using the standard method [25]. Earlier authors [36, 37] have observed a direct correlation between antioxidant activities and reducing power of certain protease. The reducing properties are generally associated with the presence of reductones [38], which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing power of protease suggest that it is likely to contribute significantly towards the observed antioxidant effect. However, the antioxidant activity of compounds has been attributed to various mechanisms, such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, and prevention of continued hydrogen abstraction [39]. Similar to the antioxidant activity, reducing power of protease increased with the increasing amount of sample. However, the reducing power of BHA was relatively more pronounced than that of protease.

3.8.5. Metal Chelating Activity. Chelation of ferrous ions by protease was estimated by the standard method [26]. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red colour of the complexes decreases. Measurement of the rate of colour reduction therefore allows estimation of the chelating activity of the coexisting chelator [40]. In the present assay, both protease and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. Absorbance of Fe^{2+}-ferrozine complex was decreased dose dependently; otherwise, the activity was increased on increasing concentration from 0 to 3.5 mg/mL. Metal chelating capacity was significant since the protease reduced the concentration of the catalyzing transition metal in lipid peroxidation [41]. It was reported that chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilising the oxidized form of the metal ion. The data obtained and shown in Figure 4(d) reveal that the protease has an effective capacity for iron binding, suggesting that its action as an antioxidant may be related to its iron binding capacity.

4. Conclusion

In the present study, feather degradation was successfully carried out by fermentation of the strain *Streptomyces* sp. MAB18. Production of protease from this strain was simple and it will be easy to scale up, as this actinobacterium grows on simple media with feathers as a sole source of carbon, nitrogen, and energy, thus allowing its enzyme production from an inexpensive substrate and a commercial potential with low production cost. When the protease obtained from MAB18 under optimum conditions was assessed for antioxidant activity using DPPH radical-scavenging activity, O_2 -scavenging activity, NO-scavenging activity, Fe^{2+} chelating activity, and reducing power, the enzyme was found to possess good antioxidant potential. Hence, the use of this protease in fish feed formulations as a source of protein and natural antioxidants would be an advantage both for the aquaculture industry and the consumers. Further research is essential to incorporate this protease in animal models to study its effect on the growth and *in vivo* lipid peroxidation. Further, the oxidative stability of the animal diet formulated using the protease should also be given due attention.

Conflict of Interests

The authors indicate no conflict of interests.

Acknowledgments

This research was supported by a grant from Marine Bioprocess Research Center of the Marine Biotechnology Program funded by the Ministry of Oceans and Fisheries, Republic of Korea. One of the authors, Kannan Sivakumar, expresses his thanks to the Director, Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, and Annamalai University authorities for facilities and encouragement.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 203735, 10 pages
http://dx.doi.org/10.1155/2013/203735

Review Article

β-Glucosidases from the Fungus *Trichoderma*: An Efficient Cellulase Machinery in Biotechnological Applications

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Received 8 May 2013; Accepted 15 June 2013

Academic Editor: Arzu Coleri Cihan

β-glucosidases catalyze the selective cleavage of glucosidic linkages and are an important class of enzymes having significant prospects in industrial biotechnology. These are classified in family 1 and family 3 of glycosyl hydrolase family. β-glucosidases, particularly from the fungus *Trichoderma,* are widely recognized and used for the saccharification of cellulosic biomass for biofuel production. With the rising trends in energy crisis and depletion of fossil fuels, alternative strategies for renewable energy sources need to be developed. However, the major limitation accounts for low production of β-glucosidases by the hyper secretory strains of *Trichoderma*. In accordance with the increasing significance of β-glucosidases in commercial applications, the present review provides a detailed insight of the enzyme family, their classification, structural parameters, properties, and studies at the genomics and proteomics levels. Furthermore, the paper discusses the enhancement strategies employed for their utilization in biofuel generation. Therefore, β-glucosidases are prospective toolbox in bioethanol production, and in the near future, it might be successful in meeting the requirements of alternative renewable sources of energy.

1. Introduction

β-glucosidases are members of cellulase enzyme complex and are promising candidates in biotechnological applications. Fungal species belonging to genus *Trichoderma* are ubiquitous in nature and classified as imperfect fungi due to absence of sexual reproduction [1]. *Trichoderma* is a saprophyte and produce diverse enzymes, a particular strain being specific for a certain type of enzyme. For example, *T. reesei* is used for cellulase and hemicellulase production, *T. longibratum* is used for xylanase, and *T. harzianum* is used for chitinase [2]. The cellulase system in *T. reesei* constitutes the combined activity of three enzymes: cellobiohydrolase, endo-β-glucanase and β-glucosidases, respectively. Cellobiohydrolases (EC 3.2.1.91) degrade cellobiose residues from the nonreducing end of the glucan, endo-β-glucanase (EC 3.2.1.4) catalyzes the breakdown of internal β-1,4-linkages, while β-glucosidases (EC 3.2.1.21) hydrolyze cellobiose to two molecules of glucose [3]. The conversion of cellulose to glucose is regarded as the rate limiting step in the production of biofuels from lignocellulosic materials, due to high cost of cellulases and their low efficiencies.

β-glucosidases, also named as (β-D-glucoside glucohydrolase, EC 3.2.1.21), catalyze the hydrolysis of the β-glucosidic linkages such as alkyl and aryl β-glucosides, β-linked oligosaccharides as well as several oligosaccharides with release of glucose [4, 5]. β-glucosidases are prominent class of enzymes and catalyze cellulose degradation acting synergistically with cellobiohydrolase and endoglucanase, respectively [6]. The specificity of β-glucosidases is variable towards different substrates depending on the enzyme source. The enzyme is ubiquitously present in nature and found in bacteria [7], fungi [8], yeasts [9], plants [10–13], and animals [14], respectively.

Some *Trichoderma* species amongst cellulolytic fungi have strong cellulose-degrading properties and therefore their cellulase systems have been widely studied. In *T. reesei*, the maximum production of cellulase component is of cellobiohydrolases I (CBHI) which is 60% of the total secreted protein [15], while cellobiohydrolases II (CBHII) and

endoglucanases accounts for 20 and 10% of the total secreted protein and this is a major limitation in cellulose saccharification by cellulases [16].

The mechanism of catalysis includes the degradation of cellobiose to glucose resulting in cellulose saccharification and release of the two enzymes from cellobiose inhibition [17, 18]. The enzymes are widely distributed in microbes, plants, and animals and play important roles in biological processes [19]. β-glucosidases, particularly from microorganisms, play a significant role in cellulose saccharification. However, microbes which produce the enzyme in low quantities lead to inefficient degradation of cellulose. While in microorganisms, β-glucosidases are involved in degradation of cellulose as compared to synthesis of beta-glucan during cell wall development, fruit ripening, defense mechanisms, and pigment metabolism [20, 21]. However, β-glucosidase-1 (BGL1) from *T. reesei* hyperproducing strain is produced in very small quantities. Over expression strategies in *T. reesei* or additional incorporation of β-glucosidase from other sources could be a possible option for enhancing and optimizing β-glucosidases mediated cellulose degradation. The products, cellobiose generated by endo- and exoglucanase act as inhibitors of both enzymes and removed by the action of β-glucosidases [22].

Several studies on β-glucosidases, time and again have highlighted their importance in biotechnological applications. Woodward and Wiseman reviewed the research on the fungal enzymes till 1982 [23]. Further, the enzymes from yeast were studied by Leclerc and coworkers [24] and thermostable β-glucosidases from mesophilic and thermophilic fungi [25]. Recently, molecular cloning studies on β-glucosidases were performed by Bhatia and colleagues [26]. Several other studies report on the isolation, cloning, and purification of β-glucosidases [27, 28].

With the present trends in rising the importance of β-glucosidases in industrial applications, this review is an update on fungal β-glucosidases particularly from *Trichoderma* species, an overview of their increasing significance, classification of the enzymes, their structure and properties, and also their prospective role in biotechnological applications. Furthermore, β-glucosidases may serve as a promising tool in meeting the energy crisis by generating an alternative renewable source of biofuels production in future.

2. Phylogenetics and Characteristics of *Trichoderma* Fungus

The genus *Trichoderma* is the best studied among fungi due to its biotechnological prospects and applications. The first report pertaining to the fungus *Trichoderma* dates back to 1794 [29]. Bioinformatics approaches such as oligonucleotide barcode (TrichOKEY) and a similarity search tool (TrichoBLAST) are mostly used in *Trichoderma* studies and can be accessed online at www.isth.info [30, 31]. Phenotype microarrays are the more reliable technique for the identification and characterization of newly isolated *Trichoderma* spp.

The cellulases produced from the *Trichoderma* species are important industrial products for biofuel production from cellulosic waste. *Trichoderma* species is widely present on cellulosic materials and results in their degradation [32]. At present, 165 records for *Trichoderma* are available in the Index Fungorum database (http://www.indexfungorum.org/Names/Names.asp). The international subcommission on *Trichoderma* includes 104 species characterized at the molecular level (http://www.isth.info/biodiversity/index.php). *Trichoderma* is among the most extensively used fungus species in industrial applications. The whole genome sequencing of the three strains, *T. reesei*, the industrial strain [33] (http://genome.jgi-psf.org/Trire2/Trire2.home.html), *T. atroviride* and *T. virens*, two other important biocontrol species (http://genome.jgi-psf.org/Trive1/Trive1.home.html) is under progress. The results showed that although *T. reesei* is considered as an important industrial strain for cellulose degradation, its genome consists of fewer genes encoding hemicellulolytic and cellulolytic enzymes [34].

Several species of *Trichoderma*, namely, *T. reesei*, *T. atroviride*, *T. virens*, *T. asperellum*, *T. harzianum*, *T. citrinoviride*, and *T. koningii* are considered important and used in various industrial applications. Studies on β-glucosidases from *Trichoderma* species ranging from protein purification and characterization and overexpression in different fungal strains to site-directed mutagenesis and molecular biology studies have been summarized in Table 1.

3. Structure of β-Glucosidases

With the increasing significance of β-glucosidases and their application in industrial biotechnology, efforts have been made to isolate a wide range of β-glucosidases from different sources and also, on the improvement of enzyme activity and thermostability. The structure of *T. reesei* β-glucosidase 2 (TrBgl2) has been elucidated by Lee and coworkers in 2012 [35] with a PDB code-3AHY. The structure of TrBgl2 consists of Glu165 as the catalytic acid/base and Glu367 as the catalytic nucleophile [36] and utilizes a β-retaining mechanism for its activity. The enzyme adopts a $(\alpha/\beta)_8$-TIM barrel fold typical of GH1 enzymes, with the active site including a deep pocket from enzyme's surface to the barrel core of the protein. Two conserved motifs, namely TFNEP and VTENG comprising of catalytic acid/base E165 and catalytic nucleophile E367 are situated opposite to each other at the bottom of active site. The amino acid residues supposed to be involved in substrate binding are as follows: glycone-binding residues: Q16, H119, W120, N164, N296, W417, N422, E424, W425, T431, and F433; aglycone binding residues: C168, N225, F228, Y298, T299, and W339) [36]. Mutational studies were carried out to determine the functional role of amino acids in active site. Two mutants (F250A and P172L/F250A) with increased enzyme catalytic efficiency and two mutants (L167W and P172L) with enhanced thermostability were generated [35]. Structural studies using bioinformatics approaches, are a key platform to decode the structural aspect of β-glucosidases and to understand its catalytic mechanisms.

4. Classification and Properties of Fungal β-Glucosidases

β-glucosidase are classified in glycosyl hydrolase family, and include 132 families according to CAZY web server [47].

Table 1: Studies on β-glucosidase from different strains of *Trichoderma* fungus.

S. no.	*Trichoderma* strain	β-glucosidase	Isolation strategies	References
1	*T. citrinoviride*	Extracellular β-Glucosidase	Protein purification, biochemical and proteomic characterization	[28]
2	*T. reesei*	TrBgl2	Mutational studies involving active site residues of the enzyme	[35]
3	*T. reesei* QM9414	bgl1	Overexpression of bgl1 from *Periconia* sp. in *T. reesei* QM9414 under *T. reesei* tef1α promoter	[37]
4	*Recombinant T. reesei strain,* X3AB1	bgl1	Construction of *T. reesei* strain expressing *A. aculeatus* bgl under control of xyn3 promoter	[38]
5	*T. reesei*	bgl I	Molecular cloning and expression in *Pichia pastoris*	[39]
6	*T. reesei* CL847	BGL1	Protein purification and kinetic characterization	[3]
7	*T. reesei*	β-Glucosidase (cel3a)	Molecular cloning and expression in *T. reesei*	[40]
8	*T. ressei*	β-Glucosidase BGLII (Cel1A)	Molecular cloning, expression in *E. coli*, and characterization	[41]
9	*T. harzianum* C-4	—	Protein purification and biochemical characterization	[42]
10	*T. reesei*	BGL2	Molecular cloning and expression in *Aspergillus oryzae*	[43]
11	*T. harzianum strain* P1	1,3-β-Glucosidase	Protein purification and characterization	[44]
12	*T. reesei* QM9414	Aryl-β-D-glucosidase	Protein purification and characterization	[45]
13	*T. viride*	β-Gluc I	Protein purification and biochemical characterization	[46]
14	*T. viride* QM9414 mutants	—	Biochemical studies (pH control)	[16]

β-glucosidases from archeabacteria, plants, and mammals are found in family 1 and usually exhibit β-galactosidase activity while family 3 consists of β-glucosidases from bacteria, fungi and plants [48]. Family 1 and family 3 include retaining enzymes that hydrolyze the substrates with retention of anomeric carbon via a double-displacement method [49, 50].

Cellulose constitute one of the most abundant organic biopolymers on earth, and the cleavage of glycosidic bonds plays a crucial role in a wide range of biological processes in all living organisms. β-glucosidases comprise of a major enzyme group and are classified into 1st and 3rd families and hydrolyze either *S*-linked β-glycosidic bonds (myrosinase or β-D-thioglucoside glucohydrolase, EC 3.2.3.1) or *O*-linked-glycosidic bonds (β-D-glucoside glucohydrolase, EC 3.2.1.21) [51].

Based on substrate specificity, β-glucosidases are classified in three classes: class I (aryl β-glucosidases), class II (true cellobiases), and class III (broad substrate specificity enzymes). Mostly, β-glucosidases belong to class III with diverse catalytic mechanisms including cleavage of β 1,4; β 1,6; β 1,2 and α 1,3; α 1,4; α 1,6 glycosidic bonds [26, 52]. The enzymes exhibit functional diversity in terms of substrate specificity and no specific catalytic mechanism has been observed. However, the fungal enzymes are classified on the basis of their relative activities toward cellobiose and P(O)NPG into two groups, namely, (1) cellobiases—enzymes which have higher activity towards cellobiose, and (2) Aryl-β-glucosidases—higher relative activities towards P(O)NPG than cellobiose or negligible activity towards cellobiose. These are further classified according to their affinities towards cellobiose and P(O)NPG into three groups: (1) β-glucosidases with higher affinities for P(O)NPG, (2) β-glucosidases which show higher affinity (lower K_m) for cellobiose and (3) β-glucosidases with affinities (K_m) similar for both substrates [53]. The values of K_m range from 0.031 (*Neocallimastix frontalis*) [54] to 340 mM (β-glucosidase II from *P. infestans*) [55] for cellobiose and from 0.055 mM (*Stachybotrys atra*) [56] to 34 mM (β-glucosidase II from *P. infestans*) [55] for P(O)NPG substrate.

β-glucosidases are biologically important enzymes and catalyze the transfer of glycosyl group between oxygen nucleophiles. Also, these enzymes exhibit activity for both natural (plant) or synthetic aryl-glucosides and a variety of aglycons [53]. A β-glucosidase purified from *A. niger* showed catalytic activities towards the disaccharides gentiobiose (β 1–6), sophorose (β 1-2), laminaribiose (β 1–3), and salicin (salicyl-glucose) [57]. The glucosidase from *P. herquei*, G1 β-glucosidase demonstrated relative activities of 82.7 and 70.3% toward gentiobiose and salicin (100% for PNPG) while the G2 isoenzymes are 8.7 and 54.5%, respectively [58]. This indicates that variations exist between enzymes from different species as well as between isoenzymes of the same microorganism. These enzymes possess high activity towards oligosaccharides with β $(1 \rightarrow 4)$ linkages; several studies indicated a higher activity towards glucans with β $(1 \rightarrow 2)$ and β $(1 \rightarrow 3)$ linkages. Examples include enzymes from *T. koningii* [59] and *A. fumigates* [60] with activity towards sophorose and laminaribiose than cellobiose. Although, the enzymes exhibit greater variability towards β-1,2/1,3 β-glucans, aryl-glucosides, and cellooligosaccharides, these enzymes are specific for β-anomeric configuration (exception β-glucosidase from *Thermomyces lanuginosus,* shows α-glucosidase activity) [5].

Mainly, β-glucosidases display optimum pH over the range 4.0 to 5.5 but enzyme activity has also been observed in low pH range (pH 2.5) to very high range (pH 8.0). The optimum temperature range for enzyme activity is from 35° to 80°C. The extracellular β-glucosidases from mesophilic fungi

are thermostable enzymes (up to 60°C). Example includes a β-glucosidase purified from *T. reesei* QM 9414 strain which shows high stability of 50–55°C [61]. Several reports indicated the role of the carbohydrates in thermostability of the enzymes as cellulases are mostly glycoproteins. Examples are β-glucosidases I, III, and IV from *T. emersonii* [62] and β-glucosidases of *Mucor miehei* [63].

Glucono-δ-lactone is a potent competitive inhibitor of many β-glucosidases, and values of Ki ranging from 0.0083 μM to 12.5 mM have been reported [53]. Steric similarities between the enzyme-bound substrate and Glucono-δ-lactone might explain the competitive inhibition by this compound [64]. Other inhibitors of the enzyme include nojirimycin and deoxy nojirimycin [65] and heavy metals such as Hg^{2+}, Cu^{2+}, Pb^{2+} and Co^{2+}, and *p*-chloromercuribenzoate [66].

β-glucosidases from *T. reesei* are found bound to the cell wall or cell membrane or in supernatants with pI ranging from 4.4 to 8.7. In *T. reesei*, most of the enzyme is bound to the cell wall [67] during fungal growth and therefore low quantities of β-glucosidase are secreted into the medium [68]. Kubicek [69] reported that the membrane-bound β-glucosidase plays a role in the formation of sophorose which acts as a potent inducer of cellulases. Studies also indicated that the enzyme may act in cell-wall metabolism during conidiogenesis and therefore, not really a true component of cellulolytic enzyme system [67]. Inglin and coworkers [70] isolated an intracellular β-glucosidase and postulated that the enzyme might be involved in transportation across cell membrane as a proenzyme and in metabolic regulation of cellulose induction.

5. Studies on β-Glucosidases from *Trichoderma* Species

Numerous studies on *Trichoderma* have indicated its importance in biotechnological perspectives. Several molecular biology and biochemical techniques have reported the improved isolation of β-glucosidases from different species of *Trichoderma* namely *T. reesei* [37–41, 43, 71], *T. atroviride* [72], *T. harzianum* [42, 44], *T. viride* [46, 73], *T. koningii* [59], and *T. citrinoviride* [28], respectively (Table 1). Some of the key studies on β-glucosidase from *Trichoderma* fungus are as follows.

5.1. Protein Purification. Biochemical studies resulting in purification and characterization of a β-glucosidase from Type C-4 strain of *T. harzianum* was performed by Yun et al. [42]. A β-glucosidase with high cellulolytic activity was purified to homogeneity through Sephacryl S-300, DEAE-Sephadex A-50, and Mono P column chromatographic steps. SDS-PAGE analysis revealed that the protein was a monomer with a molecular mass of 75 kDa. The enzyme properties were established in terms of optimum activity at pH 5.0 and 45°C. *p*-Nitrophenyl-β-D-cellobioside and *p*-Nitrophenyl-β-glucopyranoside served as substrates and glucose and gluconolactone acted as competitive inhibitors, respectively. Similar studies by Chandra and coworkers [28] reported the homogenous purification, kinetics, and MALDI-TOF assisted proteomic analysis of an extracellularly secreted β-glucosidase of *T. citrinoviride*. The enzyme had a molecular weight of 90 kDa, consisted of a single polypeptide chain, optimal activity at pH 5.5 and 55°C. Further, the enzyme was not inhibited by glucose (5 mM) and possess transglycosylation activity (catalyze conversion of geraniol to its glucoside).

Another study reported the comparative kinetic analysis of two fungal strains, β-glucosidase from *Aspergillus niger* and BGL1 from *T. reesei* through an efficient FPLC technique. 95% purification was obtained for BGL1 from *T. reesei* and cellobiose was used as substrate for kinetic characterization of the enzyme. The study revealed that β-glucosidase, SP188 from *Aspergillus niger* (K_m = 0.57 mM; K_p = 2.70 mM), has a lower specific activity than BGL1 (K_m = 0.38 mM; K_p = 3.25 mM) and more sensitive to glucose inhibition. Furthermore, a Michaelis-Menten model was generated and revealed comparative substrate kinetics of β-glucosidase activity of both enzymes [3]. Chirico and Brown [45] purified a β-glucosidase from the culture filtrate of *T. reesei* QM9414 strain to homogeneity and the purified enzyme exhibited activity towards cellobiose, p-nitrophenyl β-D-glucopyranoside and 4-methylumbelliferyl β-D-glucopyranoside.

A new type of aryl-β-D-glucosidase with no activity towards cellobiose was isolated and purified from a commercial cellulase preparation derived from *T. viride*. The purification techniques included Bio-Gel gel filteration, anion exchange on DEAE-Bio-Gel A, cation exchange on SE-Sephadex, and affinity chromatography on crystalline cellulose. The enzyme had a molecular weight of 76,000 Dalton and showed high activity with on *p*-nitrophenyl-β-D-glucose and *p*-nitrophenyl-β-D-xylose and moderate activity towards crystalline cellulose, xylan, and carboxymethyl cellulose [46].

5.2. Genomics Studies

5.2.1. Promoter Analysis. Although *T. reesei* have been explored extensively for cellulase production, the major limitations are the low β-glucosidase activity and inefficient biomass degradation, respectively. The xyn3 and egl3 promoters were used to enhance the expression of β-glucosidase 1 (BGL1) through homologous recombination. The recombinant strains showed 4.0- and 7.5-fold higher β-glucosidase activity under the control of egl3 and xyn3 promoters as compared to native strains. Furthermore, Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry determination revealed that BGL1 was over expressed. The increased level of BGL1 was adequate for cellobiose and cellotriose degradation [74].

5.2.2. Mutational Studies. The mutants of *T. reesei* capable of cellulase overproduction have been considered significant and economical for saccharification of pretreated cellulosic biomass [75]. Low BGL activity in *T. reesei* results in cellobiose accumulation leading to reduced biomass conversion efficiency and cellobiose-mediated product inhibition of CBH I (Cel7A) [76]. Exogenous supplementation of BGL in *T. reesei* cellulase preparations has been used as an alternative strategy to overcome this problem [77, 78].

Nakazawa and coworkers [38] constructed a recombinant *T. reesei* strain, X3AB1 that was capable of expressing an *Aspergillus aculeatus* β-glucosidase 1 with high specific activity under xyn3 promoter control. The study involved the isolation and harvesting of the culture supernatant from *T. reesei* X3AB1 grown on 1% Avicel (as carbon source). It exhibited 63- and 25-fold higher β-glucosidase activity against cellobiose compared to those of the parent strain PC-3-7 and *T. reesei* recombinant strain expressing an endogenous β-glucosidase I, respectively. The study further demonstrated that xylanase activity was 30% less when compared to due to the absence of xyn3 promoter. X3AB1 strain when grown on 1% Avicel-0.5% xylan medium, produced 2.3- and 3.3-fold more xylanase and β-xylosidase, respectively, than X3AB1 grown on 1% Avicel.

Furthermore, a mutant strain of *T. citrinoviride* was developed by multiple exposures to ethidium bromide and ethyl methyl sulphonate [79]. The mutants secreted FPase, endoglucanase, β-glucosidase and cellobiase 0.63, 3.12, 8.22, and 1.94 IU mL^{-1} which was found to be 2.14-, 2.10-, 4.09-, and 1.73-fold higher compared to the parent strain. Further studies indicated that under submerged fermentation conditions, glucose (upto 20 mM) did not led to inhibition of enzyme production. Comparative fingerprinting revealed the presence of two unique amplicons suggesting genetic uniqueness of the mutants.

5.2.3. Molecular Cloning and Heterologous Expression. A novel fungal β-glucosidase gene (bgl4) and its homologue (bgl2) have been cloned from *T. reesei* [43]. This enzyme reportedly showed homology with plant β-glucosidases classified in β-glucosidase A (BGA) family. The BGL2 protein from *T. reesei* showed an amino acid composition of 466 on SDS PAGE and exhibited 73.1% identity with β-glucosidase from fungus *Humicola grisea.* Both the genes have been expressed in *Aspergillus oryzae* and purified. Furthermore, β-glucosidases of *Humicola grisea* have been used in combination with *Trichoderma* cellulases to improve the saccharification of cellulose. The study also demonstrated that the recombinant BGL4 from *Humicola grisea* showed strong activity towards cellobiose and the incorporation of the recombinant BGL4 led to improvement in cellulose saccharification by 1.4–2.2 times. Overexpression of recombinant BGL4 gene from *Humicola grisea* in *T. reesei* or *T. viride* has been reported *to* improve the saccharification of cellulose by cellulases complex [43].

A β-glucosidase cloned from *T. reesei* and its expression studies have been reported in *Pichia pastoris* GS115 strain [39]. *T. reesei* produced β-glucosidase in very low amounts [27] which acted as a limiting factor in cellulose degradation. To overcome this, it has been reported that a β-glucosidase from *T. reesei* (bglI) was over expressed in *Pichia pastoris* GS115 under the control of methanol-inducible alcohol oxidase (AOX) promoter and *S. cerevisiae* secretory signal peptide (a-factor). The expression of β-glucosidase in the culture medium has been reported to reach the productivity of 0.3 mg/mL and the maximum activity was reported as 60 U/mL. Furthermore, the protein purification yielded a recombinant β-glucosidase of molecular weight 76 kDa, a 1.8-fold purification with 26% yield, and a specific activity of 197 U/mg was achieved. The optimum activity of the enzyme was at 70°C and pH 5.0.

Several studies aimed at the improvement of the fungus *T. reesei* for β-glucosidase production since the yield is reported to be quite low and it is also required for conversion of cellobiose to glucose which hampers cellulase production. Dashtban and Qin [37] successfully engineered a β-glucosidase gene from the fungus *Periconia* spp. into the genome of *T. reesei* QM9414 strain. As compared to the parent strain (2.2 IU/mg), the *T. reesei* strain showed about 10.5-fold (23.9 IU/mg) higher β-glucosidase activity after 24 h of incubation. The recombinant enzyme was thermotolerant and was completely active when incubated at 60°C for two hours. Also, a very high total cellulase activity (about 39.0 FPU/mg) was found in comparison to the parent strain which did not show any total cellulase activity at 24 h of incubation. Furthermore, enzyme hydrolysis assay using untreated NaOH or Organosolv pretreated barley straw showed that the recombinant *T. reesei* strains released more reducing sugars compared to the parental strain. Such studies would benefit the bioconversion techniques, namely, biomass conversion using cellulases.

5.3. Bioinformatics Studies

5.3.1. Site Directed Mutagenesis. Another approach of mutational studies was performed by Lee and coworkers [35] and it showed that induced mutations in the active site of β-glucosidase from *T. reesei* lead to improved enzyme activity and thermostability of the enzyme. The study involved mutations in the outer channel of the active site of the enzyme. The mutants, P172L and P172L/F250A showed enhanced enzyme activity in terms of 5.3- and 6.9-fold increase in K_m and k_{cat} values towards 4-nitrophenyl-b-D-glucopyranoside (p-NPG) substrate at 40°C as compared to the wild type. Also, L167W or P172L mutations lead to higher thermostability of the enzyme as demonstrated by their melting temperature, Tm. Furthermore, the mutant, L167W, showed an effective synergistic activity together with cellulases in cellulose degradation. These mutational studies hold prospects in engineering enzymes having industrial applications such as biofuel production.

5.3.2. Biochemical Studies. Several inhibitors were used to study the enzyme activity of β-glucosidase from *T. reesei* QM 9414 strain. Diethylpyrocarbonate (DEP) at a concentration above 10 mM completely inhibited the enzyme activity while the presence of substrate or analog protected the enzyme from inactivation. The enzyme showed a pseudo-first-order reaction kinetics, having a second-order rate constant of 0.02 mM^{-1} min^{-1}. The presence of 1 M hydroxylamine restored the enzyme activity which indicated the modification of histidine residues. Also, statistical analysis of residual fractional activity compared to the number of modified histidine residues exhibited that presence of one histidine residue is important for catalysis. Other inhibitors of β-glucosidase include *p*-hydroxymercuribenzoate which completely inhibited the enzyme at concentration above

2 mM. The modified enzyme when treated with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) showed that presence of one cysteine residue was essential for enzyme activity. Also, various other inhibitors like 2-ethoxy-l-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) were used to study the effect of chemical modifications on enzyme kinetics [79].

6. Biotechnological Applications of β-Glucosidases

Studies on β-glucosidases have been carried out from different sources, namely, microbes, plants, and animals [7–14]. Amongst these, fungal sources are immensely explored due to their better prospects in commercial applications. β-glucosidases are promising candidates of glycosyl hydrolase family and catalyze the selective cleavage of glucosidic bonds. The enzyme is found in all living organisms and involved in diverse biological processes, namely, cellular signaling, oncogenesis, host pathogen interactions, degradation of structural and storage polysaccharides, and processes of industrial relevance [26]. Due to the rising significance of β-glucosidases in industrial biotechnology, emerging trends focus on the maximum exploitation of this category of enzymes. In plants, the enzyme catalyzes the beta-glucan synthesis during cell wall development, fruit ripening, pigment metabolism, and defence mechanisms [20, 21] while in microorganisms, these are involved in cellulose induction and hydrolysis [80, 81]. In humans and mammals, the enzyme catalyzes the hydrolysis of glucosyl ceramides [82]. Biosynthesis of glycoconjugates such as aminoglycosides, alkyl glucosides, and fragments of phytoalexin-elicitor oligosaccharides which play a role in microbial and plant defence mechanism is an important application of β-glucosidases [26]. However, the saccharification of cellulosic biomass for biofuel production is the most extensive area of research and application. The fungal β-glucosidases, being an efficient biocatalysts, finds applications in various industrial processes. The major applications of β-glucosidases from *Trichoderma species* are as follows.

Bioethanol Production. The rising energy demands and depletion of fossil fuels initiated research on alternative sources for energy production. Lignocellulosic biomass is the abundant component of plants and renewable in nature therefore utilized for bioethanol production. Cellulase enzyme complex catalyzes cellulose degradation and comprises of three different enzymes: exoglucanase, endoglucanase, and β-glucosidase (BGL) which acts synergistically for complete hydrolysis of cellulose [83, 84]. The initial steps include the cleavage of cellulose fibers by endoglucanase releasing small cellulose fragments which are acted upon by exoglucanase resulting in small oligosaccharides, cellobiose which is hydrolysed into glucose by β-glucosidases. The cellulolytic enzyme complex secreted by fungus, *T. reesei* is most widely used in industrial bioethanol applications. The conversion of cellobiose to glucose is regarded as the rate limiting step in bioethanol production from lignocellulosic biomass due to low efficiency and high costs of cellulases. Also, hyperproducing strains of *T.reesei* produce β-glucosidase in very low amounts [27]. Alternative methods such as cocultivation fungal strains producing cellulose and β-glucosidase, namely, *T. reesei* and *A. phoenics* or *A. niger* was used to enhance the activity of β-glucosidase [85].

Several alternatives strategies have been utilized such as heterologous expression of β-glucosidase in other systems for enhanced production [85], supplementation of exogenous β-glucosidase to the cellulase complex of *T. reesei* [86], engineering β glucosidase for overexpression and production [37], promoter use for enhanced expression [74], and site directed mutagenesis [35]. Enzyme preparations consisting of extracellular β-glucosidase produced by *T. atroviride* mutants and cellulase producing *T. reesei* were found to be better than commercial preparations for saccharification and of pretreated spruce [87]. Furthermore, studies also indicated that enzyme mixtures from different fungal strains exhibited better activity than commercial preparations namely celluclast 1.5 L, novozyme 188, and accellerase 1000 [86]. Delignified bioprocessings from *Artemisia annua* (known as marc of Artemisia) and citronella (*Cymbopogon winterianus*) have been utilized for bioconversion by six species of *Trichoderma* and cellulase production [88]. Among six species, *T. citrinoviride* was found to be most efficient producer of cellulases and a high amount of β glucosidase. Also, *T. virens* was not capable of producing complete cellulase enzyme complex on any test waste or pure cellulose, except on marc of *Artemisia*, where it produced all three enzymes of the complex [89]. Table 2 exhibits various enhancement studies and the possible outcome in terms of fold enhancement obtained for production of β-glucosidases from different strains of *Trichoderma*.

7. Conclusion

Biofuel production from lignocellulosic biomass comprising cellulose complex is the most important application and accounts for maximum exploitation of enzyme in industrial processes. However, slow enzymatic degradation rate and feed-back inhibition of the enzyme (particularly β-glucosidase) limit their commercialization. Current β-glucosidase applications involve manipulation strategies such as development of glucose-tolerant β-glucosidase and external administration together with other cellulases. Development of mutants and genetic engineering studies is an emerging area with good prospects in enzyme development with desired properties.

Commercially, companies such as Novozymes and Genencor have developed cellulolytic enzymes cocktails for biomass hydrolysis such as Cellic series of enzymes [90] and Accellerase series of enzymes [91]. Although, the details of enzyme mixture is not disclosed, but it was assumed that the enzymes preparations were from genetically modified *T. reesei* with high β-glucosidase activity. With the tremendous progress on β-glucosidases with an aim to improve its production and catalytic activity, it is likely that in near future, these would cease to be a limiting factor in biofuel production. Further, expectedly with the ongoing research efforts in this field, the management of energy crisis and fuel

Table 2: Studies comprising of the enhancement strategies used for β-glucosidase production.

S. no.	Strain used and enzymes	Enhancement strategies	Conclusion	Reference
1	*Aspergillus aculeatus* β-glucosidase 1	A recombinant *T. reesei* strain, X3AB1 under the control of xyn3 promoter	63- and 25-fold higher β-glucosidase activity against cellobiose	[38]
2	β-glucosidase from *Periconia* spp.	Heterologous expression in *T. reesei*	Around 10.5-fold (23.9 IU/mg) higher β-glucosidase activity A very high total cellulase activity (about 39.0 FPU/mg)	[63]
3	*T. reesei*, Bgl2	Mutational studies and engineering of active site residues	Mutants, P172L, and P172L/F250A showed enhanced k_{cat}/K_m and k_{cat} values by 5.3- and 6.9-fold Also, mutant L167W had the best synergism with *T. reesei* in cellulosic biomass degradation	[32]
4	*T. reesei* (bglI)	Overexpression in *P. pastoris* GS115 under methanol-inducible alcohol oxidase promoter and *S. cerevisiae* secretory signal peptide.	β-glucosidase expression was 0.3 mg/mL and the maximum activity was 60 U/mL	[39]
5	β-glucosidase 1 (BGL1)	Use of xyn3 and egl3 promoters through homologous recombination	4.0- and 7.5-fold higher β-glucosidase activity	[70]
6	*T. citrinoviride* mutants	Mutational studies, use of ethidium bromide and ethyl methyl sulphonate as mutagens	Secretion of endoglucanase, β-glucosidase and cellobiase was found to be 2.14-, 2.10-, 4.09-, and 1.73-fold higher	[28]
7	Thermostable β-glucosidase (*cel3a*)	*cel3a* from *Talaromyces emersonii* was expressed in *T. reesei*	High specific activity against *p*-nitrophenyl-β-D-glucopyranoside (V_{max}, 512 IU/mg) and was competitively inhibited by glucose (*k*i, 0.254 mM) and displayed transferase activity	[40]
8	BGL4 from *H. grisea*	Overexpression of BGL4 in *T. reesei* or *T. viride*	Improvement in cellulose saccharification by 1.4–2.2 times	[43]
9	*T. reesei* Rut C-30	Temperature and pH profiling studies	0.02% Tween-80 concentration was optimum, pH 5.0 and temperature (31°C) initially (for 18 h) was optimum for maximum production of cellulase and β-glucosidase	[89]

demands to a certain extent would be balanced by the biofuels generation and management.

Abbreviations

CBHI: Cellobiohydrolase I
BGL1: β-Glucosidase-1
CBHII: Cellobiohydrolase II
CAZY: Carbohydrate active enzyme database
TrBgl2: *T. reesei* β-glucosidase 2
GH1: Glycosyl hydrolase family 1
P(O)NPG: *p*-Nitrophenyl b-D glucopyranoside
K_m: Michaelis constant
pI: Isoelectric point
Ki: Inhibitor's dissociation constant
KDa: Kilo dalton
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
MALDI-TOF: Matrix-assisted laser desorption/ionization- time of flight
FPLC: Fast protein liquid chromatography
V_{max}: maximum rate of reaction

Acknowledgments

The authors are thankful to Director CIMAP for constant encouragement and CSIR, New Delhi for the NWP09 grant. Pragya Tiwari thanks CSIR for the award of senior research fellowship.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 954542, 9 pages
http://dx.doi.org/10.1155/2013/954542

Research Article

Improvement of Medium Chain Fatty Acid Content and Antimicrobial Activity of Coconut Oil via Solid-State Fermentation Using a Malaysian *Geotrichum candidum*

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Received 8 April 2013; Revised 19 June 2013; Accepted 28 June 2013

Academic Editor: Bidur Prasad Chaulagain

Coconut oil is a rich source of beneficial medium chain fatty acids (MCFAs) particularly lauric acid. In this study, the oil was modified into a value-added product using direct modification of substrate through fermentation (DIMOSFER) method. A coconut-based and coconut-oil-added solid-state cultivation using a Malaysian lipolytic *Geotrichum candidum* was used to convert the coconut oil into MCFAs-rich oil. Chemical characteristics of the modified coconut oils (MCOs) considering total medium chain glyceride esters were compared to those of the normal coconut oil using ELSD-RP-HPLC. Optimum amount of coconut oil hydrolysis was achieved at 29% moisture content and 10.14% oil content after 9 days of incubation, where the quantitative amounts of the modified coconut oil and MCFA were 0.330 mL/g of solid media (76.5% bioconversion) and 0.175 mL/g of solid media (53% of the MCO), respectively. MCOs demonstrated improved antibacterial activity mostly due to the presence of free lauric acid. The highest MCFAs-rich coconut oil revealed as much as 90% and 80% antibacterial activities against *Staphylococcus aureus* and *Escherichia coli*, respectively. The results of the study showed that DIMOSFER by a local lipolytic *G. candidum* can be used to produce MCFAs as natural, effective, and safe antimicrobial agent. The produced MCOs and MCFAs could be further applied in food and pharmaceutical industries.

1. Introduction

Coconut oil, which is a very important source of medium chain fatty acids (MCFAs), exhibits good properties due to its different metabolism pathway [1]. Three valuable MCFAs exist in coconut fat, namely, caprylic (C8:0), capric (C10:0), and lauric (C12:0) acids, where lauric acid makes up about 50% of the total FAs content. The antimicrobial effects of MCFAs against bacteria, fungi, viruses, and protozoa have been investigated extensively [2–5]. MCFAs are even preferable to polyunsaturated fatty acids (PUFAs), because some bacteria such as Lactobacilli are stimulated by the presence of these fatty acids [6]. Among MCFAs, lauric acid and its derivatives have been demonstrated as the most effective antimicrobial agents for foods and cosmetics. In addition, they are effective in alteration of ammonia concentration, methane production, and milk fatty acids composition of ruminants [4, 7–9]. Furthermore, Hristov et al. (2009) [9] showed that administration of free lauric acid and coconut oil together exhibits stronger antimicrobial effects compared to a single application.

Production of fatty acids from fats and oils is important due to its wide application as raw materials in food, cosmetic, pharmaceutical, and oleochemical industries [10]. The

current techniques for production of fatty acids are based on chemical, physical, and enzymatic methods [11, 12]. The use of commercial lipases would be preferable due to the mild processing conditions and less energy used [12] however, it is not a cost-effective way at large scale. On the other hand, as stated by Sado Kamdem et al. (2008) [2], among the compounds naturally presented in high-fat foods, free fatty acids (FFAs) produced by lipolysis during storage can be regarded as potential bactericides and/or bacteriostatics. The limiting aspect of food fatty acids is generally due to their amount, which is lower than the minimal inhibitory concentration (MIC) and inactivation of pathogens [13]. Hence, effective and natural FFAs production needs to be improved to enhance the antimicrobial activity.

Fermentation is an important process to increase the availability of important nutrients by enzymatic hydrolysis of raw substrates especially in solid-state system (SSF). Filamentous fungi are the most widely applied microorganisms in SSF [14]. *G. candidum*, which is Generally Recognized As Safe (GRAS) [15, 16], has been employed in cheese industry for many years [15].

Many processes have been developed that utilize raw materials for the production of chemicals and value-added fine products [17, 18]. The application of coconut as solid material for SSF has been suggested by Pandey et al. (1995) due to its high nutritional values [19]. In the present investigation, the potential application of SSF process in directly producing MCFAs from coconut fat has been studied. This process, generally named as direct modification of substrate through fermentation (DIMOSFER), was applied for the first time in oil modification. Therefore, the idea of this work would be to use a GRAS microorganism (*G. candidum*) to produce GRAS antimicrobial agents (MCFAs) particularly lauric acid through a green, clean, and cost-effective method (DIMOSFER).

2. Materials and Methods

2.1. Microorganism. Local *G. candidum* strain was purchased from Malaysia Agriculture Research and Development Institute (MARDI, Serdang, Selangor, Malaysia). The fungus was maintained on potato dextrose agar (PDA) slants at 4°C and periodically subcultured.

2.2. Inoculum Preparation. Inoculum suspension was prepared from the fresh, mature culture (7 days old at 30°C) of local *G. candidum* on potato dextrose agar slant. The spores were harvested with sterile distilled water containing 0.01% tween 80, transferred to a sterile tube, and the resulting suspension was homogenized for 15 s with a gyratory vortex mixer at 2000 rpm. Appropriate concentration (inoculum size = 10^5 spores/mL), counting in a cell-counting haemocytometer, was inoculated into potato dextrose broth (PDB) (modified from [20]).

2.3. Solid-State Fermentation and Optimization of MCFAs Production. Solid-state fermentation was carried out in 250 mL conical flask containing 10 g of coconut flakes [19]. Independent variables and ranges were selected based on the preliminary studies, where the level of moisture content, which was adjusted by distilled water, varied from 10 to 50%, and the level of external coconut oil content varied from 0 to 50% (v/w). The flasks were sterilized by autoclaving at 121°C for 20 min. After cooling down, the flasks were inoculated with 2 mL of 3-day-old PDB culture of local *G. candidum* strain. The content of each flask was mixed thoroughly with sterile spatula for uniform distribution of fungal spores in the medium. Flasks were incubated for a period of 3 to 30 days at 30°C. Samples were withdrawn for analysis according to the experimental design (five-levels three factor central composite rotary design, CCRD) at different periods of time (Table 1). Samples were then kept in the freezer for oil extraction and chemical analysis. Response surface methodology (RSM) was employed to build the best model and optimize the fermentation system using Design Expert version 6.06 (Stat Ease Inc. USA) (Table 1). One-way ANOVA was employed to study the main effects and intractions between parameters selected on coconut oil bioconversion.

2.4. Lipolytic Activity: A Time Course Study. Fermentation was carried out in 250 mL conical flasks each containing 10 g of coconut flakes, where the effective parameters were adjusted at the point of maximum MCFAs production. The flasks were incubated at 30°C and harvested every 24 h (for the period of 40 days). In order to evaluate the extracellular lipolytic activity [21], the content of each flask was soaked with 100 mL of aqueous solution of phosphate buffer 100 mM, pH 7 and shaken on a rotary shaker (200 rpm) for 1 h at 30°C. Finally, the suspension was squeezed through a double-layer muslin cloth and solution was centrifuged at 4000 ×g for 20 min at 4°C, and the supernatant was filtered through a membrane filter (pore size of 0.22 μm). The clear filtrate obtained was assayed for extracellular lipolytic activity [22]. Furthermore, intracellular lipolytic activity was assayed after breakage of the cells using different common methods and followed by filtration. The clear filtrate obtained was assayed for intracellular and cell debris on the filter for cell-bound lipolytic activities. Cell breakage efficiency was assessed using microscopic tests. One unit of lipolytic activity was defined as 1.0 μmol of free fatty acid liberated min^{-1} and reported as Uml^{-1}. All reported data were the average of triplicate experiments.

2.5. Product Characteristics Analysis

2.5.1. Coconut Oil Extraction from Solid Culture. Fermented coconut samples were kept in freezer after ending the incubation period based on the CCRD. Subsequently, 2 g of samples from each flask was placed in the round bottle of the soxhlet extractor. Petroleum ether (200 mL) was added to each sample, and oil extraction was performed for 10 h under moderate temperature (40–50°C). The solvent from oil/solvent mixtures was evaporated to get the extracted coconut oil after fermentation, and the product is called

Table 1: CCRD for coconut oil hydrolysis and the level of derivatives in the form of MCFA, MCDG, and MCMG produced by local *G. candidum* lipase in SSF.

Run no.	Moisture (additional) (%)	Oil (%)	Time (day)	Coconut oil hydrolysis (%)	MCFA (%)	MCDG (%)	MCMG (%)
1	18	10	16.0	40.00	6.70	26.00	7.32
2	42	10	16.0	62.00	480	7.30	6.74
3	18	40	8.5	7.67	3.67	3.85	0.16
4	42	40	8.5	2.43	0.17	2.36	0.00
5	18	10	24.5	7.40	1.24	6.15	0.00
6	42	10	30.0	46.00	22.40	20.50	2.64
7	18	40	24.5	13.12	4.64	8.48	0.00
8	42	40	24.5	27.30	18.30	9.00	0.00
9	10	25	16.5	30.00	7.77	21.60	0.70
10	50	25	16.5	55.80	47.00	7.80	1.00
11	30	0	16.5	54.13	40.00	6.78	7.43
12	30	50	16.5	39.70	14.18	14.80	0.63
13	30	25	3.0	1.00	0.00	1.00	0.00
14	30	25	30.0	61.00	46.40	14.30	0.50
15	30	25	16.5	28.00	7.50	20.30	0.22
16	30	25	25.0	11.42	2.10	9.32	0.00
17	30	25	16.5	28.00	17.80	9.70	0.50
18	30	25	16.5	14.73	7.73	7.00	0.00
19	30	25	16.5	20.00	7.00	13.00	0.00
20	30	25	16.5	15.00	5.00	10.00	0.00

modified coconut oil (MCO). All treatment combinations were conducted in triplicates.

2.5.2. Acylglycerol Composition Analysis. The acylglycerol composition of the extracted oil sample was determined by using reverse-phase high-performance liquid chromatography (RP-HPLC) (Alliance model Waters e2695 Separation Modules, UK) equipped with ELSD (Alliance model Waters 2424 ELS Detector, UK). Samples were dissolved in acetone (5% v/v) and after filtration through a 0.45 μm PTFE membrane filter were injected onto Merck KGaA (Darmstadt, Germany) LiChrospher 100 RP-18e 5 μm (250 mm × 4 mm) column under gradient condition [23]. The mobile phase used was a gradient of acetone and acetonitrile mixture (from 90% acetonitrile-10% acetone to 85% acetonitrile-15% acetone within first 15 min, then to 20% acetonitrile-80% acetone within next 20 min; and to 90% acetonitrile-10% acetone for last 10 min), where the flow rate was adjusted at 1 mL/min. The column temperature was maintained at 35°C. The drift tube and nebulizer of detector were set at 55°C and 36°C, respectively. The nitrogen gas pressure was 35 psi and the total time for a HPLC run was 45 min. The retention time was 3–7 min for FFA and MG peaks, 8–18 min for DG peaks, and 23–37 min for TG peaks. Each fraction was quantified based on the area normalization approach. TG peaks were identified based on the retention time of TG standards. Each sample was analyzed three times, and the data were reported as mean ± SD of percentage areas.

2.5.3. Antibacterial Activity Studies. Antibacterial activity of the modified coconut oils was evaluated using both Gram-negative and Gram-positive bacteria. Selected Gram-negative bacterium *Escherichia coli* (ATCC 10536) and Gram-positive bacterium *Staphylococcus aureus* (ATCC 25923) were cultivated aerobically at 37°C for 12 h in trypton soy broth (TSB) medium. Bacterial inoculums were prepared at the midlogarithmic phase of their growth containing approximately 10^8 colony-forming units per mL (cfu/mL). It was achieved by diluting the overnight cultures of bacteria with the fresh TSB medium until constant absorbance at 630 nm was gained ($OD_{630} = 0.5$) [24].

Antibacterial activity of the modified oils was evaluated following the method described by Patgaonkar et al. (2011) [24] with some modifications by Ghanbari et al. (2012) [25]. The sample was prepared by mixing the bacterial inoculum (10 μL), TSB medium (120 μL), and modified oil (120 μL) in each well of the 96-well microplate in triplicates. Control samples contained media and bacterial culture, with and without oil (nonmodified). After incubation of samples at temperature of 37°C for 12 h, their absorbance was measured at 650 nm using microplate reader (Power wave, Biotek). The percentage of inhibition was calculated as $[(OD_{control} - OD_{sample})/OD_{control}) \times 100]$.

3. Results and Discussion

3.1. Lipolytic Activity: A Time Course Study. In order to study the direct hydrolysis process of coconut oil through SSF

(DIMOSFER process), the lipolytic activity of the culture was evaluated in a time course study. Results demonstrated no extracellular lipolytic activity. Therefore, the high rate of hydrolysed coconut oil could be associated with intracellular or cell-bound lipolytic activities [26]. However, no lipolytic activity was detected in the cell filtrate after cell disruption using homogenization, sonication, and normal solvent extraction methods, and only cell-bound associated lipolytic activity was responsible for *in situ* coconut oil modification. Likewise, a membrane bound lipase in *G. candidum* GC-4 [27] and a mycelial lipase in a *G. candidum* strain [28] have been reported.

3.2. Modeling and Optimization of DIMOSFER Process for MCFAs Production. Hydrolysis of coconut oil by local *G. candidum* lipolytic activity in SSF was studied, and the yield of corresponding MCFAs production was optimized using response surface methodology (RSM). The selected effective variables were moisture content (%), coconut oil (%), and incubation period of time (day). Shredded coconut meat as an oily source was used as solid support in the solid culture, which originally contained 33.5% of oil (internal coconut oil) and 50% of moisture (internal moisture content). Similarly, the same levels of oil and moisture contents in the coconut were reported by Pandey et al. (1995) [19].

Among effective parameters chosen, moisture content was very essential for this *G. candidum* growth and coconut oil hydrolysis in DIMOSFER process. Based on preliminary study, the original moisture content (internal moisture) of the coconut flakes was not sufficient for direct hydrolysis reaction of the oil. Therefore, additional water (10–50% v/w) was added into the coconut-based medium (external moisture). The solid culture oil content was the second essential parameter in this study. Coconut flakes considered as oily substrate originally contained 33.5% oil in their parenchyma (internal oil). Additional oil (external oil) would be necessary for more efficiently hydrolysis by the fungal lipolytic activity in the solid culture and eventually to produce more MCFAs. The external coconut oil level was 0 to 50% (v/w), and incubation period of time was 3 to 30 days based on the preliminary study. The rate of coconut oil hydrolysis in DIMOSFER process was reported as response in this modeling and optimization study (Table 1). The extracted modified coconut oils after fermentation processes (based on the conditions of CCRD), which contained less triglycerides and more FFAs, have been shown in Table 1.

A reduced cubic model (1) was found to be the best-fitted model to explain the functionality of the system. Coefficient of determination ($R^2 = 0.8728$) and significant F-test analysis ($F_{\text{model}} = 4.99$) and probability value ($P_{\text{model}} > F = 0.0154$) indicated that the model was highly reliable. The model also showed insignificant lack of fit as shown by probability value ($P_{\text{model}} > F = 0.6705$) at 5% significance threshold for MCFAs-rich coconut oil production (Table 2).

Table 2: ANOVA analysis of reduced cubic model.

Source	Sum of squares	DF	Mean square	F value	Prob > F	
Model	6489.22	11	589.93	4.99	0.0154	Significant
A	7.03	1	7.03	0.059		
B	265.65	1	265.65	2.25		
C	601.29	1	601.29	5.09		
A^2	0.061	1	0.061	$5.14E-04$		
B^2	1262.8	1	1262.8	10.68		
C^2	45.34	1	45.34	0.38		
AC	271.67	1	271.67	2.3		
BC	1012.97	1	1012.97	8.57		
A^3	215.65	1	215.65	1.82		
C^3	804.19	1	804.19	6.8		
A^2C	568.14	1	568.14	4.81		
Residual	945.57	8	118.2			
Lack of fit	234.22	3	78.07	0.55	0.6705	not significant
Pure error	711.34	5	142.27			
Cor total	7434.79	19				

A: Moisture content (M); B: oil content (O); C: time (t).
R-squared = 0.8728; Adj R-squared = 0.8044.

Final equation obtained from the reduced cubic model to get the maximum coconut oil hydrolysis in DIMOSFER process (after fermentation) was as follows (1):

$$\begin{aligned}\left[\text{Coconut oil hydrolysis } (\%) = -7144.59\right.&\\ +207.66M - 4.64O + 332.75t&\\ -1.83M^2 + 0.047O^2 - 3.15t^2&\\ -7.43M \cdot t + 0.12O \cdot t + 4.41E&\\ \left.-3M^3 + 0.064t^3 + 0.047M^2 \cdot t\right],&\end{aligned} \quad (1)$$

where M, O, and t were moisture content (%), oil content (%), and incubation time (day), respectively.

3.2.1. Main Effects and Interactions between Parameters. Figure 1 shows the interaction between moisture content (M) and incubation time (t) on coconut oil hydrolysis by local lipolytic *G. candidum* through SSF. The moisture parameter revealed significant changes in yields of oil hydrolysis. By increasing the moisture content, the rate of coconut oil hydrolysis increased significantly until M reached to the middle of the applied range. Increasing M after the middle value decreased the yield of response drastically. On the other hand, the yield of coconut oil hydrolysis was maximized at the beginning of incubation period (time) while, after that, the response decreased slightly. Based on the model achieved and its related ANOVA, M was more effective than t on the oil bioconversion.

Other studies [29, 30] also demonstrated that the optimum moisture level in SSF has a great impact on the physical properties of the solid substrate as well as the enzyme

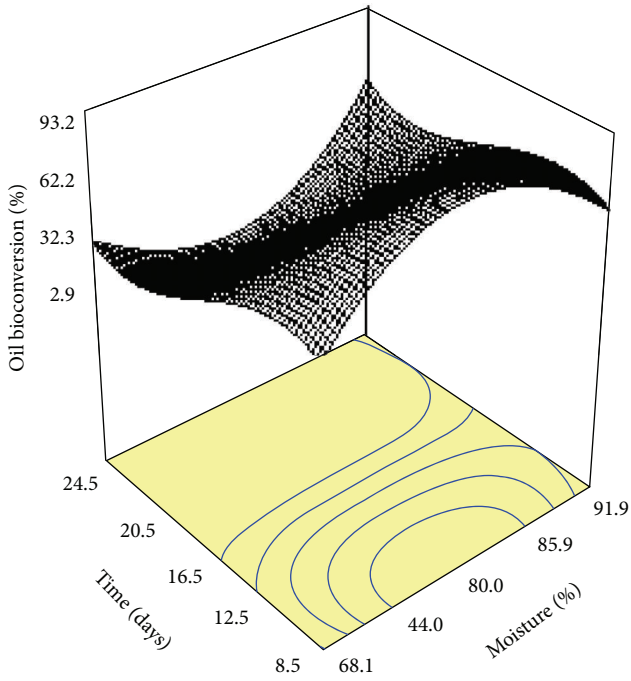

Figure 1: Three-dimensional graphs for the solid-state coconut oil hydrolysis by local *G. candidum*.

production. It has been stated that lower moisture than optimum decreases the solubility of the solid substrate, lowers the degree of swelling, and produces a higher water tension. Likewise, higher moisture levels than optimum value cause decreased porosity, lower oxygen transfer, and alteration in solid-state particle structure [29]. In our previous study, RSM revealed good understanding in complicated biological systems [31]. A reduced cubic-fitted model (Table 2) revealed its potential to determine the best operative conditions for *G. candidum* local strain lipolytic activity towards coconut oil hydrolysis and MCFA production. Finally, six different conditions of MCOs (MCO_1–MCO_6) were compared together in the case of antibacterial activity (Table 3).

3.2.2. Optimum Condition. In order to obtain the maximum lipolysis and MCFAs production in DIMOSFER, process conditions were optimized. The optimum lipolytic function of local *G. candidum* on coconut oil was found to be 29% moisture content and 10.14% oil content, after 9 days of incubation. Maximum coconut oil hydrolysis was 76% under the optimum condition, which consisted of 53% total MCFAs. As shown in Figure 2, the level of medium chain triglycerides (MCTG) content dropped after fermentation, where medium chain monoglycerides (MCMGs), medium chain diglycerides (MCDGs), and medium chain fatty acids (MCFAs) were produced. However, the level of generated lauric acid was obviously the highest compared to other compounds (Figure 2). Hence, the function of this local nonextracellular *G. candidum* lipase in SSF on coconut oil hydrolysis was effective.

The optimum values obtained for this fungus in the solid culture indicated that the local *G. candidum* was able to produce MCFAs under economic conditions. The optimum oil content of the solid culture was found to be 10.14%, which was less than the center level (25%). Time was kept at 9 days after incubation, which was also lower than the center point (16.5 days) to obtain maximum level of coconut oil hydrolysis. Our finding also showed that the optimum level of moisture content required was 29% which was close but still lower than the center point (30%). Thus, from an industrial point of view, to get the optimum lipolytic reaction at the lowest possible level of time, the local *G. candidum* strain could be a good choice as all parameters were optimized at low amounts. Based on our observation, the low water activity required could improve the function of local *G. candidum*, where this characteristic is very crucial in large-scale production to prevent a sticky culture [29].

3.3. Feasibility of Direct Modification of Coconut Oil Process. According to the results obtained in this work, DIMOSFER process could be applied for modification of oil substrate in SSF. As shown in Figure 2, MCTGs content of coconut oil was partially hydrolysed into MCMGs, MCDGs, and particularly MCFAs after fermentation process, where eventually coconut oil turned into the MCAFs-rich coconut oil. To the best of our knowledge, the feasibility of this method in any oil modification and fatty acid production has never been reported. Fernandes et al. (2004) [32] and Martínez-Ruiz et al. (2008) [33] used dried SSF preparations as economical biocatalysts for synthetic reactions in organic solvents. They demonstrated the feasibility of using dried fermented solids, containing lipases without expensive extraction, purification, and immobilization processing. Moreover, Parfene et al. (2013) [34] produced MCFAs using yeast lipase through an agar-based solid culture on plate. The use of DIMOSFER process in a natural plant-based solid culture for MCFAs production using lipolytic *G. candidum* strain was examined for the first time in this study.

3.4. Antibacterial Effects of Modified Coconut Oils (MCOs). Based on the suggested conditions by the reduced cubic model, MCO_1 to MCO_6 in Table 3 were compared to the normal coconut oil. The results demonstrated that all MCOs (MCO_1–MCO_6) extracted from the local *G. candidum* solid cultures revealed significant improved levels of antibacterial activities (ABAs) compared to the control (Table 3). MCOs, which were produced from hydrolysis of coconut oil during DIMOSFER process, contained different proportions of MCTGs, MCDGs, MCMGs, and MCFAs (Figure 2(b)) compared to the normal coconut oil, which contained MCTGs (Figure 2(a)). Detailed analysis of the lipid classes by RP-ELSD-HPLC (Figure 2) showed that the amount of MCFAs particularly C_{12} (lauric acid) played important role in inactivation of the growth of both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*E. coli*) bacteria selected (Table 3). Similarly, results obtained by Carroll (1980) [35] indicated that high amount of fatty acids content play important role in broadening the antimicrobial spectrum of

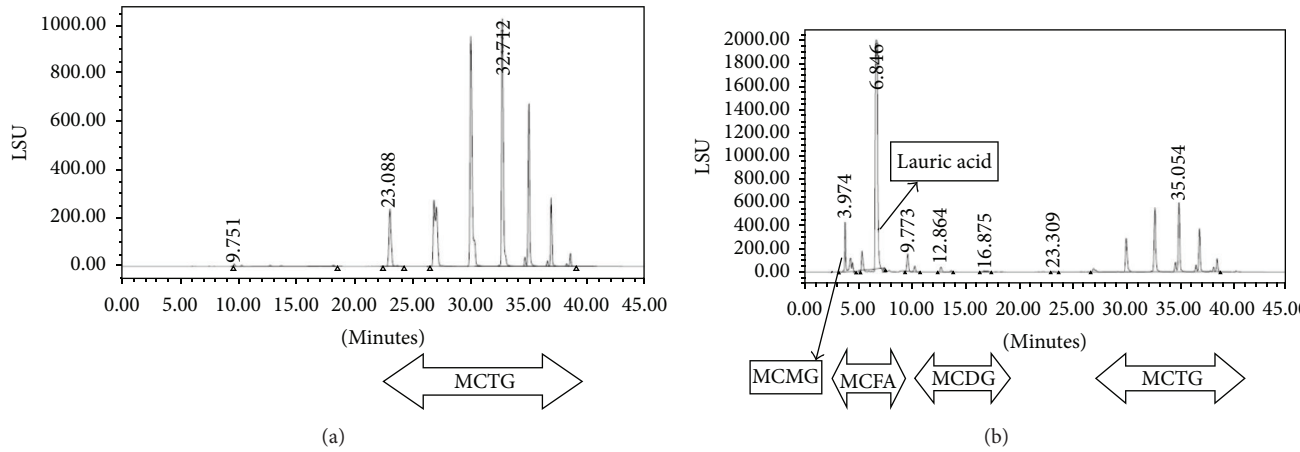

Figure 2: Coconut oil (a) and optimum modified coconut oil (MCO_6), (b) glycerides profiles analyzed by ELSD-RP-HPLC.

Table 3: Composition of the selected modified coconut oils (MCO_1–MCO_6) produced by local *G. candidum* through DIMOSFER process along with their antibacterial activities.

Sample	SSF condition			Product composition (%)				ABA (%)	
	Moisture content (%)	Oil content (%)	Incubation time (day)	MCTG	MCDG	MCMG	MCFA	*E. coli*	*S. aureus*
MCO_1	32	50	30	80	15	1	14	15	20
MCO_2	42	10	10	54	20.52	2.64	22.4	71	75
MCO_3	30	0	16	54.5	9.8	4.65	31.14	60	63
MCO_4	30	25	10	38	20.32	0.5	40.5	75	80
MCO_5	50	25	16	44.5	7.8	1	47	78	85
MCO_6 (opt)	30	10	9	24	16.55	6.45	53	80	90
Coconut oil				99	1	0	0	3.1	4.8
Without oil				—	—	—	—	0*	0*

MCO_1–MCO_6: modified coconut oils obtained from DIMOSFER by local *G. candidum* strain.
Opt: optimum condition obtained from optimization of coconut oil hydrolysis through DIMOSFER process.
Composition: MCTG: medium chain triglyceride; MCDG: medium chain diglyceride; MCMG: medium chain monoglyceride; and MCFA: medium chain fatty acid.
ABA: antibacterial activity.
Pathogenic bacteria: *Escherichia coli* (ATCC 10536) and *Staphylococcus aureus* (ATCC 25923).
* *Escherichia coli* (1.44×10^8 CFU/mL) and *Staphylococcus aureus* (1.30×10^8 CFU/mL).

modified oils. In addition, it has been demonstrated that the MCFAs and their corresponding monoglycerides and diglycerides have antimicrobial effects against *S. aureus* [7, 36] and *E. coli* [37–40]. Hayashi (1995) [38] indicated that the combination of MCTG, MCDG, MCMG, and MCFA revealed broad range of antimicrobial properties against human pathogens and enveloped viruses. Moreover, these compounds are known to have antimicrobial effects against food-borne pathogens like *L. monocytogenes* [41] and *C. botulinum* [42].

As shown in Table 3, the level of MCFAs, mostly composed of free lauric acid, was the most important factor for antibacterial activity. The highest antimicrobial activities (90% against *S. aureus* and 80% against *E. coli*) obtained under maximum level of MCFAs (53%) produced particularly lauric acid. The effectiveness of free lauric acid in antibacterial activity against Gram-positive and Gram-negative bacteria was also demonstrated by Khoramnia et al. (2013) [43]. It has been well established that lauric acid represents the strongest antimicrobial activity among all fatty acids [44].

Sado-Kamdem et al. (2009) [3] indicated that the antimicrobial mechanism of MCFAs is to increase cell membrane fluidity when added to foods. As stated by Sado Kamdem et al. (2008) [2], FFAs' affect the division intervals of single cells which bring about an inhibitory effect compared to the control medium's longer division intervals. The antimicrobial action of lauric acid is due to the penetration of acid in the lipid membrane of the bacterial cell. The corresponding cellular acidic pH leads cell death by suppressing cytoplasmic enzymes and nutritional transport systems as well as uncoupling ATP driven pumps [45, 46]. Similar mechanism has been proposed for MCFA, MCDG, and MCMG [40], whereby these functional lipids kill bacteria by disrupting the permeability barrier of cell membrane. For instance, it

was reported that lauric acid-rich feed prevented the death of infected mice, Guinea pigs, and cows by inhibiting the growth of *Mycobacterium* by interfering with the enzymatic systems [35].

Nakatsuji et al. (2009) [47] found that lauric acid has the potential of becoming an innate, safe, and effective therapeutic medication for all *P. acnes*-associated diseases. The safety of lauric acid and its esters when consumed in large doses and over extended periods of time was indicated to be safe [4]. Therefore, they could be considered as natural GRAS antimicrobial components. Since coconut oil has been widely used in cosmetic products and also approved for clinical applications, this lauric acid enriched oil developed from this work has the potential to be used in therapeutic applications. Similarly Hristov et al. (2009) [9] indicated that combination of lauric acid and coconut oil revealed even higher antimicrobial activity, better milk fatty acid alteration, and lower methane production *in vivo* compared to the individual application of these elements.

Moreover, as stated by Kitahara et al. (2006) [48], MCFAs particularly lauric acid are suitable for external application for infection control and medical treatment in hospitals. Soni et al. (2010) [8] reported that application of lauric acid in cheese enhanced bactericidal activity without affecting sensory quality. In addition, Soni et al. (2012) [49] illustrated that the mixture of MCFAs enhanced the antimicrobial activity and methane formation suppression in ruminants effectively. All these studies are testament to the potential application of the produced MCO.

4. Conclusions

In the present study, the use of DIMOSFER in production of value-added coconut oil by a Malaysian strain of lipolytic *G. candidum* and the chemical characterization of the modified oils and their antimicrobial activities were investigated. The variation in glycerides composition of MCOs and the extracted coconut oils after fermentation process was evaluated by ELSD-RP-HPLC analysis. The yield of coconut oil hydrolysis and MCFAs production through DIMOSFER process were optimized by a reduced cubic model at 76% and 53%, respectively. The fungal lipolytic activity on coconut fat hydrolysis was maximized at 29% of moisture content and 10.14% of oil content after 9 days of incubation in SSF. Antimicrobial activities of MCOs were evaluated against some food-borne bacteria, and an increase in inhibitory activity with increasing concentration of MCFAs particularly lauric acid was noted. Therefore, DIMOSFER process accompanied with the use of a novel local lipolytic *G. candidum* as a GRAS microorganism can be considered as a "green" process. This process was found to be advantageous in MCFAs production as GRAS antimicrobial agents. The produced MCOs, rich in free MCFAs content, could be further applied for food, cosmetic, and pharmaceutical purposes. Natural enrichment of lauric acid in an edible culture offers a new approach to increase lauric acid intake in human populations with the potential to improve long-term human health.

Conflict of Interests

The authors wish to declare that there is no known conflict of interests associated with this publication and there has been no other significant financial support for this work that could have influenced its outcome.

Acknowledgment

The financial support by Universiti Putra Malaysia is gratefully acknowledged.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 175012, 14 pages
http://dx.doi.org/10.1155/2013/175012

Research Article

Production and Characterization of Keratinolytic Protease from New Wool-Degrading *Bacillus* Species Isolated from Egyptian Ecosystem

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Received 2 December 2012; Revised 7 March 2013; Accepted 28 March 2013

Academic Editor: Periasamy Anbu

Novel keratin-degrading bacteria were isolated from sand soil samples collected from Minia Governorate, Egypt. In this study, the isolates were identified as *Bacillus amyloliquefaciens* MA20 and *Bacillus subtilis* MA21 based on morphological and biochemical characteristics as well as 16S rRNA gene sequencing. *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 produced alkaline keratinolytic serine protease when cultivated in mineral medium containing 1% of wool straight off sheep as sole carbon and nitrogen source. The two strains were observed to degrade wool completely to powder at pH 7 and 37°C within 5 days. Under these conditions the maximum activity of proteases produced by *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 was 922 and 814 U/ml, respectively. The proteases exhibited optimum temperature and pH at 60°C and 9, respectively. However, the keratinolytic proteases were stable in broad range of temperature and pH values towards casein Hammerstein. Furthermore the protease inhibitor studies indicated that the produced proteases belong to serine protease because of their sensitivity to PMSF while they were inhibited partially in presence of EDTA. The two proteases are stable in most of the used organic solvents and enhanced by metals suggesting their potential use in biotechnological applications such as wool industry.

1. Introduction

Keratins are classified as fibrous proteins called scleroproteins that occur abundantly in epithelial cells. These proteins are insoluble in water, weak acid and alkali, and organic solvents and are insensitive to the attack of common proteolytic enzymes such as trypsin or pepsin [1]. The animal remains rich in α-keratin such as animal skin, hair, claws, horns, and wools.

The important property of these proteins is the presence of high cystine content that differentiates keratins from other structural proteins such as collagen and elastin. Both a high cystine content as well as a high content of glycine, proline, serine, and acidic amino acids and a low content of lysine, histidine, and methionine (or their lack) as well as the absence of tryptophan are also characteristic of keratins [2, 3]. Numerous disulfide cystine bonds present in keratin to bind peptide chains and packed as α-helices as in hair and wool or in β-sheet arrangements as in case of feathers. The disulfide linkage and the tight secondary structure of keratins make them difficult to be hydrolysed by common proteolytic enzymes [4].

The major problem of α-keratin hydrolysis is the presence a high numbers of disulfide bonds that make it insoluble in nature and resistant to proteases hydrolysis [5]. Keratinolytic protease enzymes are spread in nature and elaborated by different groups of microorganisms that can be isolated from polluted area with keratin wastes [6]. A vast variety of Gram-positive bacteria including *Bacillus*, *Lysobacter*, *Nesternokia*,

Kocuria, and *Microbacterium* as well as a few strains of Gram-negative bacteria such as *Xanthomonas*, *Stenotrophomonas,* and *Chryseobacterium* are confined as keratin degraders [7–10]. Most of keratin degrading bacteria belong to the genus of *Bacillus* [11].

The keratinous substrate such as feather and wool can be degraded in basal medium by microorganisms which are capable of utilize keratin as sole carbon and nitrogen source [12]. Keratinolytic proteases have broad substrate specificity where they have the ability to hydrolyze soluble protein such as casein, gelatin, and bovine serum albumin. Additionally, they can hydrolyze the insoluble protein including feather, silk, and wool [13]. Keratinolytic proteases mostly belong to serine or metalloproteases showing sequence similarity with subtilisin group of proteases [14, 15]. In recent years, more demands to keratinolytic proteases are increasing due to their multitude in industrial applications such as the feed, fertilizer, detergent, and textile industries. The present study describes the isolation and identification of new *Bacillus amyloliquefaciens* MA20 and *B. subtilis* MA21 strains from Egyptian ecosystem that grow well on wool as sole carbon source. Moreover the two strains are able to degrade wool and their enzymes can be used to improve the wool quality. This paper includes full characterization of the keratinolytic protease which explains that the best environmental conditions can be used to improve the wool quality in industry.

2. Materials and Methods

2.1. Sample Collection. Different types of samples were collected from different Governorates of Egyptian ecosystem included Alexandria, Behera, Qaliubia, Mania, and Asiut. These fresh samples were varied such as soil, sand soil, humus, waste wool, and rhizosphere under olive trees. These samples were collected in sterile falcon tubes and transported to the microbiological lab in (City of Scientific Research and Technological Applications).

2.2. Strains Isolation. The bacterial strains were isolated by suspending 1 g of soil samples in a 10 mL sterile 0.85% (w/v) saline solution and then treated for 20 min at 80°C. This will enable the isolation of the spore-forming bacteria. Luria-Bertani (LB) agar medium with 1% (w/v) skim milk was used for their cultivation by spreading 0.1 mL of each 10^{-5} and 10^{-6} dilutions. The plates then were incubated for 24 hours at different temperatures [4, 16]. The colonies which give clear zones formed by hydrolysis of skim milk were picked. Pure bacterial isolates were obtained by reculturing individual colonies several times on fresh LB agar medium to produce single colony from each.

2.3. Strains Selection. Twelve selected strains isolated according to the diameter of clear zone were cultured on medium containing 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g KH_2PO_4, and 10 g wool per liter; pH 7 and incubated for 5 days at 37°C. The wool was used as sole carbon and nitrogen source for detecting potent strains that have the ability to degrade the wool completely. Three strains degraded the wool and the supernatants of their culture were assayed on plate containing 1% gelatin powder which is soluble in phosphate buffer pH 7. After determining the existence of the activity (by the clear zone of the supernatants), *Bacillus* sp. MA20 and *Bacillus* sp. MA21 were selected and preserved for further investigation.

2.4. Bacterial Identification. While the phenotypic characteristics and isolation method of the two selected isolates indicate that they are related to *Bacillus* group but further identification was conducted.

The strains identification are included the spore morphology, Gram stain, and motility. The morphological and physiological characteristics of the bacterial isolates were compared with the data from Bergey's Manual of Determinative Bacteriology [17].

2.5. Scanning of Bacillus sp. MA20 and Bacillus sp. MA21 by Scanning Electron Microscope. The bacterial smear was prepared by centrifuging the bacterial cultures at 12,000 rpm for 20 min. The pellets were washed 2 times by saline solution. The pellet was suspended in sterilized distilled H_2O. The bacterial film was prepared and fixed on glass slides till complete drying. The smear was coated with gold using sputter coater. The golden coated sample was scanned at 20 KV acceleration voltages at room temperature.

2.6. Genetic Identification and Differentiation

2.6.1. DNA Extraction. The genomic DNA of *Bacillus* strains was isolated using modification method from Sambrook et al. [18].

2.6.2. Identification by 16S Ribosomal RNA (rRNA)

PCR Amplification according to Sambrook et al. [18]. The PCR amplification reactions were performed in a total volume of 50 μL. Each reaction mixture contained the following solutions: 2 μL of DNA (40 ng), 1 μL of 10 pmol forwarded 16S-rRNA primer (5′-AAATGGAGGAAGGTGGGGAT-3′); 1 μL of 10 pmol reverse 16S rRNA primer (5′-AGGAGGTGATCCAACCGCA-3′); 0.8 μL of 12.5 mM (dNTP's); 5 μL of PCR buffer included $MgCl_2$, and 0.2 μL Taq polymerase (1 Unit) and water-free DNAse and RNAse were added up to 50 μL. The PCR apparatus was programmed as follows: 3 min denaturation at 95°C, followed by 35 cycles that consisted of 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C, and a final extension was 10 min at 72°C. The products of the PCR amplification were analyzed by agarose gel electrophoresis (2%).

2.6.3. PCR Cleanup and 16S rRNA Sequencing. The PCR products were cleaned up for DNA sequencing following the method described by Sambrook et al. [18]. Automated DNA sequencing based on enzymatic chain terminator technique, developed by Sanger et al. [19], was carried out using 3130X DNA Sequencer (Genetic Analyzer, Applied Biosystems, Hitachi, Japan).

2.6.4. Phylogenetic Analysis. Similarity analysis of the nucleotides was performed by BLAST searches against sequences available in GenBank. For phylogenetic tree construction, multiple sequences were obtained from GenBank and the alignments were performed using MEGA 5 software version 5.1 [20].

2.7. Keratinolytic Protease Production. Bacillus sp. MA20 and *Bacillus* sp. MA21 were first inoculated in liquid LB medium to produce large amount of cells. After 18 hrs, the colony forming units (CFU)/mL culture were $3 * 10^6$, and 2% volume of the liquid medium was transferred to the production medium using 250 mL flask containing 100 mL of the production medium. The production medium containing (w/v) NaCl, 0.5 g/L; K_2HPO_4, 0.3 g/L; KH_2PO_4, 0.4 g/L; wool, 10 g/L; and the pH was adjusted 7.0–7.2 using 2N of NaOH and HCl [16]. The cultivated media were incubated at 37°C and 200 rpm for 5 days. Culture supernatants were obtained by centrifugation at 12,000 rpm and 4°C for 30 min. The different supernatants which contain the crude enzymes were used in assay and analysis of enzymes.

2.8. Enzymes Assay

2.8.1. Detection of the Proteolytic Activity on Plates. The crude enzyme of *Bacillus* sp. MA20 and *Bacillus* sp. MA21 was screened for their proteolytic activity using agar well diffusion plate method described by Amara et al., after modification [21]. One gram of gelatin powder was suspended in 100 mL phosphate buffer pH 7 and autoclaved. After sterilization, the soluble component was added to sterile water containing agar (18 gm agar/L). The suspension then stirred gently and distributed in Petri dishes (25 mL/plate). After complete solidification of the agar on plates, wells were punched out of the agar, by using a clean sterile cork borer. The base of each hole was sealed with a drop of melted sterile water agar (15 g agar per liter H_2O) using sterile Pasteur pipette. Fifty μL of each bacterial supernatant was added to each well and preincubated at 4°C for 2 hrs and then overnight incubated at different temperatures.

2.8.2. Visualization of the Enzyme Clear Zone. Coomassie blue (0.25%, w/v) in methanol-acetic acid-water 5 : 1 : 4 (v/v/v) was used in plates staining to visualize the gelatin hydrolysis where 10 mL was added to each plate and incubated in room temperature for 15 min followed by removing the staining solution from the plates surfaces and washing gently by distilled water. Then the plates were destained using destining solution (66 mL methanol, 20 mL acetic acid, and 114 mL H_2O_{bidest}) for a suitable time [22]. Also extracellular protease detection was determined according to Vermelho et al., [23] with modification. Staining was performed with 0.1% amido black in methanol-acetic acid-water 30 : 10: 60 (v/v/v) for 5–20 min (according to the quality of amido black stain) at room temperature. Regions of enzyme activity were detected as clear areas, indicating that hydrolysis of the substrates has been occurred.

2.8.3. Enzyme Assay Spectrophotometrically

Preparation of Casein in Different Buffers with Different pH. Casein Hammarstein was weighted in a quantity of 0.325 g and dissolved in 50 mL of different buffers at different pH. The mixture was dissolved by heating gently to 80–90°C without boiling in water bath according to Amara and Serour [24] or using hot plate with stirring for more accurate because casein is highly stick with the walls of the container. The following buffers were used:

(i) sodium phosphate, pH 6-7-8 (0.1 M)
(ii) glycine/NaOH pH 9-10 (0.1 M)
(iii) sodium phosphate dibasic/NaOH pH 11 (0.05 M)
(iv) KCl/NaOH pH 12 (0.2 M).

2.8.4. Optimum Temperature of the Enzymes. Ten μL of each supernatant which contains the crude enzyme was added to 490 μL of the casein Hammarsten soluble in buffer pH 7. The enzymes-substrate mixture was incubated at different temperatures of 20, 30, 40, 50, 60, 70, and 80°C using water bath for 15 min. After the incubation period the enzyme reaction stopped by adding 500 μL of 10% trichloroacetic acid (TCA). The mixture was allowed to stand in ice for 10 min and then centrifuged at 13,000 rpm for 10 min. The absorbance of each sample was determined spectrophotometrically at 280 nm and their tyrosine content derived from the tyrosine standard curve which was carried out according to Amara and Serour [24] and the enzyme activity was determined as Unit/mL.

2.8.5. Optimum pH of the Enzymes. Ten μL of each supernatant which contains the crude enzyme was added to the 490 μL of the casein Hammarsten soluble in different pH values. The enzymes-substrate mixture was incubated at 60°C which acts as optimum temperature in a water bath for 15 min. At the end of incubation period the enzyme reaction stopped by adding 500 μL of 10% trichloroacetic acid (TCA). The mixture was allowed to stand in ice for 10 min and then centrifuged at 13,000 rpm for 10 min. The absorbance of each sample was determined spectrophotometrically at 280 nm and their tyrosine content derived from the tyrosine standard curve and the enzyme activity determined as Unit/mL.

2.8.6. Confirmation of Optimum pH and Temperature. The previous reaction was performed using casein Hammarsten soluble in Glycine/NaOH pH 9. The enzyme substrate mixture was incubated at various temperature of 20, 30, 40, 50, 60, 70 and 80°C in water bath for 15 min. The enzyme activity was calculated as mentioned previously.

2.8.7. The Optimized Enzymes Reaction. The optimized reaction is a mixing between 10 uL of crude enzyme and 490 μL of the casein Hammarsten soluble in Glycine/NaOH pH 9. The mixture was incubated at 60°C in water bath for 15 min. The reaction was stopped with 500 μL of 10% TCA. The mixture was allowed to stand in ice for 10 min and then was

centrifuged at 13,000 rpm for 10 min. The enzyme activity was determined as mentioned above using tyrosine standard curve.

2.8.8. Thermal Stability. The thermostability was carried out by preincubating the crude enzyme solution at a temperature range of 4°C to 80°C for 0.0 to 24 hrs. The residual activity was measured with standard enzyme reaction. The control is enzyme reacted at zero time (consider as 100%) [16].

2.8.9. pH Stability. The pH stability was determined by preincubating the enzyme solution in buffers with different pH values (3–12) at room temperature from 0.0 to 48 hrs. The residual activity was measured with standard enzyme reaction. The control is enzyme reacted at zero time and considered as 100% [16]. The following buffers were used:

(i) citrate buffer pH 3-4-5-6 (0.1 M)

(ii) sodium phosphate pH 7-8 (0.1 M)

(iii) glycine/NaOH pH 9-10 (0.1 M)

(iv) sodium phosphate dibasic/NaOH pH 11 (0.05 M)

(v) KCl/NaOH pH 12 (0.2 M).

2.8.10. Effect of Inhibitors. The inhibitors were added to supernatants which contain the produced enzyme and were incubated for 30 min at 30°C before being tested for proteolytic activity. Protease inhibitors phenylmethanesulphonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol, and the detergent sodium dodecyl sulfate (SDS) were used. The inhibitors stocks were prepared in distilled water except that PMSF was prepared by using isopropanol. The final concentrations of PMSF, EDTA, and β-Mercaptoethanol are 5 mM and 1 mM while SDS is 0.5% and 0.1% (w/v). The control was enzyme mixed with distilled water instead of inhibitors. Control activity was considered to be 100% [25].

2.8.11. Effect of Metal Ions. The effect of metal ions on protease activity was investigated using two concentrations of 5 mM and 10 mM (final concentration). The metal stock solutions were prepared in distilled water and diluted to the appropriate concentrations. The enzyme solution was mixed with the different metal solutions and incubated for 30 min at 30°C before assay. A control was also included where the enzyme was mixed with distilled water instead of metal solution. Control activity was considered to be 100% [25]. $ZnCl_2$, $MgCl_2$, $CuSO_4$, Urea, $HgCl_2$, $CaCl_2$, $BaCl_2$, Guanidin HCl, and $MnCl_2$ were used.

2.8.12. Effect of Solvents. The effect of the different solvents (Methanol, Ethanol, DMSO, Isopropanol, Tween 20, and Triton X100) on protease activity was investigated using a concentration of 1% and 0.5% (final concentration) [25].

2.9. First-Dimension Protein Electrophoresis

2.9.1. Sodium Dodecyl Sulfate Polyacrylamid Gel Electrophoresis (SDS-PAGE). Characterization of proteins and evaluation of the protein enrichment process SDS-PAGE was performed in a discontinuous SDS-PAGE vertical slab gel electrophoresis apparatus as described by Laemmli [26]. Discontinuous SDS-PAGE consisted of a stacking gel (5%, w/v, pH 6.8) and a separating gel (12%, w/v, pH 8.8). The separating gel was prepared in a 1 mm slab gel (10 × 10 cm).

2.10. Gel Staining Using Silver Stain. The gels were stained using silver nitrate staining methods as described by Blum et al. [27].

2.11. Zymogram for the Detection of Protease Activity Using SDS-PAGE. As above in the case of protease zymography using SDS PAGE and the separating gel concentration was 12%. The samples were mixed with 5X sample buffer without β-mercaptoethanol and without boiling.

After running the gel at 80 V the SDS-PAGE gel was stripped off from the gel plate and soaked in 1% triton X100 for 2 hrs to change the solution every 1 hour or in 2.5% triton X100 for 30 min for removing SDS and renaturating the enzymes.

The gel was washed three times with tape water and soaked in 1% gelatin powder solubilized in Glycine/NaOH buffer pH 9 for 90 min at 60°C. The gel was washed with buffer for 10 min before staining.

Then the gel was stained with Coomassie blue stain for 2 hrs. After that the gel was destained till the active bands appear.

2.12. Zymogram for Detecting Protease Activity Using Native PAGE. The gel was carried out using all components of the SDS-PAGE and the same conditions but without SDS as well as the buffers described above. The samples were prepared by mixing with 5X sample buffer for native PAGE and without boiling. The gel was washed with tap water and soaked into 1% gelatin powder as mentioned previously. Then the gel was stained with Coomassie blue stain for 2 hrs. After that, the gel was destained till the active bands appear.

2.13. Two-Dimension Polyacrylamide Gel Electrophoresis (2D-PAGE). First-dimension isoelectric focusing (IEF) was performed using Ettan IPGphor3 and the second-dimension SDS-PAGE was applied using vertical electrophoresis of Ettan DALTtwelve System. The experiment was carried out using the operation instruction of manufacturer (GE healthcare *company*). The gels were stained with Coomassie stain where Coomassie blue R-250 (0.02 g) in methanol-glacial acetic acid-distilled water (10 mL, 5 mL, and 100 mL resp.) was used.

3. Results and Discussion

3.1. Strain Isolation. Microbial keratinolytic protease has been described for various biotechnological applications in food, detergent, textiles, and leather industries, and yet the

growing demand for these enzymes necessitates the screening for novel keratinolytic microorganisms with potential applications [28, 29]. Keratinolytic protease has been described for several species of *Bacillus* [30, 31] due to the broad distribution of keratinase among these genera, and this study focused on keratinolytic protease production from them.

A total of 48 pure cultures of spore-forming bacteria were isolated and purified which obtained from different samples collected from Governorates of Egypt. All isolates were screened using selective method for *Bacillus* isolation. The proteolysis activities of all the isolates were detected using the plate test method containing LB agar medium with 1% (w/v) skim milk. Among the isolates analyzed, 12 isolates exhibited proteolytic activity in which they had a halo diameter of fivefold longer than the colony diameter. All isolates have the proteolytic activity but do not have the ability to degrade wool, for that the twelve selected isolates were grown using a medium which contain (w/v) NaCl, 0.5 g/L; K_2HPO_4, 0.3 g/L; KH_2PO_4, 0.4 g/L; wool, 10 g/L; and the pH was adjusted at 7.0–7.2 using 2N NaOH and HCl. The flasks were incubated for 5 days until the wool has been completely degraded by some isolates. Three *Bacillus* isolates show the ability to degrade the wool and gained the names *Bacillus* sp. MA20, *Bacillus* sp. MA21, and *Bacillus* sp. MA10. In the last screening step, the obtained supernatants from the cultivation of three above *Bacillus* strains culture media were assayed using agar well diffusion in Petri dishes including gelatin powder plate which suspended in phosphate buffer pH 7 as in Figure 1. *Bacillus* sp. MA20 and *Bacillus* sp. MA21 were selected for studying the keratinolytic protease enzyme based on the diameter of clear zone (Figure 1). By refering to their isolation source, the two selected strains were found to be isolated from Minia Governorates. This method is simple but proves to be efficient for determining the best enzyme-producing isolates. Out of the three strains, the two which were given the best result have been subjected to further identification.

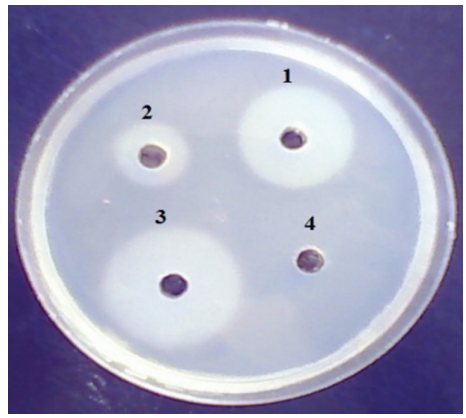

Figure 1: Proteolytic activity of the supernatants obtained from three *Bacillus* sp. on gelatin suspended in phosphate buffer pH 7. The well number (1) is supernatant from *Bacillus* sp. MA21, number (2) is from *Bacillus* sp. MA10, number (3) is from *Bacillus* sp. MA20, and (4) is control free of enzyme.

3.2. Strains Identification

3.2.1. Identification by Morphological and Biochemical Tests. The morphological and physiological characteristics of the isolated strains were compared with the data from Bergey's Manual of Determinative Bacteriology [17] who revealed that *Bacillus* sp. MA20 and *Bacillus* sp. MA21 are matching with those of *B. subtilis* group. In previous articles on taxonomy, species included in the *B. subtilis* group are the following: *B. velezensis*, *B. atrophaeus*, *B. mojavensis*, *B. malacitensis*, *B. axarquiensis*, *B. nematocida*, *B. vallismortis*, *B. subtilis*, and *B. amyloliquefaciens* [32–34].

The two strains are aerobic, motile, and Gram-positive rods. The smears of *Bacillus* sp. MA20 and *Bacillus* sp. MA21 were scanned by scanning electron microscope which indicates the bacterial size of the two *Bacillus* strains which was measured by slime view program software.

The results of biochemical tests of the *Bacillus* sp. MA20 and *Bacillus* sp. MA21 indicated that they are related to *B. subtilis*, and *B. amyloliquefaciens* which summarized in Table 1.

3.3. Identification Based on Genetic Materials (DNA)

3.3.1. DNA Extraction. The isolated DNA was analyzed by gel electrophoresis and the quality of the DNA for each sample has been identified for further investigation.

3.3.2. Identification by 16S Ribosomal RNA (rRNA). The amplified 16S rRNA gene from the DNA of *Bacillus* sp. MA20 and *Bacillus* sp. MA21 was determined using 2% agarose gel. The size of the amplified fragments was determined by using size standard (Gene ruler 50 bp–1031 bp DNA ladder). The PCR products were visualized under UV light and photographed using gel documentation system. Approximately 380 bp of 16S rRNA gene was amplified.

The PCR products were purified and sequenced using 16S forward primer. The sequences of *Bacillus* sp. MA20 and *Bacillus* sp. MA21 were deposited in national center for biotechnology information (NCBI GenBank) under the Accession numbers (HQ115599.1–HQ115600.1), respectively. The basic local alignment search tool (BLAST) algorithm was used to retrieve for homologous sequences in GenBank.

The *Bacillus* sp. MA20 revealed 98% identity to *Bacillus amyloliquefaciens* and *Bacillus subtilis* while *Bacillus* sp. MA21 revealed 97% identity to *Bacillus subtilis* and *Bacillus amyloliquefaciens*. Based on the morphological, biochemical, and molecular characteristics, the *Bacillus* sp. MA20 and *Bacillus* sp. MA21 were designated as *B. amyloliquefaciens* MA20 and *B. subtilis* MA21, respectively.

A phylogenetic tree based on the comparison of 16S rRNA sequences of reference strains was constructed. The phylogenetic analysis was performed with 341 bp sequences using the software MEGA 5 [20], using the neighbour-joining method and based on Jukes-Cantor distances. The branching

Table 1: Morphological and biochemical properties of *Bacillus* sp. MA20 and *Bacillus* sp. MA21.

Characteristics	*Bacillus* sp. MA20	*Bacillus* sp. MA21
Morphological		
Shape	Rods	Rods
Gram stain	G+ve	G+ve
Motility	Motile	Motile
spore formation	+ve	+ve
Growth		
Growth temperature	15°C–50°C	15°C–60°C
Growth pH	5–8	5–8
Biochemical tests		
Oxidase	+ve	+ve
Catalase	+ve	+ve
Voges-Proskauer	+ve	+ve
Indol production	−ve	−ve
Nitrate reduced to nitrite	+ve	+ve
Hydrolysis of		
Casein	+ve	+ve
Gelatin	+ve	+ve
Wool	+ve	+ve
Starch	+ve	+ve
Acid from		
Glucose	+ve	+ve
Arabinose	+ve	+ve
Xylose	+ve	+ve
Mannitol	+ve	+ve
Gas from glucose	−ve	−ve
Utilization of		
Citrate	+ve	+ve
Propionate	−ve	−ve

pattern was checked by 500 bootstrap replicates (Figures 2 and 3).

3.4. Media Screening for Keratinolytic Protease Production. Keratinolytic proteases are largely produced in a basal medium with keratinous substrates, and most of the organisms could utilize keratin sources such as feather and wool as the sole source of carbon and nitrogen [35, 36]. *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 were tested on eight nutrient media. The selected media is medium (1) which is containing (w/v) NaCl, 0.5 g/L; K_2HPO_4, 0.3 g/L; KH_2PO_4, 0.4 g/L; wool, 10 g/L, and the pH was adjusted 7.0–7.2 using 2N of NaOH and HCl.

Korniłłowicz-Kowalska, (1997) and Amara and Serour (2008) reported that the mass loss of the keratin substrate is the most reliable indicator of microbial keratinolytic abilities. *B. amyloliquefaciens* MA20 and B. *subtilis* MA21 have the ability to degrade wool completely after incubation for 5 days and remain it as powders in the bottom of flasks [24, 37].

3.5. Enzyme Production and Characterization. The keratinolytic protease enzyme was produced using production medium mentioned above for each of *B. amyloliquefaciens* MA20 and *B. subtilis* MA21, and the crude enzymes were characterized.

3.6. Detection of the Proteolytic Activity on Plates. The crude enzymes of *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 were assayed by agar well diffusion methods on plates containing gelatin soluble in glycine/NaOH buffer of pH 9. The Coomassie blue and amido black bind to gelatin and whole plate giving the colour of dye except the hydrolysis areas which appear as transparent without dye (Figure 4).

3.7. Characterization of Keratinolytic Protease Enzyme

3.7.1. Influence of pH and Temperature. The effect of temperature and pH on enzymes activity and stability was determined. The optimum temperature and pH for protease activity of enzymes produced by *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 was found to be 60°C, 9.0, respectively.

The enzymes activity was investigated at pH 7 and different temperatures (Figure 5). The proteolytic activities were determined at optimized temperature 60°C and different pH (Figure 6). For confirmation from the optimized reaction, the enzymes activity was assayed at pH 9 with different temperatures (Figure 7).

Results revealed that the keratinolytic protease from *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 is similar to those produced using bacteria, actinomycetes, and fungi and has a pH optimum in a neutral-to-alkaline range [38, 39]. The optimal temperature for activity was also found in the usual range for keratinolytic protease (30–80°C).

The maximum activity of protease enzyme produced by *B. amyloliquefaciens* MA20 was 922 U/mL at pH 9 and 60°C while the maximum activity of protease enzyme produced by *B. subtilis* MA21 was determined as 814 U/mL at the same conditions. The alkaline pH of the keratinolytic protease enzyme from *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 suggests a positive biotechnological potential.

The enzymes were stable at temperatures between 4 and 70°C. The enzyme produced by *B. amyloliquefaciens* MA20 is more thermostable than enzyme produced by *B. subtilis* MA21 (Figures 8 and 9). The results of thermal stability indicated that the keratinolytic protease from *B. amyloliquefaciens* MA20 is more thermostable than the enzyme produced by *B. subtilis* MA21.

The thermal stability studies give the enzyme from *B. amyloliquefaciens* MA20 the advantage for using in industrial applications which are the main objective of this study.

The crude enzyme from *B. amyloliquefaciens* MA20 is described as stable over a broad pH range of 4.0–12.0, but the best stable pH is 9 and 10 (Figure 10). Figure 11 indicates that the enzyme from *B. subtilis* MA21 was stable in pH range (5.0–12.0) with high stability at pH 9. The stability of keratinolytic proteases produced by *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 has been suggested to offer great advantages for

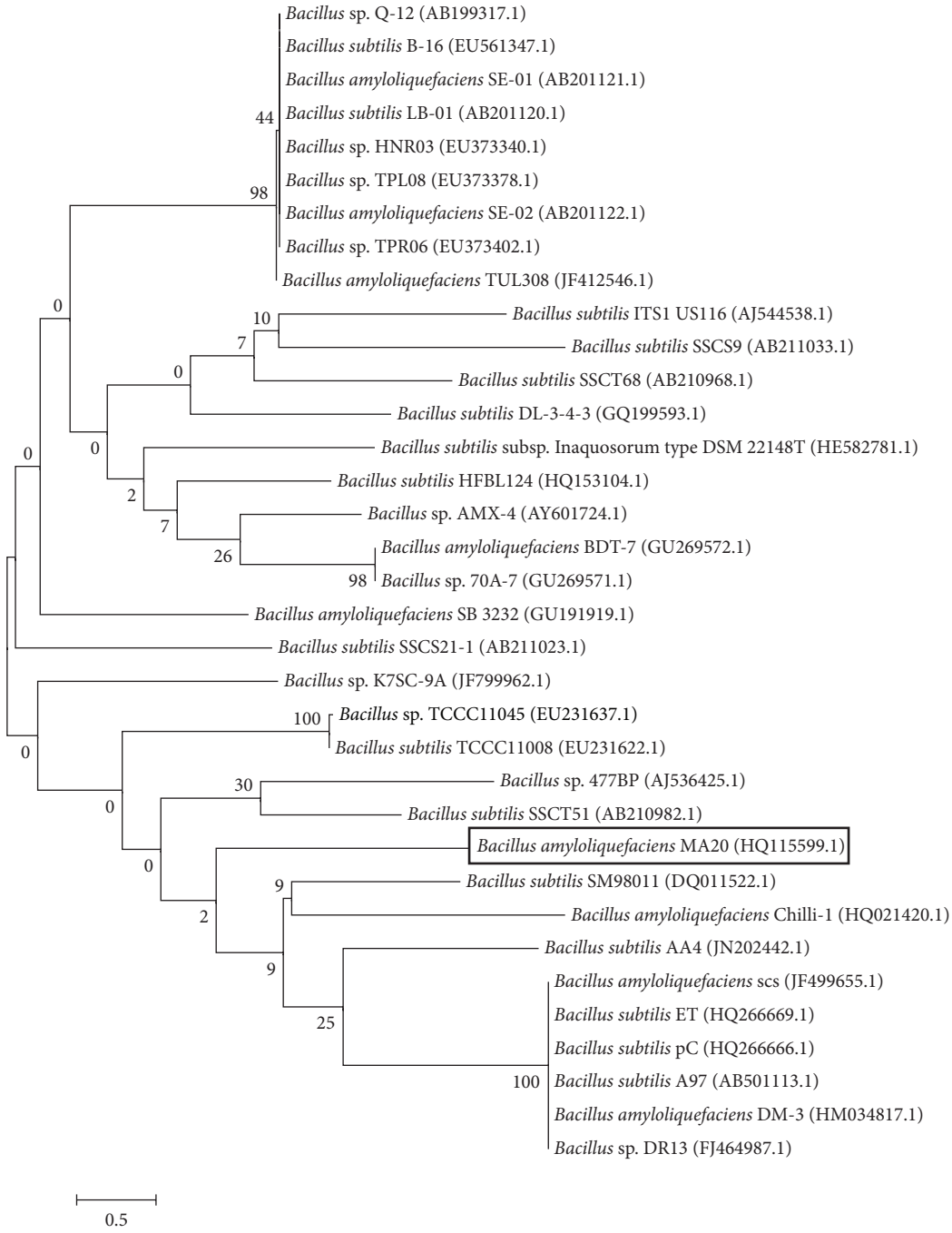

Figure 2: Phylogenetic position of *Bacillus amyloliquefaciens* MA20 within the genus *Bacillus*. The branching pattern was generated by neighbor-joining tree method. The Genbank accession numbers of the 16S rRNA nucleotide sequences are indicated in brackets. The number of each branch indicates the bootstrap values. The bar indicates a Jukes-Cantor distance of 0.5.

industrial purposes such as wastewater treatment and leather tanning [40].

3.8. Influence of Protease Inhibitors, Solvents, and Metal Ions on Enzymes Activity. Mostly keratinolytic proteases belong to the subtilisin family of serine proteases with cysteine proteases, which have higher activity on casein [41]. The keratinolytic proteases produced by *Bacillus* sp. are often serine-proteases, such as the enzymes produced by *B. licheniformis* [42], *B. pseudofirmus* [43], and *B. subtilis* [44, 45]. Protease activity of enzymes prepared from *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 was completely inhibited by serine protease inhibitor (PMSF). The result indicated the presence

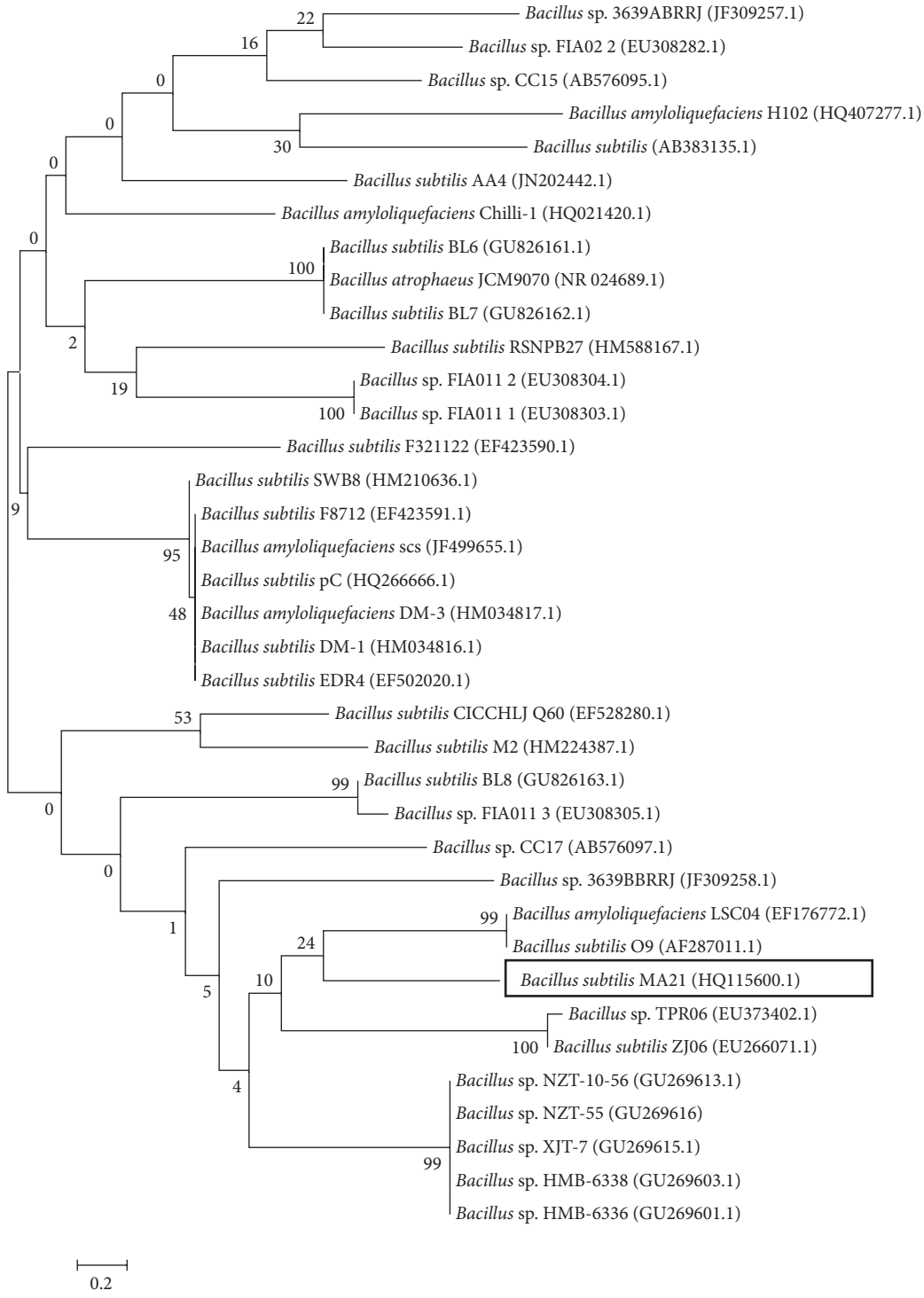

Figure 3: Phylogenetic position of *Bacillus subtilis* MA21 within the genus *Bacillus*. The branching pattern was generated by neighbor-joining tree method. The Genbank accession numbers of the 16S rRNA nucleotide sequences are indicated in brackets. The number of each branch indicates the bootstrap values. The bar indicates a Jukes-Cantor distance of 0.2.

of the serine group in the enzyme active site. The enzymes activity was partially inhibited by EDTA (Tables 2 and 3). This suggests that the keratinolytic protease from the *Bacillus* strain belongs to keratinolytic serine protease family.

The stability of keratinolytic proteases in presence of SDS acts as a positive advantage of enzymes feature because it indicated the possibility of using them in different industrial purposes as detergent industry, leather industry, and wool

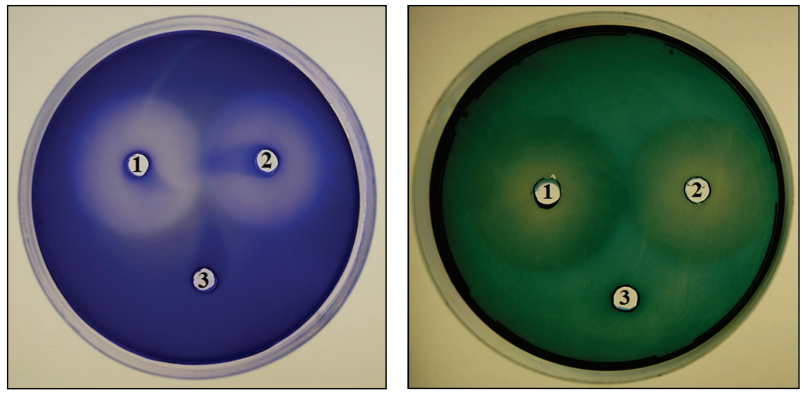

Figure 4: Proteolytic activity of crude enzymes produced by *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 using gelatin as substrate and both of Coomassie blue and amido black stains. Blue plate stained with Coomassie blue while green plate stained with Amido black. The well (1) refers to enzymes produced by *B. amyloliquefaciens* MA20, (2) refers to enzymes produced by *B. subtilis* MA21, and (3) is inactive enzyme.

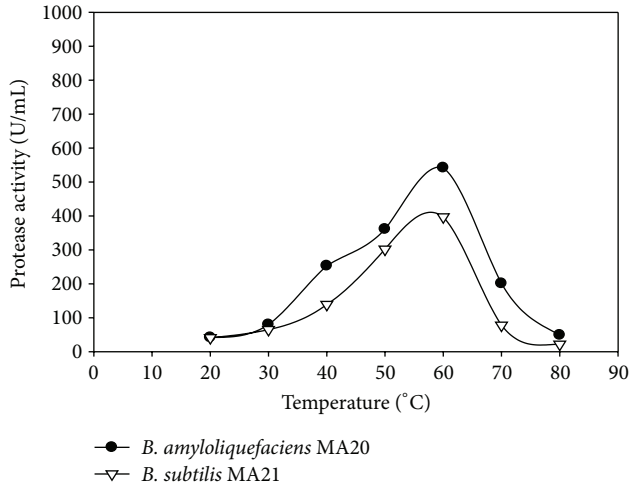

Figure 5: Effect of temperatures at pH 7 on protease activity of crude enzymes produced by *B. amyloliquefaciens* MA20 and *B. subtilis* MA21.

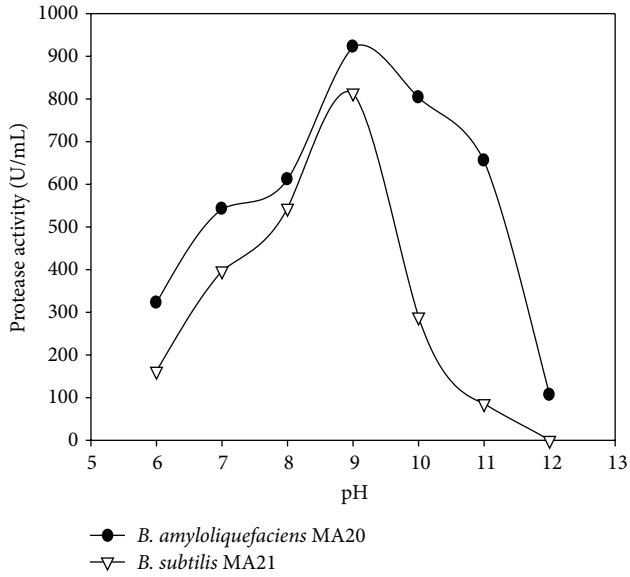

Figure 6: Effect of pH at 60°C on protease activity of crude enzymes produced by *B. amyloliquefaciens* MA20 and *B. subtilis* MA21.

improvement. The stability toward SDS is important because a few authors reported that SDS-stable enzymes are also not generally available except for a few strains such as *Bacillus clausii* I-52 [46] and *Bacillus* sp. RGR-14 [47]. The effects of various solvents and metal ions on enzyme activity were examined in order to find which ions are stimulators and which are inhibitors of the catalytic process.

The metal ions were used with two final concentrations (5.0 mM and 10 mM) while the used solvents with final concentrations (1% and 0.5%). The effects of metal ions and solvents on enzyme activities are summarized in Tables 2 and 3.

3.9. First-Dimension Protein Electrophoresis. The production of extracellular keratinolytic proteases by *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 was evaluated by SDS-PAGE and zymogram analysis. Giongo et al. found multiple bands after zymogram of keratinolytic protease produced by three strains of *Bacillus sp*. P6, P7, and P11 using feather degrading medium [25]. Growth of the two strains in presence 10 g/L of wool resulted in the production of multiple proteases, as observed by a zymogram on gelatin (Figures 12 and 13). Multiple clear zones were observed and detected using (PAGE Ruler Prestained Protein Ladder) which indicated that the proteolytic activity was not due to a single protein. The native-PAGE zymogram of enzymes from the 2 strains was performed and compared with protein ladder (Figures 14 and 15). The zymogram using native page was carried out for detecting the bands of protein where the purification performs using native protein. Multiple bands could be detected on native-PAGE zymogrm.

3.10. Two-Dimension Protein Electrophoresis. The two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Table 2: Effect of protease inhibitors, metal ions, and solvents on proteolytic activity of *B. amyloliquefaciens* MA20.

Substance	Final concentration	Relative activity (%)	Final concentration	Relative activity (%)
Control	0	100	0	100
β-mercaptoethanol	5 mM	18	1 mM	98
PMSF	5 mM	0	1 mM	0
EDTA	5 mM	35	1 mM	97
SDS	0.5%	54	0.1%	122
$ZnCl_2$	10 mM	153	5 mM	156
$MgCl_2$	10 mM	119	5 mM	95
$CuSO_4$	10 mM	266	5 mM	204
Urea	10 mM	114	5 mM	139
$HgCl_2$	10 mM	100.4	5 mM	107
$CaCl_2$	10 mM	104	5 mM	107
$BaCl_2$	10 mM	115	5 mM	116
Guanidin HCl	10 mM	103	5 mM	132
$MnCl_2$	10 mM	71	5 mM	162
Methanol	1%	90	0.5%	100
Ethanol	1%	103	0.5%	100
DMSO	1%	90	0.5%	103
Isopropanol	1%	98	0.5%	108
Tween 20	1%	86	0.5%	94
Triton X100	1%	61	0.5%	65

Table 3: Effect of protease inhibitors, metal ions, and solvents on proteolytic activity of *B. subtilis* MA21.

Substance	Final concentration	Relative activity (%)	Final concentration	Relative activity (%)
Control	0	**100**	0	100
β-mercaptoethanol	5 mM	48	1 mM	70
PMSF	5 mM	0	1 mM	0
EDTA	5 mM	13	1 mM	96
SDS	0.5%	25	0.1%	99
$ZnCl_2$	10 mM	**92**	5 mM	93
$MgCl_2$	10 mM	**22**	5 mM	98
$CuSO_4$	10 mM	**23**	5 mM	73
Urea	10 mM	**24**	5 mM	52
$HgCl_2$	10 mM	**27**	5 mM	37
$CaCl_2$	10 mM	**65**	5 mM	142
$BaCl_2$	10 mM	**29**	5 mM	69
Guanidin HCl	10 mM	**42**	5 mM	49
$MnCl_2$	10 mM	**64**	5 mM	67
Methanol	1%	92	0.5%	99
Ethanol	1%	92	0.5%	97
DMSO	1%	78	0.5%	96
Isopropanol	1%	98	0.5%	101
Tween 20	1%	78	0.5%	86
Triton X100	1%	57	0.5%	133

Table 4: Two-dimension protein gel electrophoresis report of crude enzymes produced by *B. amyloliquefaciens* MA20 and *B. subtilis* MA21.

Gels	Spots	Minimum gray	Maximum gray	Columns	Rows	Pixel width	Pixel height
B. amyloliquefaciens MA20	237	23	209	2512	1510	353	353
B. subtilis MA21	291	0	255	2336	1452	353	353

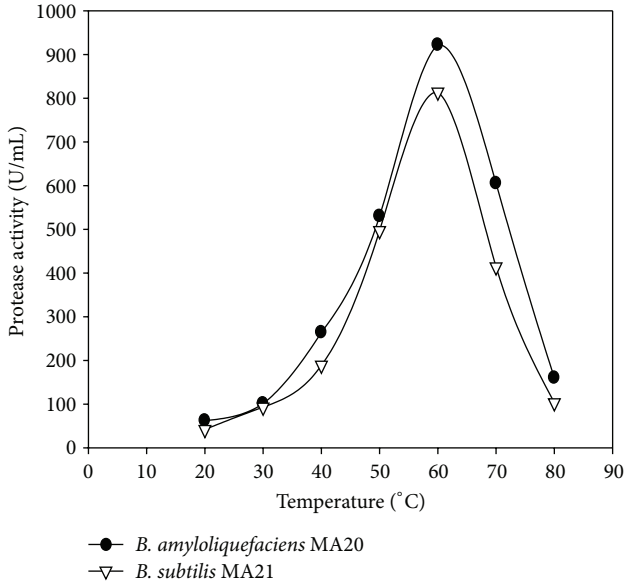

Figure 7: Effect of temperatures at pH 9 on protease activity of crude enzymes produced by *B. amyloliquefaciens* MA20 and *B. subtilis* MA21.

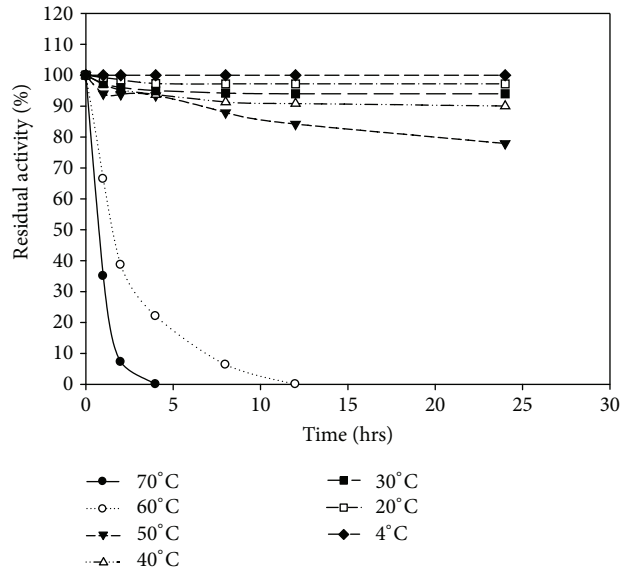

Figure 8: Thermal stability of crude enzyme produced by *B. amyloliquefaciens* MA20.

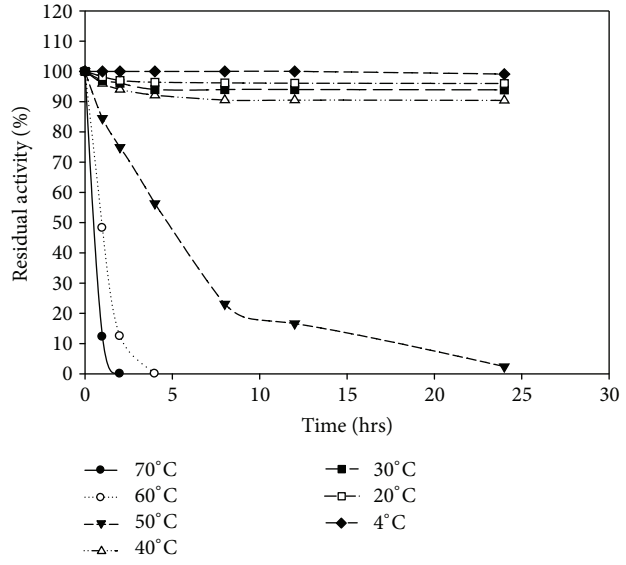

Figure 9: Thermal stability of crude enzyme produced by *B. subtilis* MA21.

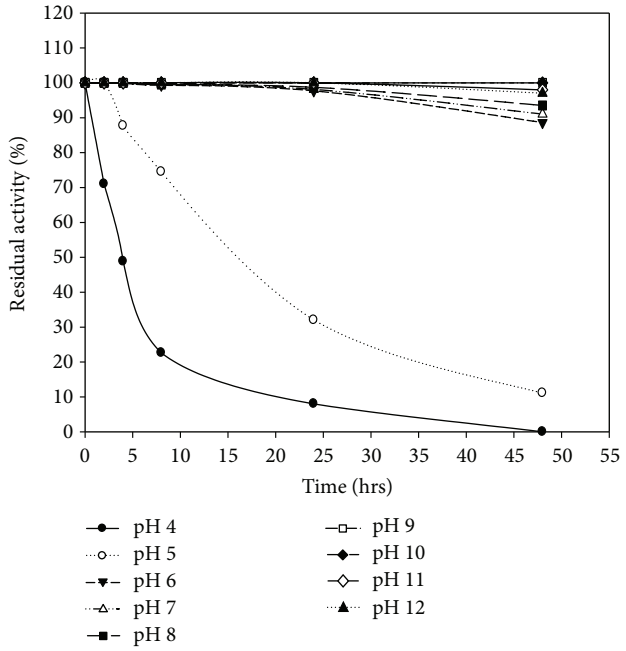

Figure 10: pH stability of crude enzyme produced by *B. amyloliquefaciens* MA20.

is an advanced technique that depends on protein separation firstly according to the pH and secondly based on the molecular weight. This technique was carried out to differentiate between the crude keratinolytic proteases obtained from *B. amyloliquefaciens* MA20 and *B. subtilis* MA21. After the separation based on the pH, the strips were applied on gel for separating according to molecular weight. The gels were stained with Coomassie blue for detecting the protein spots.

In order to classify the keratinolytic protease enzyme, a systemic comparison of 2D maps of proteases was conducted with image master 2D Platinum 6 software. The two gels were photographed by high quality scanner image (Figure 16).

The results were analyzed using image master 2D Platinum 6 software. The spots were detected before matching between the two gels and the report was obtained (Table 4). Every spot refers to the presence of one protein. The gel which was loaded with keratinolytic protease from *B. amyloliquefaciens* MA20 had 237 spots while the gel applied with *B. subtilis* MA21 had 291 spots.

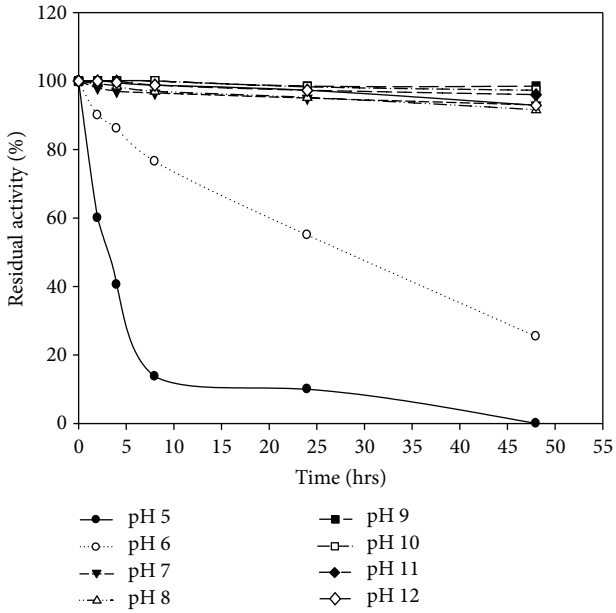

Figure 11: pH stability of crude enzyme produced by *B. subtilis* MA21.

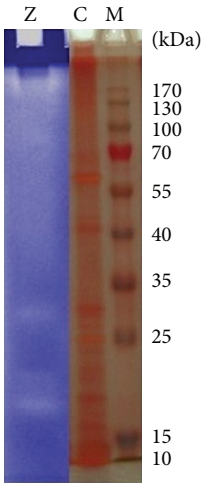

Figure 12: SDS-PAGE and zymogram analysis of keratinolytic protease enzyme from *B. amyloliquefaciens* MA20. The lane (M) is prestained protein ladder, lane (C) is protein pattern of crude enzyme, and lane (Z) is zymogram of enzyme.

The 2 gels were matched and consider gel resulted from crude enzymes of *B. amyloliquefaciens* MA20 as reference and the final report that summarizes the difference between 2 gels was obtained (Table 5). The 2 gels were matched and the percent of matching between them was 13.25%.

4. Conclusion

This paper described in details different methods that lead to the production of keratinolytic protease from two *Bacillus* sp. strains. Different methods and assays ranging from simple to advanced were carried out to prove that the enzymes have

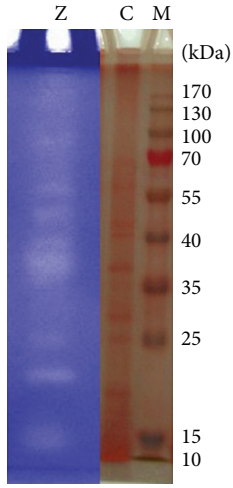

Figure 13: SDS-PAGE and zymogram analysis of keratinolytic protease enzyme from *B. subtilis* MA21. The lane (M) is prestained protein ladder, lane (C) is protein pattern of crude enzyme, and lane (Z) is zymogram of enzyme.

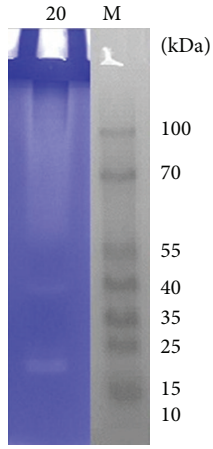

Figure 14: Native-PAGE zymogram analysis of keratinolytic protease enzyme from *B. amyloliquefaciens* MA20. The lane (M) is prestained protein ladder, and lane (20) is zymogram of enzyme.

Table 5: Match statistics report of crude enzymes produced by *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 after two-dimension protein gel electrophoresis.

Gel name	Gel name	Number of matches	Percent matches
B. amyloliquefaciens MA20 (reference)	*B. subtilis* MA21	35	13.2576

the ability to degrade wool. The two used strains have been selected based on that they belong to *Bacillus* sp. and show the best keratinolytic activities. The study included molecular and bioinformatic tools to identify the two *Bacillus* sp. strains. 16S rRNA, phylogenetic tree, Blast search for nucleotide similarity, scanning electron microscope, SDS-PAGE, and 2D-PAGE have been used to differentiate the both strains

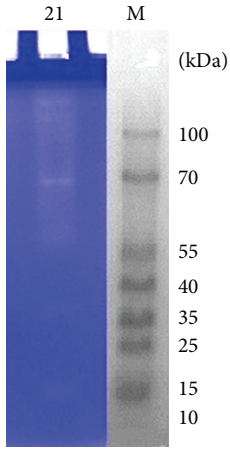

Figure 15: Native-PAGE zymogram analysis of keratinolytic protease enzyme from *B. subtilis* MA21. The lane (M) is prestained protein ladder, and lane (21) is zymogram of enzyme.

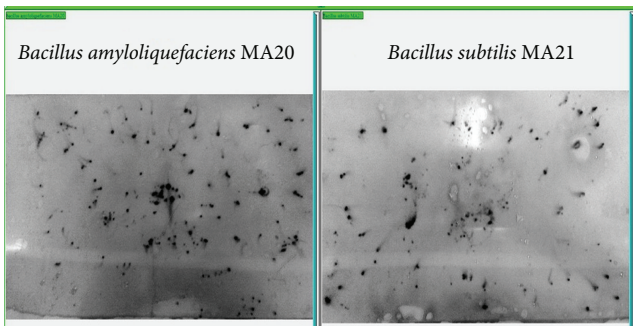

Figure 16: Two-dimension gel of enzymes from *B. amyloliquefaciens* MA20 and *B. subtilis* MA21.

and their produced enzymes. The study succeeded in characterization of *Bacillus* sp. strains which were named *Bacillus amyloliquefaciens* MA20 and *Bacillus subtilis* MA21. The enzyme activities either on agar diffusion plates or through the enzyme activity bioassays under different experimental conditions proved that the both strains are able to produce different keratinolytic protease enzymes. *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 could be used in large-scale production of keratinolytic protease enzymes where these enzymes were stable at temperature range between 4 and 70°C, and over a wide range of pH values (4–12), as well as, stable against organic solvents and detergents. The characters of keratinolytic enzymes produced by *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 could play an important role especially in industrial applications; therefore this research acts as preliminary studies for applying the keratinolytic proteases in wool quality improvement.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 154549, 10 pages
http://dx.doi.org/10.1155/2013/154549

Review Article

Strategies to Characterize Fungal Lipases for Applications in Medicine and Dairy Industry

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Received 3 May 2013; Accepted 6 June 2013

Academic Editor: Bidur Prasad Chaulagain

Lipases are water-soluble enzymes that act on insoluble substrates and catalyze the hydrolysis of long-chain triglycerides. Lipases play a vital role in the food, detergent, chemical, and pharmaceutical industries. In the past, fungal lipases gained significant attention in the industries due to their substrate specificity and stability under varied chemical and physical conditions. Fungal enzymes are extracellular in nature, and they can be extracted easily, which significantly reduces the cost and makes this source preferable over bacteria. Soil contaminated with spillage from the products of oil and dairy harbors fungal species, which have the potential to secrete lipases to degrade fats and oils. Herein, the strategies involved in the characterization of fungal lipases, capable of degrading fatty substances, are narrated with a focus on further applications.

1. Introduction

Lipases are defined as triacylglycerol acyl hydrolases (EC 3.1.1.3) and are involved in the hydrolysis of fats and oils to yield glycerol and free fatty acids [1] (Figure 1(a)). Lipases belong to the class of serine hydrolases and do not require any cofactor. Under natural conditions, lipases catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase where the enzyme remains dissolved [2] (Figure 1(b)). Lipases are involved in conversion reactions, such as esterification, interesterification, transesterification, alcoholysis, acidolysis, and aminolysis [3]. Many microorganisms such as bacteria, yeasts, molds, and a few protozoa are known to secrete lipases for the digestion of lipid materials [1, 4–12]. Microbes, being ubiquitous in distribution, are highly successful at surviving in a wide range of environmental conditions owing to their great plasticity and physiological versatility and have been the subject of several reviews [13, 14]. Due to efficient enzyme systems, microbes thrive well in inhospitable habitats [15]. With mechanisms for adapting to environmental extremes and for the utilization of their trophic niche, the ability of microorganisms to produce extracellular enzymes is of great survival value [16]. Among different microbial enzymes, lipases are widely documented among bacteria, fungi, plants, and animals [17, 18].

Extracellular secretion has been well studied for a number of fungi, primarily zygomycetes [19], hyphomycetes [20], and yeasts [21, 22]. Lipase production has also been reported for some ascomycetes [23] and coelomycetes [24]. Lipolytic activity has been observed in *Mucor* spp. [25, 26], *Lipomyces starkeyi* [27], *Rhizopus* spp. [26, 28–30], *Geotrichum candidum* [25, 31–34], *Pencillium* spp. [9, 28, 35, 36], *Acremonium strictum* [37], *Candida rugosa* [38], *Humicola lanuginosa* [39], *Cunninghamella verticillata* [40], and *Aspergillus* spp. [11, 41]. Considering the importance of fungal lipases, their applications are discussed and the techniques involved in lipase generation have been gleaned recently [1].

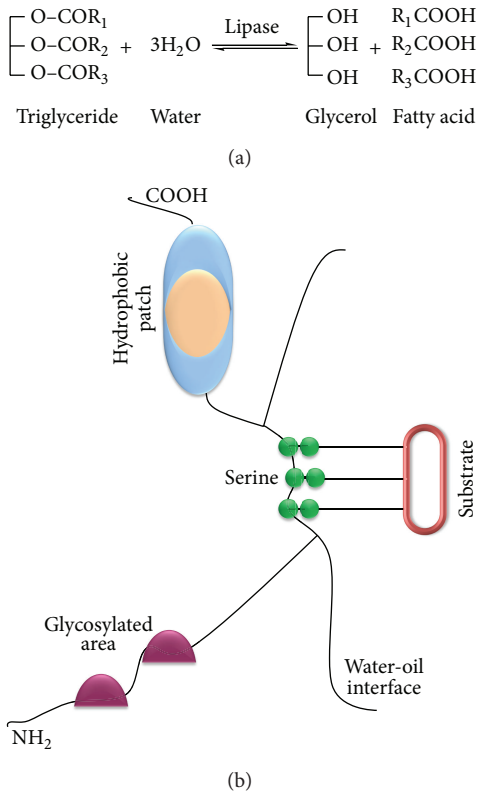

FIGURE 1: (a) Hydrolysis of triglyceride by lipase [1]. Upon hydrolysis triglyceride converts into glycerol and fatty acid. (b) Representation of a molecule of lipase with its features. The substrate can be any triglyceride [2]. Substrate interactive regions are displayed.

Fungi are involved in the degradation of undesirable materials or compounds converting them into harmless, tolerable, or useful products. The undesirable materials include sewage waste from domestic and industrial complexes and plant, animal, and agricultural wastes, oil spills, and dairy waste. The role of fungi in bioremediation processes in varied environments has been well documented [16, 30, 42, 43]. There has been an increasing awareness of potentially harmful effects of the worldwide spillage of oil and fatty substances in both saline and fresh waters. Domestic waste is also considered as the pollutants as it has a high amount of fatty and oil substances. Industrial and domestic wastes harbor fungal species of greater potential in degrading fats and oils. Besides waste disposal, bioconversion by fungal activity results in the production of a vast number of useful substances. Thus, waste can be converted into a resource. Bearing in mind the importance of lipolytic fungal enzymes from different disposal sources, this overview focuses on strategies to characterize the fungal lipases with an emphasis on a wide range of applications.

2. Lipolytic Fungal Species from Oil-Spill Wastes

Due to usage of vegetable oils for cooking, these oils are released into the open environment both at the production level and by domestic users. To keep the environment clean, these oils should be degraded by using environmentally-friendly technology. Several oil-industries have been established at both small scale (Figure 2(a)) and large scale (Figure 2(b)). Oil spillages from these production points (Figure 2(c)) cause a hindrance in ensuring environmental hygiene due to the formation of clogs in drain pipes [44]. Cleanup and recovery of oil wastes is difficult and depends upon many factors, including the type of oil spilled, the temperature of water affecting evaporation, and biodegradation. Microbial degradation is one of the most important events to ameliorate oil pollution in the environment. Fungi that produce lipases are found in diverse habitats including oil-contaminated soils, wastes around oil processing factories, domestic waste points, and dairy products [27]. Gopinath et al. [16] have isolated 34 fungal species from oil-spill contaminated soils, collected in major cities of India. These species were tested for their survival with the changes in seasons. Twelve fungal species from oil-mill effluent composts at Nsukka have been studied and it was found that *Aspergillus* spp. are more common; however, the higher lipase producers are *Trichoderma* sp. followed by *Aspergillus* spp. [9]. D'Annibale et al. [45] used olive mill waste water as the substrate to determine lipase production. Lipase producing fungal species were also recovered from compost heaps, coal tips, and industrial wastes [43]. Cihangir and Sarikaya [42] have isolated *Aspergillus* sp. from the soil samples collected in Turkey. Extracellular lipase of *Rhizopus* sp. isolated from oil-contaminated soil was recently characterized [30].

3. Screening Lipase Production on Agar Solid Surface

Studies on mycoflora are significant as they could harbor species of the highest potential for degradation. The industrial demand for new lipase sources continues to stimulate the isolation and screening of new lipolytic microorganisms. In view of the interesting applications of microbial lipases, it could be of tremendous value to screen and identify microorganisms of highest potential for the biodegradation of oils and fats. Although, different screening strategies have been proposed for the determination of lipase activity, assays using agar plates are highly recommended, because it is an easier method with lower cost. Assay using agar plates are performed due to the fact that activities for lipases are hard to determine because of the water-soluble enzyme acting on substrates which are insoluble [46, 47].

To isolate fungal species from the oil-spill contaminated soils, screening studies were performed by Gopinath et al. [16, 40] using different substrates on agar plates. These methods with different substrates include Tween-20 (Figure 3(a)), tributyrin (Figure 3(b)), and vegetable oil in the presence of Rhodamine (Figure 3(c)). Due to the oil rich environments of the substrates, special attention was given to screening of lipolytic enzymes. On the Tween-20 substrate, a visible precipitate appeared due to the deposition of calcium salt crystals formed by the liberated fatty acid by the action of lipase or by clearing of such a precipitate due to complete

(a) (b) (c)

Figure 2: Oil production and spillage. (a) Crushing system in small-scale oil production. (b) Crushing system in large-scale oil production. (c) Spillage from oil production points. These releasing points are potential sources of environmental issues.

degradation of the fatty acid salts. Brockman [48] suggested that the primary role of calcium ions is to remove fatty acids formed during hydrolysis as insoluble calcium soaps, and thus changing the substrate-water interface relationship to favorable conditions for enzyme action. The development of a clear crystal zone of Tween-20 around the fungus was also an indication of lipolytic activity, and this zone can be measured. Using Tween-20 as the substrate, Salihu et al. [36] screened different fungal species for the production of lipases. Another substrate, tributyrin, is convenient because it is easily dispersed in water by shaking or stirring without the addition of any emulsifiers. Tributyrin is a very strong surface-active substance, and its hydrolysis can be followed by measuring the increase in the diameter of the clear zone. Nevertheless, the observed zones of clearing could be the activity response of nonspecific esterases, which may have little or no activity against the long-chain triglycerides [49]. Hence, it is imperative to use another method to confirm true lipase activity. Tributyrin agar plates were used to investigate lipase production by new strains and 18 strains were found to be positive [42]. Using tributyrin formation of the clear zone around the fungal colony showed different mutant strains that produced extracellular lipases [50]. By using Tween-20 and tributyrin substrates, lipolytic activity (high and moderate activity) was evidenced by 19 and 32 species, respectively [51]. The lipolytic potential of this fungus was also confirmed by the Rhodamine method because the enzyme will fluoresce with orange compound (Figure 3(c)) as reported by Kouker and Jaeger [52]. Furthermore, Hou and Johnston [53] as well as Lee and Rhee [54] proved that this method is highly sensitive and reliable as a lipase assay. In a recent study, the Rhodamine method with olive mill wastewater was used to determine the production of lipases by *Aspergillus ibericus* [11]. Savitha et al. [3] used Rhodamine fluorescence-based assay to screen 32 fungal species from different sources. Our previous results provide very useful information about

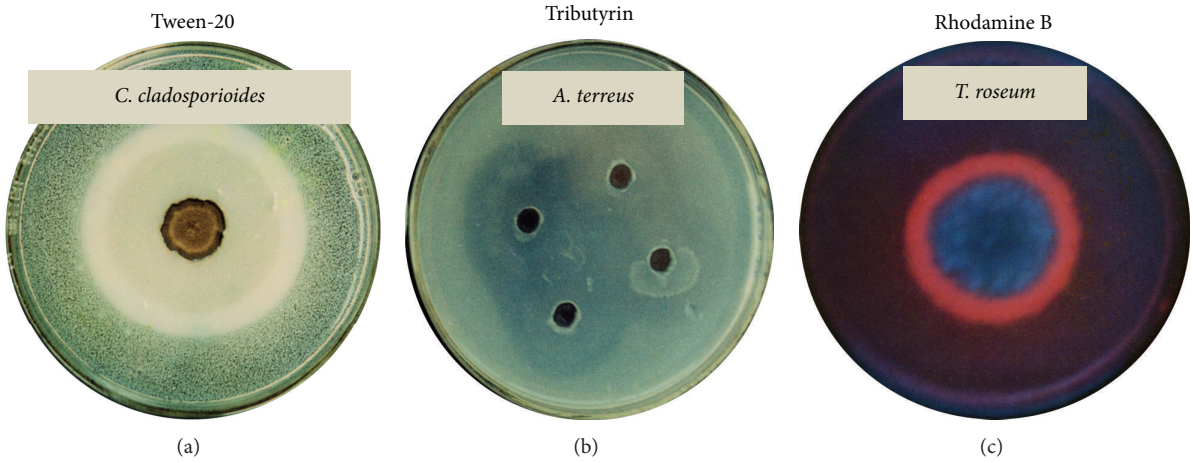

Figure 3: Agar plate screening for lipases. Using the substrates (a) Tween, (b) tributyrin, and (c) vegetable oil. In the Tween method, formation of calcium crystals was observed. The tributyrin method shows a clear zone, whereas in the Rhodamine method, formation of fluorescence with fatty substrate was observed under UV illumination. Active zones are increasing with a period of incubation time and these zones can be measured.

the degradation of vegetable oils by *Cunninghamella verticillata* in the presence of Rhodamine [51]. Based on the above screening strategies, Gopinath et al. [16] revealed the following fungi as potential candidates that secrete enzymes lipases, *Absidia corymbifera, Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans, Aspergillus terreus, Cunninghamella verticillata, Curvularia pallescens, Fusarium oxysporum, Geotrichum candidum*, *Mucor racemosus*, *Penicillium citrinum*, *Penicillium frequentans*, *Rhizopus stolonifer*, and *Trichoderma viride*. They also conducted screening studies for other enzymes and confirmed that some of the isolated fungal species could also secrete amylases, proteases, and cellulases in addition to lipases, representing the ability of these species to survive in a wide range of environmental substrates [16]. These observations provided interesting perspectives, demonstrating that fungi isolated from oil-rich environments represent a source of several enzymes potentially exploitable for biotechnological purposes. In another study, 59 fungi were screened by measuring the formation of halos on the agar plate used for lipase screening [55]. Preliminary screening studies for lipase production by fungi were also carried out on agar plates using olive oil or emulsified tributyrin by gum arabic [56]. Kumar et al. [57] screened fungi with bromophenol blue dye supplemented agar plates with olive oil as the substrate.

4. Degradation of Oils by Lipases

The use of specific microbial lipases to catalyze interesterification reactions became considerable interest because of its advantages over chemical catalysts. Traditionally, fatty acids are manufactured by the hydrolysis of oils at high temperature and pressure. However, lipase hydrolysis is an energy saving process because oil degradation of fatty acids (the reaction) can be carried out at room temperature and pressure [58]. Industrially important chemicals manufactured from fats and oils by chemical processes could be produced by lipases with rapidity and better specificity under mild conditions [59, 60]. Lipases are one among several kinds of extracellular enzymes that perform the function of recycling large quantities of insoluble organic material in nature [61]. Apart from numerous applications such as transesterification [62, 63], ester synthesis, production of biosurfactants [64], and application in food and dairy industry [35, 65, 66], the enzyme lipase has a proven role as a useful interesterification catalyst. Interesterification is a technology by which fatty acids within a triacylglycerol molecule can be interchanged with regard to their positional distribution. The process of fat splitting, along with interesterification, is an essential tool in the manufacture of new tailor made fats and oils. Enzyme-catalyzed reactions of lipids are of considerable interest in view of their possible applications in the biotechnology of fats and oils. The technique of fat splitting plays an important role in the manufacture of soaps and other industrial products like candles from conventional minor oils. Different isolates from oil mill effluent have been tested for their ability to degrade the different oils and the potential of individual species varied with the type of fatty acid residues in the oil (Table 1). From this study, it was revealed that the behavior of lipases from different fungal species is different in terms of their biochemical characteristics. Teng and Xu [67] analyzed the production of lipase from *Rhizopus chinensis* under experimental conditions and Bapiraju et al. [68] performed a similar study with mutants of *Rhizopus* sp. Studies have also been documented with lipase from *Penicillium* spp. [69, 70]. Extensive review on the production of lipases from different microbes has been published [71].

5. Purification Strategies for Lipases

Knowledge of the purified lipase activities to be used for biotechnological purposes is mandatory, and it can be the basis or other applications. Various purification strategies have been reviewed for the lipase enzyme [72–74]. In the case

Table 1: Degree of utilization (%) of vegetable oils by fungal species.

Organism	Olive	Soybean	Groundnut	Cottonseed	Sunflower
A. strictum	20	60	40	90	90
C. verticillata	80	80	90	40	90
G. candidum	90	10	90	40	80
M. racemosus	50	60	60	10	50
R. miehei	30	90	90	80	90
R. stolonifer	60	70	80	20	60
T. roseum	90	60	80	80	80
T. viride	50	20	30	50	30

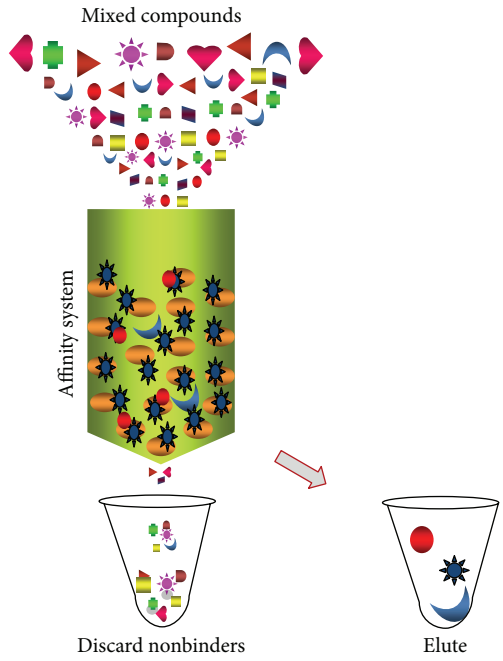

Figure 4: Purification of lipase using an affinity system. Separation of lipase from mixed compounds is indicated. Bound lipase can be eluted by creating stringent conditions.

of extracellular lipases, it is primarily important to remove other contaminants from the compound mixture containing lipase by suitable strategy (Figure 4). The conventional purification strategies give a low yield due to a large hydrophobic surface near the active site. Novel purification steps are mandatory to increase overall enzyme yields and it could be achieved by opting an appropriate chromatography system. One of the choices is hydrophobic interaction chromatography and it is considered as a common strategy [75–77]. In addition to this, ion exchange and gel filtration chromatography are commonly preferred methods [75, 78–80]. A reversed micellar system, membrane processes, immunopurification, hydrophobic interaction chromatography with an epoxy-activated spacer arm (ligand), column chromatography using polyethylene glycol (PEG)/sepharose gel, and aqueous two-phase systems are also recommended [81]. Kumarevel et al. [82] reported a stepwise purification strategy for fungal lipases to remove other components released from the fungus *Cunninghamella verticillata* extracellularly, using acetone precipitation as the important step. To avoid many steps in this study and to minimize the impurities as much as possible the experiment was repeated with 50% acetone saturation with a gradual increments of 5% acetone. Using the above methods, many lipases from different microorganisms have been reported, and molecular masses of 31 and 19 kDa have been reported for the lipase of *Aspergillus niger* by Hofelmann et al. [83], 21.4 kDa for *Rhodotorula pilimena* [84], 30 kDa for *Rhizopus japonicus* [85], 51 kDa for *Pichia burtonii* [86], 25 kDa for *Aspergillus oryzae* [87], 49 kDa for *Mucor hiemalis* [88], 35.5 kDa for *Aspergillus niger* [89], 49 kDa for *Cunninghamella verticillata* [40], and 32 kDa for *Geotrichum candidum* [34]. Different strategies for lipase purification with the varied sources were recently described in detail by Singh and Mukhopadhyay [1], and it seems that the production of lipases from fungal species results in different molecular sizes, due to variations in the number of amino acid residues. Saxena et al. [73] summarized the purification strategies for microbial lipases. Overall, traditional purification strategies are considered time consuming with lower yields and the trends are moving towards aqueous two-phase extraction, and purification in ionic liquids and purification based on lipase-lipase interaction [10].

6. Statistical Calculations

Statistical calculations were focused in the past, due to their reliable prediction for the experimental conditions for enzyme studies to be optimized [34, 40, 90–92]. In statistics, response surface methodology (RSM) has referred the relationships between several explanatory variables and one or more response variables. This method was introduced initially by Box and Wilson [93]. The main idea of RSM is to get an optimal response by using a sequence of designing experiments, and it was suggested to use a second-degree polynomial model to perform RSM. Box-Behnken design experiments are one of the most common, and this is an independent quadratic design without an embedded factorial design. Different combinations of midpoints are used for experiments; for example, with 3 experimental parameters, 17 experiments can be run and it yields a predicted result (Figure 5). The Box-Behnken design is where the outcome unit (Y) is related to experimental variables by a response equation,

$$Y = f(X_1, X_2, X_3, \ldots, X_k). \quad (1)$$

As mentioned above a second-degree quadratic polynomial is used to represent the function in the range of interest,

$$Y = R_0 + \sum_{i=1}^{k} R_i X_i + \sum_{i=1}^{k} R_{ii} X_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} R_{ii} X_i X_j + \varepsilon, \quad (2)$$

where $X_1, X_2, X_3, \ldots, X_k$ are the input variables which effect the response Y, R_0, R_i, R_{ii}, and R_{ij} ($i = 1-k$, $j = 1-k$) are the known parameters, and ε is the random error. A second-order model is designed such that the variance of Y is constant for all points equidistant from the center of the design

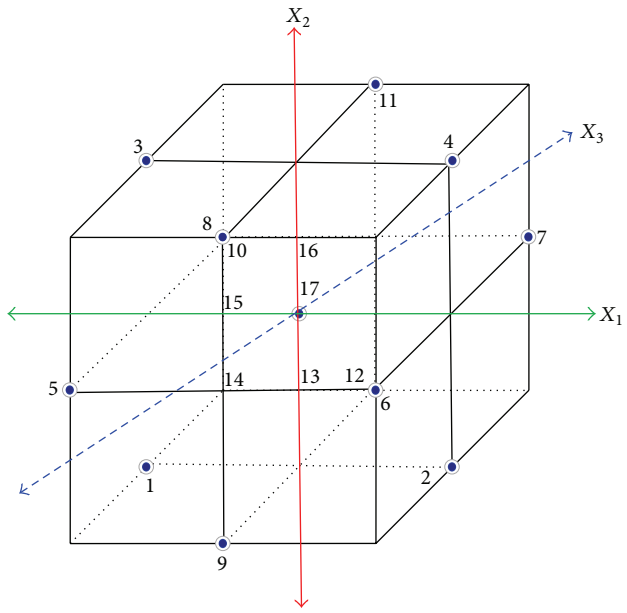

Figure 5: Box-Behnken design for experiments. Different combinations at the midpoints used for experiments are shown with 3 experimental parameters and 17 experiments run. A number of experiments vary with the number of experimental parameters.

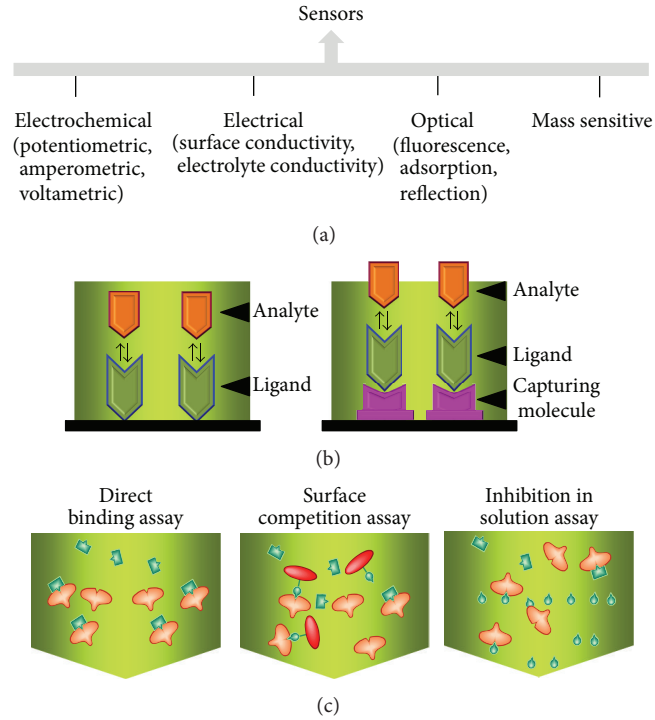

Figure 6: Sensing systems. (a) Types of sensors. (b) Strategy of immobilizing ligand and analyte. (c) Methods involved with ligand and analyte binding. Sensitivity depends on the interactive molecules.

(Figure 5). The validity of the model can be determined based on Student's t-test. The Fisher-test, P value, t-test, and R^2, and so forth can be used to evaluate the model as well as to determine the optimal processing conditions. The Fisher-test with a very low probability value ($P_{\text{model}} > F = 0.0001$) showed that the regression model had a very high significance. The model reliability of fit was checked by means of the determination coefficient (R^2). This model fits the experimental range studied perfectly when the value of R^2 is adjusted to nearly one. Using the Box-Behnken design, conditions were optimized for lipase production by *Geotrichum candidum* [34] and optimization of purified lipase from *Cunninghamella verticillata* for physical parameters was also shown [40]. Using these optimized conditions, the purification steps were reduced and purified lipase was used for crystallization studies [82]. Other than Box-Behnken, the Plackett-Burman design and the Luedeking-Piret model can also be used for different optimization studies. Statistical design experiments by Plackett-Burman were used to evaluate the production of lipase by *Candida rugosa* [1].

7. Biosensors for Lipase

A biosensor is a combination of a biological component with a physicochemical detector and it assists with analysis of biomolecular interactions. Development of an analytical system will help us to find a minute amount of biological agents within mixed compounds. Different types of sensing systems have been proposed for determining biomolecular interactions and environmental monitoring [94–98]. In general biosensors may be classified as electrochemical, electrical, optical, or mass sensitive (Figure 6(a)). The core design for sensors mainly includes three components, probe-target recognition, signal transduction, and physical readout [99]. On the sensor surfaces the lipase can be either ligand or analyte. Ligand can be immobilized directly on the sensor surface or indirectly via an immobilized surface chemical linkage (Figure 6(b)). The direct adsorption of the molecules on the sensor surface leads to rapid, simple, and cheaper strategies compared to immobilization by chemical means. Using these strategies the interactions of lipase with oil or molecules for the purpose of interactive analysis and environmental monitoring can be done by different assay formats (Figure 6(c)). Various detection and measurement methods or strategies are discussed on microbial lipases [100]. Phospholipases are potential markers for diagnosing diseases in the pancreas and coronary arteries [101, 102]. In all, free and phosphatidyl-bound choline in milk and a dietary supplement can be determined quantitatively, using a phospholipase D packed bioreactor [103]. A surface acoustic wave sensing system was generated to measure pancreatic lipase [104]. Lipase activity based on glycerol dehydrogenase/NADH oxidase was reported based on amperometric sensor [105]. Lipases can be immobilized on the sensing surface and can function as lipid biosensors for blood cholesterol determinations [106].

8. Conclusions

Fungi are capable of producing several enzymes for their survival within a wide range of substrates. Among those

enzymes, lipases are predominantly used in several applications. These fat-splitting enzymes are attractive because of their applications in fields relevant to medicine and dairy industry. Lipases play a major role as the biocatalysts and microbial lipases can be produced in large scale by overexpression. The disadvantage lipase enzyme is that it continues to be active due to turnover reaction and may need to optimize the reaction condition and specificity with different sources of lipase [107]. Indeed, various methods have been proposed for the different lipases to survive under variant physical and chemical conditions. The strategies involved in the characterization of lipases were discussed here, suitable for large-scale production. The great advantage of fungal lipases is that they are easily amenable to extraction due to their extracellular nature, which will significantly reduce the cost and makes these lipases more attractive than those bacteria. Furthermore, with available sources such as LIPABASE (database for true lipases), which provides taxonomic, structural, and biochemical information, genetically engineered lipase sequences from fungal species will hasten the production, especially in the dairy industry.

Acknowledgment

P. Anbu would like to thank Inha University, Republic of Korea, for the financial support from Inha University Research Grant.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 728735, 8 pages
http://dx.doi.org/10.1155/2013/728735

Research Article

Production, Purification, and Characterization of a Major *Penicillium glabrum* Xylanase Using Brewer's Spent Grain as Substrate

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Received 3 April 2013; Accepted 20 April 2013

Academic Editor: Arzu Coleri Cihan

In recent decades, xylanases have been used in many processing industries. This study describes the xylanase production by *Penicillium glabrum* using brewer's spent grain as substrate. Additionally, this is the first work that reports the purification and characterization of a xylanase using this agroindustrial waste. Optimal production was obtained when *P. glabrum* was grown in liquid medium in pH 5.5, at 25 °C, under stationary condition for six days. The xylanase from *P. glabrum* was purified to homogeneity by a rapid and inexpensive procedure, using ammonium sulfate fractionation and molecular exclusion chromatography. SDS-PAGE analysis revealed one band with estimated molecular mass of 18.36 kDa. The optimum activity was observed at 60 °C, in pH 3.0. The enzyme was very stable at 50 °C, and high pH stability was verified from pH 2.5 to 5.0. The ion Mn^{2+} and the reducing agents β-mercaptoethanol and DTT enhanced xylanase activity, while the ions Hg^{2+}, Zn^{2+}, and Cu^{2+} as well as the detergent SDS were strong inhibitors of the enzyme. The use of brewer's spent grain as substrate for xylanase production cannot only add value and decrease the amount of this waste but also reduce the xylanase production cost.

1. Introduction

Xylan is a major structural polysaccharide of plant-cell walls being the second most prevalent in nature after cellulose. It is a heterogeneous polymer constituted primarily by a linear β-(1,4)-D-xylose backbone, which is partially acetylated and substituted at different degrees by a variety of side chains, mainly α-D-glucuronosyl and α-L-arabinosyl units. Due to its structural complexity, several hydrolases are required for complete xylan degradation. The key enzyme in this process is endo-β-(1,4)-xylanase (EC 3.2.1.8), which cleaves the xylan backbone to xylooligosaccharides [1, 2].

Interest in xylanolytic enzymes has increased in recent years due to their potential application in biotechnology. Xylanolytic enzymes have applications in conversion of lignocellulosic materials to chemicals and fuels, animal feed digestion, food and textile industries, and as bleaching agents in the pulp and paper processing [3, 4].

Industrial production of enzymes on large scale is associated mainly with the substrate. The use of agroindustrial wastes as low-cost substrates for the industrial enzymes production is a significant way to reduce production cost. Thus, lignocellulosic substrates have now received considerable interest because of their possible use in secondary fermentation process [5]. Various lignocellulosic substrates involving agroindustrial waste materials like corn meal, corn cob, wheat bran, wheat straw, rice straw, sugarcane bagasse, and coffee by-products are being used as substrates for the production of fungal xylanases [6–8].

Brewer's spent grain (BSG), the main residue of brewing industry, is rich in cellulosic and noncellulosic polysaccharides. It is produced at large amounts during the year and

represents around 85% of the total by-products generated [9]. In Brazil, the world's fourth largest beer producer (8.5 billion litres/year), approximately 1.7 million tonnes of BSG were generated per year. Despite the large amounts produced, BSG has received little attention as a low-cost by-product and its use is still limited, mainly as animal feed [10].

Penicillium glabrum is a filamentous fungus that is distributed worldwide, frequently involved in food contamination. Due to its ability to disperse a large number of spores in the environment, *P. glabrum* is frequently found in the food manufacturing industry [11]. Despite its large implication in food contamination, few studies have been conducted to investigate the enzyme production by this fungus. Only tannase production by *P. glabrum* has been described in the literature [12]. Since many agroindustrial wastes are a potentially valuable resource for industrial exploitation, this work aimed to evaluate the xylanase production by *P. glabrum* using different agroindustrial wastes, establish the best fungal growing conditions for xylanase production with the best substrate, and biochemically characterize the major enzyme purified.

2. Materials and Methods

2.1. Organism and Growth. P. glabrum used in the present work is available in the Culture Collection of Environmental Studies Center—CEA/UNESP, SP, Brazil. Conidia were obtained from cultures in Vogel solid medium [13] containing 1.5% (w/v) glucose and 1.5% (m/v) agar at 25 °C for 7 days. Liquid cultures were prepared in the same medium with 1% (w/v) carbon source and the pH was adjusted to 6.5. Erlenmeyer flasks (125 mL) containing 25 mL of medium were inoculated with 1.0 mL of spore suspension containing 5×10^7 spores/mL and incubated at 30 °C for 5 days in stationary condition.

2.2. Preparation of Agroindustrial Wastes. The agroindustrial wastes were obtained locally. The residues were prepared by exhaustive washing with distilled water, dried at 80 °C for 24–48 h, and milled (35 mesh).

2.3. Enzyme Preparations and Assays. Cultures were harvested by filtration. The culture filtrate was assayed for extracellular activity and protein. The mycelium was washed with distilled and sterilized water, frozen, and ground with sand in 0.05 M sodium phosphate buffer pH 6.5. The slurry was centrifuged at 3.900 g at 4 °C, and the supernatant was used as intracellular protein source.

Xylanase activity was determined at 50 °C using 1.0% (w/v) birchwood xylan (Sigma, st. Louis, MO, USA) in McIlvaine buffer pH 6.5. This buffer is prepared by a mixture of 0.1 M citric acid and 0.2 M sodium monohydrogen phosphate. After 5 and 10 min of incubation, the reaction was interrupted by the addition of 3,5-dinitrosalicylic acid (DNS), and the reducing sugars released were quantified [14], using xylose as standard. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 μmol of reducing sugar per min, under assay conditions. Specific activity was expressed as unit per milligram of protein. All enzymatic assays were developed in triplicate, and the results are presented through mean values.

2.4. Protein Determination. Total protein was determined by modified Bradford method [15], using bovine serum albumin (BSA) as standard.

2.5. Culture Conditions for Xylanase Production

2.5.1. Enzyme Production on Different Carbon Sources. Vogel's liquid medium was supplemented with various carbon sources at a concentration of 1% (w/v). The inoculated flasks were incubated at 28 °C under stationary condition, for five days. Xylanase activity was determined as described previously.

2.5.2. Effect of Incubation Period, Initial pH, and Temperature on Xylanase Production. The incubation period's influence on xylanase production by *P. glabrum* was studied under standing culture and under shaking culture (120 rpm) for 8 days. The effect of initial pH on the enzyme production was assayed from 3.0 to 8.0, and the temperature influence was verified from 15 to 40 °C. The initial pH values were adjusted by the addition of 1.0 M sodium hydroxide or phosphoric acid solutions.

2.6. Purification of a Major P. glabrum Xylanase

2.6.1. Ammonium Sulfate Fractionation. The crude enzyme (50 mL) was fractionated by ammonium sulfate precipitation (0–90%, w/v). The supernatant of 90% ammonium sulfate saturation obtained after centrifugation (6.000 g, 20 min., 4 °C) was extensively dialyzed against 0.05 M ammonium acetate buffer pH 6.8 before analyses.

2.6.2. Exclusion Molecular Chromatography. The protein sample obtained in the step above was chromatographed on Sephadex G-75 column (2.6 × 64.0 cm), equilibrated, and eluted with 0.05 M ammonium acetate buffer, pH 6.8, flowing at 18 mL/h. Fractions (3 mL) whose protein content was estimated by reading absorbance at 280 nm and xylanase activity assayed as described previously. To determine xylanase molecular mass through gel filtration chromatography, the column was calibrated using blue dextran for the void volume determination and ribonuclease (15.4 kDa), chymotrypsin (25.0 kDa), ovalbumin (43.0 kDa) and bovine serum albumin (67.0 kDa) as standards. The molecular weight of xylanase was estimated from a regression curve ($R^2 = 0.993$), by plotting log of the molecular weights of the standards against the ratio between elution volumes of the standards and the void volume of the column.

2.6.3. Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a gradient of 8–18% (w/v) polyacrylamide according to Laemmli [16]. The resolved protein bands were visualized after staining with 0.1% (w/v) Coomassie Brilliant Blue R-250 in methanol, acetic acid, and distillated water (4 : 1 : 5, v/v/v). The proteins phosphorylase b, bovine serum

albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin (SDS-LMW markers-Sigma, st. Louis, MO, USA) were used to plot the standard curve log of molecular weight against relative mobility in the gel.

2.7. Enzyme Characterization

2.7.1. Temperature and pH Optima, Thermal and pH Stability. To determine the optimum pH, the purified xylanases were assayed at 50 °C, in different pH values using 0.05 M glycine-HCl buffer from pH 1.6 to 2.5 and McIlvaine buffer from 3.0 to 7.0. The optimum temperatures were determined by performing the reaction at temperatures ranging from 15 °C to 75 °C in McIlvaine buffer pH 3.0.

For pH stability assays, the purified enzymes were diluted (1 : 2 v/v) in 0.05 M glycine-HCl buffer from pH 1.6 to 2.5 and McIlvaine buffer for pH range from 3.0 to 7.0. The samples were incubated at 4 °C for 24 h. After this period, the xylanase activity was assayed under optimal conditions. To evaluate the thermal stability, the purified enzyme was incubated at 50 °C, 55 °C, and 60 °C at the optimal pH determined above for different periods.

2.7.2. Effect of Substances. The effect of metallic ions and other compounds on the xylanase activity was evaluated at concentrations of 2 mM and 10 mM. The residual activities were measured in relation to the control without substances by performing the enzyme assay at the optimal conditions.

2.7.3. Substrate Specificity. Specificity of xylanase against birchwood, beechwood, and oat spelt xylans, carboxymethyl cellulose, (CM-cellulose) and Avicel were assayed. Substrate solutions of 1% (w/v) were prepared in a buffer of optimum pH activity for the enzyme.

2.7.4. Kinetic Parameters. The enzyme was incubated with oat spelt, beechwood, and birchwood xylans, at concentrations between 4.0 and 30 mg/mL. The Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were estimated from the Lineweaver-Burk reciprocal plots, using "GraFit" 5.0 software.

3. Results and Discussion

3.1. Influence of the Carbon Source on Xylanase Production. Distinct substrates, such as pure carbohydrates and some agroindustrial wastes, were evaluated for xylanase production (Table 1). Among the pure carbohydrates, highest values of xylanase activity were obtained with oat spelt xylan, corresponding to 25.44 U/mL and 64.96 U/mg protein. In general, higher levels of xylanolytic enzymes can be achieved with substrates derived from xylan. According to Kulkarni et al. [1], xylanase activity is inducible and xylan-rich substrates play an important role in xylanase induction. When *P. glabrum* was cultivated in medium with lactose, sucrose, cellobiose, Avicel, and CM-cellulose, enzyme activity was not detected. Conversely, lactose and sucrose increased xylanase production by *Penicillium canescens* 10-10c [17]. In the presence of glucose, xylose, and maltose only low levels of enzyme activity were verified, when compared to the cultures with oat spelt xylan.

Table 1: Influence of pure carbohydrates and agroindustrial wastes on xylanase production by *P. glabrum*.

Carbon source (1% w/v)	Intracellular protein (mg)	Xylanase activity (U/mL)	Xylanase activity (U/mg protein)
Pure carbohydrates			
Glucose	1.75	0.04	0.07
Xylose	1.04	0.25	0.60
Maltose	0.89	0.06	0.14
Lactose	0.34	ND	ND
Sucrose	1.34	ND	ND
Cellobiose	1.11	ND	ND
Avicel	0.25	ND	ND
CM-cellulose	0.34	ND	ND
Oat spelt xylan	1.98	25.44	64.96
Agroindustrial wastes			
Sugarcane bagasse	0.53	8.34	25.14
Wheat bran	1.84	20.54	43.35
Oat bran	1.14	13.54	28.31
Rice straw	1.24	17.65	30.34
Brewer's spent grain	2.10	34.32	102.65
Soybean meal	0.76	8.23	20.43
Corn cobs	0.83	7.32	18.23
Citrus pectin	0.34	0.10	0.34
Orange bagasse	0.38	0.16	0.65

Among the agricultural and agroindustrial wastes, highest xylanase production was observed with brewer's spent grain (34.32 U/mL and 102.65 U/mg protein). These values were higher than those obtained with oat spelt xylan. The production of xylanolytic enzymes by *Penicillium janczewskii* with brewer's spent grain as substrate was previously described by Terrasan et al. [18]. However, lower levels of xylanase activity were obtained when compared to this study, corresponding to 15.8 U/mL, under optimized conditions. Xylanase activity was also observed in the presence of wheat bran, oat bran, and rice straw; however, the values obtained, were lower than that observed by brewer's spent grain. Only low levels of xylanase activity were verified with sugarcane bagasse, soybean meal; and corn cobs. Orange bagasse and citrus pectin provided minimal fungal growth, with absent or no significant levels of xylanase produced. The different production levels observed among the lignocellulosic materials are probably related on differences in composition and the accessibility of the substrates to the fungi. Considering the elevated xylanase production obtained with BSG, this substrate was selected for the subsequent optimization experiments.

3.2. Effects of Culture Conditions on Xylanase Production. Cultivation conditions are essential for the successful production of an enzyme, and optimization of parameters such

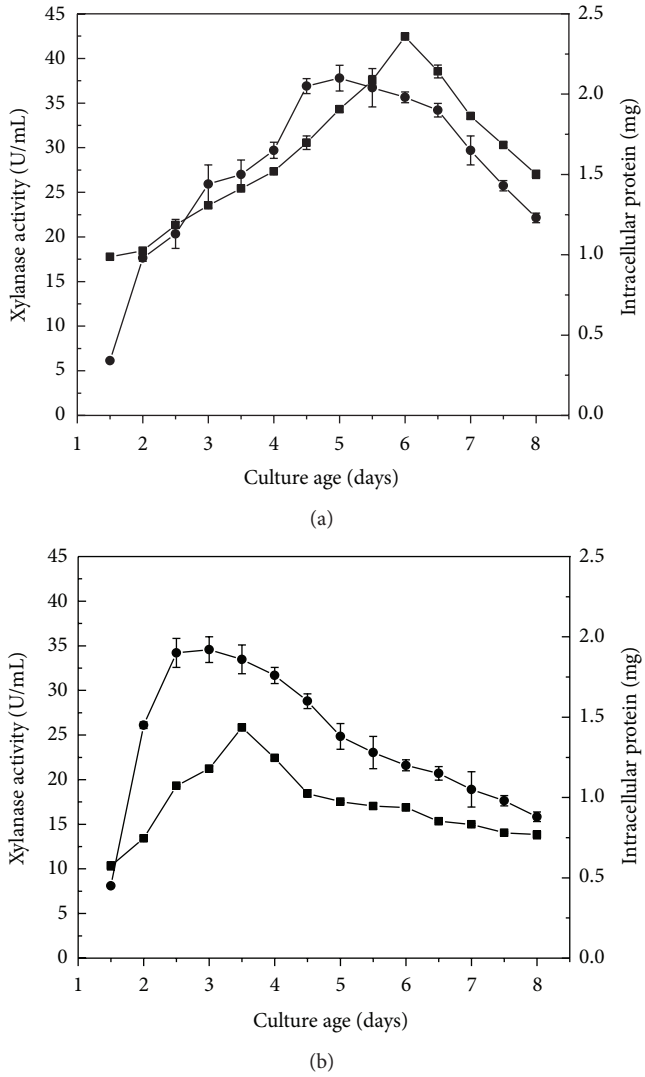

Figure 1: Time course of xylanase production by *P. glabrum* in stationary (a) and shake culture at 120 rev min^{-1} (b). Culture conditions: Vogel medium with 1% (w/v) brewer's spent grain, pH 6.5 at 28 °C. (■) Xylanase activity (U/mL), (•) intracellular protein (mg).

as period of cultivation, pH, and temperature is important for a process development. In standing culture (Figure 1(a)) with brewer's spent grain, the highest xylanase production was verified in 6.0 day-old cultures (42.45 U/mL). In shaking condition (Figure 1(b)), maximal xylanase activity was observed on 3.5-day-old cultures, corresponding to the values of 25.85 U/mL. The highest *P. glabrum* growth, measured by the intracellular protein concentration, occurred on the fifth day in standing culture and on the third day in shake culture. Under shaking, as well as under standing condition, xylanases were expressed during the stationary phase, reaching the decline phase (Figure 1).

Temperature and pH are important environmental parameters that determine growth rates of microorganisms and significantly affect the level of xylanases produced. The influence of pH culture on xylanase production during *P. glabrum* cultivation is presented in Figure 2(a). Xylanase activity was detected in all pH evaluated. The highest activity was observed at initial pH 5.5, corresponding to the values of 48.54 U/mL. With rare exceptions, xylanase production by filamentous fungi occurs in cultures with an initial pH under 7.0. *Trichoderma harzianum* [19] and *Penicillium janczewskii* [18] showed enhanced xylanase production at pH 5.0 and 5.5, respectively. *P. glabrum* could grow in media with initial pH between 3.0 and 8.0 (Figure 2(a)), with maximal growth in the range of 4.5 to 6.5. This result clearly indicates the acidophilic nature of this fungus.

The effect of temperature on xylanase production by *P. glabrum* is presented in Figure 2(b). The highest xylanase activity was verified at 25 °C, corresponding to 51.43 U/mL. Similarly, maximum xylanase production by *Trichoderma viride* was achieved at 25 °C [20]. The optimal growth verified at 25 °C is in accordance with the literature that describes this temperature as ideal for *P. glabrum* [11]. The maximal temperature for this filamentous fungus was 35 °C, which is in agreement with some data reporting the absence of growth above 37 °C [21, 22].

3.3. Purification of Xylanase. The major *P. glabrum* xylanase was purified by protein precipitation with ammonium sulfate and molecular exclusion chromatography. High enzyme proportion (about 84%) was observed in the 90% ammonium sulfate saturation supernatant. The molecular exclusion chromatography elution profile resulted in one xylanase activity peak (Figure 3). The fractions corresponding to this peak were pooled and the sample was submitted to SDS-PAGE, showing electrophoretic homogeneity (Figure 4).

The electrophoretic analysis revealed that xylanase corresponded to a single molecular mass band of 18.36 kDa. Native enzyme molecular mass of 21.3 kDa was estimated for *P. glabrum* xylanase by molecular exclusion chromatography, showing monomeric form. The molecular mass of the *P. glabrum* xylanase is in agreement with those found for the catalytic domain of low molecular mass xylanases, belonging to family 11 [23]. Sanghvi et al. [7] partially purified a xylanase from *T. harzianum* with 29.0 kDa molecular mass, while 27.0 kDa was the molecular mass estimated for the xylanase from *Penicillium* sp. [8].

The summary of the *P. glabrum* xylanase purification is presented in Table 2. The procedure resulted in an overall yield of 76.94%, and the specific activity increased 5.10-fold. The purified xylanase exhibited high specific activity corresponding to 457.89 U mg^{-1} protein.

3.4. Properties of Purified Xylanase

3.4.1. Effects of pH and Temperature, Thermal and pH Stabilities. The effects of temperature and pH on the activity of the purified xylanase were investigated. The xylanase showed optimal activity at pH 3.0 (Figure 5(a)). Similarly, *Laetiporus sulphureus* xylanase exhibited optimum activity at pH 3.0 [24]. However, most xylanases present optimal activity in pH between 5.0 and 7.0 [25], and among the acidophilic xylanases, the majority of them showed high activity only under slight acid conditions.

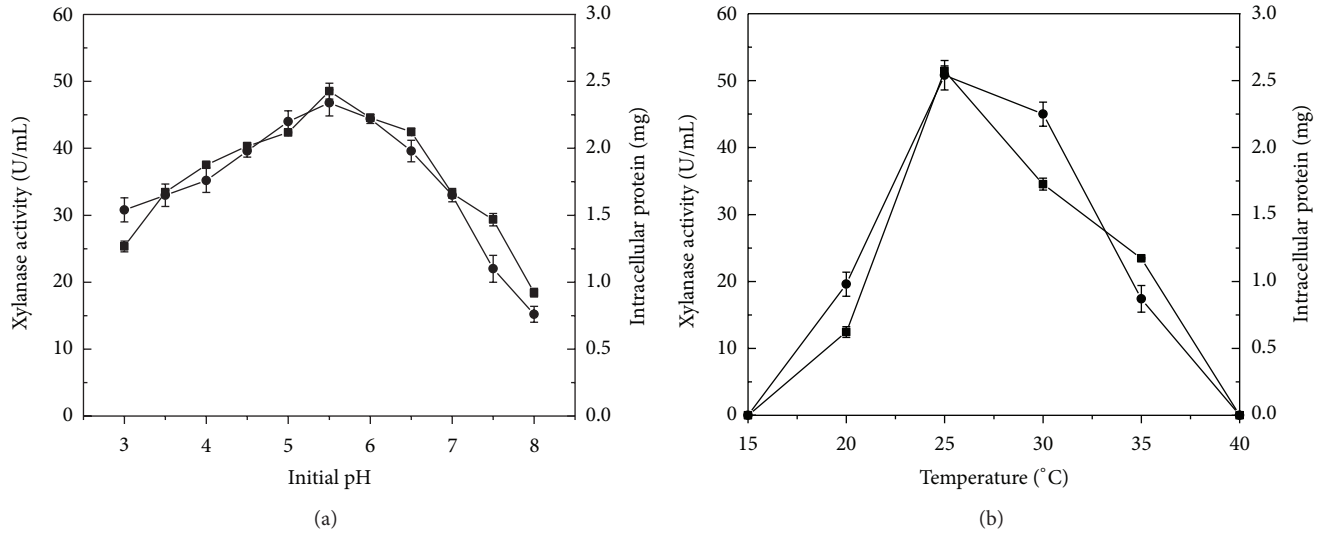

Figure 2: Effect of initial pH (a) and temperature (b) on xylanase production by *P. glabrum*. Culture conditions: Vogel medium with 1% (w/v) brewer's spent grain under stationary condition for six days at 28 °C (a) and pH 5.5 (b). (■) Xylanase activity (U/mL), (•) intracellular protein (mg).

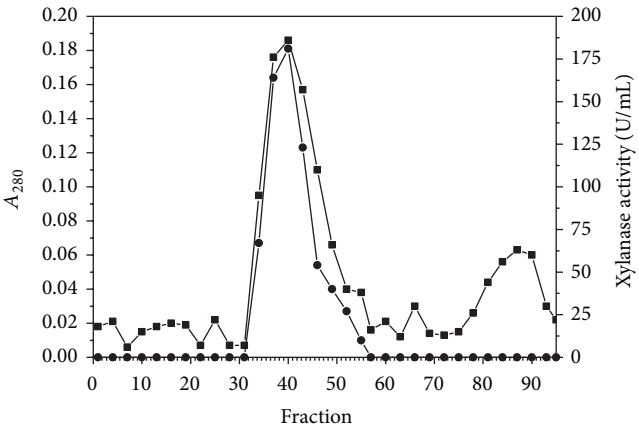

Figure 3: Gel filtration on Sephadex G-75 of the xylanase from *P. glabrum*. The column was equilibrated and eluted with 50 mM ammonium acetate buffer pH 6.8. The flow rate and fraction size were 18 mL/h and 3.0 mL, respectively. (■) A_{280} and (•) xylanase activity.

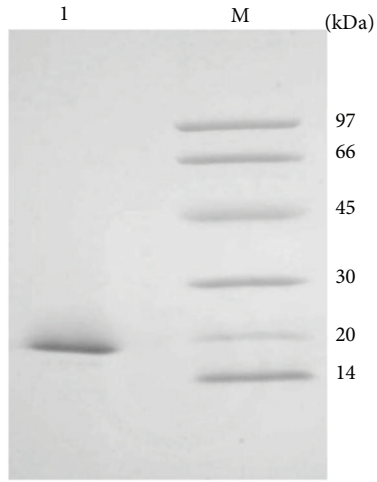

Figure 4: SDS-PAGE (8–18%) of the purified *P. glabrum* xylanase. Lane M: low molecular weight standard proteins; lane 1: purified enzyme.

The optimum temperature for xylanase activity was 60 °C (Figure 5(b)). Values of optimum temperature of xylanase hydrolysis vary according to the producing microorganism. Usually, xylanases from filamentous fungi show optimum temperature between 40 °C and 55 °C [26–28]. Nevertheless, other fungal xylanases show optimum temperature at 60 °C or above [24, 29].

A pH stability study is an essential part of an enzyme characterization before it can be exploited commercially. The xylanase produced by *P. glabrum* was stable over a broad pH range (Figure 6(a)). Xylanase activity was maintained over 80% at pH from 2.5 to 5.0. Microbial xylanases are usually stable over a wide pH range (3–10) [1]. The optimum activity in very acidic conditions and pH stability exhibited by *P. glabrum* xylanase make this enzyme attractive for some industrial applications, such as in feed and food industries.

Thermal stability is an interesting enzyme property due to the great industrial importance. Then, enzyme stability analyses were carried out. The purified xylanase from *P. glabrum* was incubated without substrate at 50 °C, 55 °C, and 60 °C (Figure 6(b)). The estimated half-lives ($T_{1/2}$) at 60 °C and 55 °C were 15 and 32 min, respectively. This enzyme was stable at 50 °C with $T_{1/2}$ of 150 min, retaining 70% of its activity over 60 min at this temperature. The *P. glabrum* xylanase is more thermostable than many fungal xylanases, such as those from *Penicillium expansum* [27] and *Aspergillus niger* B03 [28], however, less thermostable than the xylanase from thermophilic *Talaromyces thermophilus* [30].

Table 2: Purification of xylanase from *P. glabrum*.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification (fold)	Recovery (%)
Culture filtrate	2571.50	28.61	89.84	1.00	100.00
Supernatant 90% of $(NH_4)_2SO_4$	2165.01	7.42	291.78	3.25	84.19
Sephadex G-75	1978.10	4.32	457.89	5.10	76.92

3.4.2. Effect of Substances. In order to verify the effect of substances on xylanase activity, the purified enzyme was incubated in the presence of several metallic ions, sodium dodecyl sulfate (SDS), tetrasodium ethylenediaminetetraacetate (EDTA), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), and β-mercaptoethanol, at 2 mM and 10 mM concentrations (Table 3). In general, the xylanase activity was enhanced with increased concentration of the substances used. Hg^{2+}, Cu^{2+}, and Zn^{2+} were strong inhibitors of the xylanase. Likewise, *Penicillium sclerotiorum* and *Aspergillus ficuum* xylanases were inhibited by these ions [26, 31]. The inhibition by Hg^{2+} seems to be a general property of xylanases, indicating the presence of thiol groups of cysteine residues in their active sites or around them [32]. Xylanase activity remains unaltered in the presence of Na^{+} and Mg^{2+}. Slight activation was observed in the presence of Ca^{2+}, Co^{2+}, and Ba^{2+}. Additionally, *P. glabrum* xylanase was remarkably stimulated when incubated with Mn^{2+}, as *A. niger* B03 xylanase [28].

EDTA, a metal chelator, decreased xylanase activity, indicating that the purified enzyme requires metal ions for their actions. Total loss of activity was observed in the presence of SDS, indicating that hydrophobic interactions must be important in maintaining xylanase structure. The reducing agents β-mercaptoethanol and DTT stimulated xylanase activity. The enzymatic activity stimulation in the presence of these thiol group-protecting agents can be explained by preventing the oxidation of sulfhydryl groups. Dutta et al. [33] and Cardoso and Filho [34] also related the involvement of cysteine residues in the maintenance of tertiary structure of the active site in *Penicillium citrinum* and *Acrophialophora nainiana* xylanases, respectively.

3.4.3. Substrate Specificity and Kinetic Studies. Specificity studies indicated that the *P. glabrum* xylanase did not hydrolyze Avicel or CM-cellulose but acted only on xylans. The purified xylanase exhibited typical Michaelis-Menten kinetics for oat spelt, birchwood, and beechwood xylans, allowing the corresponding kinetic constants to be calculated. *P. glabrum* xylanase showed K_m values of 5.3, 3.1, and 1.2 mg mL^{-1} and V_{max} values of 212.10, 194.21, and 393.17 μmol min^{-1} mg^{-1} of protein, for birchwood, beechwood, and oat spelt xylans, respectively. The K_m and V_{max} values exhibited by *P. glabrum* xylanase are in agreement with the values presented by other fungal xylanases which range from 0.09 to 40.9 mg mL^{-1} for K_m and from 0.106 to 10.000 for V_{max} [4]. The values of K_m for these substrates indicated that this enzyme has higher affinity for oat spelt xylan. Similarly, the xylanases from *A. nainiana* [34] and *Fusarium oxysporum* [35] showed highest value of K_m for oat spelt xylan.

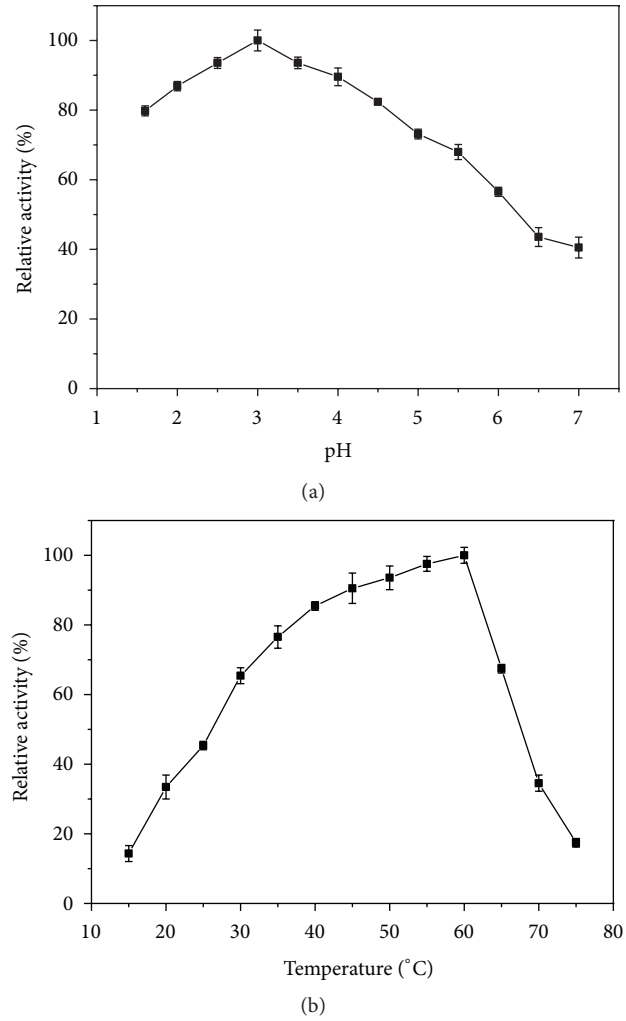

Figure 5: Influence of pH (a) and temperature (b) on the purified *P. glabrum* xylanase. Assay conditions: 0.05 M glycine-HCl buffer from pH 1.6 to 2.5 and McIlvaine buffer from 3.0 to 7.0; 50 °C (a); McIlvaine buffer pH 3.0 (b).

4. Conclusions

In this study, a *P. glabrum* strain was able to produce high levels of xylanase using brewer's spent grain as substrate. Conventional purification methods were effective to purify the major xylanase from *P. glabrum*. The purification procedure resulted in higher overall yields as compared to others

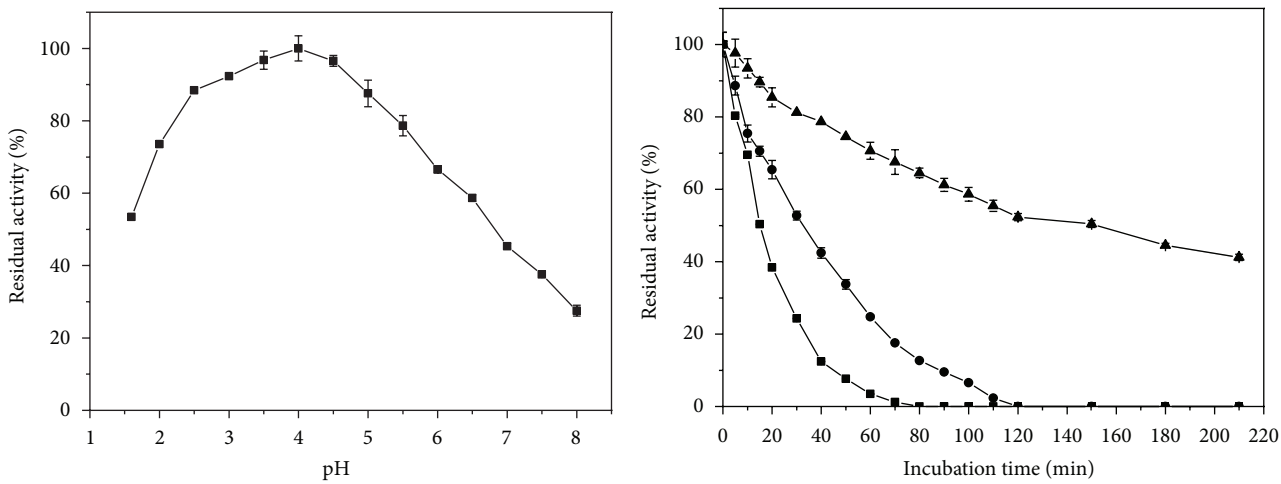

Figure 6: pH (a) and thermal (b) stabilities of purified *P. glabrum* xylanase. The enzymatic preparation was incubated without substrate with glycine-HCl buffer from pH 1.6 to 2.5 and McIlvaine buffer from pH 3.0 to 7.0 at 4 °C for 24 h (a); the enzymatic preparation was incubated at (▲) 50, (•) 55, and (■) 60 °C, without substrate (b). In both assays, the residual xylanase activity was assayed with McIlvaine buffer, pH 3.0, at 60 °C.

Table 3: Effect of different substances on relative activity (%) of purified xylanase from *P. glabrum*.

Substance	Relative activity (%) Concentration	
	2 mM	10 mM
Control	100	100
$CuCl_2$	69.7 ± 0.3	21.3 ± 0.3
$ZnSO_4$	84.4 ± 2.0	75.2 ± 1.1
$MnSO_4$	178.9 ± 1.6	206.7 ± 2.2
$BaCl_2$	117.5 ± 1.5	124.9 ± 1.7
$CaCl_2$	122.0 ± 1.4	126.5 ± 1.2
NH_4Cl	102.0 ± 1.55	134.3 ± 1.7
NaCl	99.3 ± 1.3	99.2 ± 1.5
SDS	ND	ND
PMSF	92.0 ± 0.9	89.3 ± 0.5
$MgSO_4$	97.2 ± 1.7	97.0 ± 1.2
DTT	113.4 ± 1.2	134.4 ± 2.0
$CoCl_2$	117.2 ± 1.6	122.2 ± 1.4
$HgCl_2$	47.1 ± 0.5	ND
$Pb(CH_3COO)_2$	68.7 ± 2.0	19.1 ± 0.6
EDTA	89.0 ± 2.0	64.8 ± 1.0
β-mercaptoethanol	124.0 ± 2.0	136.0 ± 2.0

described in the literature [24, 26, 36]. Furthermore, the specific activity of purified xylanase is higher than those of previously purified xylanases [26, 31, 37]. *P. glabrum* xylanase was active at very low pH, with optimum at 3.0, and it was stable in an acid pH range. These characteristics make it potentially useful in some biotechnological processes such as for animal feed, clarification, and maceration of juices and wines. Additionally, the use of brewer's spent grain as substrate for xylanase production can not only add value and decrease the amount of this waste but also reduce xylanase production cost.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

The authors would like to thank CAPES (Coordination for the Improvement of Higher Education Personnel) and CNPq (National Council of Technological and Scientific Development) for the financial support and the scholarship awarded to the authors.

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