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Research Article

Optimization of Protease and Amylase Production by *Rhizopus oryzae* Cultivated on Bread Waste Using Solid-State Fermentation

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This research was carried for the coproduction of two industrial enzymes: α-amylase and protease via SSF by *Rhizopus oryzae* on humidified bread waste. Fermentation time, inoculum size, initial moisture content, salt solutions, and the thickness of the substrate were investigated one by one. Fungus culture was carried out in sterile aluminum trays, and pH was adjusted to 5.5. The main results showed that the highest levels of enzyme production were obtained at 120 h, 65% relative humidity, height media of 1 cm, 10^5 spore/g, and M-9 solution (g/L): NaH_2PO_4, 12.8; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1; $MgSO_4$ $7H_2O$, 0.5; $CaCl_2$ $2H_2O$, 0.01. α-Amylase (100 U/g) and protease (2400 U/g) produced by SSF from *Rhizopus oryzae* (CH4) on BW as substrate are of great interest in industries and could be valorized as enhancers of the bread making process.

1. Introduction

Food waste occurs at different stages of production, processing, retailing, and consumption since it is produced throughout the food life cycle [1]. The amount of food waste has increased in the last 25 years due to population and economic growth. These wastes could be considered valuable by-products and could be a valuable resource for the production of value-added molecules [2, 3]. Food waste consists mainly of six types according to their source or origin: vegetable trimmings and pulp, starch-based waste, fruit peels and pulp, spent grains, meat and fish waste, and dairy waste [4, 5].

The high carbohydrate content and the additional nutrients of food waste make them ideal media for microbial growth and enzyme production as well as other high value-added molecules [6]. Bread is among the major food waste in many countries around the world. It is estimated that 1.2 million tons of bread are wasted every year, globally [7]. Actually, in Tunisia, more than 45 billion breads, subsidized by millions of Tunisian dinars (34 million USD), are thrown, annually in the street [8]. Wastage occurs at bakeries, retail outlets with leftovers, and consumer households. So, there is a real need to develop processes for reducing the associated problems with bread waste formation. BW is a good fermentation feedstock also since it is available year round and its availability is not associated with the harvest season [9]. It is used for its high yielding bioenergy substrates mainly for ethanol and biohydrogen production [10]. The work of Pleissner et al. [11] suggested hydrolysed BW for bioplastic production by *Halomonas boliviensis*. According to the findings of Zhang et al. [12], bakery waste was successfully used as the nutrient-complete hydrolysates for the fermentative production of succinic acid on *Aspergillus awamori and A. oryzae*. This substrate has also been used to produce amylase [13]. Many studies have tried BW as raw

materials for enzyme production such as glucoamylase and protease [9, 13–18] using *Bacillus*, *Thermomyces* sp., *Aspergillus, or Monascus purpureus*. In fact, BW is a successful material for enzyme production for the following advantages: availability, cost, and reduced contamination. Recently, Benabda et al. [19] reported that they successfully used BW containing valuable components as media for microbial growth.

Solid-state fermentation (SSF), an environmentally friendly technique, which uses solid substrates at low moisture levels, requires suitable solid substrate among agroindustrial materials [20]. Various raw materials are used for SSF, for example, winery and brewery waste, olive mill waste, sunflower cake, sugarcane bagasse, fruit's peels and pulps, cereal brans, and BW [21–25]. In this process, microorganisms grow and produce a wide variety of products such as mushrooms [26], aroma [21, 27], microbial oil [28, 29], preservatives like fumaric acid [30, 31], enzymatic production of wax esters [32], and enzymes [14, 27], thus reducing the cost of production, as reported by these studies.

Among microorganisms, filamentous fungi are the best adapted for SSF due to their mycelia mode of growth as well as their neutral physiological capabilities [33]. In the present work, we used *Rhizopus oryzae* as filamentous fungi for amylase and protease by SSF on BW. This fungus is able to efficiently grow on a wide variety of substrates under operational conditions, thus furnishing numerous bioproducts of interest, such as enzymes, organic acids, aromatic compounds, and colorants. In previous study, M'hir et al. [34] reported that *Rhizopus oryzae* produces proteases in mixture with *Enterococcus* decreasing gluten concentration for celiac persons. Proteases produced could be alternatives to detoxify gluten used in food biotechnology [35]. Given these data, we were interested to use *Rhizopus oryzae* in SSF for the production of proteolytic enzymes. Moreover, the addition of amylases in bakery products contributes to improve consumer acceptance [36]. Fungal α-amylases were considered as safe additives [37]. Their application is gaining an increasing interest since it produces milder processing conditions, reduced the formation of byproducts, and lowered the refining and recovery costs [38]. Therefore, BW can be used as a substrate for enzyme production as it is carbohydrate-rich waste source. To the best of author's knowledge, this study is the first considering *Rhizopus oryzae* for enzyme production on BW.

In this work, we attempt the coproduction of two important industrial enzymes: α-amylase and protease by *Rhizopus oryzae* through SSF using BW as a substrate. The objective of this study was to optimize the parameters such as fermentation time, initial moisture content, height of substrate, inoculum size, and salts' addition. All of these affect concomitant hydrolytic enzyme production in solid-state fermentation of BW. These parameters were evaluated by step wise/single parameter optimization.

2. Materials and Methods

2.1. Microorganism. *Rhizopus oryzae* CH_4, belonging to the Culture Collection of the Laboratory of Ecology and Microbial Technology, was used in this study. The culture was propagated on potato dextrose agar (PDA) (Oxoid) and stored at 4°C.

2.2. Inoculum Preparation. For inoculum preparation, *Rhizopus oryzae* was grown on the PDA plate at 30°C for 7 days for complete sporulation [34]. 9 mL of sterile distilled water containing 9 g/L NaCl was mixed and was added to the plate, and the spores were scraped with an inoculating loop under aseptic conditions. The spore suspension was used as the inoculum for subsequent fermentation. The spore count was carried out using a Malassez counting chamber. Fermentations were inoculated with spores at different levels.

2.3. Substrate Preparation. Commercial white bread waste was purchased from a local bakery and was aged from 6 days (BW was stored at room temperature in the bakery) (moisture: 15.2/100 g dry basis, carbohydrate 49/100 g db, and starch 47.2/100 g db), ground with a mortar, sieved to obtain a fine powder, and then filtered with a colander (5 mm mesh size). BW was used at the same day of collection to conduct following experiments.

2.4. Fungal Solid-State Fermentation and Condition Optimization. BW powder was taken in rectangular trays of approximate dimension 286 mm $\times$ 120 mm $\times$ 80 mm (about 250 g of BW in each one). Trays were closed with aluminum foil, autoclaved at 121°C for 15 min, cooled to room temperature, and mixed thoroughly with sterilized distilled water to reach appropriate moisture content. The moistened powder was spread evenly on the tray in layers, typically between 0.5 and 2.5 cm deep in tray with an open top and then inoculated with fungal spore suspension of *Rhizopus oryzae* CH_4 as described above and recovered with aluminum foil. 1 mL of cryopreserved spores solution of *Rhizopus oryzae* (1×10^5 spores/mL) was inoculated in the surface of bread waste. Then, the mixture was homogenized with sterile spatula. The operation was carried out aseptically. Trays were incubated at 30°C under static conditions. Figure 1 illustrates the bread waste SSF flow chart.

Factors like incubation period, moisture content, inoculum size, pH, substrate height, and salt effects influencing the secretion of enzymes by *R. oryzae* under SSF were investigated by adopting the search technique-varying parameters one at a time. Optimization of the fermentation parameters for SSF was carried out to determine the effect of each parameter on enzyme production. α-Amylase and protease production was studied in SSF of BW, by altering various physicochemical and cultural conditions with one at the time.

2.5. Enzyme Recovery and Assay. For isolation of enzymes produced in SSF samples, 5 g of the fermented substrate was suspended in 50 mL of distilled water at room temperature using a kitchen-type blender to form a suspension of 100 g/L. After agitation in a rotary shaker (170 rpm) for 1 hour at 30°C, the slurry was squeezed through wet cheese cloth

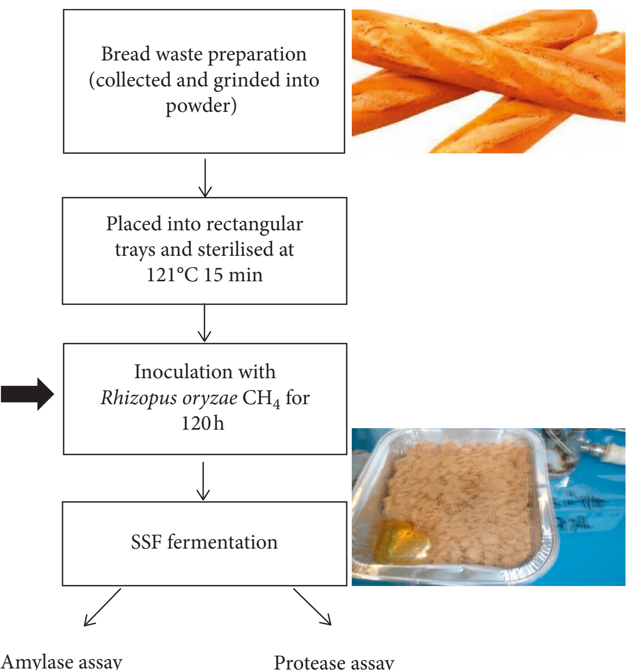

Figure 1: Bread waste valorization flow chart in the present study.

followed by the centrifuging the whole content at 8000 g, 15 min, and 4°C to remove the insoluble matter, and the clear supernatant obtained was used as the source of enzymes [39].

2.6. α-Amylase Assay. The amylolytic activity was assayed using the DNS method after crude extraction of fermented bread waste powder [40]. α-Amylase activity was determined at 55°C for 30 min by measuring the release of reducing sugar from 1% soluble starch (w/v) as substrate in 25 mM sodium acetate buffer at pH 5 where D-glucose was used as standard. One unit of alpha amylase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars, expressed in glucose equivalent, per minute per g of fermented substrate under the specified conditions.

2.7. Protease Assay. The method mentioned by Han et al. [10] was used to quantify protease activity with modification. Exactly, the reaction mixture containing 0.3 mL of 20% (w/v) azocasein (Sigma), 0.1 mL of enzyme preparation, and 0.2 mL of citrate buffer at pH 5.2 was incubated for 4 h at 45°C. The reaction was stopped by adding 2 mL of 10% TCA and stored at 4°C for 10 min, and the test tubes were centrifuged at 3500 g for 5 min at 4°C. One unit of the enzyme activity (U) was defined as an absorbance (440 nm) change of 0.01 after 4 h at 45°C [34, 41].

2.8. Statistical Analysis. The data for all experiments have been calculated from three replications, with the values presented as the mean ± SE (standard error).

3. Results and Discussion

3.1. Effect of Incubation Period. To investigate the effect of the incubation time on the enzymatic activity of *R. oryzae* under SSF fermentation, the temperature was 30°C, the inoculum size 10^5 spores/g, the initial moisture content 60%, the substrate height at 1 cm, and the pH at 5.5. In SSF fermentation, several factors are to be optimized since they control the microbial growth and thus the enzymatic production, namely, temperature, fermentation time, moisture content, pH medium, inoculum size, carbon and nitrogen sources, and salt concentrations.

Figure 2 shows the effect of fermentation time on the production of both amylase and protease for 144 h. The amounts of protease and amylase production were of 1260 U/g and 16.2 U/g on 24 h, respectively. The highest activity was achieved within 120 h of fermentation for both enzymes (2412 U/g and 100 U/g, respectively, for protease and amylase). After this fermentation time, the activity fell drastically. In general, long incubation period results in an enzyme production decrease consequent to the reduction of nutrients in the substrate as well as to the release of proteases and the drop in the pH in the substrate [42, 43]. M'hir et al. [34] reported a protease activity value of 2.50 U. According to Hashemi et al. [44], SSF with *Bacillus* sp. recorded an optimal amylase activity of about 3800 U/L, whereas Ferreira et al. [45] reported a value of 6500 UI/L on SSF using *R. oryzae* on wheat bran. Enzyme production by microorganisms is highly influenced by media components and physical factors such as temperature, pH, incubation time, and inoculum density [46]. Trying to produce protease by *A. flavus* using SSF, Johnvesly et al. [47] reported that the maximum protease production occurred during the 7th day of incubation. Protease production from rice mill wastes by *Aspergillus niger* in SSF increased with the incubation period [48], in accordance with our results. In addition to incubation time, protease production strongly depends on the extracellular pH which affects several metabolic and enzymatic processes and transports various components across the cell membranes, thus influencing the cell growth and metabolite production [49]. Moreover, Nafisa et al. [50] reported a gradual decrease in protease units correlated to an increasing incubation period. This indicated the enzyme's role as a primary metabolite, being produced in the log phase of the growth of the fungus for using nitrogenous source of BW [51].

Concerning α-amylase, the maximal productivity of α-amylase 100 U/g was achieved after 120 h (Figure 2(b)), which is two times higher than the reported result of Han et al. [14] on the 5th day by *A. awamori* using BW. Glucoamylase production by *A. awamori* showed similarity to our results, using pastry waste without nitrogen supplements [52]. In fact, variation levels of glucoamylase activity were influenced by a good balance of carbon, nitrogen, and phosphor [52]. Furthermore, Melikoglu and Webb [25] mentioned that near 95% of the starch has been consumed by 144 hours. The highest enzymatic activities (protease and glucoamylase) were measured during this period.

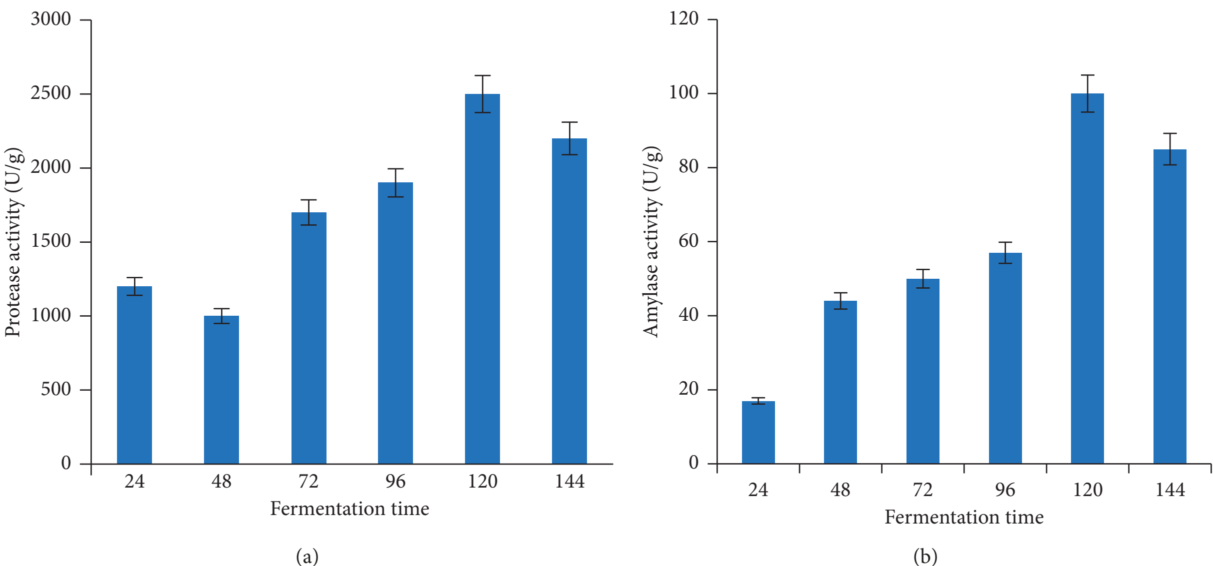

Figure 2: Effect of incubation period (*h*) on the production of protease (a) and α-amylase (b) by *R. oryzae* CH_4 at 30°C under static conditions.

3.2. Initial Moisture Effect. Initial moisture content of bread waste was adjusted to 45%, 50%, 55%, 60%, 65%, and 70% levels. Incubation period was 120 h, and the temperature was 30°C with an inoculum size of 10^5 spores at an inoculation height of 1 cm. The pH was fixed at 5.5. Our results showed that the protease activity ranged from 1150 to 1550 U/g, whereas that of α-amylase varied from 63.42 to 216.66 U/g (Figure 3). A concomitant production for optimal protease and amylase was, respectively, of 1550 U/g and 216.66 U obtained with an initial moisture content of 65%. The lowest ones were associated with a common initial moisture content of 45%.

These results were consistent with levels mentioned by Haque et al. [18], where optimum moisture content was about 65% for protease and 55–60% for glucoamylase activities. These authors reported a maximum of 8 U/g of glucoamylase and 116.6 U/g for protease observed with filamentous fungus *Monascus* cultivated in SSF on bakery waste. The protease activity showed in our study was more than tenfolds higher than those reported by Haque et al. [18]. However, protease values obtained by Haque et al. [18] are six times higher that reported by Liang et al. [53], with the shrimp and crab shell powder medium by *Monascus*. The most important benefit of using bread waste is its high nutrient content, making it useful without the addition of supplements [19].

Higher initial moisture (180%) content has been reported by Meligoklu et al. [16] with BW using *A. awamori,* where protease production increased up to 71.6 U/g, decreasing after.

According to Sivaramakrishnan et al. [54], α-amylase production increases with the increase of initial moisture content. A low moisture level is associated with an early sporulation and a low enzyme yield. This may be explained by the nonavailability of nutrients. A high moisture level caused a decrease of particles porosity and a stickiness of substrate, thus resulting in agglomeration and reduction of gas volume and gaseous diffusion inducing as a consequence of low oxygen transfer [16, 55].

Hence, it is important to provide the moisture content at an optimum level. In SSF, water must be available in the fermentation medium for microbial growth and biochemical activity. Indeed, water content influences the physical state of the substrate, nutrient availability, diffusion of nutrients, and oxygen-carbon dioxide exchange in a complex way [56].

3.3. Effect of the Inoculum Size. Inoculum amount is an important biological factor, which determines biomass production in fermentation [57, 58]. Maximum yield of enzyme production should be result of a balance between the proliferating biomass and available nutrients [59]. Figure 4 illustrates enzyme activity. BW was inoculated with varied levels of spore suspension (10^4, 5.10^4, 10^5, and 5.10^5 spores/g) with a moisture content of 65% and inoculation height of 1 cm with pH 5.5. The results showed maximal activities of 2400 U/g db and 100.32 U/g for both protease and amylase obtained with an inoculum size of 10^5 spores/g. Tiann and Yuan [43] reported that the inoculum level had no significant influence on enzyme productivity in SSF. However, Meligoklu et al. [16] reported 1.0 million spores/g dry as optimum size. Demir and Tarı [60], determined that 10^7 spores/g is optimum for enzyme production in wheat bran SSF by *Aspergillus*. Paranthaman et al. [48] have mentioned that further increase in inoculum volume resulted in a decrease in protease production. This fact could be explained by high amount of inoculum that caused overcrowding of spore thus and decreasing enzyme production [59]. The maximum protease production was noticed at an inoculum size of 2×10^6 spores/mL [61].

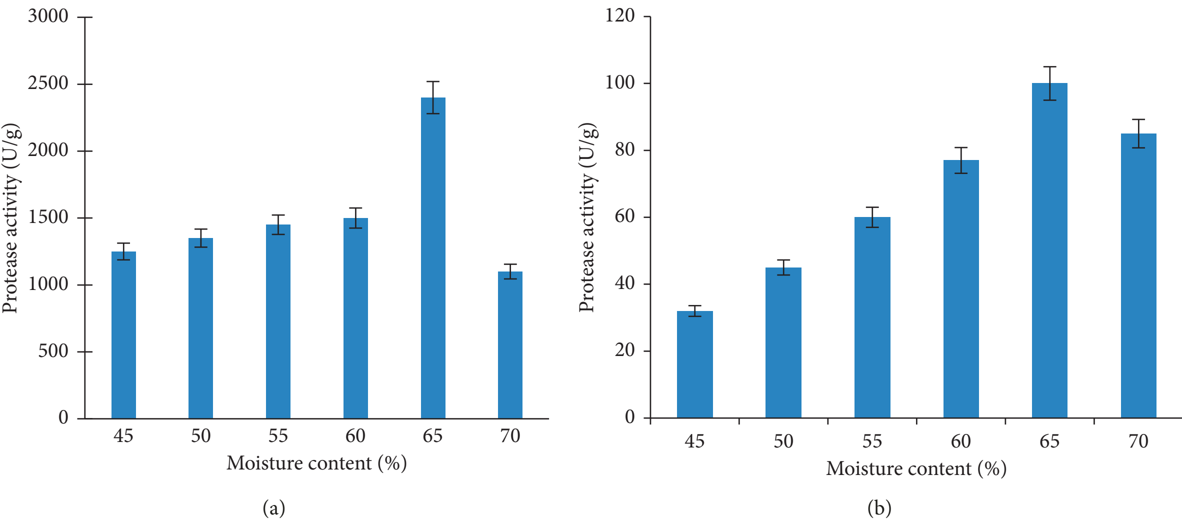

Figure 3: Effect of the initial moisture content (%) on the production of protease (a) and α-amylase (b) by *R. oryzae* CH_4 at 30°C for 120 h under static conditions.

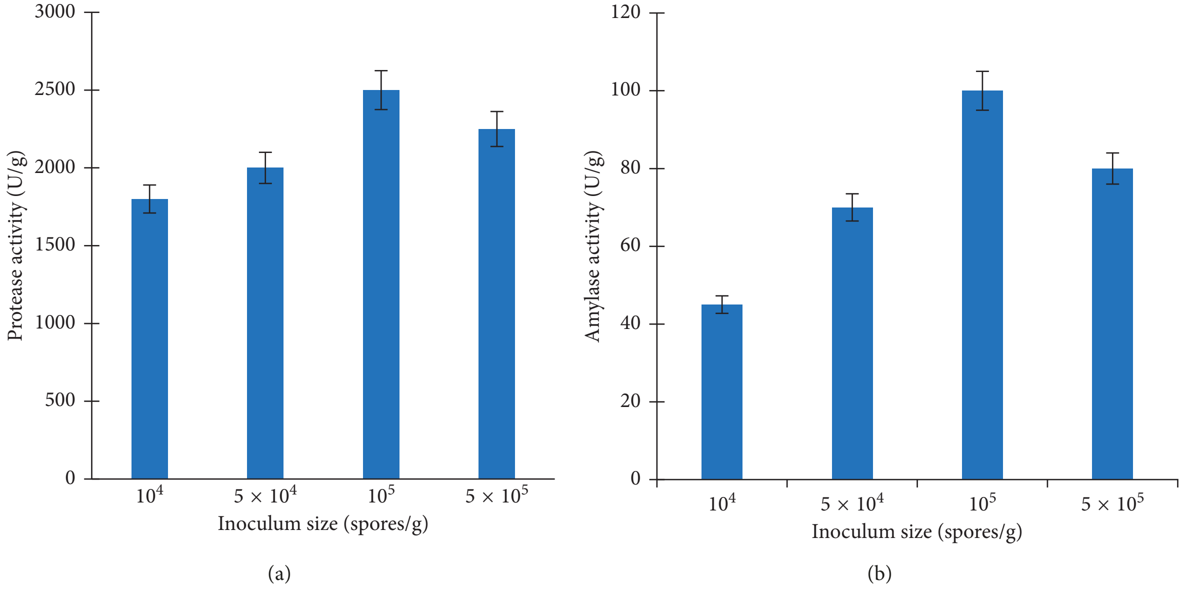

Figure 4: Effect of inoculum size on protease activity (a) and amylase activity (b).

3.4. Effect of Salts. Experimental studies shown in Figure 5 presented that the optimal values of protease and amylase were 1493 U/g and 90.06 U/g, respectively, for salt solution S3. Previously studies [62–64] have reported that NaCl (only present in S3) enhanced protease production. This salt plays an important role in the stabilization and the protection of the enzyme from undergoing denaturation [64]. Earlier studies reported that other inorganic nitrogen sources increase enzyme production. In fact, when supplemented with potassium nitrate, *Aspergillus funiculosus* produced maximum protease [65]. Furthermore, El Safey and Abdul Raouf [66] and Karataş et al. [67] reported that ammonium sulfate was the best nitrogen source for protease production. However, Mukhtar and Haq [68] reported peptone as the best nitrogen supplier for protease production by *Rhizopus oligosporus*. Salt solution S3 with ammonium chloride increases protease production by three times. This result is in agreement with the finding of Rathod and Pathak [69]. Presence of inorganic nitrogenous supplements may improve nutritional quality and consequently affect α-amylase synthesis. α-Amylase yield increased with proper supplementation of C and N sources as they are absolutely necessary for microbial growth [70]. Sindhu et al. [71] have tried solid-state fermentation by *Penicillium janthinellum* (NCIM 4960) using wheat bran. They reported that α-amylase production was enhanced with ammonium sulfate, whereas ammonium nitrate, ammonium chloride, sodium nitrate, potassium nitrate, and sodium nitrite showed inhibitory effect on its production. However, ammonium chloride induces production of amylase [67]. The inhibitory effects of

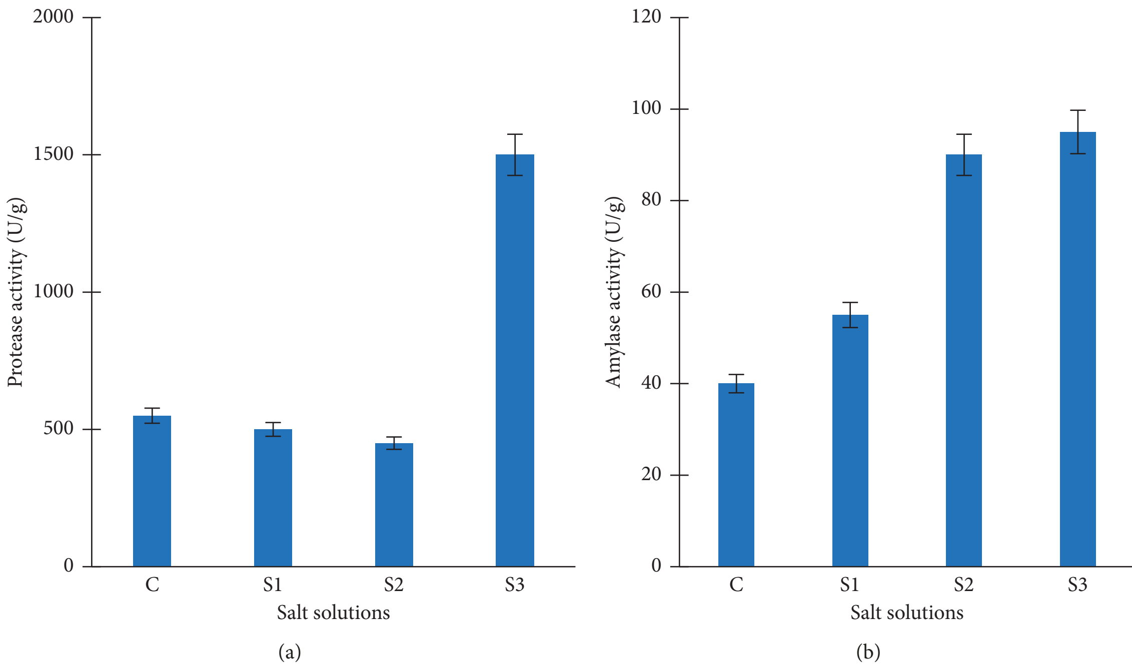

Figure 5: Effect of salts on protease activity (a) and amylase activity (b). S1: Czapek–Dox solution (g/l): $NaNO_3$, 2.5; KH_2PO_4, 1; $MgSO_4$ $7H_2O$, 0.5; KCl, 0.5. S2: M-15 solution (g/l): NH_4NO_3, 3; KH_2PO_4, 1; $MgSO_4$ $7H_2O$, 1; $FeSO_4$ $5H_2O$, 0.01. S3: M-9 solution (g/l): NaH_2PO_4, 12.8; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1; $MgSO_4$ $7H_2O$, 0.5; $CaCl_2$ $2H_2O$, 0.01. C: control: bread waste and sterilized distilled water.

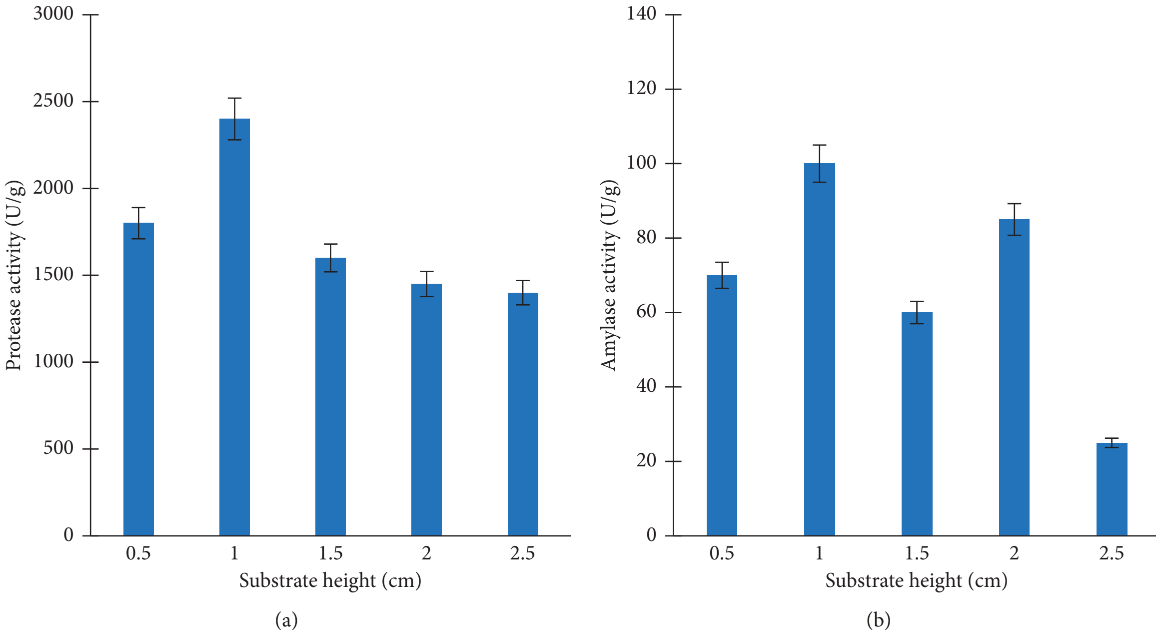

Figure 6: Effect of substrate height on protease activity (a) and amylase activity (b).

some of the salts could be due to the pH changes in the medium.

3.5. Effect of Substrate Layer Thickness. The thickness of the substrate is an important factor and should be optimized for controlling aerobic conditions for fungi. Protease and amylase activities varied with substrate thickness that ranged from 0.5 cm to 2.5 cm according to Figure 6. The maximum protease and amylase activities were recorded at the layer thickness of 1 cm with respective values of 2400 U/g and 100 U/g. Similarly, results were observed (11 mm thickness) by Demir and Tari [72] with wheat bran SSF by *Aspergillus sojae* for polygalacturonase production. In fact, an increase of the layer thickness may reduce oxygen for fungus inducing a poor growth [73]. However, the increase of

substrate thickness cannot promote fungal growth. On the contrary, the lower thickness has been reported to be unable to support the complete growth of fungus [74]. Dojnov et al. [75] reported that high quantities of substrate enhance fungal growth. The highest α-amylase activity was detected on 32 g of triticale grains (medium substrate height during SSF). According to these authors, glucoamylase production was in correlation with fungal growth and was the highest on 48 g of triticale grains (highest substrate height).

4. Conclusion

BW is a good culture medium in SSF in terms of composition and safety. Enzymes production by *Rhizopus oryzae* such as α-amylase and protease under optimized cultural conditions were as follows: 65% moisture content, inoculum 10^5 spores/g, and incubation period of 120 h (5 days). Further studies should be continued to test protease and α-amylase stability (thermal, pH, and detergent stability) with the goal of performing their production in pilot scale. Several other biochemical and biophysical parameters like sensitivity towards ions, inhibitors, reaction kinetics, and structural studies should be performed to validate their industrial applications.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

This research did not receive specific funding but was performed at the Laboratory of Ecology and Microbial Technology (LETMi).

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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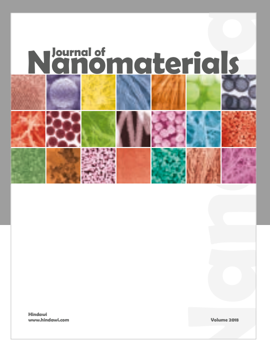

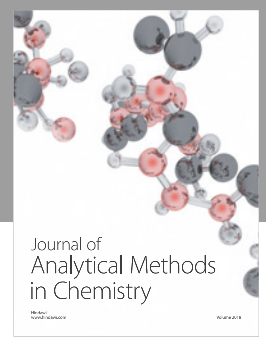

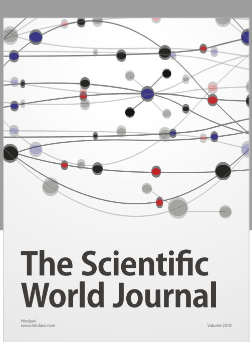

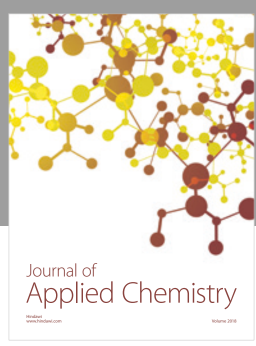

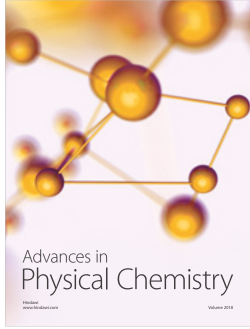

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