Screening and optimization of ligno cellulose degrading fungi using agro wastes as substrates

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ABSTRACT

Waste cellulosic materials (potato peel, onion peel, carrot peel and sugar beet peel) are inducers for cellulase, xylanase and ligninase production in wild strains of *Penicillium crustosum* and *Penicillium* sp. which were isolated from agro wastes collected from different localities of Bangalore (India). The results obtained indicated that the production of these enzymes started from the fourth to seventh day. The degradation of carboxymethyl cellulose (CMC), and xylan was observed with a yellow opaque layer formation around the colony in case of cellulase enzyme detection, whereas lignin was observed with the formation of brown oxidation zones around the colonies. Among all of fungi studied, *Penicillium* sp. showed highest clearance zone for 0.67cm whereas, highest clearance zone for xylanase was *Penicillium crustosum* (0.74 cm). Lignin degradation was observed by *Penicillium* sp. The brownish black colour was the characteristic feature of positive test and *Penicillium crustosum* could not degrade lignin. A submerged type of fermentation was carried out for the enzyme production with the substrate concentration of 5.8 % (w/v). The optimization conditions such as incubation time, pH, temperature and effect of different carbon and nitrogen sources were studied. The highest cellulase activity was observed with potato peel as substrate on 6th day and for onion peel, carrot peel and sugar beet peel maximum cellulase activity was on 7th day of incubation. The highest cellulase activity was observed with sugar beet at the pH of 6.5 (1.56 U/ml) and at temperature 20°C (1.28U/ml). With different carbon and nitrogen sources, the highest enzyme activity was observed with urea and onion peel as substrate (2.58U/ml) and with rhamnose and sugar beet peel as substrate (1.49 U/ml).

Keywords: fungi, agro wastes, carboxymethylcellulose (CMC), xylan, lignin

INTRODUCTION

Fungi grow in diverse habitats in nature and are cosmopolitan in distribution requiring several specific elements for growth and reproduction. A wide range of media are used for isolation of different groups of fungi that influence the vegetative growth and colony morphology, pigmentation and sporulation depending upon the composition of specific cultures medium, pH, temperature, light, water and surrounding atmospheric gas mixture [1,2]. Agro wastes which include potato peel, carrot peel, onion peel and sugar beet peel are among wastes that cause of environmental pollution. Their conversion to useful products may ameliorate the problems they cause. Proper biotechnological utilization of these wastes in the environment will eliminate pollution and convert them into useful byproducts [3]. Cellulose and hemicellulose which contain in those agro wastes are commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms commonly bacteria and fungi [4]. Cellulose, the world's most abundant carbohydrate polymer, is mainly broken down by cellulases. Cellulases can be divided into 3 groups

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based on their activity on cellulose: endoglucanase (*endo*-1, 4-ß-D-glucanase, EG, EC 3.2.1.4); cellobiohydrolase (*exo*-1,4-ß-D-glucanase, CBH, EC 3.2.1.91) and ß-glucosidase (1,4-ß-D-glucosidase, BG, EC 3.2.1.21) [5,6]. Cellulases, usually produced by fungi, bacteria and protozoans [7], but also have important role in industrial and commercial applications such as textile, laundry, pulp and paper, fruit juice extraction, and animal feed additives as well as in bioethanol production [8]. Majority of the reports on commercial production of cellulase utilize submerged fermentation (SmF), because of its ease of controlling the conditions. In this study fungal species were isolated from degrading potato peel, carrot peel, onion peel and sugar beet peel and tested for their cellulase, xylanase and ligninase, by measuring the zone of clearance and cellulase activity.

MATERIALS AND METHODS

Isolation and Screening of Cellulolytic Fungi

Potato peel, onion peel, sugar beet peel and carrot peel were sun dried for 3 to 5 days to reduce the moisture content and were grinded into power using Marlex, Excella Mixer Grinder (Mumbai, India). The fine powder was passed through 150 µm mesh size screen, and the fractions so obtained were stored in polyethylene bags for further use. During screening process 12 fungi were obtained from agro wastes. Only two strains were *Penicillium crustosum* and *Penicillium* sp. had lignocellulolytic properties. The two strains were selected for enzyme production that showed the largest diameter of clearing zones in plate assays. Basing on the zone of clearance, Cellulolytic, Xyanolytic and lignolytic activity was determined [9].

Serial dilution of sample was prepared in sterile water and 0.1 ml of diluted sample was spreaded on the surface of potato dextrose agar (PDA) and incubated for 7 days at 28°C. Colonies with different morphological form was picked and subculture to obtain pure culture. Stock was maintained on PDA agar at 4°C for further studies. Screening of cellulase producing fungi was done on CMC selective agar containing: 0.02% $NaNO_3$; 0.1% K_2HPO_4; 0.05% $MgSO_4$; 0.05KCl; 0.2% (CMC) sodium salt; 0.02% peptone and 1.7% agar. Plates were incubated at 28°C. After 3 days of incubation, plates were flooded with Gram's iodine solution for 3 to 5 minutes [10]. A clear zone around the colony, indicating cellulose utilization and enzyme activity of fungal cultures were observed and zone of clearance measured [11]. The disappearance of original color in the fungus treated medium was observed. The evaluation of degradation was assessed as the disappearance of color from the Petri plate, during the growth of the fungal mycelium.

Screening of Xylanolytic Degrading Fungi

Xylanolytic degrading activity was measured by using xylanolysis basal medium (XBM) incorporated with 0.25% w/v and 1.6% w/v agar and autoclaved. The plates were inoculated with the fungi and incubated at 28°C in dark (3-4 days) of incubation the plates were flooded with Gram's iodine solution for 5 minutes. Xylan degradation around the colonies appears as yellow-opaque areas against brown/reddish color for undegrade [11]. The disappearance of color and change in original color in the fungus treated medium was observed. The evaluation of degradation was assessed as the disappearance of color from the Petri plate, during the growth of the fungal mycelium.

Screening of Ligninolytic Fungi (Bavendamn test)

Ligninolytic method as described [12] was applied for the isolation of ligninase producing fungi. Fungal isolates were cultured were on Malt extract agar medium .For screening purpose malt extract

agar medium (3%) was substituted with respective enzyme substrates tannic acid (TA) for ligninase at pH 5.8 [13]. The Petri plates were incubated at 28°C for 7 days. Ligninolytic activity was assessed by observing the brownish colored zone around the colony.

Cellulase Activity Assay

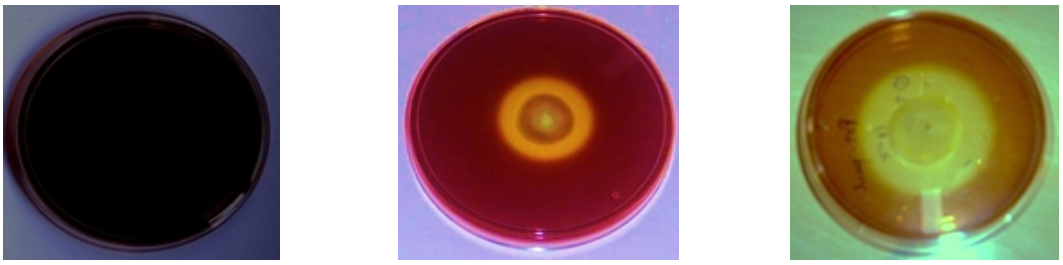
The one mL enzyme reaction mixture was composed of 0.5 mL of diluted enzyme and 0.5 mL of 1% (w/v) carboxymethyl cellulose (CMC; Sigma, St. Louis, MO, USA) in 0.05M citrate buffer of pH 4.8 The reaction mixture was incubated at 100°C for 10 min and the released reducing sugar was determined by the 3, 5-dinitrosalicylic acid method [9]. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μM of glucose from CMC per min under the assay conditions.

Optimization of Cultural Conditions

Penicillium sp. was inoculated in ammonium nitrate (NH_4NO_3), 150mL conical flask and incubated at 28°C for a period of 7 days. The quantity of cellulase produced varies with type of microorganism and fermentation condition. To determine the effect of incubation time, fermentation was carried out for various time periods. Submerged fermentation was carried out to study the effect of various physico-chemical parameters required for the maximal production of cellulase by *Penicillium* sp. The parameters that were optimized were substrates (potato peel, onion peel, carrot and sugar beet peel). Incubation time 6 days for potato peel and 7 days for onion peel, sugar beet peel and carrot peel; pH (4.5-7.5); incubation temperature (20-50°C). Studies were also conducted to examine the effect on cellulase production of various additives supplemented into agro wastes culture. The examined additives were carbon sources such as xylose, glycerol and rhamnose and nitrogen sources were urea, peptone, and nutrient broth at 0.5 w/v. Triplicates were maintained for each experiment.

RESULTS AND DISCUSSION

Screening fungal cultures were identified as *Penicillium crustosum* and *Penicillium sp*. by preparing a wet mount using lacto phenol cotton blue and confirmed by National Fungal Culture Collection of India (NFCCI), Pune. It was observed that both the fungi cultures were positive for the production of cellulase, xylanase and ligninase enzymes. The formation of clearing zones indicated that *Penicillium crustosum* and *Penicillium* sp. are one of the best producers of cellulase and xylanase. The ability to produce cellulase and xylanase were further confirmed by the formation of brownish clear zone of hydrolysis in Gram's iodine solution. Zone of clearance was observed after 3 days of incubation for fungal growth before flooding Gram's Iodine solution for observing decolorization zone (Figure 1 and Figure 2) all fungi showed decolorization zone around the mycelial growth on CMC and Xylan. *Penicillium* sp. showed the largest decolorization zones as compared to *Penicillium crustosum*. The formation of clearing zone around the colonies confirms the secretion of extracellular cellulase and xylanase. Diameters of the colony and the clear zone were measured. Clear zone was observed by naked eyes or documented by taking a photograph. The color of Gram's iodine solution contained media changed from brownish to yellow-orange depending on fungal species. The results with two strains fungal species showed that the clarity and detection rate of clear zone (enzyme activity) is apparent with the medium contained Gram's iodine solution.



a b c

Figure 1. Iodine plate assay showing cellulase activity on 4th day of incubation at 28°C, screening of xylanase producing fungal isolated. (a) Control; (b) *Penicillium crustosum*; (c) *Penicillium* sp.

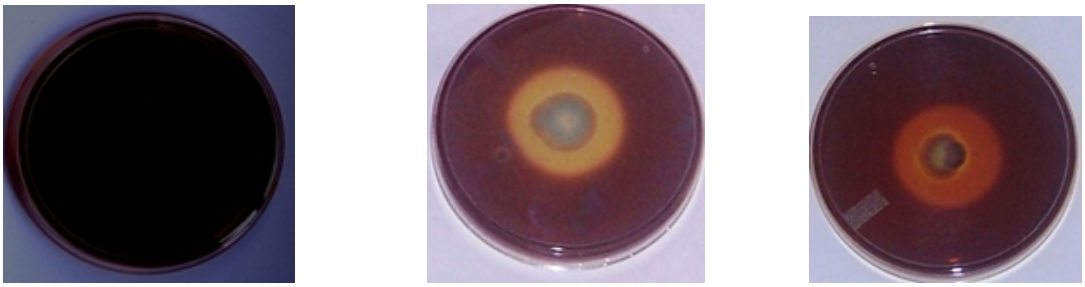


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Figure 2. Iodine plate assay showing xylanase activity on 4th day of incubation at 28°C, screening of xylanase producing fungal isolated. (a) Control; (b) *Penicillium crustosum*; (c) *Penicillium* sp.

The successful use of Gram's Iodine solution in the detection of fungal cellulolytic and xyanolytic activity was reported in *Bacillus sp*., *Pseudomonas sp* and *Penicillium chrysogenum* [10] and in fungi strain KS1, KS2 and KS5, N.K [14]. These reports also support our work. The addition of Gram's Iodine solution is used to extend the detection zone of clearance with different fungi and different substrates

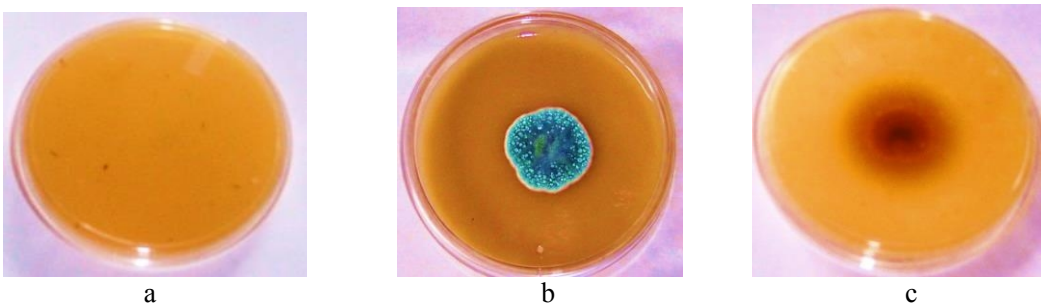

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Figure 3. Fungal isolates showing clear zone around their colonies. (a) Control; (b) *Penicillium* sp.; (c) *Penicillium crustosum*.

When cultivated on nutrient agar containing certain phenolic compounds as gallic acid or tannic acid, the white rot fungi produce a deeply brown coloured zone around the mycelium. The data of Bavendamn test is proved for the presence of ligninase activity of fungi which was observed as zone of clearance without flooding any dye. Except *Penicillium crustosum* which was unable to grow or produce activity zone under these conditions of this assay was observed (Figure 3). The fungi isolated were able to produce cellulolytic, lignolytic and xylanolytic enzymes having application in paper industry because lignolytic enzymes degrade lignin and leaves cellulose and hemicellulose from which xylan is degraded by xylanase enzyme produced by fungi and cellulose remains free and quality of paper depending up amount of cellulose [15].The isolated fungi were selected by several screening methods by plate assay, to evaluate the lignocellulolytic properties like cellulolytic, xylanolytic, and polyphenoloxidase activity and diameter of hydrolysis zones were measured.

In the current experiment, the rate of cellulase production increased with increase incubation time (Figure 4). Cellulase production increased gradually during the fermentation period. The maximum amount of cellulase was recorded on 6th day for potato peel with maximum cellulase activity (8.89 U/ml) was noted. Onion peel, carrot peel and sugar beet peel showed maximum cellulase at 7th day with maximum cellulase activity of (29.6 U/ml); (26.88 U/ml) and (23.31 U/ml) respectively.

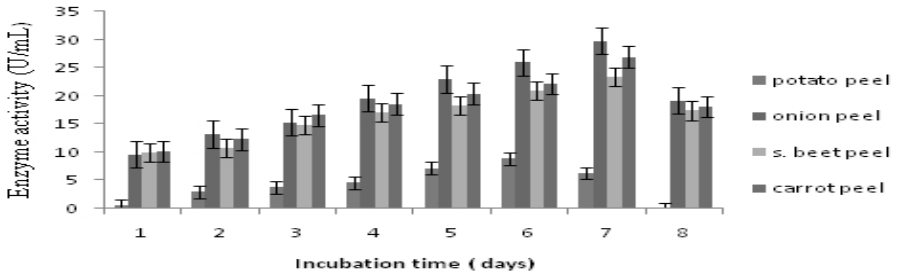

Figure 4. Effect of incubation time enzyme activity by using different substrates.

The production of enzyme was related to the incubation period. Onion peel produced large amount of cellulase enzyme within 7 days of incubation, whereas potato peel produced small amount of cellulase enzyme upon 6 days of incubation. Zhang et al. [16] also reported the maximum time of CMCase production of 7days of fermentation period for *T. viride* which was similar to our findings. Zhang et al. [17] reported the maximum production of cellulase after 6 days of fermentation period. Ogel et al. [18] reported that time course required to reach maximum levels of activity may be affected by several factors, like the presence of different ratios of amorphous to crystalline cellulose.

The optimum pH of the substrate is an important factor for growth and metabolic activities of microorganism that affects the performance of submerged fermentation. This implies that the successful production of cellulase depend on maintenance of initial pH of the production medium. A range of initial pH 4.5 to 7.5 was examined to investigate the effect of pH on cellulase production. The pH was adjusted using 1N HCl and 1N NaOH solutions. The enzyme activity varied with the pH (Figure 5). For the substrate potato peel the activity of the enzyme increased up to pH 6.5, which recorded the highest activity (1.04 U/ml) and decreased at the pH of 7.5.Similarly the enzyme activity for the onion peel substrate increased up to the pH 7.5 with the highest activity (1.28 U/ml) and decreased at the pH of 4.5.The sugar beet peel as substrate the enzyme activity was highest with the pH of 6.5 (1.56U/ml) and after which the activity decreased with the increase in pH. With the carrot peel as the substrate the enzyme activity showed a gradually increase till the pH 6.5 which

had the highest activity (1.28U/ml).In comparison with the other substrates the sugar beet peel with pH 6.5 recorded the highest activity of the enzyme cellulase.

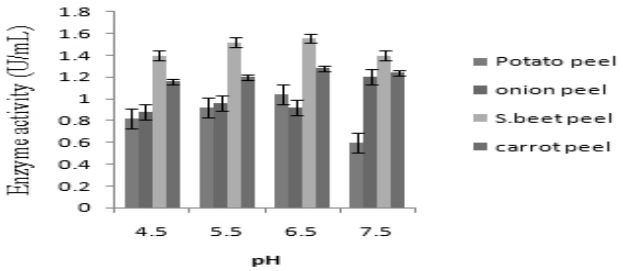

Figure 5. Effect of pH on enzyme activity by using different substrates.

The optimal pH for fungal cellulases varies from species to species. Pečiulytė [19] isolated cellulolytic fungi from waste paper gradual recycling materials and stated the optimum pH of 4.5, 5.5, 6and 6.5 for *Aspergillus niger* DPK-cl-12, *Gliomastixrorum, Stachybo tryschartarum* DPK-cl-111 and *Penicillium funiculosum* DPK-cl-19 respectively at 30°C. Soni et al. [20] reported that optimum pH of CMCase produced by different fungi such *Aspergillus sp* showing optimum pH of 6.0, *A. terreus* pH 6.0 and *M. fergusii* T41 showing the optimum pH of 4.0. Lee at al. [21] purified and characterized the cellulase produced by *Bacillus amyoliquefaciens*DL-3 utilizing rice hull and reporwhich were isolated from marine bacterium *Bacillus subtilis*s ub sp. *Subtilis* A-53. Pandal et al. [22] also reported cellulase activity with *A. niger* isolated from Similipal Bioreserve Forest. The wide variation in pH might be due to the different substrates and different microbial origin.

Temperature is a critical factor for cellulase production which varies according to the organism involved. The effect of temperature on the cellulase activity of the cellulase was determined at various temperatures ranging from 20, 30, 40 and 50°C and the results were recorded (Figure 6). In the present study, maximal cellulase production by the *Penicillium spp* was obtained at temperature of 20°C.

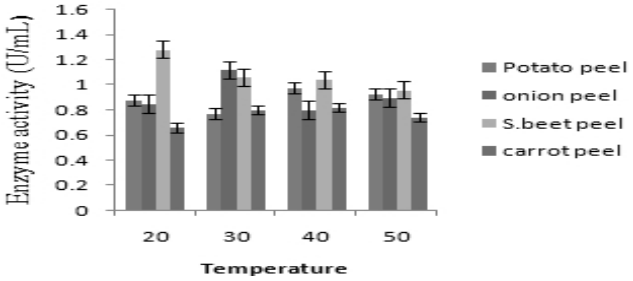

Figure 6. Effect of temperature on enzyme activity using different substrates.

The effect of temperature on cellulase activity is another important factor. For the substrate potato peel and carrot peel, the enzyme activity increased at 40°C, which showed the highest activity (0.98U/ml) and (0.82 U/ml) respectively and decreased for both at the 30°C and 20°C. Similarly for sugar beet peel, the enzyme activity showed (1.28 U/ml) at 20°C and (1.12 U/ml) at 30°C for onion peel. In comparison with other substrates, sugar beet peel exhibited highest cellulase activity at 20°C. Siva et al. [23] reported the optimum fermentation temperature of 20^{o}C for endoglucanase production from *Aspergillus niger*.In this study, 20°C temperature was found

optimum to support maximum production of cellulase as observed by the researcher. At higher temperature, the organisms have to spend a lot of energy and at lower temperature, transport of nutrients is delayed [24].

Rhamnose, xylose, and glycerol were supplemented to the different substrates, each at 0.5% (w/v). Each additive differentially affected cellulase production. Onion peel and sugar beet peel slightly enhanced cellulase production, while the other carbon sources did not enhance cellulase production (Figure 7). Sugar peel and carrot peel displayed highest enzyme activity (1.49 U/ml), 0.42 U/ml with Rhamnose and the lowest enzyme activity found with xylose and glycerol at (1.3 U/ml), and (0.32 U/ml) respectively. Potato peel, onion peel showed enzyme activity with glycerol (0.34 U/ml), (1.25U/ml) respectively. The lowest enzyme activity with potato peel and onion peel was found at (0.21 U/ml) and (1.12U/ml) with xylose respectively. Sugar beet peel was the highest in enzyme activity with Rhamnose of all substrates (Figure 7).

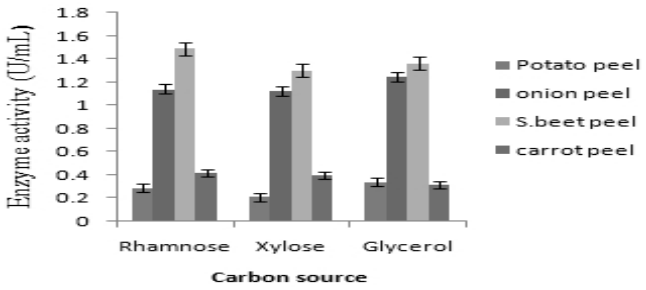

Figure 7. Effect of carbon sources on enzyme activity by using different substrates.

Kuyper et al. [25] reported expression in *S. cerevisiae* of a xylose isomerase gene of the anaerobic fungus *Piromyce s*sp. E2, but both recombinant strains the rates of xylose utilized have been very low. Twerdochlib et al. [26] observed the utilization of L-Rhamanse used as carbon source within *P. stipites.*

Urea, peptone and nutrient broth significantly enhanced the production of cellulase with onion peel and sugar beet peel while potato peel and carrot peel markedly decreased cellulase production (Figure 8). The potato peel, sugar beet were the highest enzyme activity with peptone at (0.24/ml), (1.6U/ml) respectively and lowest enzyme activity with potato peel and sugar beet peel were (0.13 U/ml) with nutrient broth and (1.54U/ml) with urea. Carrot peel presented highest enzyme activity with urea at (1.33 U/ml) and the lowest enzyme activity found at (0.53U/ml) with nutrient agar. Onion peel was the highest in enzyme activity with urea (2.58 U/ml) and (2.08 U/ml) with peptone. It was reported that good cellulase yield can be obtained with ammonium compound as the nitrogen source.

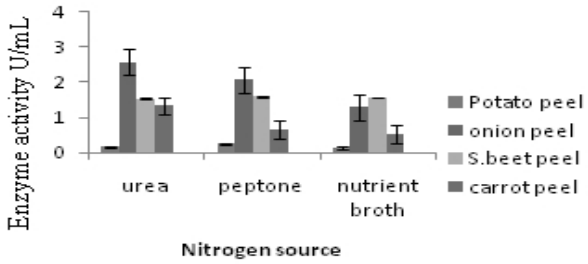

Figure 8. Effect of nitrogen sources on enzyme productivity by using different substrates.

Ammonium compounds are reported to be the most favorable nitrogen compounds for protein and enzyme production [27]. Generally, the results confirmed that urea; a low cost fertilizer, supported the maximum production of the enzyme as compared to peptone and nutrient broth. Though the addition of organic nitrogen sources such as nutrient broth and peptone resulted in increased growth and enzyme production, it has been reported that they are not an effective replacement for inorganic nitrogen sources because of their higher cost [28].

CONCLUSION

Microorganisms are rich sources of cellulase, ligninase and xylanase enzymes, which are produced by diverse genera and species of fungi. Fungi are known to produce cellulase and xylanases which are of high industrial importance. *Penicillium crustosum and Penicillium sp* were compared to determine maximum clear zone of cellulase and xylanase. The factors such as incubation time, pH, and temperature, carbon and nitrogen source need to be considered in the cultivation of these fungi since it affects the cellulase production. Demonstrating cellulase production at different fermentation parameters with the strains, the results are informative in determining culture conditions for scale-up in a commercial production process. The study also demonstrated the promising utilization of agro wastes for enzymes production.

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