Extracellular Production of Amylase and Protease by *Penicillium Purpurogenum* BKS9

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Abstract In the present study, *Penicillium purpurogenum* BKS9 was used with different agro waste substrates i.e. starch, wheat bran, soya powder, boiled rice, unboiled rice and milk powder for the production of both amylase and protease by liquid static surface fermentation (LSSF). Among the various substrates tested, wheat bran (WB) was found to be the best substrate for maximum (112.64 U/ml) amylase production whereas soya powder (121.23 U/ml) for production of protease. Immobilization study also revealed that the highest amylase was observed (137.6 U/ml) when wheat bran was used as substrate whereas maximum protease production (130.73 U/ml) with soya seed powder. Maximum biomass production was observed (4.4 ± 0.2 g/50ml) when unboiled rice was taken as a substrate in comparison to other substrate.

Keywords Biotechnological Agro Food Waste Utilization, Fungi Immobilization, Natural Substrates, Wastes Fermentation

1. Introduction

Exploitation of enzymes as bio-catalyst is becoming the best option which gradually replacing chemical catalysts in many areas of industry especially in textile and detergent industries [1]. Further, microbial enzymes are gaining biotechnological importance for their technical and economical advantages [2]. Among all enzymes the fungal amylase and protease are two most imperative enzymes in contemporary biotechnology as they satisfy the industrial needs and almost replaced the chemical hydrolysis of starch and protease in food processing industries and have utmost significance demand. Therefore they hold approximately 25% of the total enzyme trading [3]. Amylase and protease as extracellular enzymes have potential applications in a number of biotechnological industrial processes such as in

food, beverage, sugar, fermentation, detergent, textiles and paper industry [4]. These enzymes are found in animals (saliva, pancreas), plants (malt), bacteria and molds [5]. Sources of amylase and protease in yeast, bacteria and molds have been reported and their properties have been described [6]. Amylase and protease of fungal origin was found to be more stable than the bacterial enzymes on a commercial scale.

Filamentous fungi exhibit numerous inimitable properties that put together them as exceptional candidates for industrial exploitations. Keeping in view of the credentials, many of them have been used for the production of bulk quantities of extracellular enzymes through fermentations [7]. The filamentous fungi generally used for the production of polymer-degrading enzymes are mostly of *Aspergillus* and *Penicillium* [7, 8]. Fungi generally produce α -amylase (dextransing enzymes), beta-amylase (saccharifying enzymes), proteases and other enzymes to survive on this orb. For that, they can utilize a variety of substrates. Among different types of substrates, the agro-substrates are easily available, cheap carbon sources required for the growth of microorganisms, including filamentous fungi and for the production of enzymes. In this context, unboiled rice, boiled rice, wheat bran and soya powders would be the rich source of both macro- and micronutrients essential for the growth of fungi.

Hence in the present study attempt has been made to produce such industrially important amylase and protease enzyme from different fungal sources using cheap and easily available agro waste like unboiled rice, boiled rice, wheat bran, soya powders etc.

2. Materials and Methods

2.1. Inoculums Development

Penicillium purpurogenum BKS9 (GenBank accession No.

KT 222270) was used for all the experiments. After receiving, the fungal culture was maintained as pure cultures on Sabouraud's dextrose agar (SDA) medium for further experiments. The differential streaking procedures was performed according to Dubey and Maheswari [9]. The isolated fungal species were also sub-cultured on SDA slants at regular intervals during the entire study period. Spore suspension (1 ml) having spore concentration of about 1×10^7 cells ml^{-1} from 7 days old culture was used as inoculum in the subsequent experiments [10].

2.2. Liquid Static Surface Fermentation for Production of Amylase and Protease

Liquid static surface fermentation (LSSF) was carried out in triplicate in 150 ml Erlenmeyer flasks containing 50 ml of sterilized fermentation medium having individually starch, milk powder (amul), wheat bran, soya powder, unboiled rice and boiled rice as substrate. The fermentation medium containing these substrate was inoculated with 1.5×10^7 cells ml^{-1} culture of *P. purpurogenum* BKS9 in triplicate and incubated at $30 \pm 2^\circ\text{C}$ static conditions for 96 h. After completion of fermentation, enzymes were extracted from the fermented media as per Alva et al. [11] with slight modifications. Harvested culture of the fermented broth was centrifuged at 10,000 rpm for 30 min at 4°C and the cell free supernatant obtained was used as crude amylase and protease enzyme for further assay.

2.3. Amylase Assay

Amylase activity was measured in triplicate following the method of Bernfeld [12]. The reaction mixture containing 0.5 ml of 1% soluble starch solution prepared in 0.2 M acetate buffer (pH 4.0) and 0.5 ml of suitably diluted enzyme solution was incubated at 50°C for 10 min. Enzyme and reagent blanks were incubated maintaining the same condition simultaneously. After 10 min of incubation the reaction was terminated by adding 1.0 ml of 3, 5 dinitrosalicylic acid (DNS) solution (1 g of DNS dissolved in 20 ml of 2 M NaOH, to which 30 g of sodium potassium tartarate and water were added to make it 100 ml). The reaction mixture was boiled for 15 min and after cooling O.D. was taken in spectrophotometer at 540 nm. One unit of enzyme activity was defined as the amount of enzyme release 1 μmol of reducing sugar per min.

2.4. Protease Assay

Extracellular protease activity was determined in triplicate according to the method of van den Hombergh et al. [13]. Each 450 μl sample was incubated with 50 μl 1% (w/v) BSA (Fraction V, Sigma) in 0.1 M sodium acetate buffer (pH 4.0) at 37°C . After 30 min of incubation, the reaction was terminated with 500 μl of 10% (w/v) trichloroacetic acid (TCA). After incubation at 0°C for 30 min, the precipitated proteins were removed by centrifugation at 6000 rpm for 5

min, and the absorbance of the TCA-soluble fraction was estimated as per Lowry et al. [14]. One unit (U) of protease activity was defined as the amount of enzyme that releases 1 μg of tyrosine per min.

2.5. Separation and Measurement of Dry Cell Biomass

The biomass content was determined by measuring the dry weight of the fungal sample. The sample was centrifuged at 10,000 rpm, at 4°C for 10 min and the fungal biomass was rinsed with sterilized double distilled water and filtered out using Whatman No.1 filter paper. The biomass obtained was dried overnight inside the hot air oven at 80°C till the constant weight was attained. Finally, the dry weight of the fungal mycelia was weighed and calculated.

Weight of biomass = weight of dry filter paper with fungal biomass - weight of blank and dry filter paper

[Dry weight of biomass= dry weight of fungus]

2.6. Immobilization of *P. Purpurogenum*

The alginate entrapment of cells was performed according method of Sharma and Satyanarayana [15]. Sodium alginate solution (3%) was prepared by dissolving sodium alginate in 100 ml boiling water and autoclaved at 121°C for 15 minutes. Both alginate slurry and cell suspension were mixed well i.e. in a ratio of 1:1 for 10 minutes to get a uniform mixture. The slurry was taken into a sterile syringe and added drop wise into chilled 0.2 M CaCl_2 solution from 5-cm height and kept for curing at 4°C for 1 h. The cured beads were washed with sterile distilled water 3 to 4 times. When the beads were not being used, they were preserved in 0.9% sodium chloride solution in the refrigerator. All operations were carried out aseptically under laminar flow unit.

Conidia immobilized in beads of calcium alginate were inoculated in fermentation medium and incubated at 30°C for 96 h. After successful fermentation, the beads were drained and rinsed with sterile saline solution (0.8% NaCl) equal to twice the volume of culture liquor. The beads were then again ready for use in the production of amylase and protease.

2.7. Production of Amylase and Protease by Repeated Batch Process

The reusability of immobilized *P. purpurogenum* cells in alginate was examined. Immobilized cells were aseptically inoculated to sterilized fermentation broth of the above agro waste and incubated at 30°C for 96 h. Finally, the fermented media were filtered on Whatman No. 1 paper and beads were washed twice with sterile saline solution (0.8% NaCl) and kept in phosphate buffer (1 M, pH 7.0). Then, the beads were again introduced into the fresh medium. After attaining maximum enzyme production, the spent medium was replaced with fresh production medium (50 ml) and the process was repeated for five batches until the beads/blocks

started disintegrating. The enzyme titers and cell leakage of each cycle were determined. Amylase activity was measured following the method described by Bernfeld [12] and extracellular protease activity was determined according to the method of van den Hombergh et al. [13].

2.8. Statistical Analysis

Statistical analysis was performed by SPSS, version 10 for windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Liquid Static Surface Fermentation for Production of Amylase and Protease

In the present study, among the various substrates tested

for amylase activity, wheat bran was found to be the best substrate for maximum (112.64 U/ml) amylase production (Fig. 1). Similarly, in case of production of protease soya powder (121.23 U/ml) was found to be the best substrate for *P. purpurogenum* (Fig. 1). Milk powder has also a high potential as a substrate because both protease and amylase activities were found to be very high when used as a substrate.

3.2. Measurement of Dry Cell Biomass

Biomass production was found to be different for different substrates. Maximum biomass was obtained from *P. purpurogenum* when cultivated with rice (4.4 ± 0.2 g/50ml) at 30°C for 96 h (Fig. 2).

Figure 1. Comparative analysis of amylase and protease production using LSSF by *P. purpurogenum*

Figure 2. Biomass produced by *P. purpurogenum* under liquid static surface fermentation

Figure 3. Calcium alginate immobilized spores of *P. purpurogenum*

Table 1. Production of amylase & protease (U/ml) using immobilized beads of *P. purpurogenum*

Batch operation	Unboiled rice		Boiled rice		Wheat bran		Soya powder	
	Amylase	Protease	Amylase	Protease	Amylase	Protease	Amylase	Protease
First	68.84	73.51	91.56	63.61	119.92	89.91	97.91	121.95
Second	78.91	80.90	97.04	69.60	137.6	101.32	109.32	130.73
Third	56.86	64.93	93.9	63.73	115.94	94.85	100.25	119.91

3.3. Immobilization of *P. Purpurogenum* Spores for Production of Amylase and Protease

The reusability of alginate immobilized spores for amylase and protease production was studied. Beads entrapping *P. purpurogenum* spores (**Fig.3**) were successfully used in 3 repeated cycles of fermentation using unboiled rice, boiled rice, wheat bran and soya seed powder as the substrates. The production of amylase and protease gradually increase from first cycle to the second cycle and decrease thereafter. Maximum production of amylase (137.6 U/ml) was attained with wheat bran whereas the production of protease (130.73 U/ml) with soya powder (**Table 1**).

4. Discussion

Bacterial amylases and proteases have long been used in industry, but, now-a-days, they are replaced by fungal amylases as these enzymes can be effortlessly extracted and separated from fungal biomass [16]. Liquid static and/or solid state fermentation can be used for the large-scale production of these enzymes. Numerous amylase-producing organisms have been documented [17, 18]. For example, many species of genus *Aspergillus* are renowned for their

ability to degrade starch. Khan and Yadav [19] have isolated four fungi from soil and were screened for alpha amylase production. Among these four fungi, *Aspergillus niger* was found to have best activity among all the four isolates.

In the present study, various agro-substrates were screened for both amylase and protease production by LSSF. Among the various substrates tested for amylase activity, wheat bran was found to be the best substrate for amylase production by *P. purpurogenum*. In the present study, due to significantly higher volumetric activity achieved with wheat bran, it was used in the modified fermentation medium. It is important to note that this substrate is economic and could be employed for industrial production of amylase. Biosynthesis of manifold forms of glucoamylase (GA) and production of acid protease with wheat bran as a substrate by different fungal sp. were also reported earlier [20, 21, 22]. Sindhu et al. [23] have studied the effect of incubation period on the production of protease using *P. godlewskii* SBSS 25 and reported the highest production of enzyme on the fourth day of cultivation in SmF which is at par with the present finding.

Immobilization technique is generally employed for the entrapment of microbial cells, biocatalysts and other metabolites for the repeated use. But, recently, it has been extended to fungi. Immobilized fungal spore/cells were first applied for the biotransformation of cortex lone to

hydrocortisone by *Curvularia lunata* and of glucose to itaconic acid by *A. terreus*. At present, immobilization of fungi is studied for numerous applications in industrial processes in which only free mycelia were used earlier. Immobilization of fungal spores/cells and their enzymes has become one of the most precious techniques in the field of modern biotechnology [24]. Furthermore, this encapsulation provides prolonged metabolic activity when microbial cells are repeatedly used and it also provides protection to the organism from inhibitory compounds or metabolites. Similarly, El-Katatny et al. [24] have also encapsulated the *Trichoderma* spp. for enhanced production of cell wall degrading enzyme.

5. Conclusions

Developing countries, like, India is rich in agricultural residues and are the most abundant renewable resources which can be used for the production of enzymes and other metabolites by different microorganisms. Production of enzymes from such residues will have an enormous potential as it is environmental-friendly and sustainable alternative to costly substrates. Further, the use of native and potent microorganisms is essential to attain economic production of enzymes. In this context, *A. terreus* and *P. purpurogenum* could be the prospective candidates for both amylase and protease production by LSSF with agro- substrates. In the present biotechnological era, α -amylases and proteases are applied in biopharmaceutical applications, though, their major applications in food and detergent industries. Further, use of immobilization techniques in enzyme production will greatly reduce the high capital investment and in alternate, it will reduce the final cost of the product.

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