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Protease Production Using Solid State Fermentation

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ABSTRACT

Proteases are enzymes that break down protein molecules through peptide bond hydrolysis. Microbial proteases are the most important industrial enzymes with considerable applications in food, medicines and pharmacy. They are extracellular enzymes that can be produced by both submerged and solid state fermentation (SSF). Aspergillus sp. are known to produce various types of proteases. Solid state fermentation processes generally use natural raw materials as carbon and energy source. Different agro-industrial waste products will be evaluated to check possibility of potential utilization of substrates in SSF. The production of enzymes by bioprocesses is a good value added to agro industry residues. A comparative study was carried out on the production of protease using Wheat bran and Rice bran as substrates in solid state fermentation by Aspergillus niger ATCC 16404. Among them Wheat bran produced higher activity protease as 1.785 U/ml than Rice bran 1.487 U/ml activity under solid state fermentation conditions. The optimized conditions for producing maximum yield of protease were incubation at room temperature (28±2)°C, 120hours, pH 6, concentration of nitrogen source 0.5% and additional carbon source is 1% sucrose. The protease production from agricultural waste products can be commercially used in detergents and leather industry and it adds great value to agro-industrial wastes.

Keywords: Bioprocess, Protease, Solid State Fermentation, Bran, Optimization.

I. INTRODUCTION

Enzymes are biocatalysts that determine the patterns of chemical and energy transformations. The most striking characteristics of enzymes are their catalytic power and specificity Proteolytic enzymes are included in a sub-class of the enzymes hydrolases. These enzymes hydrolyse proteins into smaller peptides and amino acids by cleavage of peptide bonds (Munawar et. al., 2014). Their involvement in the life cycle of disease-causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS (Sawant et. al., 2014).

A. Classification of proteases

On the basis of the site of action on protein substrates, proteases are broadly classified as endopeptidases or exopeptidases enzymes (Sawant et. al., 2014). The endopeptidases are divided into four subgroups based on their catalytic mechanism, serine protease, aspartic protease, cysteine protease, and metalloproteases (Rawlings and Barrett, 1993).

Several species of strains including fungi (Aspergillus flavus, Aspergillus melleus, Aspergillus niger, Chrysosporium keratinophilum, Fusarium graminarum, Penicillium griseofulvin, Scedosporium apiosermum) and bacteria (Bacillus licheniformis, Bacillus firmus, Bacillus alcalophilus, Bacillus amyloliquefaciens, Bacillus proteolyticus, Bacillus subtilis, Bacillus thuringiensis) are reported to

produce proteases. Fungi elaborate a wide variety of proteolytic enzymes than bacteria and also offer an advantage as the mycelium can be grown on cheaper substrate, broad range of pH and the mycelium can also be easily removed from the final product (Sri lakshmi et. al., 2014).

Protease production under solid-state fermentation has been investigated using Bacillus sp. (Prakasham et. al., 2005; Akcan et. al., 2011, Imtiaz et. al., 2013, Mukhtar et. al., 2013; Sathyavrathan et. al., 2014; Pant et. al., 2015; Ortiz et. al., 2016). **Solid state**

fermentation

Solid-state fermentation has been defined as the process with solid materials as substrate with near-absence of water content, generally using natural raw materials as carbon and energy source. Solid-state fermentation has many advantages including superior volumetric productivity, use of inexpensive substrate, simpler downstream processing, lower energy requirement and low wastewater output (Özkan et. al., 2011; Sri lakshmi et. al., 2014). Solid-state fermentation is especially suited for the growth of fungi because of their lower moisture (30 to 80%) requirements compared with the bacteria (Sankeerthana et. al., 2013; Mukhtar et. al., 2009). Rice bran was used as the substrate for screening nine strains of Rhizopus sp. for neutral protease production by Solid state fermentation (Alagarsamy et. al., 2006; Chutmanop et. al., 2008).

The growth and production of protease by Aspergillus has been studied by using rice bran, rice mill waste, vegetable waste, oil cakes, wheat bran, fruit waste (Mukhtar et. al., 2009; Paranthaman et. al., 2009; Madhumithah et. al., 2011; Kranthi et. al., 2012; Sankeerthana et. al., 2013; Dutta et. al., 2014; Munawar et. al., 2014; Santhi, 2014).

Protease production by Fusarium oxysporum on rice bran under solid-state fermentation was studied (Ali et. al., 2013).

Protease production by a thermophilic fungus Humicola grisea was evaluated in solid state fermentation (SSF) with different substrates and also under the influence of different activators and inhibitors. The environmental conditions of the fermentation play a vital role in the growth and metabolic production of microbial population (Sri lakshmi et. al., 2014). Optimization of different media components can greatly affect the enzyme production cost (Joo et. al., 2003; Wang et. al., 2008; Schrickx et. al., 1995).

Applications of proteases

Viral proteases have gained importance due to their functional involvement in the processing of the proteins of viruses that cause diseases such as AIDS and cancer (Rawlings and Barrett, 1993). All of the virus-encoded peptidases are endopeptidases. Retroviral aspartyl proteases that are required for viral assembly and replication are homo-dimers and are expressed as a part of the poly protein precursor. The mature protease is released by autolysis of the precursor (Kuo et. al., 1994).

Besides extended application for nutritional and pharmaceutical purposes, proteases from natural sources are also widely used tools in biotechnolgical practices. Their degradative properties make them useful for general protein digestion in tissue dissociation, cell isolation, and cell culturing. The specificity and the predictability of cleavages by proteases enables their use for more specific tasks such as antibody fragment production, the removal of affinity tags from recombinant proteins and specific protein digestion in the proteomics field mainly for protein sequencing (Mótyán et al., 2013). Due to their key role in the life-cycle of many hosts and pathogens they have great medical, pharmaceutical, and academic importance (Li et al., 2013; Gupta and Khare, 2007). In view of the recent trend of developing environmentally friendly technologies, proteases are envisaged to have extensive applications in leather tanning industries and in several bioremediation processes (Gupta et

al., 2002). Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations; whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used (Bholay and Niranjan, 2012).

In the present work we have studied the production of protease by solid-state fermentation using different agro-industrial waste products like rice bran and wheat bran as cheap substrates and the effect of physico-chemical parameters like pH, temperature, concentration of nitrogen and different carbon sources on protease production.

II. MATERIALS AND METHOD

The organism used in the present study was Aspergillus niger ATCC 16404 obtained from Microbial Type Culture Collection & Gene Bank and maintained on potato dextrose agar slants.

1. Collection of substrates:

Different agro-industrial waste residues- rice bran and wheat bran were used. Rice bran was collected from rice-mill from the area nearby Kalyan, India in polythene bag. The sample was grounded in dry blender until the size of the particles reached the desired size and stored at ambient condition for further use. Wheat bran was purchased from online store amazon.in and used as it is as substrate.

Figure 1: Substrates used for solid state fermentation

2. Inoculum Preparation:

The inoculum was prepared by dispersing the spores from a week-old fungal slant culture in 0.1 % Tween-80 solution with a sterile inoculation loop (Paranthaman et. al., 2009).

3. Solid-State Fermentation:

Ten grams of each substrate (rice bran and wheat bran) was taken in a 250 ml Erlenmeyer flask separately, moistened with 15 ml of the salt solution (Ammonium chloride-0.5g; Sodium nitrate-0.5; Potassium dihydrogen orthophosphate-0.2g; Magnesium sulphate-0.2mg; Sodium chloride-0.1g per 100ml distilled water). The flasks were plugged tightly with cotton wool, sterilized at 121.5°C for 15 min, cooled, inoculated with 1 ml of fungal spore suspension (100 spores/ml) and incubated at 37°C for 120 hr (Paranthaman et. al., 2009).

4. Extraction of Crude Enzyme:

A solution of Tween-80 (0.1 %) was added in to the 100 ml of distilled water. 50 ml, of this water was added to the fermented substrate and homogenized the substrate on a rotary shaker at 120 rpm for 12 h. The content of flask was filtered with help of 4-fold muslin cloth. The suspended solids were removed by centrifuging the homogenate at 8000 x g at 4°C for 15 min and the resultant supernatant was used for analytical studies (Paranthaman et. al., 2009).

5. Protein Estimation in Crude Enzyme

Amount of protein in crude enzyme was determined by Lowry's method of protein estimation, in which 0.5ml of crude enzyme, 0.5ml of distilled water was reacted with 5ml of Lowry's reagent C (Folin-

Ciocaltaeu reagent) and 0.5ml of Reagent D (0.5N NaOH) and the absorbance was read at 660nm. Absorbance was compared with the standard graph prepared by reacting known concentration of protein ranging from 50 µg/ml to 250 µg/ml with the Lowry's reagents and plotting a graph between concentration of protein Bovine Serum Albumin and OD at 660 nm (Lowry et.al., 1951).

6. Measurement of enzymatic activity

Protease activity in the culture supernatant was determined according to the method of Tsuchida et. al., (1986) using casein as a substrate. A mixture of 500 µl of 1% (w/v) of casein in 50 mM phosphate buffer, pH 7 and 200 µl crude enzyme extract were incubated in a water bath at 40°C for 20 minutes. After 20 minutes, the enzyme reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid (TCA) and was kept at room temperature for 15 minutes. Then, the reaction mixture was centrifuged to separate the unreacted casein at 10,000 rpm for 5 minutes. The supernatant mixed with 2.5 ml of 0.4M Na2CO3 and 1 ml of 3-fold diluted Folin Ciocalteau reagent. The resulting solution was incubated at room temperature in the dark for 30 minutes and absorbance of the blue color developed was measured at 660 nm against a reagent blank using a tyrosine standard. One international unit (IU) of protease activity is the amount of enzyme, which liberates 1 µmol of tyrosine per min. (Mohapatra et. al., 2003).

7. Optimization of fermentation conditions

Production of protease from Aspergillus niger was optimized by controlling different physico chemical parameters like pH, temperature, carbon source and concentration of nitrogen source.

8. Initial pH of medium

The effect of initial pH on protease production was studied by changing the initial growth medium pH from 2, 3 and 6 with 1N Lactic acid/ NaOH before sterilization at 121°C for 15 min. and optimum pH for protease production was determined.

9. Incubation temperature:

The effect of temperature was studied by incubating the inoculated flasks at different temperatures viz. room temperature (28±2)°C, 37°C and 55°C.

10. Effect of concentration of supplementary nitrogen sources

Whether the addition of supplementary nitrogen sources could enhance the production of protease was tested by supplying an inorganic nitrogen source ($NaNO_3$ and NH_4Cl) at a level of 0.5% w/w in the medium. The flasks were then incubated for 5 days at room temperature. At the end of the incubation period, protease production in cell free supernatant was determined. Further, nitrogen source was optimized with different concentrations [0.3, 0.5, 0.7% (w/v)].

11. Effect of carbon sources

The effect of carbon sources on enzyme production was investigated by supplementing the basal salt solution, pH 7, with 1% (w/v) of different carbon sources, viz., maltose, lactose, and sucrose. The flasks were then incubated for 5 days at room temperature. At the end of the incubation period, protease production in cell free supernatant was determined.

12. Partial purification of Protease:

About 20 ml of the crude enzyme extract prepared was brought to 80% saturation by adding solid ammonium sulphate salt with continuous stirring. The mixture was left overnight at 4°C. The mixture was centrifuged at 10,000 rpm for 20 minutes and the pallete was dissolved in 30ml of 50mM phosphate buffer (pH 7) which was then subjected to dialysis (Shanmugapriya et. al., 2012).

The precipitate dissolved in phosphate buffer at pH 7 is dialyzed against the same buffer overnight at 4°C (Immanuel et. al., 2006). The partially purified sample was assayed for enzyme activity and protein content.

III. RESULT AND DISCUSSION

1. Protease production by solid state fermentation

The production of protease by the standard culture Aspergillus niger ATCC 16404 in solid state fermentation using solid substrates that have been moistened with a salt solution is done. The enzyme activity of protease produced was calculated using tyrosine standard by Folin-Lowry method.

The selection of an ideal agro-biotech waste for enzyme production in solid state fermentation process depends upon several factors, mainly related with cost and availability of substrate material (Paranthaman et. al., 2009).

2. Effect of different substrates on protease production

Two different agro-waste substrates were used for solid state fermentation viz., wheat bran and rice bran. Five grams of each substrate were weighed and hydrated with 10 ml of basal salt solution containing (g): NaCl; 0.1g, $MgSO_4.7H_2O$; 0.2g, KH_2PO_4; 0.2g, NH_4Cl_2; 0.5g and $NaNO_3$; 0.5g dissolved in 100ml of distilled water (Coral et al. 2003). Flasks containing fermentation medium were autoclaved, cooled and inoculated with 1.0ml of Aspergillus niger ATCC 16404 spore suspension prepared in 0.1% Tween 80 solution, incubated for 120 hrs at 30°C. Rice bran and Wheat bran were used as substrate. Wheat bran was the suitable substrate for production of protease at pH 6 and (28±2)°C, enzyme activity 0.850 U/ml. When grown on rice bran, the fungus produced protease showing 0.799 U/ml enzyme activity [Table 1]. The protein content of wheat bran (14-16%) is higher compared to rice bran (7-8%). This could be the factor for lower production of protease when rice bran was used as a substrate.

Table 1: Enzyme activity of protease produced by the culture on rice bran (RB) and wheat bran (WB) by SSF

Substrate	Extinction at 670nm	Tyrosine formed (µmole)	Enzyme activity (units/ml)
RB 1	0.34	0.95	0.20
RB 2	0.30	0.85	0.18
RB 3	0.35	1.00	0.21
WB 1	0.59	1.65	0.35
WB 2	0.62	1.75	0.37
WB 3	0.68	1.90	0.40

3. Optimization of fermentation conditions for protease production by SSF

Different fermentation conditions that may affect production of protease from *A. niger* were optimized as follows:

Production of the enzymes by mold culture mostly depends on the pH of medium. Therefore, the effect of different pH values on the production of protease by *A. niger* was studied. Production of the protease has increased with pH. Maximum enzyme activity [0.439units/ml] was observed at pH 6 for wheat bran and [0.255U/ml] (Table 2). Generally, protease production by microorganism depends on the extracellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production (Paranthaman *et al.*, 2009). Changes in the pH may also cause denaturation of enzyme resulting in loss of catalytic activity. It may also cause change in the ionic state of substrate which may result in the formation of charged particles which may not correspond with the ionic active sites of enzyme (Karuna and Ayyanna, 1993).

Table 2: Enzyme activity of protease using different substrates at different pH values

Substrate	pH 2	pH 4	pH 6
Enzyme activity WB	0.323	0.429	0.439
Enzyme activity RB	0.17	0.201	0.255

Maximum protease production was obtained at room temperature (28±2)°C and protease activity was found to be 0.850 Units/ml for wheat bran and 0.799 Units/ml for rice bran (Table 3). In contrast, by reports of Paranthaman *et. al.,* (2009), fermentation carried out at 35°C was best suited for protease production. Protease activity was decreased with increase in temperature. Higher temperature (55°C) is found to have some adverse effect on metabolic activities of microorganisms and cause inhibition of growth of the fungus. The enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds within enzyme structure.

Table 3: Enzyme activity of protease using different substrates at different temperatures

Substrate	(28±2)°C	37°C	55°C
Enzyme activity WB	0.850	0.828	0.00
Enzyme activity RB	0.799	0.733	0.00

The effect of the concentration ammonium chloride and sodium nitrate on the protease production was studied using the range of 0.3-7.0% of nitrogen sources. A gradual increase in protease activity was observed with the increment of nitrogen source concentration and it declines after 0.5% (Table 4).

Table 4, Enzyme activity of protease using different concentrations of nitrogen sources during SSF

Substrate	Concentration of nitrogen source		
	0.3%	0.5%	0.7%
Enzyme activity WB	1.615	1.402	1.36
Enzyme activity RB	1.402	1.44	0.977

The highest protease activity (1.8Units/ml) was obtained when sucrose was used as a carbon source while the least protease activity (1.59Units/ml) was produced when maltose was used (Table 5). Madzak *et al*. (2000) also recorded that sucrose was a good substrate for production of extracellular proteases.

Table 5: Enzyme activity of protease using different carbon sources during SSF

Substrate	Carbon source		
	Sucrose	Maltose	Lactose
Enzyme activity WB	1.785	1.46	1.657
Enzyme activity RB	1.487	1.402	1.326

IV. CONCLUSION

Proteases are specialized proteolytic enzymes that are widely distributed nearly in all plants, animals and microorganisms. Proteases from microbial sources are preferred to those from other sources since they possess almost all the characteristics desired for their biotechnological applications. Fungal proteases are more preferred by researchers due to high diversity, broad substrate specificity, and stability under extreme fermentation conditions. In this study, the conditions for protease production by *A.niger* in solid state fermentation medium were optimized.

Wheat bran is a promising substrate for the production of protease by *A.niger* under solid state fermentation conditions. Optimum conditions for protease production by *A.niger* under solid state fermentation were: pH 6.0, 0.5% of a nitrogen source, (28±2)°C temperature during 120hrs incubation period.

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