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Production of protease by *Aspergillus flavus* under solid state fermentation

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Aspergillus flavus, isolated from spoiled sample of casein produced 1.6 U of extracellular protease/g of solid substrate in 10 days of incubation period. Enzyme production in submerged fermentation (0.12 U/ml) was found to be less than solid state fermentation (SSF; 0.99 U/ml). Culture conditions were optimised for maximal yield of enzyme.

Microbial proteases are the enzymes account for about 60% of the total enzyme market[1]. Proteases are used in pharmaceutical, food processing, meat tenderisation, dairy, tanning, brewing, baking, animal feed, detergent, textile and other industries. Numerous molds, especially those belonging to *Aspergillus*, *Pencillium*, *Rhizopus* and *Mucor* groups have been known to produce variety of proteases. Solid state fermentation (SSF) is preferred to submerged fermentation (SmF) due to its numerous advantages[2]. The present paper deals with the production of protease under SSF by *A. flavus*. Optimisation of various culture parameters to enhance the enzyme production has been investigated.

Microorganism and media—*Aspergillus flavus* isolated from spoilt casein sample was maintained on PDA slants at 4°C. *A. flavus* was identified by using the standard methods of Barnett and Berry (1972)[3].

Submerged fermentation—Broth Czapek-dox medium (100 ml) was taken in Erlenmeyer flask (250 ml) sterilised at 121°C for 30 min and inoculated with loopful of spores from PDA slant. Submerged fermentation was carried out for four days at 37°C on orbital shaker rotating at 150 rpm.

Solid state fermentation—Solid substrate , wheat bran (20 g) was taken in Erlenmeyer flask (500 ml) and moistened with mineral salt solution[1]. The substrate was sterilised at 121°C for 1 hr. The sterilised substrate was inoculated with 2 ml of preculture prepared in Czapek-dox medium and incubated at room temperature 37°C for 3-14 days. Phosphate buffer (200 ml; *p*H 6.9; 0.2 *M*) was added to the fermented bran and kept on orbital shaker at 200 rpm for 1 hr. Using two fold cheese cloth the enzyme was filtered from moldy substrate. The extract was centrifuged and filtered through Whatman no 1 filter paper. Thus obtained filtrate was used as source of protease. Each experiment was carried out in duplicate. Protease was assayed by Kunitz's method[4]. Culture filtrate (1 ml) was mixed with 1 ml of casein substrate (1% w/v) prepared in phosphate buffer (*p*H 6.9; 0.1*M*) and digested with TCA (5% w/v) and incubated for 20 min at 45°C. The contents of the test tubes were filtered, added 2 ml of 0.5 *N* NaOH to 1 ml of filtrate and colour reaction was carried out with 0.6 ml of Folin Ciocultue reagent (FCR). The optical density was measured at 660 nm and compared with standard curve of tyrosine. One unit of enzyme was defined as the amount of enzyme required to liberate one microgram of tyrosine in 20 min at 45°C.

Optimisation of culture parameters—Various solid substrates were screened for higher protease production. Wheat bran was chosen as best solid substrate and further experiments were carried out using this substrate. Wheat bran (10-50 g) was fermented in separate Erlenmeyer flasks (500 ml) and was tested for high enzyme titres. Effect of incubation period (3-14 days) on protease production was studied. Inoculum volume (1-6 ml) was varied in order to test for high enzyme production. Different eluting solvents such as phosphate buffer (*p*H 6.0; 0.2 *M*), acetate buffer (*p*H 5; 0.2 *M*), distilled water (*p*H 7.0) and citrate buffer were used to optimise the effect of eluting solvents on recovery of the enzyme.

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Effect of volume of moistening agent (10-50 ml) on protease production was studied. Different sugars as carbon sources at 3.3% level were used as inducers for high protease yield. Wheat bran was supplemented with organic nitrogen sources such as yeast extract, casein, gelatin, peptone and soybean meal at 10% concentration. Inorganic nitrogen sources such as ammonium nitrate, ammonium sulphate, potassium nitrate and urea at 1.5% concentration were tested for high protease production. Concentrations taken above for inducers, organic and inorganic nitrogen sources is based on preliminary studies.

Among various solid substrates screened for protease yield wheat bran, rice bran + wheat bran (10+10 g) gave maximum enzyme titres (Table 1). Battaglino *et al*[5] have reported production of high yield of protease on rice hulls/ rice bran (7:3) with *A. oryzae* in SSF. Isolation of two different extracellular proteases, an alkaline and neutral protease from *A. flavus* using wheat bran and soybean as solid substrates was carried out by Impoolsup *et al*[6]. Protease production by *A. flavus* was greater at 30 g of solid substrate as compared to that of high or low substrate levels. Effect of bran concentration on protease production in SSF was studied by George *et al*[7]. Ten days of incubation period was optimal to yield high enzyme titres (Table 2). Malati and Chakraborty[8] have reported maximum alkaline protease by *A. flavus* isolate after 56 hr of incubation. Moistening agent (40 ml) was sufficient to moisten wheat bran (30 g) to give high enzyme titres. Ghildyal *et al* [9] in their studies have reported that at higher moisture content there is faster growth of organism and early entry into stationary phase as well as early initiation of enzyme production. Lactose as inducer, gelatin as organic nitrogen source and potassium

Table 1—Effect of solid substrates on protease production by *A. flavus*

Solid substrate	Protease activity (Unit/g substrate)
Rice straw	0.21
Red gram husk	0.28
Wheat bran	1.15
Rice bran	0.97
Redgram husk(coarse)	0.08
Rice straw+wheat bran	0.01
Red gram husk+wheat bran	0.63
Rice bran+ wheat bran	1.16
Rice straw+ rice bran	0.16

Table 2—Effect of incubation period on protease production by *A. flavus*

Incubation period (Day)	Protease activity (Unit/ g substrate)
3	0.06
4	0.08
5	0.13
6	0.17
7	0.19
8	0.12
9	0.15
10	0.25
11	0.16
12	0.06
13	0.08
14	0.14

Table 3—Influence of various parameters on production of protease by *A. flavus* on wheat bran

Parameters	Protease activity (Unit/ g substrate)
Substrate concentration(g)	
10	0.63
20	0.95
30	1.20
40	1.16
50	0.98
Inoculum volume(ml)	
1	0.13
2	0.27
3	0.14
4	0.14
5	0.12
6	0.20
Moistening agent(ml)	
10	0.19
20	0.65
30	0.54
40	0.98
50	0.72
Inducers(Sugars) (3.3%)	
Sucrose	0.95
Glucose	0.73
Mannose	0.67
Fructose	0.94
Lactose	1.09
Inorganic nitrogen sources(1.5%)	
Ammonium sulphate	0.53
Ammonium nitrate	1.02
Potassium nitrate	1.00
Urea	0.80
Organic nitrogen sources (10%)	
Yeast extract	0.74
Casein	0.72
Gelatin	1.02
Peptone	0.66
Soybean meal	0.66

nitrate as inorganic nitrogen source yielded high enzyme titres. (Table 3). Malati and Chakraborty [8] have reported that lactose acted as best inducer of enzyme as compared to sucrose, starch, fructose, dextrin and maltose in *A. flavus*. The present investigation could be scaled up to higher levels of protease production using wheat bran as solid substrate. Further work is in progress to characterise and purify protease.

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