

Optimization of Extraction of Fpase from the Fermented Bran of *Aspergillus niger* in Solid State Fermentation

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A local isolate of *Aspergillus niger* was cultivated under optimal growth conditions on wheat bran in solid state fermentation. Filter paperase from fermented bran was separately extracted with different solvents to test the recovery of the enzyme. Among solvents tested, distilled water served as the best leachate, thus the conditions were further optimized with distilled water. After two washes of fermented bran with distilled water for 1.5 h each under stationary conditions at 1 g wheat bran : 5 mL distilled water, the maximum recovery of 13.5 U g⁻¹ of wheat bran was obtained.

Key words: *Aspergillus niger*, Fpase, extraction, solid state fermentation, wheat bran

Cellulases are important industrial enzymes with potential applications in several industrial processes [Bhat and Bhat, 1997]. The main potential applications are in food, animal feed, textile, fuel and chemical industries [Aristidou and Pentilla, 2000; Chandra *et al.*, 2007]. The cellulose-rich plant biomass is one of the foreseeable sources of fuels, single cell protein, and feed stock due to its continuous synthesis using CO₂ and solar energy at an annual rate of 72×10¹⁰ tons through photosynthesis by plants [Oksanen *et al.*, 2000; Lynd *et al.*, 2002; Chandra *et al.*, 2007]. Effective utilization of cellulosic materials through bioprocesses will be an important key and a challenge to overcome the shortage of foods, feed and fuels, which the world may face in the near future due to the explosive increase in human population [Ohmiya *et al.*, 1997]. Cellulases act synergistically to convert the complex carbohydrates present in the lignocellulosic biomass into glucose, which can be subsequently fermented into ethanol, butanol, acetone or 2,3-butanediol on a large scale [Gadgil *et al.*, 1995; Hoshino *et al.*, 1997; Van Wyk, 2001] for use as

biofuels. However, because large quantities of active cellulose preparations are required, in some of the applications, the enzymatic degradation is not cost-effective at present [Lynd *et al.*, 2002]. To overcome this short-coming, efforts are being directed continuously to reduce the cost of production of cellulase by searching for high-yielding cellulose-producing strains, mutants that are resistant to catabolite repression, by genetic engineering methods and optimizing the fermentation conditions [Kotchoni and Shonukan, 2002]. To date, the production of cellulase has been intensively studied in submerged fermentation with different microorganisms in comparison to the solid-state fermentation [Lynd *et al.*, 2002]. Solid- state fermentation is an attractive process for the economical production of cellulose due to its low capital investment and facile operating conditions [Pandey *et al.*, 1999]. Extraction of enzyme from the fermented bran is a key factor in the production of enzyme in solid-state fermentation [Lonsane and Krishnaiah, 1992]. Up to now, the cellulolytic enzymes have been arbitrarily extracted using solvents without a systematic approach [Ramakrishna *et al.*, 1982]. The present investigation addressed the extraction of Fpase (EC 3.2.1.91), one of the components of the cellulase complex, as a model enzyme produced by *Aspergillus niger* through solid-state fermentation.

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Abbreviations: Fpase, filter paperase

Materials and Methods

Microorganism. A local isolate of *A. niger* isolated

from the soils contaminated with the effluents of cotton ginning mills in Nandyal, India [Narasimha *et al.*, 1999] was used in the present study. The fungal culture was maintained on a Czapek Dox medium. The spore suspension of *A. niger* was prepared by adding 2 mL of sterile distilled water to the 7-day grown slants [Narasimha *et al.*, 1999].

Method of fermentation. The fermentation was carried out in 250-mL Erlenmeyer flasks. Six milliliters of the modified Czapek Dox medium was added to the 10 g wheat bran in the flasks at the beginning, and the remaining balance for achieving 40% moisture level was provided to the respective matrix in the form of distilled water. The modified Czapek Dox liquid medium contained g/L 2.0 g NaNO₃, 1.0 g K₂HPO₄, 0.5 g MgSO₄ · 7H₂O, 0.5 g KCl, 0.01 g FeSO₄ · 7H₂O, 30.0 g sucrose, 25 g (NH₄)₂SO₄ and 5.0 g cellulose. Flasks were autoclaved for 30 min at 121°C and inoculated with spores at 2 × 10⁶, followed by incubation at ambient (30 ± 2°C) temperature. At regular intervals, sterile distilled water was added to each flask to maintain the initial moisture content during incubation [Chandra *et al.*, 2007].

Enzyme extraction. Ten grams of the fermented bran in a 250-mL conical flask was extracted with the selected solvents by soaking the fermented bran at 30°C for 30 to 150 min. The crude extract was then filtered through a cotton cloth and the filtrate was centrifuged at 9,000 × g for 20 min. The clear filtrate obtained was used for the Fpase assay. The parameters selected for this study were type of solvent, volume of solvent, soaking time, physical state of leaching and number of washes. In the experiment on the parameter pertaining to the number of washings, fresh lots of the solvent were added to the fermented bran recovered at the end of the previous extraction. Leachates from these extractions were collected separately and tested for the recovery of Fpase.

Enzyme assay. Flasks were withdrawn daily for 5 days. The activity of Fpase in the culture filtrate/leachate was quantified following the method of Mandels and Weber [1969]. One unit is the amount of enzyme in the culture filtrate releasing 1 μmole of reducing sugar from the Fpase per min. The production of the enzyme on solid matrix is expressed as the number of Fpase units per gram of bran.

Statistical analysis. All measured values are the averages of three replicates. Values in the figures are means of 3 replicates ± standard deviation.

Results and Discussion

Solvent Selection. The extraction efficiency is critical to the recovery of the enzyme from the fermented

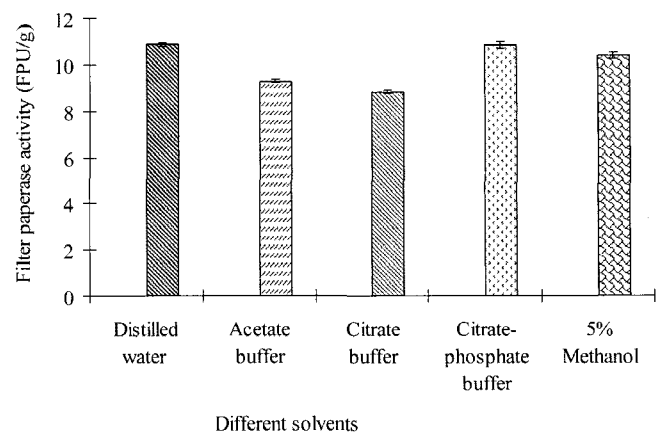


Fig. 1. Selection of solvent for Fpase extraction.

biomass; hence selection of a suitable solvent is necessary. Different solvents selected for this study were distilled water, 0.2 M acetate buffer (pH 5.0), 0.2 M citrate buffer (pH 5.0), 0.2 M citrate-phosphate buffer (pH 5.0), and 5% methanol (v/v). A single wash with 20 mL distilled water was used for extraction, because, among all the solvents used, distilled water gave the highest yield of 10.87 U g⁻¹. Therefore, subsequent optimization experiments were carried out only with water.

Leaching, a process for recovering the solute from the solids in the form of crude extract using the appropriate solvent, is an important unit operation [Treybal, 1981]. Various factors are known to influence the degree of leaching of the product from the fermented substrate produced by solid-state fermentation [Lonsane and Krishnaiah, 1992]. The major factors include solvent efficiency, solid to solvent ratios, incubation time for soaking, physical state of leaching, and number of washes.

One of the important steps in leaching of any product in the solid-state fermentation system is the selection of a suitable solvent. Different solvents have been used separately for the extraction of cellulolytic enzymes from the fermented bran including citrate buffer (0.1 M, pH 4.8) [Fadel, 2000], sterile distilled water [Singh and Garg, 1995], and water, which was allowed to stand at room temperature (25 to 28°C) for 1 h, as well as for the recoveries of cellulases and β-glucosidase [Shamala and Sreekantiah, 1987], and cellulase and xylanase [Heck *et al.*, 2002]. Of all solvents tested in the present study, distilled water served as the best leachate in extracting Fpase from the fermented bran. The use of citrate-phosphate buffer (pH 4.0) to leach other enzymes such as α-galactosidase from the fermented mass has been reported by Annunziato *et al.* [1986]. Distilled or tap water alone or with glycerin or sodium chloride gave the highest yield in the amyloglucosidase extraction from the

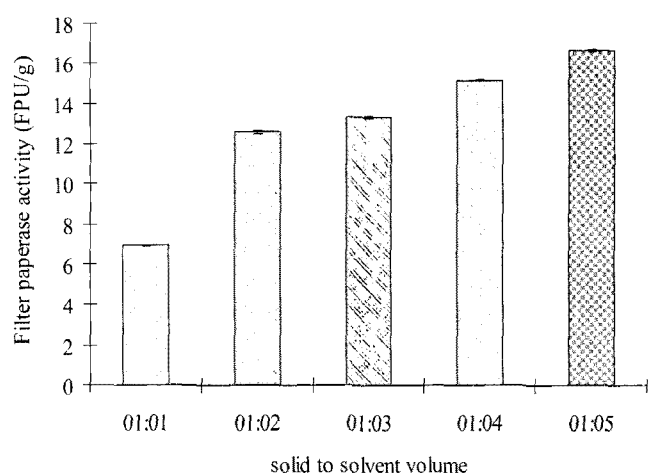


Fig. 2. Effect of solvent volume on Fpase extraction.

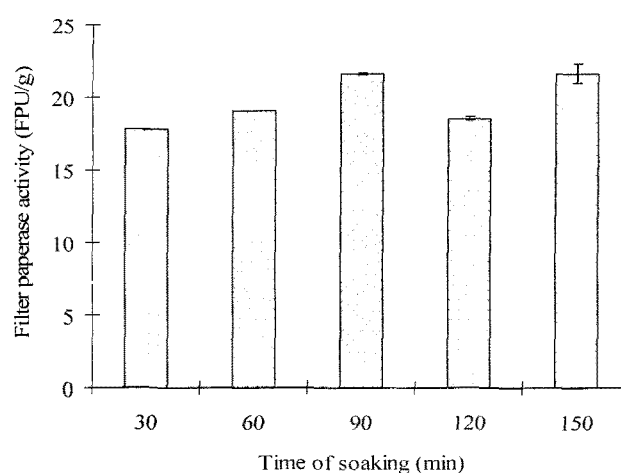


Fig. 3. Effect of soaking time on the activity of Fpase.

mould-wheat bran [Ramakrishna *et al.*, 1982], whereas alcoholic solvents were less efficient than the aqueous solvents. Phosphate and acetate buffers (pH 5.9) were found to be more suitable for alpha-amylase extraction. Qadeer *et al.* [1980] and Fernandez-Lahore *et al.* [1998] reported that 0.5 M sodium chloride was more suitable for acid protease recovery than distilled water.

Solid-to-Solvent Ratio. In the solid-state fermentation system, free flowing solvent is very much limited. Thus, adequate amount of the solvent is required to leach out the enzyme present. The volume of the solvent, i.e. distilled water, varied from 10 to 50 mL, and extraction was carried out immediately after the addition of the solvent. The results of extraction of the enzyme are presented in Fig. 2. At the solid-to-solvent ratio of 1:5, 50 mL solvent was maximal for the extraction of enzyme from 10 g of fermented bran. The total activity decreased when lower volume of the solvent was used for extraction.

The ratio of solvent-to-solid plays a vital role in the extraction of Fpase from the fermented bran. Recovery of the low yield of enzyme from the fermented bran with lower volume of solvent might be due to the insufficient solvent volume to penetrate the solid fermented mass. Higher solvent to solid ratios also causes the solute to be more dilute in the final extract. The increase in leaching efficiency gained at higher ratios must, therefore, be balanced against the extra effort for concentration of the dilute extract. However, in many cases, it may not be possible to achieve an economic balance [Lonsane and Krishnaiah, 1992]. Information on the extraction of cellulolytic enzymes from the fermented bran is scant. In the present study, 50 mL solvent could achieve the maximum extraction of the Fpase from the bran. However, Palit and Banerjee [2001] reported that maximum amount of amylase was extracted using 30 mL

solvent at solid-to-solvent ratio of 1:3 from 10 g of fermented bran. The efficiency of leaching increased as the ratio increased [Lonsane and Krishnaiah, 1992]. An increase in the solvent-to-solid ratio from 2:1 to 9:1 improved the efficiency of the leaching of α -amylase from 41 to 100% [Ramesh and Lonsane, 1988]. The ratios used by other workers in leaching of the enzymes were in the range of 1:1 to 10:1 [Arima, 1964; Meyrath and Volavsek, 1975; Ramakrishna *et al.*, 1982]. In literature, the use of 1:50 and 1:30 ratios of bran-to-solvent for leaching of other enzymes have also been reported [Minagawa and Hamaishi, 1962]. The leachates in these cases were very dilute and thus involved large volumes of leachates for downstream processing. Hence, the use of low bran to solvent ratio is necessary to maintain the product in a concentrated form to achieve the highest leaching efficiency.

Incubation time for soaking. The volume of water in relation to the solid matrix was retained at the optimal level, and the incubation time for soaking was further optimized for maximum enzyme recovery from the fermented bran. The time period was varied from 30 to 120 min (Fig. 3). Ninety minutes soaking time was optimum, and thereafter no beneficial effect on the Fpase extraction was observed, indicating the minimum time for total penetration of the solvent through the fermented biomass. Further increase in the contact time slightly decreased the leaching of the enzyme.

Solid-solvent contact time is another factor governing the efficiency of leaching. In the present study 90 min contact time between the solvent and the solid achieved maximum recovery of the Fpase. Drop in the recovery of Fpase at a higher contact time may be due to the inactivation of the enzyme. Soaking of the mouldy substrate in water at room temperature (25 to 28°C) for 1 h was used for the recovery of cellulases and β -

glucosidase [Singh and Garg, 1995; Fadel, 2000]. Smiley *et al.* [1976] employed 1 h contact time with occasional agitation for the leaching of α -galactosidase from the fermented bran, whereas 150 min soaking for amylase extraction was reported by Palit and Banerjee [2001].

Physical state of leaching. Two different leaching conditions viz. stationary and shaking were employed during extraction of the enzyme from the fermented bran using water. Shaking conditions were achieved by adding the mixture of fermented bran and solvent in the flasks and placing them on an orbital shaker at 150 rpm at 30°C. The results showed that the stationary condition was more effective for the leaching process. The recovery of the enzyme was 14.07 and 12.41 FPU/g under the stationary and the shaking conditions, respectively; the stationary leaching condition gave 13.4% more enzyme activity compared to the shaking condition.

Number of washes. In earlier experiments, extraction of the enzyme from the fermented bran was carried out by a single washing with the solvent. In order to determine whether a single wash could completely recover the enzyme, fermented bran collected from the previous extraction was extracted again with the fresh lots of solvent in repeated manner for enzyme. The enzyme was recovered in large amounts from the first two washings only, indicating two washings were sufficient for maximum leaching of the enzyme. The first and the second washings recovered 12.× and 2.× FPU/g of FPase activity, respectively, indicating two washings were sufficient for optimal leaching of the enzyme. The third washing leached only 1.× FPU/g activity

Complete recovery of the Fpase from the bran improves the efficiency of the fermentation process. In the present study, the first two washes together could accounted for 95% of the total recovery. Similar observation has been made by Palit and Banerjee [2001] on the extraction of the amylase.

Conclusion. The present study showed that the distilled water with two washes for 90 min under stationary conditions at solvent to solid ration of 5 mL distilled water to 1 g of wheat bran resulted in the optimal extraction of the Fpase from the fermented bran.

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