Optimization of extraction of β -endoglucanase from the fermented bran of *Aspergillus niger*

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Abstract A local isolate of *Aspergillus niger* was cultivated under optimal growth conditions on wheat bran in solid state fermentation. β -endoglucanase from fermented bran was separately extracted with different solvents to test recovery of enzyme. Among solvents tested, distilled water served the best leachate. Conditions were further optimized with this leachate. Two washes of fermented bran with the leachate for 30 min each under shaking conditions in a ratio of 1 g of wheat bran : 4 ml of distilled water together yielded maximum recovery of 16.7 U/g of wheat bran.

Keywords *Aspergillus niger* · Endoglucanase · Extraction · Solid state fermentation · Wheat bran

Cellulose-rich plant biomass is one of the foreseeable sources of fuels, single cell protein and feed stock to chemical synthesis due to its continuous synthesis from CO₂ and solar energy at annual rate of 72×10^{10} tons through photosynthesis by plants [1–3]. Individual enzyme components in cellulases act synergistically to convert complex carbohydrates present in lignocellulosic biomass into glucose, which can be subsequently fermented to ethanol, butanol, acetone or 2,3-butanediol on large scale [4, 5] which may be used as biofuels. Enzymatic saccharification of lignocellulosic mass is not cost-effective at present, due to high cost of production of cellulase by fermentation methods [2]. To overcome this short-coming, different approaches – searching for high-yielding cellulase producing strains, mutants that are resistant to catabolite repression, genetic engineering methods and optimizing fermentation conditions [6]. The production of cellulase has been intensively studied in submerged fermentation with different microorganisms in comparison to solid state fermentation [3]. However, solid state fermentation is an attractive process to produce cellulase economically due to its lower capital investment and its lower operating conditions [7]. Extraction of enzyme from the fermented bran is an important unit operation in downstream processing in solid state fermentation [8] and holds key for recovery of enzyme. Cellulolytic enzymes have been arbitrarily extracted with solvents without systematic approach [9]. The present investigation addresses the extraction of β -endoglucanase (EC 3.2.1.4), one of the component in cellulase complex as a model enzyme produced by *Aspergillus niger* in solid state fermentation.

A local isolate of *A. niger* from the soils contaminated with the effluents of Cotton Ginning Mill, Nandyal, Andhra Pradesh [10] was used in the present study. This fungal

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culture was maintained on Czapek Dox medium. Spore suspension of *A. niger* was prepared by adding 2 ml of sterile distilled water to 7-day grown slants.

The fermentation was carried out in 250 ml Erlenmeyer flasks containing 10 g of wheat bran. The wheat bran was initially moistened to 40% (w/v) level with 6 ml of modified Czapek Dox liquid medium + 6 ml of sterile distilled water. The modified Czapek Dox liquid medium contained ingredients (g/l) NaNO_3 - 2.0 g, K_2HPO_4 - 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.5 g, KCl - 0.5 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.01 g, sucrose - 30.0 g, $(\text{NH}_4)_2\text{SO}_4$ - 25 g, and cellulose - 5.0 g. Flasks were autoclaved for 30 min at 121°C and inoculated with spore density at 2×10^6 and incubated at ambient ($30 \pm 2^\circ\text{C}$) temperature. At regular intervals, sterile distilled water was added to each flask for maintaining initial moisture content during the course of incubation [11].

Entire 10 g fermented bran in 250 ml conical flask was extracted with chosen solvents by soaking the fermented bran at 30°C temperature for a desired period. The crude extract was then squeezed out through a cotton cloth and the filtrate was centrifuged at $9,000 \times g$ for 20 min. The clear filtrate obtained was used for β -endoglucanase 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 an experiment on parameter pertaining to number of washings, fresh lots of solvent were added to fermented bran recovered at the end of previous extraction. Leachates from these extractions were collected separately and tested for recovery of β -endoglucanase.

Flasks were withdrawn at daily intervals for 5 days and processed for endoglucanase (CMCase) activity. Activity of endoglucanase in the culture filtrate/leachate was quantified by carboxymethyl cellulase method [12]. One unit of endoglucanase activity was defined as the amount of enzyme releasing 1 μM of reducing sugar per minute. The production of enzyme on solid matrix is expressed as number of units per gram of bran.

Data presented are the averages of three replicates. Duncan's Multiple Range (DMR) test for all data was carried out [13].

The extraction efficiency is critical to the recovery of the enzyme from the fermented 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% (v/v) methanol. A single wash of the solvent with volume of 20 ml was used for extraction in the experiment. It is clear from Fig. 1 that among all the solvents used distilled water gave the best result yielding about 9 U/g. Because of maximum recovery of enzyme from the bran, with water, subsequent experiments were carried out with only water for further optimization.

Fig. 1 Selection of solvent for β -endoglucanase extraction. Means, in each bar, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.

Different solvents were separately used for extraction of cellulolytic enzymes from the fermented bran in different studies. For instance, citrate buffer (0.1 M, pH 4.8) was tried to leach cellulase from the fermented bran by Fadel [14]. Soaking the moldy substrate in water at room temperature ($25\text{--}28^\circ\text{C}$) for 1 h yielded good recovery of cellulases β -glucosidase and xylanase [15, 16]. Of all solvents tested in the present study, distilled water served the best leachate in extracting β -endoglucanase from fermented bran. This might be due to dissolution of the all fermented bran by distilled water which then becomes salt solution and hence able to extract enzyme from fermented bran. Distilled water is a common extracting solvent (available, save and low cost) used by other workers [17–19]. Distilled or tap water alone or with glycerin or sodium chloride was found to be best for amyloglucosidase extraction from moldy-wheat bran [9], whereas alcoholic solvents were found to be less efficient than aqueous solvents. Fernandez-Lahore et al. [20] found that 0.5 M sodium chloride was more suitable for acid protease recovery than distilled water. For this reason, subsequent experiments were carried out with only water for further optimization. Various factors are known to influence the degree of leaching of the product from fermented substrate produced under solid state fermentation [8]. The major factors include solvent efficiency, solid to solvent ratio, incubation time for soaking, physical state of leaching and number of washes.

In solid state fermentation (SSF) system free flowing solvent is very much limited. Thus adequate amount of solvent is required to leach out the enzyme present in the moldy bran. Extraction of enzyme from the bran was carried out with the solvent, i.e. distilled water within a range of 10–50 ml. The results of extraction of the enzyme are presented in Fig. 2. Forty milliliter solvent quantity was

Fig. 2 Effect of solvent volume on β -endoglucanase extraction. Means, in each bar, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.

sufficient enough for extraction of enzyme from 10 g of fermented bran, at solid to solvent ratio of 1:4. There was a decrease in total activity when lower volume of solvent was used for extraction.

The ratio of solvent to solid plays a vital role in extraction of β -endoglucanase from the fermented bran. Recovery of low yields of enzyme from the fermented bran with lower volume of solvents might be due to insufficient solvent volume to penetrate the solid fermented mass. Higher solvent to solid ratios also cause the solute to be more dilute in the final extract. Lower moisture content cause a reduction in solubility of nutrients provided to organism by fermented bran, a lower degree of swelling and higher water tension [21]. The reduction in enzyme yields at high initial moisture content might be due to the steric hinderance in interparticle spaces and impaired oxygen transfer [22]. The increase in leaching efficiency gained at higher ratios must therefore be balanced against extra effort for concentration of dilute extract. However, in many cases, it may not be possible to achieve an economic balance [8]. Information on extraction of cellulolytic enzymes from the fermented bran is scanty. In the present study, 40 ml solvent could achieve maximum extraction of β -endoglucanase from the bran. But Palit and Banerjee [23] reported that 30 ml solvent could extract optimally amylase at solid to solvent ratio of 1:3 from 10 g of fermented bran. The efficiency of leaching increases as the ratio increases [8]. The ratios used by other workers in leaching of the enzymes are in the range of 1:1 to 10:1 [9, 24, 25]. The leachate in these cases will be very dilute and thus involves large volumes of leachate for downstream processing. Hence, in order to have the product in concentrated form, it is always preferable to use low bran to solvent ratio with highest leaching efficiency.

Fig. 3 Effect of incubation time on soaking. Means, in each bar, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.

Using the best solvent, water and keeping its volume in relation to solid matrix at optimal level, incubation time for soaking was further optimized for maximum enzyme recovery from the fermented bran. The time period was varied from 30 to 150 min as shown in Fig. 3. Thirty minutes soaking time was optimum and beyond that it did not have any beneficial effect on endoglucanase extraction indicating the minimum time for the total penetration of solvent through the fermented biomass. Further increase in contact time resulted in slight decrease in the leaching of the enzyme.

Solid-solvent contact time is another factor governing the efficiency of leaching. In the present study 30 minutes contact time between solvent and solid could achieve maximum recovery of β -endoglucanase. Drop in the recovery of β -endoglucanase at higher contact time may be due to inactivation of enzyme. Other enzymes levansucrase needed 90 min contact time for extraction in SSF [17]. Contact time within a range of 1–3 h with occasional agitation was employed for leaching of enzymes [14, 26].

Two different leaching conditions viz. stationary and shaking were employed during course of extraction of enzyme from the fermented bran with water. Shaking conditions were provided by placing the mixture of fermented bran and solvent in the flasks on an orbital shaker at 150 rpm at 30°C temperature. The results are compared in Fig. 4 and shaking condition was effective for the leaching process.

Provision of shaking conditions during extraction in the present study, improved recovery of β -endoglucanase by 70% over stationary conditions. Similarly, Palit and Banerjee [23] reported beneficial nature of both agitation and recirculation conditions on extraction of amylase from the fermented bran. Beneficial nature of shaking conditions

Fig. 4 Effect of different leaching conditions on β -endoglucanase extraction.

Means, in each bar, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.

might be attributed to uniform distribution of fermented bran in the continuous phase of solvent, reducing concentration polarization [27].

In earlier experiments, extraction of enzyme from the fermented bran was carried out with a single washing of the solvent. In order to check whether a single wash could completely recover enzyme, fermented bran collected from the previous extraction was extracted again with the fresh lots of solvent in repeated manner for enzyme recovery. Yields of enzyme in repeated washings are presented in Fig. 5. As enzyme was recovered in large amounts in the first two washings only, two washings were sufficient for maximum leaching of the enzyme.

Complete recovery of β -endoglucanase from the bran improves the efficiency of the fermentation process. In the

Fig. 5 Effect of number of washes on β -endoglucanase extraction.

Means, in each bar, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.

present study, the first and second wash recovered 70% and 25% of the β -endoglucanase from the bran on the basis of total yields by three washes together. The first two washes together could account for 95% of total recovery. Similar observation was made by Palit and Banerjee [23] on extraction of the amylase.

Conclusions can be clearly drawn from the results of the present study that the distilled water in two washes for 30 min under shaking conditions at 4 ml of distilled water: 1 g of wheat bran ratio of solvent to solid could extract β -endoglucanase from the fermented bran.

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