IMPROVING THE EXTRACTION CONDITIONS OF ENDOGLUCANASE PRODUCED BY *Aspergillus niger* UNDER SOLID-STATE FERMENTATION

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Abstract - Production of cellulases under solid-state fermentation (SSF) is a promising technique that can help to reduce costs. Besides optimizing the production process, it is also important to consider enzyme recovery during the extraction step. Here, an experimental design methodology was used to investigate the effects of the operational parameters solid to liquid ratio (1:3, 1:6 and 1:9), stirring rate (80, 120 and 160 rpm), and temperature (10, 22 and 35 °C) on the recovery of endoglucanases produced by *Aspergillus niger* cultivated under SSF. Statistical analysis revealed that only the solid to liquid ratio had a significant influence on endoglucanase extraction. The highest endoglucanase recovery (35.7 U/g) was achieved using 0.2 mol/L acetate buffer at pH 4.8, together with a solid to liquid ratio of 1:9 and an agitation time of 10 minutes. In sequential extraction experiments, it was shown that most of the enzyme was recovered during the first extraction. The procedure adopted increased the efficiency of endoglucanase extraction by 70%, emphasizing the importance of selection of suitable operational conditions during SSF processes.

Keywords: Endoglucanase; Solid-state fermentation; Extraction; *Aspergillus niger*; Cellulase.

INTRODUCTION

One of the main obstacles in the bioconversion of lignocellulosic biomass into fuels and chemicals is the large quantity of expensive enzymes required for the breakdown of cellulose into simple sugars. These enzymes can be produced by the cultivation of microorganisms, especially filamentous fungi, in either liquid (submerged fermentation, SmF) or solid (solid-state fermentation, SSF) media. The SSF technique has increasingly received attention for the production of microbial enzymes, since it offers several advantages over SmF (Holker *et al.*, 2004; Raimbault, 1998).

Although numerous studies have been published describing strategies for improved production of fungal cellulases under SSF (see Singhanian *et al.*

(2010) for a review), reports concerning the recovery step are scarce. Identification of the optimum operational conditions for enzyme extraction after cultivation using SSF involves consideration of several different variables. The factors that most influence enzyme extraction are the type of solvent, the solid to liquid ratio, the temperature, the type of agitation, and the contact time, as well as the interrelations between these variables (Castilho *et al.*, 2000).

Some previous studies have investigated the influence of operational parameters on the extraction of enzymes following SSF. The enzymes considered include pectinase (Castilho *et al.*, 2000; Singh *et al.*, 1999), xylanase (Heck *et al.*, 2005; Maciel *et al.*, 2009; Pal and Khanum, 2010; Rezaei *et al.*, 2011), lipase (Menoncin *et al.*, 2010), inulinase (Bender

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et al., 2008; Chen *et al.*, 2011), and manganese-peroxidase (Arantes *et al.*, 2011). However, there have been few studies concerning the optimum conditions for cellulase extraction from SSF cultivations. Rezai *et al.* (2011) described the influence of the extraction buffer composition on the recovery of xylanase and cellulase. Chandra *et al.* (2010) investigated the extraction of endoglucanase produced under SSF; however, each individual variable was evaluated separately.

Analysis of the relationships among a number of parameters that affect the overall process can be achieved using statistical experimental design. This procedure can be very effective for identification of the optimum operational conditions for enzyme extraction. Here, we report the use of an experimental design methodology to investigate the effects of the operational parameters solid to liquid ratio, stirring rate, and temperature on the recovery of endoglucanase produced by *Aspergillus niger* under SSF. Sequential extractions were also performed in order to determine the efficiency of endoglucanase recovery.

MATERIALS AND METHODS

Microorganism

The microorganism used in this study was the wild-type strain *A. niger* 12 from the Embrapa Food Technology collection (Rio de Janeiro, Brazil), which had been isolated from black pepper (Couri and Farias, 1995). The culture was kept on dry sand under freezing conditions (-18 °C). Microorganism activation was carried out by incubation for 7 days at 32 °C using basic medium agar slants, with the following composition (g/L): citric pectin (10.0), NaNO₃ (3.0), KH₂PO₄ (1.0), MgSO₄·7H₂O (0.5), KCl (0.5), FeSO₄·7H₂O (0.01), agar-agar (20.0). The final pH of the medium was 4.2.

After the incubation period, the spores were harvested by adding 10 mL of 0.3% Tween 80 to the slant, according to Couri and Farias (1995).

Solid-State Fermentation (SSF)

Solid-state fermentations were carried out in 500 mL Erlenmeyer flasks as described by Farinas *et al.* (2011), using 10 g (dry weight) of wheat bran moistened at 60% initial moisture content with a solution of 0.9% (w:v) ammonium sulfate in 0.1 mol/L HCl. The flasks containing the solid medium were sterilized at 121 °C for 20 minutes before

inoculation. A spore suspension volume corresponding to 10⁷ conidia/g of dry solid medium was inoculated into the flasks. The cultures were then incubated at 32 °C for 72 hours in a BOD type incubator (Tecnal, Brazil).

Selection of Extraction Time and Solvent Type

A set of preliminary experiments was carried out in order to identify the best solvent type and extraction time for recovery of endoglucanase. The solvents used were distilled water, 0.2 mol/L acetate buffer at pH 4.8, 0.05 mol/L citrate buffer at pH 4.8, and 0.1% Tween 80 solution. After the cultivation period, 60 mL of solvent was added to each flask containing the solid medium, at a solid to liquid ratio of 1:6. The mixtures were stirred at 120 rpm and 22 °C, using an orbital shaker incubator (Solab, Brazil). Three different agitation times (10, 30, and 60 minutes) were tested for each solvent. The solid fraction was subsequently separated from the extract by filtration followed by centrifugation at 10000 x g for 20 minutes at 4 °C, in order to obtain a clear liquid. The supernatant was assayed for endoglucanase activity. All the experiments were performed in triplicate.

Sequential Extractions

The sequential extractions employed the solvents described previously. Each solvent was added to the flasks containing the cultivated solids, at a solid to liquid ratio of 1:6, and the mixtures were stirred at 120 rpm and 22 °C using the orbital shaker. After an extraction period of 10 minutes, the contents of the flasks were squeezed using cheese-type gauze. The squeezed material was placed in a second flask, solvent was again added, the mixture was agitated, and the solids were again separated with gauze. This procedure was repeated three times, in order to complete three sequential extractions. The extracts were filtered, centrifuged at 10000 x g for 20 minutes at 4 °C, and the clear supernatants were used for measurements of endoglucanase activity.

Experimental Design

A full factorial design was used to evaluate the effects of solid to liquid ratio, stirring rate, and temperature on endoglucanase extraction. The experimental design selected was a 2³ full factorial design comprising eleven runs, corresponding to eight axial points and three central points, with the experiments carried out in random order. Values of the independent variables and their coded levels are

given in Table 1. The response variable was the endoglucanase activity. The Statsoft (v. 7.0) statistical software package was used for analysis of the experimental data.

Enzyme Activity

Endoglucanase activity was measured using an assay based on the Ghose methodology (Ghose, 1987). Briefly, a volume of the appropriately diluted enzyme extract was incubated at 50°C for 10 minutes with a 1% carboxymethylcellulose (CMC) (Sigma, USA) solution prepared using 0.050 mol/L citrate buffer (pH 4.8). One unit of endoglucanase activity corresponds to 1 μ mol of glucose released per minute at pH 4.8 and 50 °C. Activity units were calculated by multiplying the activity value measured in the enzymatic extract (in IU/mL) by the total volume used for extraction (which varied according to the solid to liquid ratio used). The results were expressed as activity units per mass of initial dry solid substrate (IU/g). Quantification of the reducing groups released was performed according to the DNS method developed by Miller (1959).

RESULTS AND DISCUSSION

Selection of Extraction Time and Solvent Type

Preliminary experiments were performed in order to determine the extraction time and solvent type most favorable for endoglucanase extraction. An ideal solvent should extract the enzyme selectively and completely at room temperature, with minimal contact time (Singh *et al.*, 1999; Pal and Khanum, 2010). It is important to mention that, in the presence of lignocellulosic materials, *Aspergillus niger* species are able to produce an extensive range of enzymes, including cellulases, xylanases, xyloglucanases, and pectinases, in order to achieve efficient degradation of the biomass (de Vries and Visser, 2001). Here, in order to simplify the evaluation, only endoglucanase activity was used to compare the performance of the different extraction conditions.

Figure 1 shows the endoglucanase activities obtained after extraction using the different solvents (distilled water, 0.02 mol/L acetate buffer at pH 4.8, 0.05 mol/L citrate buffer at pH 4.8, and 0.1% Tween 80 solution). Solvent selection was based on cost (in the case of distilled water), buffering capacity at pH values near pH 5.0 (the optimum pH for most cellulase enzymes) and, finally, Tween 80 solution was selected based on the fact that it could contribute

to increase the permeability of the cell membrane, allowing more enzyme secretion. The extractions were performed for 10, 30, and 60 minutes, at 22 °C, with stirring at 120 rpm. The influence of time (within the range tested) on endoglucanase extraction was similar for all solvents. The endoglucanase activity recovered ranged from 27.8 IU/g (using the citrate buffer for 60 minutes) to 36.6 IU/g (using the sodium acetate buffer for 10 minutes). The use of distilled water as solvent, which would have cost advantages in industrial applications, resulted in recovery values that were of the same order of magnitude as those obtained using the other solvents (34.2 IU/g for 10 minutes extraction). Nevertheless, the use of a buffer solution as solvent is preferable, since it can improve enzyme stability. Castilho *et al.* (2000) also found that the use of acetate buffer resulted in improved extraction of pectinases produced by *A. niger* under SSF. In other studies of endoglucanase production employing SSF, the solvent used has been either water (Jecu, 2000; Gao *et al.*, 2008) or a buffer solution (Jatinder *et al.*, 2006; Camassola & Dillon, 2007).

Figure 1: Endoglucanase activity recovered using different solvents, after 10, 30, and 60 minutes of stirring at 120 rpm and 22 °C.

For all solvents tested, highest enzyme recoveries were achieved for an extraction time of 10 minutes, with no further increases in endoglucanase recovery using longer extraction times. A 10 minute extraction time was therefore chosen for subsequent experiments using sequential extractions.

Sequential Endoglucanase Extractions

Sequential extraction showed that the majority of the enzyme was recovered in the first extraction step

(Figure 2). Using acetate buffer, an additional 28% of endoglucanase was recovered in the second extraction step, while only 13% more endoglucanase was recovered using distilled water. Adding a third extraction step did not significantly increase endoglucanase recovery for any of the solvents tested. Singh *et al.* (1999) found that subsequent extractions did not have any significant effect on the recovery of pectinase produced under SSF, while Heck *et al.* (2005) reported that sequential extractions did not improve xylanase recovery.

Figure 2: Sequential endoglucanase extraction using different solvents, with 10 minutes of stirring at 120 rpm and 22 °C.

In terms of the economics of the process, it can therefore be concluded that a single extraction step should be satisfactory for most industrial applications.

Based on the above findings, one 10 minute extraction step using pH 4.8 acetate buffer was selected for extraction optimization using the experimental design methodology.

Experimental Design

The effects of the operational parameters solid to liquid ratio, stirring rate, and temperature on the efficiency of endoglucanase extraction were evaluated using a 2³ full factorial design. Table 1 presents the experimental conditions and the responses for endoglucanase activity recovery. Endoglucanase activity recovered ranged from 21.0 IU/g (run 2) to 35.7 IU/g (run 8).

The significant factors were determined by Pareto chart analysis (Figure 3). Of the three variables analyzed, only the solid to liquid ratio showed a significant effect on endoglucanase recovery ($p < 0.05$). Neither stirring rate nor temperature showed any statistically significant effect, within the ranges studied. This finding is important for industrial applications, since it allows flexibility in the selection of these variables, depending on process conditions, which could help to reduce energy consumption. The statistical analysis resulted in a high correlation coefficient of 0.9701 (the surface plot is not presented, since only one variable was statistically significant). Arantes *et al.* (2011) also found that stirring rate and temperature did not significantly influence the extraction of manganese-peroxidase produced by *Lentinula edodes* under SSF of eucalyptus residue. Similarly, Singh *et al.* (1999) found that agitation during extraction did not increase the recovery of pectinase.

Table 1: Full factorial design for endoglucanase recovery under different operational conditions, using 0.02 mol/L acetate buffer at pH 4.8, and a 10 minute extraction time.

Run	Levels			Response
	Stirring rate (rpm)	Solid to liquid ratio (m:v)	Temperature (°C)	Endoglucanase (IU/g)
1	-1(80)	-1(1:3)	-1(10)	21.2
2	1(160)	-1(1:3)	-1(10)	21.0
3	-1(80)	1(1:9)	-1(10)	34.4
4	1(160)	1(1:9)	-1(10)	35.2
5	-1(80)	-1(1:3)	1(35)	23.0
6	1(160)	-1(1:3)	1(35)	21.6
7	-1(80)	1(1:9)	1(35)	33.9
8	1(160)	1(1:9)	1(35)	35.7
9	0(120)	0(1:6)	0(22)	25.1
10	0(120)	0(1:6)	0(22)	27.0
11	0(120)	0(1:6)	0(22)	27.0

Figure 3: Pareto chart for the effects of the variables solid to liquid ratio (m:v), stirring rate, and temperature on the recovery of endoglucanase, using 0.02 mol/L acetate buffer at pH 4.8, and a 10 minute extraction time.

The positive effect of the solid to liquid ratio obtained for endoglucanase recovery means that an increase in the value of this variable should improve the extraction yield. The positive influence can be attributed to the mass transfer process, which is driven by the concentration gradient between the solid (substrate) and liquid (solvent) phases. A higher concentration gradient between the two phases facilitates transfer of the solutes to the liquid medium, which favors extraction of the enzyme. Bender *et al.* (2008), in a study that evaluated the extraction of inulinase produced under SSF of sugarcane bagasse, achieved maximum enzyme extraction using a solid to liquid ratio of 1:10.

Even though higher enzyme recovery is achieved when using a higher solid to liquid ratio, it is important to consider the concentration of the enzymatic extract required for each specific application, specially when no further concentration step will be used. Our results showed that when using a 1:9 ratio, a total of 357 IU of endoglucanase is recovered (run 8), which corresponds to an enzymatic activity of 3.97 IU/mL in a 90 mL extract volume. This final recovery value is 70% higher than the final recovery achieved when using a 1:3 ratio (run 2), when a total of 210 IU of endoglucanase is recovered with a concentration of 7.0 IU/mL in a 30 mL extract volume. Thus, a compromise between a higher final enzyme recovery and the enzymatic extract concentration should be considered when

selecting the optimum solid to liquid ratio. An ability to adjust the volume of solvent used, in order to obtain a more concentrated enzymatic extract, is an important advantage of SSF compared to SmF (Holker *et al.*, 2004).

In terms of the influence of the other variables, possible explanations for the fact that temperature did not show any significant influence on the extraction of endoglucanase are the range selected and the short contact time (10 minutes). It is known that solute solubility and diffusivity increase with temperature, which can result in higher recovery. However, enzyme thermal stability should also be considered, since deactivation can occur at higher temperatures (Castilho *et al.*, 2000). Similarly, the absence of any influence of stirring rate (within the range used) on the recovery of endoglucanase could also have been due to the short contact time. Intense agitation increases the rate of the mass transfer process. On the other hand, high stirring rates can lead to shear stress, resulting in enzyme deactivation (Arantes *et al.*, 2011).

CONCLUSIONS

The most favorable conditions for endoglucanase extraction were obtained using a buffer solution of 0.2 mol/L sodium acetate at pH 4.8, an agitation time of 10 minutes, and a solid to liquid ratio of 1:9.

Temperature and stirring rate did not influence the process, within the ranges tested. Significantly, a single extraction step was shown to provide efficient endoglucanase recovery. A 70% improvement in the recovery of endoglucanase produced by *Aspergillus niger* was indicative of the importance of correct selection of the operational parameters.

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