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ORIGINAL ARTICLE

Exoglucanase production by *Aspergillus niger* grown on wheat bran

Exoglucanase by *A. niger* in SSF

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Abstract β-Exoglucanase production on the lignocellulosic material, wheat bran, by *Aspergillus niger* under solid state fermentation (SSF) on a laboratory scale was investigated. Different fermentation parameters, such as moisture content, initial pH, temperature, depth of the substrate, and inoculum size on exoglucanase production were optimized. Moisture content of 40 %, pH of 7.0, substrate depth of 1.0 cm, inoculum size of 2×10^6 spores/g of wheat bran, and temperature at 30 °C were optimal for maximum production of exoglucanase. Maximum yields of exoglucanase with 28.60 FPU/g of wheat bran were obtained within 3 days of incubation under optimal conditions.

Keywords Solid state fermentation · Exoglucanase · *Aspergillus niger* · Wheat bran

Introduction

Cellulases are among the industrially important hydrolytic enzymes and are of great significance in present-day biotechnology (Shafique et al. 2004). Microbial conversion of cellulosic/lignocellulosic biomass into useful products is a complex process involving the combined action of three enzymes namely endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) or filter paperase (FPase), and β-glucosidase (EC 3.2.1.21) (Erikson and Patterson 1975). Amongst the cellulases, exoglucanase or avicelases are found to have potential applications in the bioconversion of agricultural waste materials to useful products, such as single cell protein, fuels, and chemical feed stocks (Kari et al. 1994; Nikolay et al. 1998; Ojumu et al. 2003). The exoglucanase have a tunnel shape and the tunnel is made up of loops which have strong interaction with the salt linkers, hydrogen bonds (Sinnott 1997). Exoglucanases are of two types; 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91) which removes cellobiose units and 1,4-β-D-glucan glucohydolase (EC 3.2.1.74) which removes glucose units, both acting from the non-reducing ends of oligosaccharides produced by the action of endoglucanase (Mullings 1985; Zhang and Lynd 2004). As, generally, exoglucanase is found to be synthesized along with other two types of cellulases, it is also important to estimate the relative ability of a particular cellulase-producing strain to release exoglucanse and the parameters affecting the synthesis.

Filamentous fungi are preferred to produce commercially important enzymes because their levels of enzyme production are higher than those obtained from other microorganisms (Bakri et al. 2003). The fungi which appear to be most promising at present are the *Trichoderma reesei* mutants with high titers of cellulase production. However, it is our interest to examine a new fungal culture for maximum exoglucanase production. Species of *Aspergillus* are major agents of decomposition and decay and have the capability to produce a broad range of enzymes which include cellulases (Berka et al. 1992; Oxenboll 1994).

In this paper, the experiments were conducted to optimize the exoglucanase production by a local strain of *Aspergillus niger* (Narasimha et al. 1999) with locally available cheap substrate, wheat bran, on SSF. The influence of moisture level, initial pH, depth of substrate, inoculum size, and growth temperature was investigated.

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Materials and methods

Microorganism and inoculum

A local isolate of *Aspergillus niger* isolated from the soils contaminated with the effluents of cotton ginning mills, Nandyal, Andhra Pradesh (Narasimha et al. 1999), was used in the present study. This fungal culture was maintained on Czapek Dox agar slants at 32 °C. Spore suspension of *A. niger* was prepared by adding 2 ml of sterile distilled water to 7-day-grown slants (Chandra et al. 2007). Spore count was measured with a hemocytometer and adjusted to 2×10^6 spores/ml by adjustment of optical density.

Substrate

Air-dried and milled wheat bran (without pre-treatment) was utilized as substrate for SSF and was purchased from a local market in Punganur, Chittoor district. The substrate was sieved through a 2-mm screen for uniform particle size (Chandra et al. 2007).

Culture media

The optimized Czapek Dox medium contained (g/l): 2.0 $NaNO_3$; 1.0 K_2HPO_4; 0.5 $MgSO_4{\cdot}7H_2O$; 0.5 KCl; 0.01 $FeSO_4{\cdot}7H_2O$; 25 glucose; 5.0 cellulose; 25 $(NH_4)_2SO_4$; pH6.0. The optimized Czapek Dox medium was used to moisten the solid substrate, wheat bran, in experiments, whereas in the case of moisture content and pH experiments, normal Czapek Dox medium (without optimization) was used to moisten the wheat bran.

Fermentation

The fermentation was carried out in 250-ml Erlenmeyer flasks containing 10 g of wheat bran. The solid substrate was initially moistened to 50 % (w/v) level with Czapek Dox liquid medium containing 0.5 % (w/v) cellulose (Aikat and Bhattacharyya 2004; Chandra et al. 2006), cotton-plugged and autoclaved at 121 °C for 30 min. Sterile solid culture medium in the flasks were inoculated with the spores of *A. niger* at a density of 2×10^6 spores/flask and incubated at ambient temperature (30± 2 °C). One ml of spore suspension containing about 2×10^6 spores/ml was used to inoculate the wheat bran for all experiments except one experiment of spore density. During the course of incubation, water loss by evaporation from the flask was aseptically replaced with addition of sterile distilled water (Chandra et al. 2007) to maintain 40 or 50 % moisture content in all experiments except one experiment of moisture level. At regular intervals, the flasks were withdrawn for further processing. The moisture content was varied by adding distilled water to obtain a moisture range of 20–80 %. Static cultivation was performed at various initial pH values: 3.0, 4.0, 5.0, 6.0, and 7.0. The cultures were incubated at different temperatures 25, 30, 37, and 40 °C. The wheat bran was filled to different depths viz., 0.5, 1.0, 1.5, and 2.0 cm in beakers. Different spore densities viz., 2×10^4, 2×10^5, 2×10^6, and 2×10^7 were inoculated in 10 g of wheat bran.

Enzyme extraction

Fresh moldy substrate were soaked in acetate buffer (0.2 M; pH5.0) or distilled water (1:4, w/v), the mixtures of fermented bran and solvent in the 250-ml Erlenmeyer flasks on an orbital shaker at 150 rpm at 30 °C temperature for 30 min, except for the moisture level and initial pH experiments (immediately extracted), and extracts were obtained by filtering through nylon cloth and the filtrate was centrifuged at 10,000 rpm for 20 min at 4 °C. The clear filtrate obtained was used for enzyme assay up to 5 days of incubation. The clarified filtrate was used for exoglucanase activity.

Enzyme assay

Flasks were withdrawn daily for 5 days. The activity of exoglucanase in the culture filtrate/leachate was quantified following the method of Mandels and Weber (1969). Exoglucanase activity was assayed in reaction mixture containing 50 mg of Whatman filter paper strip (1×6 cm), 0.05 M sodium citrate buffer (pH 4.8) and appropriately diluted enzyme solution. After incubation at 50 °C for 60 min, the reaction was stopped by addition of 3,5-dinitrosalycilic acid reagent (DNS). The amount of reducing sugar released was determined using dinitrosalicylic reagent (Miller 1959). One unit is the amount of enzyme in the culture filtrate releasing 1 μmole of reducing sugar from the filter paper per min. The production of the enzyme on solid matrix is expressed as the number of filter paper units per gram of bran.

Statistical analysis

Data presented are the averages of three replicates. Duncan's Multiple Range (DMR) test for all data was carried out (Megharaj et al. 1999).

Results and discussion

Effect of culture initial pH

The influence of medium pH was studied by adjusting the pH of Czapek Dox medium used to wet the substrate. It was observed that an initial medium pH of 7.0 with exoglucanase activity, 3.19 FPU/g of wheat bran, and specific activity of 0.32 FPU/mg protein (data not shown) was found to

Table 1 Effect of initial pH on the production of FPase in wheat bran by *A. niger* in SSF

Initial pH	FPase[a] (FPU/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
3.0	0.85 a	1.96 a	2.63 a	1.59 bd	2.15 b
4.0	0.74 a	1.81 a	2.71 a	1.15 b	1.96 b
5.0	0.93 a	2.00 a	2.67 a	1.56 bc	2.26 bc
6.0	1.19 c	2.56 b	2.74 a	1.37 b	2.11 b
7.0	0.96 ab	2.52 b	3.19 b	0.86 a	1.74 a

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

[a] Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 μmole of reducing sugar from filter paper per min

be ideal for enhanced production of exoglucanase in SSF (Table 1). Singhania et al. (2006) also reported that an initial medium pH of 7.0 is most suited for cellulase production in wheat bran. The similar trend was observed in the present study. Most of the fungi are capable of growing in a wide range of pH (4–8) (Chahal 1983). The maximum activity of cellulolytic enzymes was obtained when the initial pH was adjusted to 7.0 for growth of *F. oxysporum* on corn stover (Panagiotou et al. 2003) and *Bacillus subtilis* on banana waste (Krishna 1999). Likewise, optimal pH for production of exoglucanase by *Cellulomonas flavigena* in liquid cultures on different carbon sources has been recorded (Rajoka 2004). Initial pH6.7 was favorable for higher production of cellulase as stated by Wen et al. (2005).

Effect of moisture level

The moisture level of culture medium affects the physiology of the microorganism, and therefore it was desired to find out the optimum moisture level of the medium for the enhancement of enzyme production. The enzyme production with moisture level varying from 20 to 80 % in wheat bran moistened with medium is shown in Fig. 1. The maximum yield of the enzyme activity at 4.22 FPU/g of wheat bran and specific activity 0.7 FPU/mg protein was obtained at 40 % moisture level. Thus, production of FPase on wheat bran with 40 % moisture level was highest when compared to the production at other moisture levels at their respective peak time intervals.

Moisture content in the solid substrate is a critical factor in SSF. The moisture level demands in SSF differ according to enzyme to be produced, substrate, microorganism, and particle size of the substrate, as well as the configuration of the particles (Muniswaran and Charyulu 1994; Fadel 1999). The higher production of cellulase in wheat bran was obtained at

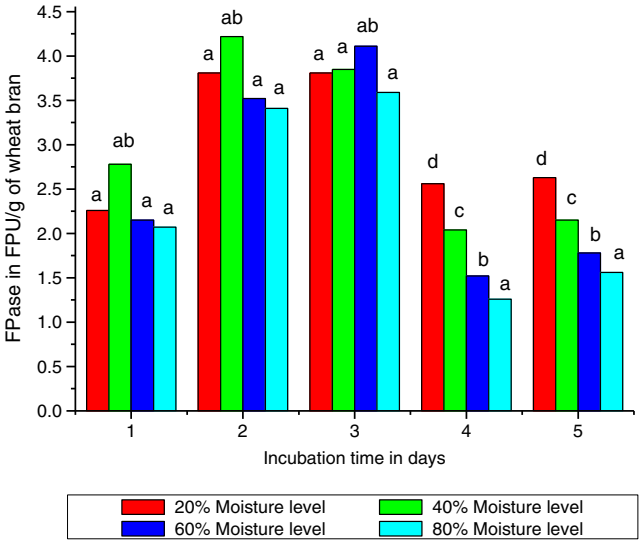

Fig. 1 Effect of moisture level on production of FPase (expressed in terms of filter paper units) in wheat bran by *A. niger* in SSF means, in each time interval, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test. One filter paper unit is the amount of enzyme in the culture filtrate releasing 1 μmole of reducing sugar from the filter paper per min

70 % moisture level as reported by Maurya et al. (2012). Cellulase production by *A. niger* and *Bacillus subtilis* on pineapple peel was found to be maximum at 75 % moisture (Sunitha et al. 2006). The reduction in enzyme yields at high initial moisture content might be due to the steric hinderance in interparticle spaces and impaired oxygen transfer (Sandhya and Lonsane 1994). Highest activities of cellulase were obtained by cultivation of *Fusarium oxysporum* on corn stover with initial moisture content of 80 % (Chahal 1983). Similar observations were made with the organisms *Trichoderma harzianum* and *Trichoderma reesei* QM 9414 on different solid substrates (Roussos et al. 1991). According to Badhan et al. (2007), optimum production of cellulolytic enzymes was observed at 80 % moisture. There was positive relationship between moisture content and cellulase production up to 70 %, but further enhancement in moisture content inversely influenced the enzyme production (Krishna 1999; Lonsane et al. 1985; Xavier and Lonsane 1994). Increasing moisture level is believed to have a reduction in the porosity of the substrate, thus limiting the oxygen transfer into the substrate (Raimbault and Alazard 1980). Likewise, a lower moisture ratio leads to reduced solubility of the nutrients of the solid substrate, a lower degree of swelling, and a higher water tension (Ikasari and Mitchell 1994). In the present study, the moisture content in solid substrate influenced the exoglucanse production in SSF by *A. niger* on wheat bran. FPase occurred in maximum amounts at 40 % moisture level. This may be due to preferential synthesis of specific proteins including proteins of cellulase enzyme at a lower moisture level than at a higher moisture level.

Depth of the substrate

An increase in exoglucanase production with an increase in depth of solid medium for fermentation of up to 1.0 cm occurred. A further increase in depth of the solid medium was not beneficial in enhancing the production of exoglucanase. Titers of exoglucanase activity in the fermented bran rose from 10.1 FPU/g wheat bran and specific activity 0.57 FPU/mg protein on the first day of incubation to 19.5 FPU/g wheat bran (specific activity 0.66 FPU/mg protein) on the second day of incubation and remained high and sustainable level until the final day of incubation. Maximum exoglucanase activity yields with 21.76 FPU/g of wheat bran (specific activity 0.68 FPU/mg protein) was obtained from the solid medium with a depth of 1.0 cm on the 5th day of incubation followed by 21.08 FPU/g of wheat bran (specific activity 0.77 FPU/mg protein) with a depth of 1.5 cm on the 4th day of incubation (Table 2).

Virtually, information on the effect of thickness of bed of solid medium on enzyme production in SSF is lacking. The influence of depth of solid medium on production of cellulolytic enzyme by *A. niger* in SSF has been assessed (Fadel 2000). According to this study, production of cellulases and β-glucosidases by *A. niger* on radicle waste increased slightly until 12 mm, was steady to 18 mm, and then decreased. The enzyme yield was reduced to less than from the maximum 74 % at 24 mm of depth. In the present study, bed thickness of 1.0 cm solid medium was optimal for production of exoglucanase by *A. niger* on wheat bran. The importance of the depth of the bed of medium on enzyme production is discussed by Muthuvelayudham and Viruthagiri (2006). Experiments were initially carried on thin beds (0.5 cm) to remove heat, and mass transport effect was observed to be more in deeper beds. Maximum xylanase on wheat bran by SSF was at 1.5 cm, whereas at 0.5 cm the yield was about 75 % of its maximum using *Trichoderma longibrachiatum* (Ramana Murthy et al. 1993).

Effect of inoculum size or spore density

The inoculum size also plays an important role in enzyme production. As shown in Table 3, maximum exoglucanase activity of 27.78 FPU/g of wheat bran (specific activity 0.99 FPU/mg protein) was obtained when the inoculum size was 2×10^6 spores/g of bran. A lower level of inoculum may not be sufficient for initiating growth and enzyme production. An increase in inoculum size ensures a rapid proliferation of biomass and enzyme production. After a certain limit, enzyme production could decrease because of depletion of nutrients due to the enhanced biomass, which would result in a decrease in metabolic activity (Kashyap et al. 2002). A balance between the proliferating biomass and available substrate material would yield maximum production of enzyme.

Maximal protein and high cellulase production, i.e., 88.1 mg/g of protein, 31.5 U/g of FPase, 46.6 U/g of CMCase and 215.2 U/g of β-glucosidase activity was achieved with inoculum size at 10 % (v/w) in SSF of radicle by *A. niger* (Fadel 2000). The optimal size of inoculum was 10,000 viable spores for dry solids in solid surface fermentation by *Chaetomium globosum, Phanerochaete chrysosporium* and *T. reesei* on soyhull (Ridder et al. 1997). In the present study, inoculum of 2×10^6 spores/g of bran was optimal for higher production of exoglucanase by *A. niger*. The enzyme activities were found to be higher with the mycelia inoculum compared to the spore inoculum (Jha et al. 1995). The inoculum volume did not affect enzyme production very much on coconut coir pith for cellulase production by *Trichoderma viride* NCIM 1051 (Muniswaran and Charyulu 1994). Larger inoculum size was detrimental to growth and production apart from adding to the fermentation cost (Muniswaran and Charyulu 1994). The marginal decrease seen at larger inoculum volumes may be due to the free excess

Table 2 Influence of depth of solid medium on production of FPase in fermented bran by *A. niger* in SSF

Depth of solid medium (cm)	FPase[a] (FPU/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
0.5	9.80 b	15.93 a	17.97 b	17.16 a	15.11 a
1.0	10.19 bc	19.44 b	19.68 b	19.91 a	21.76 b
1.5	9.46 b	19.70 bc	18.12 b	21.08 a	20.89 b
2.0	5.56 a	16.11 a	14.44 a	16.25 a	11.94 a

Means, in each column, followed by the same letter are not significantly different ($P\leq0.05$) from each other according to DMR test

[a] Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 μmole of reducing sugar from filter paper per min

Table 3 Effect of spore density on production of FPase on wheat bran by *A. niger* in SSF

Spore density (spores/g of wheat bran)	FPase[a] (FPU/g of wheat bran)				
	Incubation time (days)				
	I	II	III	IV	V
2×10^4	3.33 a	21.67 b	19.44 b	19.99 c	14.44 c
2×10^5	8.89 b	25.56 c	22.78 b	20.11 c	16.67 d
2×10^6	16.67 c	27.78 c	21.67 b	10.56 b	11.67 b
2×10^7	17.78 c	16.11 a	11.11 a	3.89 a	7.50 a

Means, in each column, followed by the same letter are not significantly different ($P\leq0.05$) from each other according to DMR test

[a] Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 μmole of reducing sugar from filter paper per min

unadsorbed liquid, which imposed an additional barrier for diffusion. Similarly, in the present study, low yields of exoglucanase occurred in fermented bran cultivated with highest spore density. Optimal production of bacterial cellulases occurred with 15 % inoculum size (v/w, based on dry weight of banana fruit stalk), but further increase resulted in reduced enzyme yield (Krishna 1999). Fermentation was carried out by inoculating the substrate using 1.25×10^7 spores/g of substrate and incubating at 32 °C for 6 days using *Rhizopus arrhizus*, whereas for *Phanerochaete chrysosporium*, the inoculum size was 1.29×10^7 spores/g of substrate and incubating at 35 °C, and for *Aspergillus* sp., 1.75×10^7 spores/g of substrate (Harikrishna et al. 2000). From the above experiments, the optimized medium and conditions in solid state fermentation were: initial pH7.0, growth temperature 30 °C, moisture level 40 %, inoculum size 2×10^6 spores/g of wheat bran, and 1.0 cm depth of the substrate. Under optimal condition for the enzyme production in SSF by *A. niger*, the exoglucanase activity was 28.60 FPU/g of wheat bran.

Effect of growth temperature

The effect of temperature on enzyme production was investigated by incubating the flasks at temperatures between 25 and 40 °C as shown in Fig. 2. At 30 °C, the maximum enzyme yield was 28.60 FPU/g of wheat bran (specific activity 0.84 FPU/mg protein), whereas the enzyme yield was reduced to 7.0 FPU/g of wheat bran (specific activity 0.23 FPU/mg protein) at a temperature of 40 °C with significant reduction in the microbial growth. Similarly, at the lower temperature of 25 °C, the yield was reduced to 8.0 FPU/g of wheat bran (specific activity 0.27 FPU/mg protein).

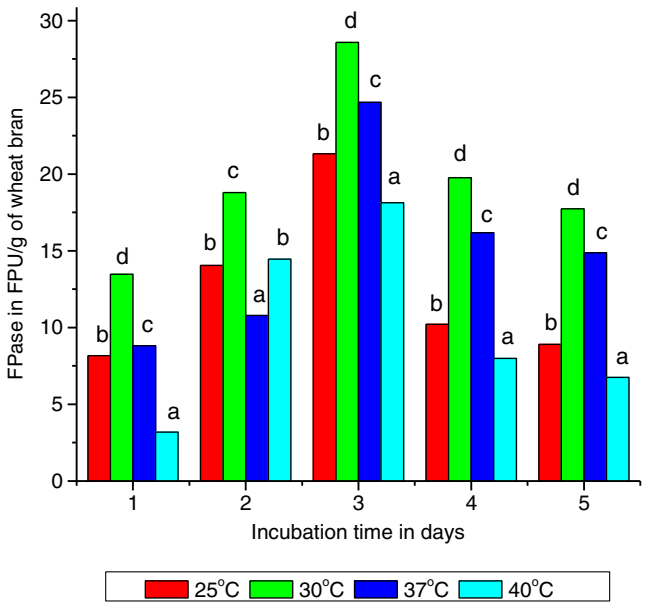

Fig. 2 Influence of growth temperature on the biosynthesis of FPase (expressed in terms of filter paper units) by *A. niger* in SSF Means, in each time interval, followed by the same letter are not significantly different ($P\leq0.05$) from each other according to DMR test. One filter paper unit is the amount of enzyme in the culture filtrate releasing 1 μmole of reducing sugar from filter paper per min

In SSF, temperature effect on production of cellulolytic enzymes from different fungi on different substrates has been reported in the literature by many investigators (Lonsane et al. 1985; Singhania et al. 2006). The optimum temperature of 32 °C has been recorded for the maximum production of cellulolytic enzymes by *A. niger* F-119 on radical wastes (Fadel 2000). At this temperature, yields of protein, FPase, CMCase, and β-glucosidase activities were 88.5 mg/g, 24.4, 36.4, and 211.12 U/g, respectively. Higher cellulase production was observed when the SSF was performed at 35–45 °C using *Penicillium chrysogenum* (Sharma et al. 1996). *Aspergillus niger* and *Bacillus subtilis* showed the maximum yields of cellulase at 35 °C (Sunitha et al. 2006; Krishna 1999). Cultivation of *Gliocladium virens* at 30 °C led to maximal production of cellulases (Singh and Garg 1995). The higher cellulase activity was observed at 30 °C (Yang et al. 2004). The incubation temperature at 45 °C was found to be optimum for production of cellulolytic enzymes by *Myceliophthora* sp. IMI 387099 (Badhan et al. 2007). Thus, occurrence of optimal production of enzyme at different temperatures may be related to the growth kinetics of the microorganism employed rather than the enzyme produced (Lonsane et al. 1985). In the present study, cultivation of *A. niger* in SSF on wheat bran at a temperature of 30 °C generated higher production of exoglucanase. Yields of exoglucanase obtained in the present study were comparable to the studies of Fadel (2000) and Singhania et al. (2006). This indicates that exoglucanase is highly sensitive towards temperature, and that high and low temperatures decrease the growth of microorganism and enzyme production.

Conclusion

Cost-effective technologies are needed for the production of this enzyme, and SSF is a suitable technology for efficient production of exoglucanase using lignocellulosic residues as substrate. Major parameters affecting the fermentation process for exoglucanase production were studied and optimal levels were identified. It is concluded from the findings that the strategy to produce exoglucanase from wheat bran was successful, as it resulted in a considerably good amount of this enzyme produced by *A. niger* under laboratory conditions (Chandra et al. 2007; Maurya et al. 2012).

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