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Full Length Research Paper

Fungal xylanase production under solid state and submerged fermentation conditions

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Seventy fungal strains were isolated from soils collected from different parts of southern Kerala, India. The strains were screened for xylanase production using Czapek's agar medium. On the basis of clearing zones formed, 34 fungal strains were selected and identified. Solid state and submerged fermentation were done to identify strains that could produce maximum amount of xylanase, as well as to identify those strains that could produce cellulase- free xylanase under these conditions. All strains produced cellulase along with xylanase in solid state fermentation, while 70% of the strains produced cellulase-free xylanase during submerged fermentation.

Key words: Xylanase, Czapek's agar, solid state fermentation, submerged fermentation.

INTRODUCTION

Xylan is the major hemicellulose constituent of hard wood and soft wood, and is the next most abundant renewable polysaccharide after cellulose. Xylan is a heterogeneous carbohydrate, consisting of a homopolymeric backbone of β-1, 4 linked D-xylopyranose units and short chain branches consisting of O-acetyl, α-L-arabinofuranosyl and α-D-glucuronyl residues. Endo-xylanase (β-1,4-D-xylan-xylanohydrolase, EC 3.2.1.8) is the key enzyme for xylan depolymerization. A large number of bacteria and fungi are known to produce xylanases (Kulkarni et al., 1999; Subramaniyan and Prema, 2002). Filamentous fungi are industrially important producers of this enzyme due to extracellular release of xylanases, higher yield com-pared to yeast and bacteria and production of several auxiliary enzymes that are necessary for debranching of the substituted xylans (Haltrich et al., 1996). However, fungal xylanases are generally associated with concurrent production of cellulases. Xylanases are produced by either solid state or submerged fermentation. Enzyme productivity in solid state fermentation (SSF) is usually much higher than that of submerged fermentation (Haltrich et al., 1996). Therefore, solid state fermentation has gained interest from researchers in recent years and has often been employed for the production of xylanases because of economic and engineering advantages (Pandey et al., 1999). Microbial xylanases are used in the animal feed, textile and food processing industries, and in the production of several valuable products like xylitol and ethanol (Salles et al., 2005). Biobleaching of pulps using xylanase is one of the most suitable applications in the pulp and paper industry to reduce and/or eliminate the use of chlorine and chlorine dioxide.

Main objectives of the study included isolation of potential xylanase-producing fungi from soil and to identify strains that secrete the maximum amount of xylanase during fermentation. The work also aimed to isolate fungal strains that could produce cellulase-free xylanase under solid state and submerged fermentation conditions.

MATERIALS AND METHODS

Chemicals

Birch wood xylan was purchased from Sigma Chemicals Co., USA, while carboxy methyl cellulose was purchased from Merck, India.

Isolation of fungi

All fungal species were isolated from soil samples collected from different places of southern Kerala, India. The dilution plate-method was employed for the isolation of fungal strains (Johnson and Curl, 1972). Sabouraud dextrose agar (SDA) medium containing 0.1% (w/v) birch wood xylan was used as the isolation medium. The plates were incubated at 30ºC for 7 days. The fungal strains were transferred to fresh SDA plates containing 0.1% (w/v) birch wood

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xylan until pure cultures were obtained. The pure cultures were identified and stored on SDA slants containing 0.1% (w/v) birch wood xylan. The identification of fungal cultures was done according to Fisher and Cook (1998) and de Hoog et al. (2000).

Screening for xylanase production on Czapek's agar medium

Seventy fungal isolates were screened for their abilities to produce extracellular xylanase during their growth on Czapek's agar medium containing xylan as the sole carbon source. The composition of the medium was ($g.L^{-1}$): birch wood xylan, 5.0; peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.2 and agar, 20.0 (Nakamura et al., 1993). The inoculated plates were incubated for 7 days at 30ºC. The clearing zones formed around the fungal growth were more visible when the plates were flooded with 0.1% (w/v) Congo Red. After 30 min of incubation, plates were washed with 1 M NaCl. A total of 34 fungal strains, which produced distinct clearing zones around their colonies, were selected. The amount of xylanase produced was quantified under solid state and submerged fermentation conditions.

Xylanase production under solid state fermentation

The fungi were cultured in Erlenmeyer flasks (250 ml) containing 10 g of wheat bran (particle size 300 - 500 m) moistened with 10 ml of mineral salts solution. The composition of the mineral salts solution was ($g.L^{-1}$): KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $(NH_4)_2HPO_4$, 2.5; NaH_2PO_4, 0.5; $CaCl_2.2H_2O$, 0.01; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.002 and birch wood xylan, 1.0. The pH was adjusted to 5. The medium was then autoclaved for 20min at 121ºC (15 lbs). After cooling, the flasks were inoculated with 1 ml of spore suspension containing $1ξ10^6$ spores ml^{-1}. The spore suspension was obtained from 7 day-old pure cultures. After mixing, flasks were incubated at 30ºC under static conditions for 7 days.

After incubation, the enzyme was harvested in sodium citrate buffer (50 mM, pH 5.3). The fermented slurry was filtered through cheese cloth and centrifuged at 10 000 x g for 20 min at 4°C. The clear supernatant was used for enzyme assays.

Xylanase production under submerged fermentation

The composition of mineral salts medium was the same as that of the solid state fermentation with birch wood xylan as the carbon source. However, wheat bran was not added. The pH of the medium was adjusted to 5.0. Fifty ml of the medium was transferred into a 250 ml Erlenmeyer flask, and after autoclaving was inoculated with 1 ml of spore suspension containing 1×10^6 spores ml^{-1}. The flasks were incubated at 30ºC on a rotary shaker (100 rpm) for 7 days. After incubation, the medium was filtered through Whatman No.1 filter paper and the filtrate was centrifuged at 10 000 x g for 15 min at 4ºC. The clear supernatant was used as source of xylanase.

Enzyme assays

Xylanase activity was determined by mixing 0.9 ml of 1% (w/v) birch wood xylan (prepared in 50 mM Na-citrate buffer, pH 5.3) with 0.1 ml of suitably diluted enzyme and the mixture was incubated at 50°C for 5 min (Bailey et al., 1992). The reaction was stopped by addition of 1.5 ml of 3,5-dinitrosalicylic acid (DNS) and the contents was boiled for 5 min (Miller, 1959). After cooling, the colour developed was read at 540 nm. The amount of reducing sugar liberated was quantified using xylose as standard. One unit of xylanase is defined as the amount of enzyme that liberates 1 μmol of xylose equivalents per minute under the assay conditions.

Cellulase (CMCase) activity was determined by mixing 0.9 ml of 1% (w/v) CMC (prepared in 50 mM Na-citrate buffer pH 5.3) with 0.1 ml of suitably diluted enzyme and incubating at 50°C for 15 min (Ghose, 1987). The reaction was stopped by addition of 1.5 ml of 3,5-dinitrosalicylic acid (DNS) and the contents was boiled for 5 min. The colour developed was read at 540 nm. The amount of reducing sugar liberated was quantified using glucose as standard. One unit of cellulase is defined as the amount of enzyme that liberates 1 μmole of glucose equivalents per minute under the assay conditions.

Determination of protein

The concentration of soluble protein was estimated using bovine serum albumin as the standard (Lowry, 1951).

RESULTS AND DISCUSSION

Among the best xylanase producers, *Aspergillus* was the most common genus and represented 55% of the selected strains. *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* (Figure 1a) and *Aspergillus ochraceous* (Figure 1b) were the most prevalent species. *A. niger* constituted 52.6% of the total *Aspergilli* isolated. Similar reports were obtained by Kadowaki et al. (1995), Das and Nanda (1995), Carmona et al. (1997) and Abdel-Sater and El-Said (2001). *Aspergillus sydowii* (Figure 1c), a rare species, was also isolated. *Penicillium* was the second most common genus and 27% of the selected fungal strains were *Penicillium* sp. (Figure 1d). *Penicillium citrinum* (Figure 1e) and *Penicillium janthinellum* were the most commonly isolated species. *Penicillium* species have been isolated frequently from soils, decomposing organic matters, agricultural and forest residues, composts and manures (Banerjee et al., 1995; Satyanarayana et al., 1988). *Trichoderma* (Figure 1f) represented the third most common genus. It constituted 11% of the selected strains. *Gliocladium* sp. was also isolated rarely.

All strains produced xylanase along with cellulase during SSF (Table 1). *Aspergillus* sp. were the best xylanase producers. Among others, *Penicillium* sp. SBS 2 secreted a high amount of xylanase (751 IU ml^{-1}) with the highest extracellular protein concentration (93 $mg.gds^{-1}$). By contrast, *P. citrinum* SBS 22 produced the least amount of xylanase (40 IU ml^{-1}).

Concomitant cellulase production with xylanase was monitored during solid state fermentation and all 34 fungal strains produced cellulase along with xylanase. This might be because of the presence of cellulose in wheat bran, the substrate for SSF. Previously it was reported that both xylanase and cellulase was produced when cellulose and hemicellulose were used together as carbon source (Kulkarni et al., 1999). Haltrich et al. (1996) reported that xylan-degrading organisms are often

Table 1. Xylanase production by different strains in solid state fermentation (SSF).

Serial No	Name of Strain	Xylanase IU gds^{-1}	Cellulase IU gds^{-1}	Protein mg gds^{-1}
1	*Aspergillus niger* SBS3	724	10	18
2	*A. niger* SBS6	689	49	19
3	*A. niger* SBS8	644	8	17
4	*A. niger* SBS14	742	43	17
5	*A. niger* SBS19	630	44	20
6	*A. niger* SBS24	639	44	21
7	*A. niger* SBS28	408	19	15
8	*A niger* SBS47	735	51	51
9	*A.niger* SBS57	753	35	13
10	*A. niger* SBS66	719	35	7
11	*Aspergillus flavus* SBS44	738	25	18
12	*A. flavus* SBS65	191	1	20
13	*A. flavus* SBS68	650	23	13
14	*Aspergillus fumigatus* SBS58	608	35	46
15	*A. fumigatus* SBS62	613	32	19
16	*A.fumigatus* SBS63	587	20	27
17	*Aspergillus ochraceus* SBS4	489	9	10
18	*A. ochraceous* SBS16	639	19	16
19	*A. ochraceous* SBS35	590	32	20
20	*Aspergillus sydowii* SBS45	543	7	26
21	*Penicillium* sp. SBS2	751	22	93
22	*Penicillium* sp. SBS10	554	24	21
23	*Penicillium* sp. SBS32	591	20	20
24	*Penicillium* sp.SBS52	696	37	49
25	*Penicillium citrinum* SBS22	40	6	15
26	*P citrinum* SBS26	739	30	17
27	*P. citrinum* SBS51	215	11	13
28	*P citrinum* SBS53	221	9	13
29	*Penicillium janthenellum*SBS18	666	20	35
30	*Trichoderma* sp. SBS56	325	3	12
31	*Trichoderma* sp. SBS60	564	37	43
32	*Trichoderma* sp. SBS64	361	5	22
33	*Trichoderma* sp. SBS67	625	32	56
34	*Gliocladium* sp. SBS13	428	6	17

IU gds^{-1} – International Unit per gram dry substance.

cellulolytic and secrete complex mixtures of xylanases and cellulases concurrently.

Xylanase production in SSF was much higher than that in submerged fermentation (SmF). Malarvizhi et al. (2003) observed 30-fold enhancement of xylanase production in solid state fermentation than liquid culture when wheat bran was used as the substrate for a culture of *Ganoderma lucidum*. Xylanase is an inducible enzyme and the xylan present in wheat bran as well as birch wood xylan acted as good inducers for enzyme production. Haltrich et al. (1996) reported that addition of small amounts of purified xylan to complex lignocellulosic substrates like wheat bran resulted in considerable enhancement of xylanase production. Wheat bran proved to be a suitable substrate along with 0.1% birch wood xylan for the production of xylanase during SSF. Several workers reported the suitability of wheat bran for xylanase production by SSF (Gawande and Kamat, 1999; Malarvizhi et al., 2003) . Commercial wheat bran consists of 30% cellulose, 27% hemicellulose, 21% lignin and 8% ash (Gawande and Kamat, 1999).

Among 10 *A. niger* isolates, six could produce cellulase

Table 2. Xylanase production by different strains in submerged fermentation (SmF).

Serial No.	Name of Strain	Xylanase IU ml^{-1}	Cellulase IU ml^{-1}	Protein μg ml^{-1}
1	*Aspergillus niger* SBS3	33	-	100
2	*A. niger* SBS6	35	1.76	100
3	*A. niger* SBS8	35	1.5	80
4	*A. niger* SBS14	24	-	85
5	*A. niger* SBS19	39	-	129
6	*A. niger* SBS24	38	-	40
7	*A. niger* SBS28	39	-	80
8	*A. niger* SBS47	32	2	130
9	*A. niger* SBS57	27	2	235
10	*A. niger* SBS66	22	-	45
11	*Aspergillus flavus* SBS 44	31	-	17
12	*A. flavus* SBS65	6	-	10
13	*A. flavus* SBS68	30	-	59
14	*Aspergillus fumigatus* SBS58	38	-	243
15	*A. fumigatus* SBS62	38	-	29
16	*A. fumigatus* SBS63	20	-	40
17	*Aspergillus ochraceous* SBS4	10	-	25
18	*A. ochraceuos* SBS16	32	2	15
19	*A. ochraceous* SBS35	36	-	80
20	*Aspergillus sydowii* SBS45	40	-	60
21	*Penicillium* sp. SBS2	2	1.76	50
22	*Penicillium* sp. SBS10	27	-	80
23	*Penicillium* sp. SBS32	1	1.2	45
24	*Penicillium* sp.SBS52	15	-	10
25	*Penicillium citrinum* SBS 22	29	-	72
26	*P citrinum* SBS26	30	1	15
27	*P. citrinum* SBS51	16	2	3
28	*P citrinum* SBS53	12	-	60
29	*Penicillium janthenellum* SBS18	26	-	80
30	*Trichderma* sp.SBS56	35	-	80
31	*Trichoderma*sp. SBS60	24	25	43
32	*Trichoderma*sp. SBS64	7	-	10
33	*Trichoderma* sp.SBS67	17	-	30
34	*Gliocladium* sp.SBS13	34	-	72

- : not detected any cellulase activity

-free xylanase (Table 2) under SmF. Previously Costa-Ferreira et al. (1994) reported that *A. niger* isolates produced cellulase-free xylanase in shake flask experiments. Maximum amount of xylanase was produced by *A. sydowii* SBS 45 (40 IU ml^{-1}). All *A. flavus* and *A. fumigatus* isolates produced cellulase-free xylanase. Arunachalam et al. (2001) isolated cellulase-free xylanase from *A. flavus* isolates, while Bailey and Viikari (1993) isolated cellulase-free xylanase from *A. fumigatus* isolates. Among the isolates of *Penicillium* species, *P. citrinum* SBS 26 exhibited maximum xylanase activity (30 IU ml^{-1}). *Trichoderma* sp. were moderate producers of xylanase. *Gliocladium* sp. SBS13 produced 34 IU ml^{-1} of xylanase without cellulase contamination.

Most researchers have used submerged cultures for xylanase production, which allows control over the degree of aeration, pH and temperature of the medium as well as control over other environmental factors required for optimum growth of organisms. In the present study, cellulase production was absent in 70% of strains under submerged fermentation (SmF) conditions. In submerged fermentation, the only available carbon source was xylan. Haltrich et al. (1996) reported that purified xylans can be excellent substrates for xylanase production and are frequently used for small- scale experiments. In a number of organisms, these pure and defined substrate increased

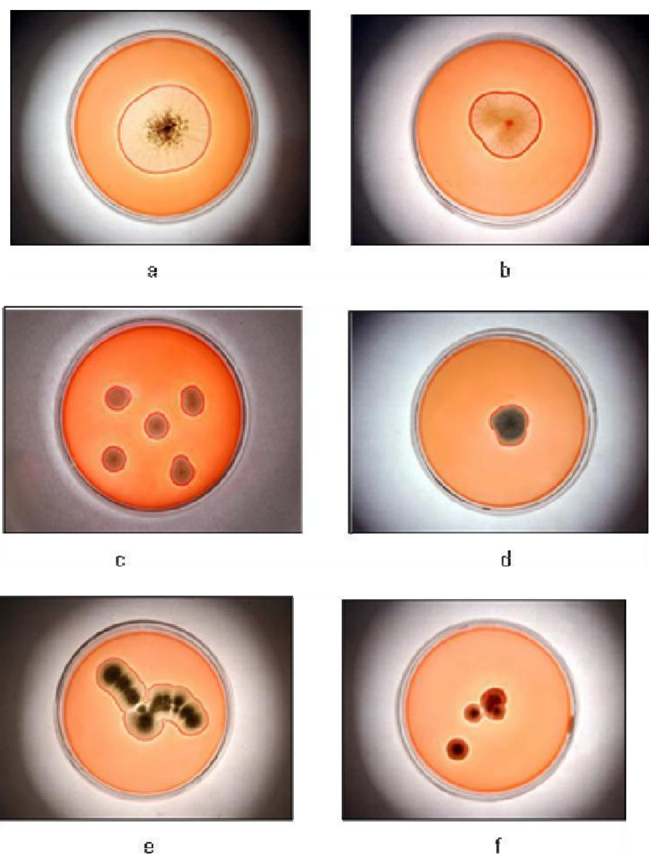

Figure 1. Growth of fungal strains on Czapek's Agar medium. After 7 days of incubation, the medium was stained in 0.1% (w/v) Congo Red. Remarkably sharp outline of each colony was due to deeply stained actively growing mycelia. (a. *Aspergillus fumigatus* SBS 58; b. *Aspergillus ochraceus* SBS 35; c. *Aspergillus sydowii* SBS 45; d. *Penicillium* sp. SBS 2; e. *Penicillium citrinum* SBS 26; f. *Trichoderma* sp. SBS 67)

the yield of xylanase, caused a selective induction of xylanase, either with complete absence or with low cellulase activities (Biswas et al., 1990; Gilbert et al., 1992).

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