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Use of *Scopulariopsis acremonium* for the production of cellulase and xylanase through submerged fermentation

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Production of cellulase and xylanase by *Scopulariopsis acremonium* through submerged fermentation was studied using shake flask cultivation media. The effect of process parameters such as effect of temperature, pH and inoculum size was investigated. The enzymes were partially purified using isopropanol and used for enzyme assays. The results indicated that the maximum cellulase and xylanase having an enzyme activity of 694.45 and 931.25 IU, respectively, were produced at 30°C incubation temperature. The pH optimum to achieve these enzyme activities was 5.5 with an inoculum size of 1 x 10^5 spores ml^{-1} of tween - 80. Cellulase was deactivated at 80°C while xylanase was deactivated at 70°C.

Key words: *Scopulariopsis acremonium*, cellulase, xylanase, submerged fermentation.

INTRODUCTION

Microorganisms in particular have been regarded as a treasure source of useful enzymes, because they multiply at extremely high rate and synthesize biologically active products which can be controlled by humans. In recent years, there has been a phenomenal increase in the use of enzymes as industrial catalysts. These enzymes offer advantage over the use of conventional chemical catalysts for numerous reasons: they exhibit high catalytic activity, a high degree of substrate specificity, can be produced in large amounts, are highly biodegradable, pose no threat to the environment and are economically viable (Gote, 2004) . Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial applications, like cellulase and xylanase whose levels in fungi are generally much higher than those in yeast and bacteria (Haltrick et al., 1996).

Submerged fermentation is used for the mass production of microorganisms with the goal of either isolating the microorganisms themselves or the metabolic product or a microbial altered substrate (e.g. in the food processing industry). Microorganisms in submerged fermentation (SmF) thrive in a liquid environment. Generally, the submerged fermentation processes are carried out in shake flasks or aerated agitated fermentor equipped with controls of fermentation parameters. Submerged fermentation has several advantages over solid state fermentation such as: it requires less man power, gives higher yield and productivity, low cost, less contamination and better temperature control during fermentation (Frost and Moss, 1987). The submerged fermentation for aerobic microorganisms is now well known and widely used method for the production of cellulase and xylanase (Garcia et al., 2002) . Cellulose being an abundant and renewable resource is a potential raw material for the microbial production of fuel and chemicals (Coughlan, 1985). Various bacteria, actionmycetes and filamentous fungi produce extra cellular cellulases when grown on cellulosic substrates though many actinomycetes have been reported to have less cellulase activity than moulds (Ishaque and Kluepfel, 1980; Kluepfel et al, 1986). Fungal cellulases are industrially important enzymes and find wide applications in foods, textiles, tissue culture etc. (Gayal and Khandeparkar, 1998) . The complete degrading of cellulose by fungi is made by a cellulolytic enzyme system. The role and action mechanism of the components of the system have been a center of many

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enzyme studies for the last three decades (Silva et al., 2005) . The cellulase enzyme system consists of three major components: endo-ß-glucanase (EC 3.2.1.4), exo-ß-glucanase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21). The mode of action of each of these being:

1. Endo-ß-glucanase, 1, 4-ß-D-glucan glucanohydrolase, CMCase, Cx: "random" scission of cellulose chains yielding glucose and cello-oligo saccharides.
2. Exo-ß-glucanase, 1, 4-ß - D-glucan cellobiohydrolase, Avicelase, C1: exo-attack on the non-reducing end of cellulase with cellobiose as the primary structure.
3. ß-glucosidase, cellobiase: hydrolysis of cellobiose to glucose.

Enzymes like cellulase have been used in the bioconversion of lignocellulosic materials into biofuels like ethanol (Wheals et al., 1999). Recently alkali stable cellulases have gained commercial importance because of their potential applications in textile, paper/pulp and detergent industries. These enzymes are also used in denim washing (Kierulff, 1997). Xylanase (EC 3.2.1.8) catalyses the hydrolysis of xylopyranosyl linkages of -1, 4-xylan, a plant polysaccharide next to cellulose in abundance. Owing to increasing biotechnological importance of hermostable xylanases, many thermophilic fungi have been examined for xylanase production (Maheshwari et al., 2000; Singh et al., 2003). Xylanases (1, 4-52 - D-xylanohydrolase; EC 3.2.1.8) are hemicellulases that hydrolyzes xylan, which is a major constituent of 1, 4-linked xylopyranose units with branches containing L-arabinofuranosyl and D-glucoronopyranose residues (Biswas, 1990).

Biotechnological uses and potential applications of xylanases include bioconversion of lignocellulose material to useful product e.g. production of whole wheat bread with improved body texture and flavour, clarify-cation of juices, improvement of the consistency of the beer and the digestibility of animal feedstock (Wong et al., 1998).

An enzyme should be stable and fast reacting during reaction, should have a low transferase activity and should be produced by organisms free of toxicity. *Scopulariopsis acremonium* is specifically recognized for cellulase and xylanase production. This is generally recognized as safe (GRAS).

MATERIALS AND METHODS

Microorganism and inoculum preparation

S. acremonium was procured from the institute of Microbial Technology, Chandigarh and was maintained at 4°C on potato dextrose agar (PDA). Spore suspension was made from ten days old cultures that had been grown on PDA slants at 30°C. It was prepared by suspending the spores from one tube in 10 ml of sterilized distilled water containing 0.1ml Tween – 80. The spore count was adjusted to10^5 spores ml^{-1} (Smith et al., 1996). One ml of inoculum was used per flask to carry out submerged fermentation.

Shake flask cultivation media

The enzymes were produced using basal medium (Manomani et al., 1983). The basal medium contained the following ingredients (gl^{-1}): $(NH_4)SO_4$ - 1.4, $CO(NH_2)_2$ - 0.3, KH_2PO_4 - 4.0, K_2HPO_4 – 0.84, $CaCl_2.2H_2O$ – 0.3, $MgSO_4.7H_2O$ – 0.3, $FeSO_4.7H_2O$ – 0.005, $MnSO_4.H_2O$ – 0.00156, $ZnCl_2$ – 0.00167, Peptone 0.25, Yeast extract – 0.10, rice bran – 4%. All the components of the basal medium except rice bran were dissolved in distilled water. Flasks having 250 ml capacity were taken and 50 ml of this solution was poured in each flask. To each flask 2.0 g of rice bran was added (Thenawadjaja et al., 1990) and autoclaved at 1.1 kg/cm^2 for 20 min. Flasks were cooled and inoculated with 1 ml of the spore suspension. The inoculated flasks were incubated at 30°C on a rotary shaker cum BOD incubator revolving at 150 rpm (Seyis and Aksoz, 2005; Meenaksui et al., 2005) . After 96 h of growth, the contents of each flask were centrifuged at 4°C and this supernatant was used as the source of crude enzyme (Nehra et al., 2002).

Partial purification of the fungal enzymes

Cellulase and xylanase were isolated and partially purified with isopropanol (Manomani et al., 1983). The mold filtrate (20 ml) was chilled at 4°C in a refrigerator and 25 ml of chilled isopropanol was added to it. The 101 precipitates were separated by centrifugation. The precipitates were dissolved in 5 ml of 0.05 M citrate phosphate buffer having pH 5.2 (Rajinder, 1992) to obtain partially purified enzymes.

Enzyme assays

Cellulase was estimated as carboxymethyl cellulase (CMCase) according to the method given by Ray et al. (1993). Xylanase activity was assayed according to the method of Geese and Gashe (1997). Reducing sugars were measured as glucose and xylose equivalents by DNS method (Miller, 1959). The absorbance was read at 575 nm. Enzyme activities of the partially purified enzymes were expressed in International Units (IU). One IU was defined as one μmol of xylose (for xylanase activity) or glucose (for carboxy methyl cellulase activity) equivalents released per minute per ml under the following assay conditions by using xylose or glucose standard curve prepared under standard assay conditions (Silva et al., 2005) . Appropriate dilution factors were used during the estimation of the enzymes.

Factors affecting enzyme production

The effect of initial culture pH on the enzyme production was investigated by adjusting the initial pH of the basal medium in the range of pH 5 - 8. To examine the effect of temperature on enzyme production the inoculated culture flasks were incubated at different temperature varying between 15 - 40°C and then the enzyme activities were determined by using the partially purified enzymes. The effect of inoculum size based on the number of spores was examined using the spore concentration of $1x10^3$, $1x10^4$, $1x10^5$ and $1x10^6$ spore ml^{-1} of Tween - 80 (Kheng and Omar, 2005) for making the spore suspension. The flasks having the sterile 119 basal medium were inoculated with the spore suspension and were incubated at 30°C on a BOD shaker cum incubator for 96 h and then the partially purified enzymes were used for the determination of the enzyme activities.

Testing of enzyme stability

To test the stability of the enzymes, the CMCase and xylanase

Table 1. Effect of temperature on the production of fungal enzymes.

Temperature (°C)	CMCase (IU)	Xylanase (IU)
15	000.50	000.24
20	010.58	008.23
25	199.83	287.50
30	694.45	931.25
35	154.76	276.04
40	119.04	173.00

Medium – Basal Medium (Manomani et al., 1983), incubation time- 96 h, temperature – 30 ± 2°C, pH - 5.5, the data represents the mean of three determinations each.

were incubated at different temperatures ranging from 40 - 80°C for 60 min (Silva et al., 2005). The reaction was stopped in ice cold water and the remaining activity was measured under standard assay conditions.

RESULTS AND DISCUSSION

The use of partially purified cellulase and xylanase improves the cost of enzyme production and is a major limitation of the economically feasible bioconversion and utilization of lignocellulosic materials. Therefore, in this study, easily available agricultural residue that is, rice bran was used as the substrate in the experiment to obtain optimum levels of cellulase and xylanase production by *S. acremonium*. The enzyme activities of the partially purified cellulase and xylanase were found to be 695.45 and 931.25 IU, respectively under the optimized conditions. Cellulase deactivated at 80°C while xylanase deactivated at 70°C.

Effect of temperature

The effect of different temperatures ranging from 15 - 40°C on the production of fungal enzymes, cellulase and xylanase by *S. acremonium* was studied. At 15°C, all enzyme activities were low which showed a gradual increase with the increase in temperature at 30°C. Further increase in temperature resulted in decrease in enzyme production indicating that 30°C was the optimum temperature for the maximum production of the enzymes (Table 1). Less activity of fungal enzymes at low temperature (15 - 25°C) and at high temperature (35 - 40°C) as compared to 30°C might be due to slow growth at low temperature and inactivation of the enzyme at high temperature. This observation was in agreement with those reported by Sudgen et al. (1994) and Biswas et al. (1990) who showed that the highest xylanase activities were obtained at temperatures that were optimum for the growth of fungi in solid state fermentation. Similarly Gupta et al. (1990) reported the optimum temperature for protein production and extracellular enzymes (cellulase and xylanase) by *Coprinus attramantarius,* utilizing cellulose to be 30°C. Kheng and Omar (2005) reported the ambient temperature for xylanase production to be 28 ± 3°C using the fungal isolate *Aspergillus niger* USM AI 1 using palm kernel cake (PKC) as substrate.

Effect of pH on the production of fungal enzymes

The negative logarithm of H_3O^+ ion concentration in moles per litre is defined as the pH. Effect of different pH values ranging from 4.0 to 9.0 on the production of fungal enzymes (cellulase and xylanase by *S. acremonium* and protease by *Rhizopus microsporus var oligosporus*) was studied by altering the pH of the basal medium that was used for carrying out the submerged fermentation. Production of the enzymes cellulase, xylanase and protease increased with increase in pH value, reaching the maximum at pH 5.5, followed by a gradual decrease thereafter (Table 2) . The decrease or increase in activities at different pH values than the optimum pH might be due to decreased production of the mycolytic enzymes. Similarly, Gupta et al. (1990) reported the optimum pH for protein production and extracellular enzymes (cellulase and xylanase) by *C. attramantarius,* utilizing cellulose to be 6. The resulting enzyme activities were endoxylanase as 7.2 IU ml^{-1}, exoglucanase as 1.0 IU ml^{-1} and xylanase as 5 IU ml^{-1}. Whereas a pH of 4.14 was reported to be optimum for the production of endoxylanase production by *Aspergillus awamori* under submerged fermentation which gave an enzyme activity of 28.25 U ml^{-1} (Li et al., 2006).

Effect of inoculum size (spore density) on the production of fungal enzymes

The effect of inoculum size based on the number of spores was examined using the spore concentration of 1 x 10^3, 1 x 10^4, 1 x 10^5 and 1 x 10^6 spores ml^{-1} on the production of fungal enzymes. It was found that 192 the increase in inoculum size resulted in rapid increase in enzyme 193 production (Table 3) due to fast degradation of the substrate. Similar observations were reported by Raimbault and Alazard (1980) who showed that maximum enzyme production and declination was achieved much faster due to the rapid degradation of the substrate a consequence of rapid growth. The enzyme activities did no the substrate. Hence, based on this the inoculum size of 1 x 10^5 was used for inoculation of basal medium. Similarly, an inoculum size of 1x10^5 spores of *Aspergillus oryzae* gave maximum - amylase activity in the range of 325 - 424 units ml^{-1} of culture filterate using agro industrial wastes (Manomani et al., 1983). However, Kheng and Omar (2005) used inoculum size of 1 x 10^4 spores of *A. niger* USM AI 1 through SSF using palm

Table 2. Effect of pH on the production of fungal enzymes.

pH	CMCase (IU)	Xylanase (IU)
4.0	058.65	104.25
4.5	191.68	535.26
5.0	587.35	810.63
5.5	694.45	931.25
6.0	588.18	813.81
6.5	456.71	744.63
7.0	326.82	546.79
7.5	175.63	283.75
8.0	045.62	099.10
8.5	010.21	015.25
9.0	000.45	000.25

Medium – Basal Medium (Manomani et al., 1983), incubation time- 96 h, temperature – 30 ± 2°C, pH - 5.5, the data represents the mean of three determinations each.

Table 3. Effect of inoculum size on production of fungal enzymes.

Inoculum size (Spores ml^{-1})	CMCase (IU)	Xylanase (IU)
1×10^3	256	256
1×10^4	338	635
1×10^5	695	930
1×10^6	680	915

Medium – Basal Medium (Manomani et al., 1983), incubation time- 96 h, temperature – 30 ± 2°C, pH - 5.5, the data represents the mean of three determinations each.

kernel cake as substrate and reported maximum xylanase activity (23.97 U g^{-1}).

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