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Research Article

OPTIMIZATION OF XYLANASE PRODUCTION BY *GLEOMASTIX INDICUS* VIA SOLID STATE FERMENTATION

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ABSTRACT

Xylanases are of great significance with tremendous potential to offer the industry. These are one of the major enzymes of the commercial sector, especially in paper and pulp industry. Major applications of xylanases also include bioconversion of lignocellulosic material and agro-wastes to fermentative products, clarification of juices, improvement in consistency of beer and the digestibility of animal feed stock. Therefore in the present study the production of xylanase by the fungus *Gleomastix indicus* under solid-state fermentation (SSF) was investigated. Optimal xylanase production (99.96 U/ml) was obtained using wheat bran as the solid substrate with 96 hours, pH 8, 28 ± 3^{0}C, 20% substrate concentration and inoculum level of 20% (w/v). Galactose and potassium nitrate are the best carbon and nitrogen sources respectively.

INTRODUCTION

Xylan, the second most abundant polysaccharide and a major component in plant cell wall consists of β-1, 4-linked xylopyranosyl residues. The plant cell wall is a composite material in which cellulose, hemicellulose (mainly xylan) and lignin are closely associated[1,2]. Three major constituents of wood are cellulose (35-50%), hemicellulose (20-30%) - a group of carbohydrates in which xylan forms the major class and lignin (20-30%). Xylan is a heteropolysaccharide containing substituent groups of acetyl, 4-Omethyl-D-glucuronosyl and α-arabinofuranosyl residues linked to the backbone of β-1, 4-linked xylopyranose units and has binding properties mediated by covalent and non-covalent interactions with lignin, cellulose and other polymers. Lignin is bound to xylans by an ester linkage to 4-O-methyl-D-glucuronic acid residues[1]. The depolymerisation action of endo-xylanase results in the conversion of the polymeric substance into xylooligosaccharides and xylose. Xylanases are fast becoming a major group of industrial enzymes finding significant application in paper and pulp industry. Xylanases are of great importance to pulp and paper industries as the hydrolysis of xylan facilitates release of lignin from paper pulp and reduces the level of usage of chlorine as the bleaching agent[3]. xylanases are applicable for delignification in bleaching process were first demonstrated by Viikari *et al.,*(1986)[4]. The applicability of xylanases increases day by day as rayon, cellophane and several chemicals like cellulose esters (acetates, nitrates, propionates and butyrates) and cellulose ethers (carboxymethyl cellulose, methyl and ethyl cellulose) are all produced from the dissolving pulp i.e. the pure form of cotton fiber free from all other carbohydrates.

Solid state fermentation (SSF) is an attractive method for xylanase production, especially for fungal cultivations, because it presents many advantages, such as higher productivity per reactor volume as well as the lower operation and capital cost[5,6]. Solid state fermentation can be performed on a variety of lignocellulosic materials, such as rice bran, wheat bran, ragi bran, corn cob, soya bran etc., which proved to be highly efficient technique in the production of xylanase[7].

Xylanolytic enzymes have several industrial applications in the paper and pulp industries; as food additives to wheat flour, for the extraction of different bioproducts and clarification of juices and wines[8]. A large number of bacteria and fungi are known to produce xylanases[9]. Filamentous fungi are industrially important producers of xylanases due to extracellular release of these enzymes. Therefore, researches in recent years have often been employed for the production of xylanases because of economic and engineering advantages[10].

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The present paper deals with enhanced production of xylanase from a fungal source *Gleomastix indicus* in SSF.

MATERIALS AND METHODS

Organism

The fungal strain *Gleomastix indicus* was obtained from culture bank of Department of Biotechnology, Meerut Institute of Engineering and Technology, Meerut. The strain was screened for xylanase activities during its growth on Czapek's agar medium containing xylan (0.1w/v) as the sole carbon source. The inoculated plates were incubated for 7 days at 28^0C. 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 1M NaCl.

Xylanase Production under solid state Fermentation

Cultivation of fungus was performed in 250 ml Erlenmeyer flask containing 20 g of sterilized wheat bran substrate with the addition of 15 ml of Mandel's medium. The Mandel's medium was prepared with the following composition (g/l) 10.0g; urea, 0.3; peptone, 0.75; yeast extract, 0.25; $(NH_4)_2SO_4$ - 1.4; KH_2PO_4 - 2.0; $CaCl_2$ - 0.3; $MgSO_4.7H_2O$ - 0.3 and trace elements (mg/l): $FeSO_4.7H_2O$ - 5; $MnSO_4$. $4H_2O$ - 1.6; $ZnSO_4.7H_2O$ - 1.4 and $CoCl_2.6H_2O$, 20.0[11]. The flasks were then inoculated with 5 ml ($1X10^3$) spore suspension and incubated at 28^0C for 5 days. The enzyme from each flask was extracted using 50 ml of distilled water. The mixture was vigorously homogenized for 30 minutes at 200 rpm and filtered through Whatmann filter paper 1. The clear supernatant was collected for enzyme assay for xylanase production.

Xylanase Assay

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 suitable diluted enzyme and the mixture was incubated at 50^0C for 5 min[12]. The reaction was stopped by addition of 1.5 ml DNSA and the contents were boiled for 5 min[13]. After cooling the color 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 liberated 1µmol of xylanase equivalent per minute under the assay conditions.

Determination of Protein

The concentration of soluble protein Protein concentration was determined with bovine serum albumin as standard[14].

Enzyme Production on Different Carbon Sources and Nitrogen Sources

The Vogel's medium was supplemented with various carbon and nitrogen sources sources at concentration of 1% (w/v). The carbon sources consist of glucan, xylan, malt extract, lactose and maltose. Similarly, the nitrogen sources were peptone, urea, $NaNO_3$, yeast extract, $(NH_4)_2SO_4$ and NH_4NO_3. Cultivation was carried out at ambient temperature 28^0C for 5 days under agitation (120 rpm). Xylanase activity was determined in each case as described previously.

Effect of Incubation Period, pH and Temperature on Xylanase Production

The effect of incubation period was determined by incubation for different periods 24, 48, 72, 96 and 120 hrs at 28^0C. Effect of pH on Xylanase production from organism under study was determined by growing of organism in fermentation media of different pH using appropriate phosphate buffer (pH 5-6), Tris HCl buffer (pH 7-8) and glycine NaOH buffer (pH 9-10). Xylanase production was measured after 5 days of fermentation period. The effect of cultivation temperature on the enzyme production was examined at ambient temperature 28, 25, 30, 35, 37 and 45^0C. Cultivation was carried out for 5 days.

Effect of Moisture level, Substrate Concentration and Inoculum size

The effect of moisture level on the enzyme production was determined by varying the (w/v) of wheat bran to moistening agent at the ratio of 1:1.0, 1:1.5, 1:2.0, 1:2.5 and 1:3.0. The moistening agent used was sterile distilled water. Effect of substrate concentration of wheat bran on xylanase production was determined by using different amount of substrate (5-40 g) with difference of 5 g inoculated with 5 ml of spore suspension and incubated at 28^0C for 5 days. The effect of inoculum size was determined by adding the spore suspension of concentration of $1X\ 10^3$, $1X\ 10^4$, $1X\ 10^5$ and $1X\ 10^6$ spores/ml prepared using the moistening agent for moisture control in the SSF system. Cultivation was carried out at ambient temperature (28 ± 3^0C) for 7 days.

Table 1 Effect of moisture content on xylanase production.

S.No	Moisture content (w/v)	Xylanase (U/ml)
1	1:1.0	20.5
2	1:1.5	28.5
3	1:2.0	31.5
4	1:2.5	37.4
5	1:3.0	36.9

Table 2 Effect of inoculum size on the xylanase production

S.No	Inoculums size	Xylanase (U/ml)
1	$1X\ 10^3$	40.3
2	$1X\ 10^4$	41.9
3	$1X\ 10^5$	47.3
4	$1X\ 10^6$	46.1

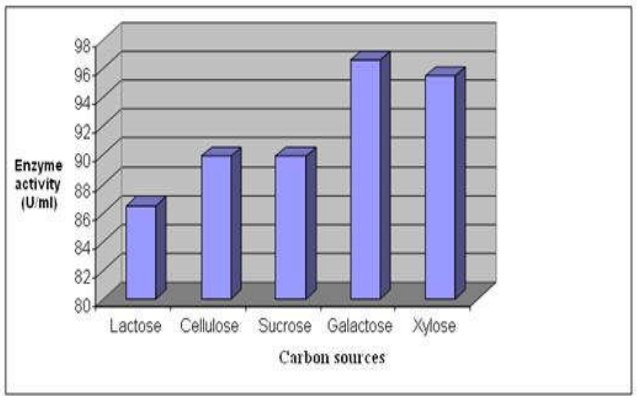

Figure 1 Effect of carbon sources on enzyme production

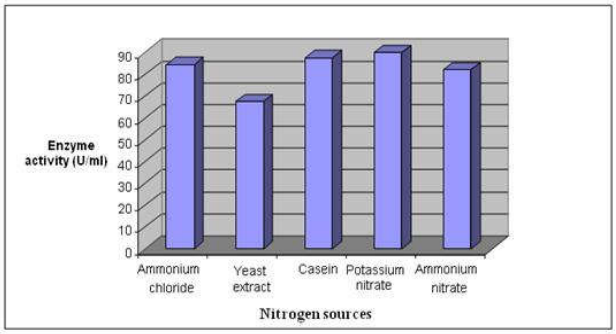

Figure 2 Effect of nitrogen sources on enzyme production

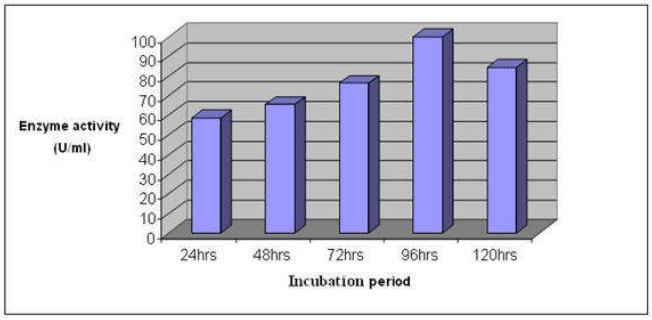

Figure 3 Effect of incubation period on enzyme production

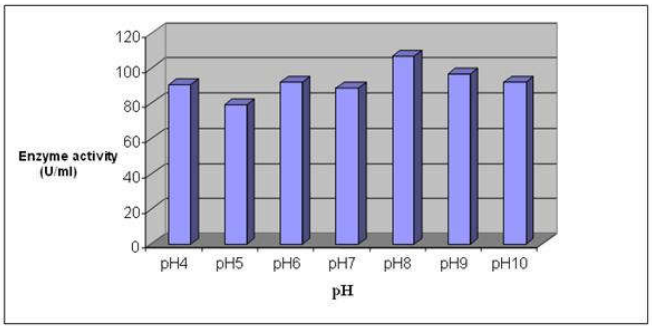

Figure 4 Effect of pH on enzyme activity

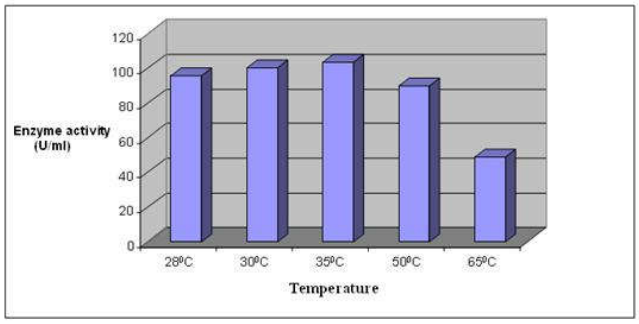

Figure 5 Effect of temperature on enzyme stability

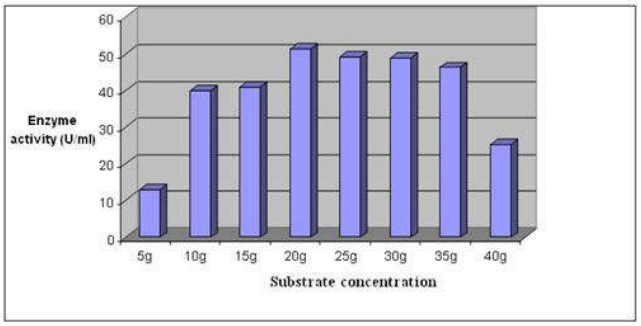

Figure 6 Effect of substrate concentration on enzyme production

RESULTS AND DISCUSSION

Xylanolytic Activity

Xylanolytic activity by *Gleomastix indicus* was investigated by cultivation in Czapek's medium containing xylan (1% w/v) as the sole carbon source. Organism was capable of exhibiting xylanolytic activities with the diameter of the clear zone of 4.7 cm and in quantitative assay organism produced 70 U/ml enzyme in solid-state fermentation (wheat bran medium).

Influence of Carbon and Nitrogen Source on Enzyme Production

The enzyme yield was affected by the carbon and nitrogen source used in the production medium and it is expected that the improvement of the nutritional value of wheat bran by the supplementation of carbon and nitrogen sources will also increase the enzyme production. Figure-1 shows the supplementation of sugars, which may act either as carbon sources or inducers. As shown in the figure, the addition of galactose resulted in an increment in xylanase production. Galactose has been described as an effective inducer and carbon source for xylanase production in several microorganisms including *Fusarium oxysporum*[15] and *Thermomyces. lanuginosus*[5]. Different nitrogen sources were studied for their effect on xylanase production by *Gleomastix indicus*. The results were depicted in Figure-2. Potassium nitrate induces maximum xylanase production (89.89 U/ml) among the organic and inorganic nitrogen sources. Use of potassium nitrate as the best nitrogen sources in the medium for xylanase production has been also reported earlier[15].

Effect of Culture Conditions on Xylanase Production

Incubation period plays a vital role in the metabolic activity of microbial cells and hence the growth. The enzyme assay was performed at every 24 hours. The enzyme production increased periodically and organism shows maximum Xylanase production at the 4th day of incubation (99.96 U/ml) Figure-3. A similar study has been reported by Sandrim *et al*., 2004[16].

The pH of the growth medium is one of the physio-chemical parameter responsible for the morphological changes in the organism and enzyme secretion. In the present study the organism was grown in the production medium having different pH ranging from 4.0 to 10.00. *G. indicus* gave maximum production (106.6 U/ml) at pH 8.0 Figure-4. Xylanase production under alkaline conditions has been reported in many bacteria[17]. On the contrary, fungal species generally favour acidic pH for the Xylanase production[18]. The initial pH of the medium has been found to influence many enzyme systems and their transportation across the cell membrane. Xylanase production by *Aspergillus terreus*[19], *Fusarium* spp.[15], *Aspergillus nidulans*[20] also showed the maximum enzyme production under alkaline conditions.

Temperature is one of the important factor in enzyme production. Therefore, the effect of temperature on Xylanase production by *G. indicus* was examined and the results obtained are shown in Figure-5. The production of Xylanase (99.96 U/ml) was maximum at the ambient temperature 28 ± 3^{0}C. The result obtained indicated that the enzyme production corresponded closely to the growth of fungus. The optimum temperature for xylanase production is similar to the optimum

temperature for the growth of fungus. Similar observations were also reported[21,22].

Solid substrate used in SSF is insoluble in water. Therefore water will have to be absorbed onto the substrate particles, which can be used by the microorganisms for the growth and metabolic activity[10] and the increasing moisture level believed to have reduced the porosity of substrate. Thus, limiting the oxygen transfer into the substrate[23]. Like wise, a lower moisture ratio leads to reduced solubility of the nutrients of the solid substrate[24]. The moisture content of the substrate was examined by adding external water in the cultivation system using distilled water at different ratio. As indicated in the Table-1 the Xylanase production was maximum using the wheat bran which was moistened with moistening agent in ratio of 1:2.5 with the production of 71.3 units.

The effect of different levels of best inducers viz., wheat bran on xylanase production by *G. indicus* inducing that 20g wheat bran (with 50ml distilled water) is the optimum for attaining maximum production (51.33 U/ml) Figure-6. However, further increase in wheat bran levels considerably decreased the Xylanase yield. This could be due to lower moisture content that reduced the solubility of the nutrients of the solid substrate[15,25].

The inoculum level is an important factor for the production of Xylanase. High inoculum concentration increases the moisture content to a significant extent. Unabsorbed excess liquid directly interferes with growth and enzyme production[26]. Lower inoculum level results in a lower number of cells in the production medium. In the present study the maximum Xylanase activity was found with $1X10^5$ spores/ml (Table-2). After this concentration no significant increase in the enzyme production has been found. This may be due to the limiting nutrients at higher inoculum size.

CONCLUSION

Fungi have great potential to produce xylanases which are of high industrial importance. Xylanases are widely used in pulp and paper making processes, food industries along with various other applications. The above study describes the optimized culture conditions such as moisture content, inoculum size, carbon and nitrogen sources incubation time pH temperature and substrate concentration for xylanase production by *Gleomastrix indicus*. Further studies will be carried out for the purification of the enzyme.

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