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Xylanase Production by *Cladosporium* sp. from Agricultural Waste

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A B S T R A C T

The objective of the present study was isolation, identification and characterization of xylanase producing fungi using Lignocellulosic Agricultural wastes as a substrate through solid state fermentation. The fungal strains were isolated from the forest soil of Himmatnagar region by serial dilution technique through birch wood Xylan Agar plate method. Conformation screening done by Congored test for xylanase, based on the reddish zone of enzyme activity formation in birch wood xylan agar plates. *Cladosporium* sp. is selected as potential xylanase producers among five isolates and optimized for xylanase enzyme production in solid state fermentation using cheaper sources like wheat husk, rice bran, and rice straw. Maximum enzyme activity was observed in rice bran among tested substrates. The maximum production of enzyme was found to be in rice bran when the volume of fermentation medium was kept as 10g/250ml conical flasks with mineral solution as moisturizing agent and moisture ratio 1:0:7. Thus the present study proved that the fungal strain used is highly potential and useful for xylanase production.

Introduction

Xylanase (EC3.2.1.8) an enzymes which degrade the linear polysaccharide β -1, 4-xylan into xylose, thus breaking down hemicelluloses, which is a major component of the cell wall of plants. Xylan consists of β -1, 4 linked xylose with substituents such as acetyl, arabinosyl and glucuronosyl residue. Endo-1, 4-Xylanase is the main enzyme responsible for the cleavage of the linkages within the xylan backbone. The xylan degrading system includes endo-1, 4-

xylanases, which release long & short xylo-oligosaccharides. Xylanases have been isolated from a wide range of microorganisms including fungi, actinomycetes & bacteria (Monti *et al.*, 1991). Xylanase have been extensively studied and could potentially employed for the production of hydrolysate from agro industrial wastes, nutritional improvement of lignocelluloses feeds (Haltrich and Steiner, 1994), Agro fiber and biobleaching

of Kraft, paper pulp, clarification of juices & wines, improving the nutritional quality of silage and green feed.

Solid state fermentation involves growth of microorganisms on moist substrate in the absence of free water and economic recovery of required product in concentrated form (Medeiros *et al.*, 2000). Production of Agricultural lignocellulosic wastes was estimated to be about 40 million tons per year (Archana and Satyanarayana, 1997). This study was concerned to develop fermentation process by culturing the *Cladosporium* sp. on various Agricultural by products and wastes as substrate for the production of xylanase enzyme.

Materials and Methods

Collection and Enrichment of soil sample

Soil samples were collected from the forest nearby Himmatnagar region, North Gujarat, India. The samples were collected aseptically and packed in sterile polythene bag. Collected soil samples were enriched with enrichment media containing Carboxy methyl cellulose: 10g/L Peptone: 5g/L, NaCl: 5g/L. Approx. 1g of soil sample was inoculated in enrichment media and incubated at 37°C for 1 week. Then enriched soil samples were used for the isolation and screening of the xylanase producing fungi.

Isolation and screening of xylanase producing fungi

Primary screening

Initial screening was conducted on media rich in Xylan. Forest soil and garden soil samples from Himmatnagar, region North Gujarat (India), were suspended in sterile water. Suspensions (0.1 ml) after serial dilution in sterile saline or water were spread onto Xylan agar plate. Incubated at

30°C for 72 hr. and observed isolated colony on xylan agar plate.

Secondary screening

The cultures isolated were spread onto xylan agar plates containing 0.5% xylan (birch wood xylan) as the carbon source while keeping all the other components of media as constant. The plates were incubated at 30°C for six days, and colonies were developed. Xylanase production was detected by flooding the plate with 0.1% Congo red for 15 min and then washed with 1M NaCl.

The colonies showing zone of xylan degradation were picked and maintained on Potato Dextrose agar slants. Confirmation screening by Congo red test for xylanase based on the reddish zone of enzyme activity formation in birch wood xylan agar plates. Screening was carried out from the different isolates by studying their ability of higher Xylanase production by Khandeparkar's selection ratio.

Ratio = D/d = Diameter of zone of clearance / Diameter of growth

Characterization of selected fungal isolate

Selected fungal isolates were characterized colonically and morphologically on Potato Dextrose Agar media, Rose Bengal Agar media and Czapek dox media. Characters of selected fungal was observed and noted down.

Enzyme spectrum of fungal isolate (Table 2)

Starch hydrolysis

Starch agar medium was prepared poured on the petriplate and labeled. A loop of fungal spores was transferred in the center of the

starch agar plate, spot inoculation was carried out. The plates were incubated at 30°C for three days. After that the plate was flooded with iodine solution. Clear zone of Starch hydrolysis was observed.

Lipid hydrolysis

Tributyrene Agar medium was prepared poured on the Petriplate and labeled. A loop of fungal spores was transferred in the center of the Tributylene Agar plate, spot inoculation was carried out. The plates were incubated at 30°C for three days, after that clear zone of Lipid hydrolysis was observed.

Casein hydrolysis

Casein Agar medium was prepared poured on the Petriplate and labeled. A loop of fungal spores was transferred in the center of the Casein Agar plate, spot inoculation was carried out. The plates were incubated at 30°C for three days, after that clear zone of Casein hydrolysis was observed.

Phosphate hydrolysis

Pikovskaya's Agar medium was prepared poured on the petriplates and labeled. A loop of fungal spores was transferred in the center of the Pikovskaya's Agar plate, spot inoculation was carried out. The plates were incubated at 30°C for three days, after that clear zone of Phosphate hydrolysis was observed.

Lignin hydrolysis

Laccase hydrolyzing medium was prepared poured on the petriplates and labeled. Laccase hydrolyzing media was supplemented with 0.02% Guaiacol. A loop of fungal spores was transferred in the center of the Laccase hydrolyzing medium plate, spot inoculation was carried out. The plates were incubated at 30°C for three days,

after that reddish brown zone of Lignin hydrolysis was observed.

Cellulose hydrolysis

Carboxy Methyl Cellulose Agar medium was prepared poured on the petriplates and labeled. A loop of fungal spores was transferred in the center of the CMC Agar plate, spot Xylanase Producing *Cladosporium* sp. from Forest Soil of Himmatnagar Region. Inoculation was carried out. The plates were incubated at 30°C for three days, after that clear zone of Cellulose hydrolysis was observed.

Xylanase production from xylan

Xylanase assay

Endo-xylanase (1, 4-B-D-xylan xylanohydrolase EC 3.2.1.8.) was assayed by the method of Bailey *et al.* (1992) with some modifications using 0.1% birch wood xylan. The substrate (xylan) was homogenized in 0.2 M acetate (CH₃COONa/CH₃COOI-I) buffer at 100°C and subjected to boiling point on a heated magnetic stirrer. The solution was cooled and kept overnight with slow stirring which was then made up to the required volume using appropriate buffers. The reaction mixture containing 1.8 ml of pre incubated birch wood xylan suspension and 200 µl of suitably diluted enzyme preparation was incubated for 10 minutes at 50°C.

Enzyme blanks were required if the dilution was rather small and/ or if the sample contained high level of reducing sugar. Enzyme blanks were prepared adding the DNS reagent prior to the enzyme addition so that only the reducing sugars present in the enzyme preparations would be answering. The reagent blank was prepared in the same manner 0.2 M acetate (CH₃COONa/

CH₃COOI-I) buffer (0.2 M pH 7) was used instead of enzyme.

The reaction was terminated by adding 1.0 ml of dinitrosalicylic acid reagent, which was then kept in boiling water bath for 10 minutes. The concentration of reducing sugars released was estimated against xylose standard by noting the absorbance at 540 nm. Released Xylose sugar concentration was measured by DNSA method (Miller, 1959). The stock solution for xylose standard was prepared in 1000 mg/ml concentration and appropriate dilutions were used as the standard. One unit of endo-xylanase activity was defined as μ mols of xylose liberated per minute per ml of enzyme preparation.

Xylanase production from agricultural wastes

Substrate preparation

Natural lignocellulosics Agricultural wastes namely Rice bran, Rice straw, Wheat husk were used as a substrates for Xylanase enzyme production. First all Agricultural wastes were washed with distilled water and then after boiled in water for 15 min. than allow to dried out in oven and milled. All the lignocellulosic wastes were passed through filters with same mesh size to provide equal surface area for fungus to grow so that Xylanase activity among the lignocellulosics would not differ due to different oxygen diffusion, Nutrient absorption and assimilation by mycelia.

Inoculum preparation

The spore suspension was prepared by adding 4 to 5 cupborrer volume of fungal growth from the 7 day incubated PDA plate in to 50 ml of sterile distilled water, and clumps were broken using inoculation

needle. The tube was shaken to make homogeneous mixture of spore suspension.

Fermentation technique

Xylanase production from the Agricultural waste was carried out by Solid State Fermentation. 10 gm of each Agricultural wastes were mixed with tap 200 ml tap water in 500 ml Erlenmeyer flasks. All flasks were sterilized for 30 min at 121°C, and then spore suspensions were inoculated in aseptic condition and incubated at 28°C under static condition for 10 days. After each 24 hrs incubation, xylose concentration was measured from filtrate by DNSA (3, 5 dinitrosalicylic Acid) method (Miller, 1959).

Enzyme extraction

After optimization period the mixture from the all Agricultural Wastes was filtered using Whattman no. 1 filter paper and partial purification was done. The partial purification of xylanase was carried out by Ammonium Sulphate precipitation.

Ammonium sulphate precipitation

The solid Ammonium Sulphate was slowly added to the supernatant of crude enzyme preparation so as to reach 20% saturation. Addition of Ammonium Sulphate was carried out with continuous stirring and then it was kept at 4°C for 1 hr. The precipitated protein was removed by centrifugation at 2000 RPM for 15 min. The pellets were collected and stored; allowed to air dry for getting crude enzyme in powder form.

Crude enzyme assay

The enzymatic reaction contained 1 ml of 1% birch wood xylan solution in sodium acetate buffer (pH = 5) and add 0.5 ml of enzyme extract and incubated at 30°C for 10 min. The reaction was terminated by adding

1.0 ml of dinitrosalicylic acid reagent, which was then kept in boiling water bath for 10 minutes. The concentration of reducing sugars released was estimated against xylose standard by noting the absorbance at 540 nm. The activity was expressed as U/ml. by using following formula.

$$\text{Xylanase Activity} = \frac{(\text{CX} - \text{CRB}) \times \text{D U/ml}}{\text{W} \times 10 \times \text{V}}$$

CX = Reading from the Standard Curve for Sample Enzyme

CRB = Reading from the Standard Curve for Reagent Blank

D = Dilution Factor of the Sample

W = Weight of Sample Taken in gm.

10 = Incubation Time in min.

V = volume of Sample Taken in ml.

Result and Discussion

Isolation and screening of xylanase producing microorganism

Five xylanase producing fungal isolates were obtained through screening from the collected soil samples. Among that KF5 exhibiting higher ratio of clear zone of Xylanase production by Khandeparkar's selection ratio. So KF5 fungal isolate was selected for the xylanase enzyme production using Agricultural wastes as a substrate.

Characterization of selected fungal isolate

Colonial Characterization of selected fungal isolate was studied on Potato dextrose Agar (PDA), Rose Bengal Agar (RBA) and Czapek dox media (Table 1).

Xylanase production from the agricultural wastes

Xylanase enzyme production from the Agricultural wastes was carried out through Solid state fermentation. Different Agricultural wastes like Rice bran, Rice straw, and Wheat husk were used for the

production of xylonite enzyme. Xylanase production was directly proportional to the xylose sugar liberation and it measured by DNSA method.

Solid State Fermentation processes are practical for complex substrates including agricultural, forestry and food processing residues and wastes, which are used as inducing carbon sources for the production of xylanase. SSF conditions are especially suitable for the growth of fungi as these organisms are able to grow at relatively low water activities, contrary to most bacteria and yeast which will not proliferate under these culture conditions. Higher enzymes titers are commonly reported as an advantage for SSF processes over SmF. Additionally, enzymes properties such as thermo stability and pH tolerance can also improved when SSF is the production techniques. Physiological growth differences which explain why filamentous fungi have lower yields, such as substrate conversion and enzyme to biomass ratio, in SmF compared to SSF have been reviewed elsewhere. In general SmF are preferred as a production process when preparations require more purified enzymes, where as synergistic effects from a battery of xylan degrading enzymes can be easily found in those preparation obtained in SSF using complex substrates.

Crude enzyme extraction

Crude Enzyme extraction carried out from the filtrate of fermented broth liquid medium using Rice bran as a raw substrate through Ammonium sulphate precipitation and Centrifugation. 0.4% of crude enzyme was obtained in dried powder form.

Enzyme activity

Enzyme activity of Crude dry form xylanase and liquid broth xylanase was measured. Crude dry form xylanase shows lesser xylanase Activity than liquid broth xylanase.

Table.1 Colonial characteristics of *Cladosporium* sp. on PDA, RBA, and Czapek dox Agar

Charactristics	PDA	RBA	Czapek Dox Agar
Surface color	Blackish grey	Cement green	Blackish grey
Margin	Entire	Entire	Entire
Reverse side	Blackish grey	Black	Black
Elevation	Raised	Raised	Raised
Growth	Slow	Slow	Slow

From the above morphological and colonial study of selected fungi was characterized as *Cladosporium* sp.

Table.2 Enzyme spectrum of *Cladosporium* sp

ENZYME	AMYLASE	PROTEASE	LIPASE	CELLULASE	PHOSPHATASE	LACCASE
	+	-	+	+	+	+

Note: + indicates the Enzyme produced.
- indicates the Enzyme not produced

Table.3 Xylose concentrations obtained from agricultural wastes by *Cladosporium* sp.

S.No.	Substrate	Concentration µg/ml
1.	Rice bran	345
2.	Wheat husk	285
3.	Rice straw	265

Table.4 Enzyme activity in U/ml from Rice bran by *Cladosporium* sp.

S.No.	Form of Xylanase	Xylanase activity
1.	Liquid broth xylanase	51.0 U/ml
2.	Crude Dry form Xylanase	25.0 U/ml

The selected fungal isolates are dominant heterologous enzyme producing microorganism which are very much attracted by industries for a varying reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete extracellular proteins. The biochemistry, physiology and generics of some of these isolates are well studied, facilitating further development and greater exploitation for industrial purposes (Alexopoulos, 1952). Low enzyme activity of the isolates on plate agar as compared to

that obtained on liquid medium might be due to the adsorption of the enzymes on the surface of insoluble xylan particles present in the solid culture medium (Irwin *et al.*, 1994). The fact that the enzyme activity of some of the isolates were higher in liquid medium is an indication that the clearing found on xylan plate agar of most of the isolates is due to the regional presence of glucanase and xylanase and or membrane bound glucanase and xylanase that has led to good clearing zone on solid agar plate (Kazuhisa, 1997).

Conclusion

Among 5 different selected fungal isolates, KF5 exhibiting higher zone of Xylanase activity on xylan agar plate was confirmed by Congo red test and colonically and morphologically characterized as *Cladosporium* sp. Selected *Cladosporium* sp. used for the Xylanase production by Solid state fermentation using Various Agricultural wastes. Among tested agricultural wastes as substrate, Rice bran shows higher xylanase production by *Cladosporium* sp. Xylose concentration in Rice bran was 610 µg/ml which were higher among the tested substrates (Table 3). Extraction and Purification of Crude enzyme carried out from Liquid medium fermented broth of Rice bran (Table 4). 0.4 % of crude enzyme was obtained in dried powder form from filtrate of Rice bran containing medium. Xylanase activity from filtrate was 51.0 U/ml and crude dry powder was 25.0 U/ml.

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