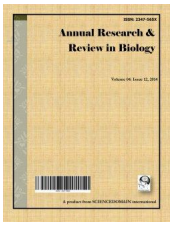

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Effect of Temperature on Production of Endoglucanase and Xylanase from Some Green Manure Fungal Decomposers

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Authors' contributions

This work was carried out in collaboration between all authors. Author RK designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author AS helped in finalization of the draft of manuscript. Authors MS and SS managed the analyses of the study. Author AC helped in statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Among the various microorganisms reported for their capabilities of cellulases and xylanases production, fungi are the most potent producers. This study reports the production of xylanolytic and cellulolytic enzymes by potential decomposing fungal isolates using a cheap medium containing green manure substrate and chemically defined basal medium under solid-state culture. A number of decomposing fungi were screened for cellulolytic and xylanolytic potential. The enzyme production with purified carboxy methyl cellulose (CMC) and birch wood xylan was studied

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and found to be promising. *Aspergillus niger* produced the highest amount of endoglucanase and xylanase *viz.,* 3.635 U mg^{-1} protein min^{-1} and 2498.20 U mg^{-1} protein min^{-1}, respectively at 25ºC. Growth and enzyme production was affected by the alterations in temperature. Highest production of endoglucanse and xylanase were noted at 25ºC and 30ºC.

Keywords: Endoglucanase; fungal decomposers; Sesbania aculeata L. and xylanase.

1. INTRODUCTION

Numerous microbial enzymes are commercially exploited and auspiciously passed down on industrial scale to catalyze several chemical processes [1]. Cellulose degrading enzymes are commercially available, but still too expensive for production of fuel ethanol [2]. In nature, several bacteria and fungi are capable of metabolizing plant cellulose aerobically and the final product is CO_2 [3]. The capability to degrade cellulose aerobically is a common feature among various bacterial and fungal communities. Cellulolytic ability is widespread in members of the *Ascomycota* and *Basidiomycota* [4-6]. Aerobic cellulolytic microbes produce freely diffusible extracellular cellulase enzyme systems consisting of endoglucanases, exoglucanases and α-glucosidases that act synergistically in the conversion of cellulose to glucose [2,7-8].

Xylans are major structural polysaccharides in plants. They belong to the group of complex structural polymers collectively referred as hemicelluloses [9]. Xylanases are group of enzymes, crucial in the bio-conversion of hemicelluloses into their constituent sugars. Variety of microorganisms, including bacteria, yeasts and filamentous fungi have been reported to produce xylanases, of which the most potent producers are fungi [10].

Solid state fermentation (SSF) offers advantages over submerged fermentation, it generally employ a natural raw material as carbon and energy source [11]. SSF processes have adequate economical potential in producing products for the food, feed, pharmaceutical and agro-industries. Latest applications of SSF include the protein enrichment of agro-industrial residues, the production of enzymes, organic acids and other fungal metabolites and spore production [11].

The production of endoglucanase and xylanases from different microbes using different substrates have been performed but little attention was paid towards the optimization for cultural conditions and particularly the possibility of a green manure substrate for its use for the production of these enzymes. Thus, the present study was undertaken to exploit the potential of green manure decomposer fungal isolates for the production of endoglucanase and xylanase.

2. MATERIALS AND METHODS

2.1 Collection of Sample

The substrate used for the study was green manure *Sesbania aculeata* L., which is a cheap and readily available source of lignocelluloses and hemicelluloses. The green manure plants (45-50 days old) were collected from local fields, cut into small pieces (1-2 cm) followed by washing and drying. These samples were stored in autoclavable plastic bags in a cold room which had its temperature set at 4ºC.

2.2 Microorganisms and Inoculum Preparation

A large number of decomposing fungi were isolated from various samples of decomposing green manure *Sesbania aculeata* L. in soil [12] and screened for their cellulolytic and xylanolytic potentials. The best enzyme producing isolates of dominant decomposing fungi were selected following the method of Kheng and Ibrahim [13]. These isolates were maintained on PDA plates. The cultured plates were washed with 100 ml of sterilized distilled water. The surface was gently rubbed with a sterilized small glass-spreader and the mycelial suspension was transferred into a sterilized 250 ml conical flask by filtration. The inoculum was kept in the refrigerator at 4ºC for enzyme assays.

2.3 Preliminary Screening of Potential Isolates of Decomposing Fungi for Enzymatic Activity through Plate Assay

The evidences for the microbial utilization of cellulose and hemicelluloses can be detected by using congo red [14]. Screening was carried out on Czapex-mineral salt agar medium [Sodium

nitrate ($NaNO_3$)-0.2%, Potassium phosphate (K_3PO_4)-0.1%, Magnesium sulphate ($MgSO_4$)-0.05%, Potassium chloride (KCl)-0.05%, Peptone-0.2%, Agar-2.0%, pH-6.5]. This medium was supplemented with 0.5% carboxymethyl cellulose (CMC) or Birch wood xylan for screening of cellulase and xylanase activity, respectively. The medium (20 ml) was poured into sterilized Petri-dishes and allowed to solidify. The different dominant decomposing fungi were separately inoculated (5 mm disc) in the middle of Petri-dishes containing solidified medium. Petri-dishes were incubated at 28±2ºC for 3-5 days. After incubation Petri-dishes were flooded with 1% congo red for 1 hour then these plates were de-stained by 1 N NaCl solution for 2-3 hour. The radius of clear zone was measured.

2.4 Enzymes Production by Potential Decomposing Fungi

Powdered green manure (*Sesbania aculeata* L.) was used as carbon source for the solid substrate production of endoglucanase and xylanase. The medium (basal salt solution) used for production contained Ammonium Sulphate [$(NH_4)_2SO_4$], 0.3%, Potassium dihydrogen phosphate (KH_2PO_4), 0.3% and Ammonium Acetate, 0.6%; and pH of the medium was adjusted to 6.0 prior to the sterilization. Each Erlenmeyer flask (250 ml) containing 5 g of substrate (particle size 2-5 mm) and 15 ml of production medium was inoculated with 2 ml (1.5×10^7spore ml^{-1}) of spore suspension prepared from 5 days old culture of test fungi grown on potato dextrose agar (PDA) medium. SSF was carried out for five days at 15ºC, 20ºC, 25ºC, 30ºC and 35ºC. The crude enzyme was extracted by addition of 50 ml sodium citrate buffer (50 mM, pH 6.0) to each flask and kept at 28ºC for 1 h under mild shaking. The fermented extract was filtered through muslin cloth, centrifuged at 11,000 rpm for 10 min [rcf = 8,928 × g] and the resultant extract was used for assay of enzyme activities. Enzyme production was studied at 15ºC, 20ºC, 25ºC, 30ºC and 35ºC.

2.5 Enzyme Assay

Endoglucanase and Xylanase activities were determined using 1% CMC (carboxy methyl cellulose) and 1% Birch wood xylan as substrates, respectively, prepared in sodium citrate buffer (50 mM, pH 6.0) [11]. The reaction was initiated by incubating equal amount of suitably diluted enzyme and substrate for 30 minute at 50ºC. The reaction was stopped thereafter, by addition of dinitrosalicylic acid (DNS) reagent followed by boiling the content for 10 minute [15] and the developed colour was read at 540 nm using Labnics LUV100A UV/VIS spectrophotometer. The amount of released sugar was quantified using the glucose (for endoglucanase) and xylose (for xylanase) standards. The protein estimation of crude enzyme extract (for both, endoglucanase and xylanase) was also done based on Bradford method [16]. One unit of enzyme activity was expressed as the amount of enzyme required to release 1µmol of glucose (for endoglucanase) and xylose (for xylanase) per mg of protein per minute under the assay conditions. The data used were the average of three replicates.

2.6 Statistical Analysis

All the data have been analyzed statistically. The enzymatic activity of different fungal agent at varying temperature and their impact on subsequent biomass reduction analyzed with factorial CRD with P= 0.01 level of significance. Different standard curves used during calculation were generated through regression analysis using different concentrations of glucose/ xylose/BSA as independent variable and corresponding OD values as dependent variable.

3. RESULTS AND DISCUSSION

3.1 Screening of Potential Decomposing Fungi for Producing Cellulolytic and Xylanolytic Enzymes

During the screening, dominant fungi were found to be exhibiting endoglucanase activity on modified czapek-mineral salt agar medium with the diameter of the clear zones ranging from 21.50 to 53.00 mm and xylanase activity with the diameter of clear zone ranging from 25.50 to 57.00 mm (Fig. 1). *Trichoderma harzianum* exhibited reproducible zone of hydrolysis of 53.00 mm diameter of endoglucanase and *Penicillium citrinum* demonstrated reproducible zone of hydrolysis of 57.00 mm of xylanases were found the best producers of endoglucanase and xylanase respectively during the plate screening. *Aspergillus flavus* was recorded as least producer of endoglucanase and xylanases with the clear zones of 21.50 and 25.50 mm, respectively.

Measurement of clear zone is an indication of capability of fungal strains for production of their

respective enzymes. This method has been employed for selection of potential endoglucanases [7] and xylanases [14] producers.

3.2 Endoglucanase and Xylanase Activity

The results from the CMC analysis for determining cellulases activities were successfully obtained for all eight fungal decomposers. The highest endoglucanase was produced by *Aspergillus niger* (3.635 U mg^{-1} protein $min.^{-1}$) at 25ºC, whereas the lowest amount of endoglucanase was produced by *Cladosporium cladosporioides* (0.212 U mg^{-1} protein $min.^{-1}$) at 15ºC (Table 1). Iyayi [17] reported production of higher amounts of cellulases by species of *Aspergillus* and *Penicillium* genera using agro-industrial by-products.

In the present study decomposer fungal strains of *A. niger, T. harzianum, P. citrinum* revealed that these strains were capable of growing not only on commercial preparation of cellulose but also on agricultural residues i.e., green manure substrate as well with high titers of enzymes. The finding of the present study is also in line

Table 1. Estimation of endoglucanase production (U mg^{-1} protein min^{-1}) by dominant decomposing fungi at different temperatures

Dominant fungi	Temperature (°C)				
	15°C	**20**°C	**25**°C	**30**°C	**35**°C
Aspergillus niger	1.397	1.563	3.635	2.341	2.122
Aspergillus flavus	0.635	0.673	1.704	1.495	1.196
Aspergillus fumigatus	0.915	1.328	1.995	1.892	1.484
Trichoderma harzianum	1.208	1.957	3.274	2.298	1.783
Penicillium citrinum	0.403	1.437	3.108	1.795	0.720
Penicillium rubrum	0.462	0.748	1.650	1.356	0.976
Cladosporium cladosporioides	0.212	0.681	1.920	1.396	0.393
Curvularia lunata	0.348	0.596	1.548	1.495	1.295
LSD (P=0.01) fungi	0.04	-	-	-	-
LSD (P=0.01) temperature	0.03	-	-	-	-
LSD (P=0.01) fungi ×temperature	0.08	-	-	-	-

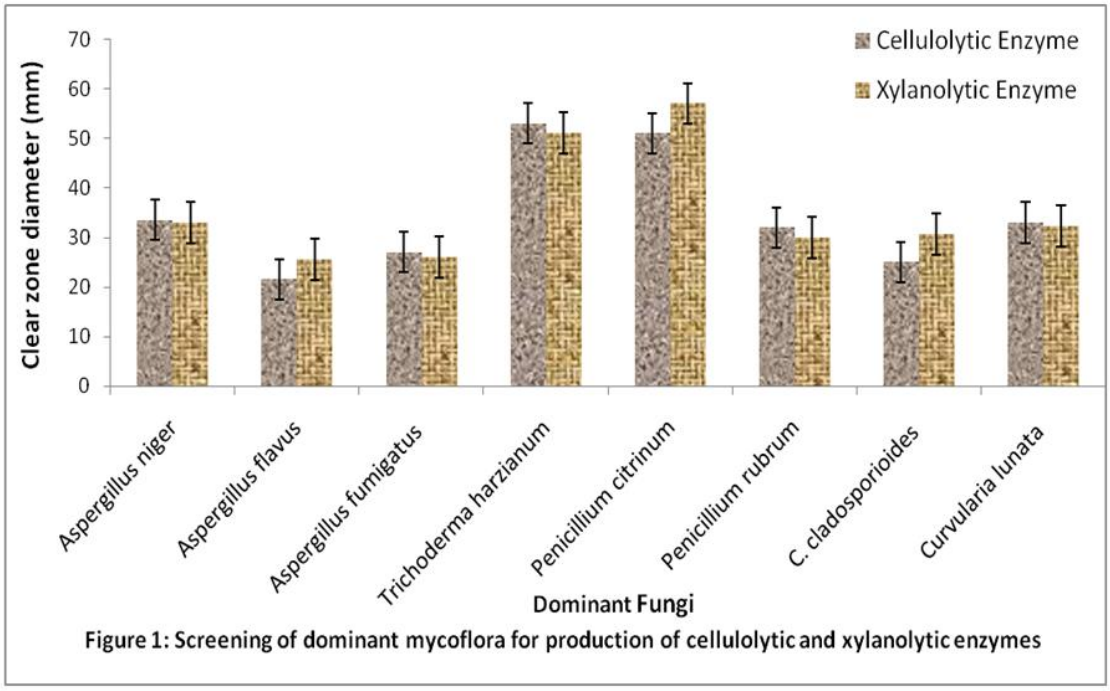

Figure 1: Screening of dominant mycoflora for production of cellulolytic and xylanolytic enzymes

with a study carried out by Kamil and Sinha [2] that indicates the considerable amount of cellulases and xylanases by indigenous *A. niger* strain under solid state culture conditions.

Economic potential in producing different enzymes using agro-waste was reported by Badhan et al. [11]. Other investigations on fermentation of agro-industrial by-products by *A. niger, A. flavus* and *Penicillium* sp. led to a conclusion that the strain of *A. niger* are better in degrading cellulose and hemicellulose in the crude natural substrates [17,18].

All eight fungal decomposers were capable to produce xylanases. The highest level of xylanases production was obtained by *Aspergillus niger* (2498.20 U mg^{-1} protein min^{-1}) at 25ºC and lowest amount of xylanases was produced by *Cladosporium cladosporioides* (133.24 U mg^{-1} protein min^{-1}) at 15°C (Table 2). A number of microorganisms such as *Trichoderma, Bacillus, Cryptococcus, Aspergillus, Penicillium, Aureobasidium, Fusarium, Chaetomium, Phanerochaete, Rhizomucor, Humicola, Talaromyces* and many more are capable to produce xylanases to a great extent [2,13,18-20].

During the experiment, among the temperatures tested for enzymes production, 25ºC and 30ºC temperature were found most suitable for the production of both the enzymes, whereas the low levels of both enzymes were recorded during the study at 15ºC, 20ºC and 35ºC temperature. This may be due to slow growth of fungal isolates at very low (15ºC, 20ºC) and higher (35ºC) temperature [2].

Table 2. Estimation of xylanase production (U mg^{-1} protein min^{-1}) by dominant decomposing fungi at different temperatures

Dominant fungi	Temperature (ºC)				
	15ºC	20ºC	25ºC	30ºC	35ºC
Aspergillus niger	894.68	1110.55	2498.20	1356.14	1054.37
Aspergillus flavus	202.79	389.60	756.55	526.85	442.70
Aspergillus fumigatus	477.53	949.88	1576.45	974.92	936.73
Trichoderma harzianum	427.26	754.12	1498.20	841.46	660.24
Penicillium citrinum	209.61	436.18	762.34	525.88	236.26
Penicillium rubrum	239.88	380.35	1174.08	478.18	315.44
Cladosporium cladosporioides	133.24	254.61	620.92	424.46	244.03
Curvularia lunata	281.33	450.44	605.27	574.21	348.70
LSD (P=0.01) fungi	83.85	-	-	-	-
LSD (P=0.01) temperature	66.29	-	-	-	-
LSD (P=0.01) fungi ×temperature	187.50	-	-	-	-

Table 3. Per cent biomass[1] reduction of *Sesbania aculeata* L. by dominant decomposing fungi at different temperatures under solid state culture conditions

Dominant fungi	Temperature (ºC)				
	15ºC	20ºC	25ºC	30ºC	35ºC
Aspergillus niger	21.04	27.86	41.20	37.60	26.20
Aspergillus flavus	17.28	27.80	34.55	32.40	24.06
Aspergillus fumigatus	15.30	23.60	28.10	27.86	26.50
Trichoderma harzianum	19.35	27.45	35.50	34.82	24.92
Penicillium citrinum	19.76	29.50	39.80	37.10	31.00
Penicillium rubrum	18.82	26.65	32.40	31.24	23.60
Cladosporium cladosporioides	14.70	18.75	26.35	25.20	22.54
Curvularia lunata	13.90	19.40	25.20	22.74	21.46
Control	3.36	4.00	5.20	4.68	3.80
LSD (P=0.01) fungi	0.48	-	-	-	-
LSD (P=0.01) temperature	0.36	-	-	-	-
LSD (P=0.01) fungi × temperature	1.08	-	-	-	-

1= Dry Weight of substrate+ Fungal Mycelium

Different fungi have the capability to use the substrate at different rates. The maximum per cent reduction in biomass of *Sesbania aculeata* L. was recorded at 25ºC by *Aspergillus niger* (41.20%) and minimum % biomass reduction (13.90%) was found at 15ºC by *Curvularia lunata* (Table 3). The per cent weight loss was found to be reduced significantly in all substrates after 5 days of incubation at various temperatures. *Aspergillus niger* recorded the maximum per cent weight loss of *Sesbania aculeata*, whereas the minimum per cent weight loss was found by *Curvularia lunata* in *Sesbania aculeata* substrate. The loss or reduction in weight is the indicator for the capabilities of the fungal species for better production of cellualses and xylanases; and utilization of substrates for mass production of its culture [21]. The level of enzymes enhanced with decomposition of green manures in the soils, is an indicator for the role of these enzymes in the decomposition of organic matter [22].

4. CONCLUSION

The present work has investigated the effect of temperature on production of endoglucanase and xylanase enzymes using a cheap medium *viz., Sesbania aculeata* L. green manure substrate. Experiment shows, that *A. niger, T. harzianum* and *P. citrinum* were able to produce significantly higher titers of endoglucanase and xylanase and may be exploited for possible biotechnological applications.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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