International Journal of Pharmaceutical Science Invention
ISSN (Online): 2319 – 6718, ISSN (Print): 2319 – 670X
www.ijpsi.org Volume 5 Issue 3 ǁ June 2016 ǁ PP.01-06

Isolation, Screening and Selection of Fungal Strains for Potential Cellulase and Xylanase Production

Sakshi Bairagi
(Department of Environmental Sciences, M.D.University, Rohtak (Haryana), India)

ABSTRACT: *The present study was aimed to isolate, screen and identify the potential cellulase and xylanase producing fungi from the soil samples collected from different areas of Haryana. Total one hundred fifty one fungal isolates were isolated from these soil samples were then screened by using selective media (i.e. CMC and Xylan agar) in order to determine the potency of microbes in producing cellulase and xylanase which were indicated by clear zones formation around the cultures. This qualitative screening which showing greater cellulase and xylanase indexes were subjected to enzyme activity tests by Dinitrosalicylic acid (DNS) method. Maximum enzyme production was achieved at 30°C, pH of 6.0 by Trichoderma atroviride on 5th day of incubation.*

Keywords: *Fungi, cellulase, screening, vegetable waste, xylanase.*

I. Introduction

Agro-industrial and food processing wastes are available in proliferate quantities over the world that causes potent threat as environment pollutants. Their conversion to useful products may improve the problems they cause. Proper biotechnological utilization of these wastes in the environment will eradicate pollution and convert them into useful byproducts [1]. Biological degradation of cellulosic biomass to fermentable sugars by utilizing enzyme producing microbes is feasible process which reduce the use of fossil fuels [2]. The majority of these wastes contain cellulose (homologous polymer of glucose molecules connected by β-1,4 linkages), hemicellulose (heterologous polymer of pentose and hexose), and lignin (complex aromatic polymer) [3]. For the complete decomposition of lignocellulosic wastes, three major groups of enzymes are required, which include cellulases, hemicellulases and lignolytic enzymes. A cellulolytic enzyme system is a complex system of enzymes composed of endoglucanase, exo-glucanase and β- glucosidase that acts synergistically to degrade cellulosic substrate [4]. On the other hand, xylanses (EC 3.2.1.18) are a complex system, it includes: xylanases and xylosidases [5]. Xylanase are a class of hydrolytic enzymes which can hydrolyze the straight polysaccharide -1, 4-xylan in hemicelluloses which is a noteworthy component of secondary cell wall of plants [6].

These enzyme is produced by several microorganisms commonly bacteria and fungi. Many microorganisms have been reported to produce cellulase and xylanase enzymes, though until now the majority of the work has focused on the use of fungal enzymes to convert celluolytic materials into sugars. *Trichoderma* spp. and *Aspergillus* spp. have most widely been used for production of these enzymes [7]. Filamentous fungi are mainly interesting as source of cellulases and hemicellulases since they secrete these enzymes into the medium and have higher activities in contrast to bacteria [8]. This aspect makes fungal enzymes more attractive for various industrial processes. Cellulase and xylanse enzymes have been used in many applications including waste treatment, chemicals production, clarification of juice, paper manufacture, animal feed and beverage industries [9]. These enzymes are the focus of several studies for their use in the bioconversion of agricultural [10]. In the paper and pulp industry, xylanase are used for biobleaching process while the enzyme in addition to cellulase is used to improve the manufacture of recycled papers [11].

High cost of production and other factors like complexity of cellulose structure, type and source of cellulose and its production by microorganisms are the major factors which influence the economics of enzyme production. Successful utilization of cellulosic materials as renewable carbon sources can be achieved by developing economically feasible technologies for enzyme production. In view of biotechnological and industrial applications of cellulase and xylanase enzymes, the present study was aimed on isolation and screening of potential cellulase and xylanase producing native fungal species from soil samples using vegetable waste as carbon source in submerged fermentation..

II. Materials And Methods

2.1 Collection of soil samples

Soil samples were collected from different regions of Haryana within a depth of 3 to 6 cm after removal of superficial layer. The sample was brought to the laboratory and maintained at room temperature for microbiological study.

2.2 Isolation of fungal colonies

Serial dilution technique was used for isolation of fungal colonies from soil samples in which each sample of 1 g soil were suspended in 10 ml of sterile distilled water and prepared stock. From this stock, various dilutions were prepared from 10^{-1} to 10^{-7}, using sterile distilled water. One milliliters of the diluted sample was poured into petriplates containing Potato Dextrose agar medium. Triplicates were maintained for each dilution. Streptomycin was added to the molten medium after autoclave and the plates were incubated at 30°C for 3- 5 days to identify the fungi. Distinct fungal colonies with different morphological form were sub-cultured to purity and were preserved on potato dextrose agar slants under refrigeration conditions.

2.3 Screening of efficient fungal isolates for cellulase and xylanase production

2.3.1 Primary screening or qualitative screening

Initially, screening was performed by plate assay method. Isolated fungal strains were primarily tested for cellulase and xylanase enzyme activity by culturing on Mandel and Reese agar medium supplemented with 1% Carboxymethyl cellulose (CMC) and 1% xylan as a carbon source. Tetracycline was added in media to control the bacterial contamination (pH 7.0). The plates were incubated at 30°C for 3 days. After incubation plates were stained with 1% congo red reagent for 15 min and destained with 1 M NaCl for 15 min and decanted it. The clear zone around the fungal colony was regarded positive for cellulase and xylanase activity. The fungal colonies having the largest clear zone were selected and studied for cellulase and xylanase production in culture broth.

2.3.2 Secondary screening or quantitative screening

Selected fungal isolates were subjected to secondary screening using Mandel and Reese media supplemented with vegetable waste as main carbon sources for cellulase and xylanase production respectively. Erlenmeyer flasks (250 ml) containing Mandel and Reese media inoculated with 72 h culture of each selected fungus (5.0 mm in diameter). These flasks were incubated at 30°C in an incubator shaker at 120 rpm for 6 days. Samples were taken after every 24 h interval. The culture medium was filtered using Whatmann no.1 filter paper, the filtrate was centrifuged at 10000 rpm for 10 min. The clear supernatant was used as the crude extracellular enzyme source.

2.4 Enzyme assay

The cellulase and xylanase were measured by dinitrosalicylic acid (DNS) method [12]. A 0.5 ml of diluted enzyme sample in 0.05 M citrate buffer (pH 4.8) was mixed with 0.5 ml of 1% of CMC or Birchwood Xylan substrate and incubated at 50°C for thirty min. The reaction was then terminated by adding 3 ml of DNS and heating at 100°C for 10 min. and the absorbance at 540 nm was measured. One unit of cellulase or xylanase was defined as the amount of enzyme producing 1 g of reducing sugar equivalent to glucose per minute under standard test conditions.

III. Result And Discussion

Fungi play a crucial role in degradation of lignocelluloseic biomass by the secretion of essential enzymes which are involved in the depolymerization of lignocelluloses. Thus, this study mainly concentrates on isolate and screens the cellulolytic and xylanolytic indigenous fungal strains from soil and to explore their hydrolytic potential enzymes for their possible future industrial applications.

3.1 Isolation of fungal isolates for enzyme production

Cellulose degrading microorganisms were isolated from fifty soil samples that are collected from different regions of Haryana (Gurgaon, Jind, Kaithal Panipat and Rohtak). In the present study, 151 fungi isolates were obtained on potato dextrose agar medium at 30°C and results are presented in Table 1. These cultures were named in numerically order from 1 to 151. Among the 151 isolates, almost 50% belong to *Aspergillus* and *Trichoderma* species followed by *Penicillim* species. These isolated fungal colonies were then subjected to primary screening for the selection of better strains.

Table 1: Fungi isolated from different regions of Haryana

S. No.	Geographical region	No. of soil samples	No of fungal isolates
1.	Rohtak	10	34
2.	Jind	10	30
3.	Panipat	10	27
4.	Gurgaon	10	31
5.	Kaithal	10	29

3.2 Screening of efficient fungal isolates for cellulase and xylanase production

Screening of microorganisms is necessary to find the appropriate starting material for enzyme production. Screening studies were done in two steps i.e primary screening and secondary screening. Lynd et al. [13] reported that the screened fungal strains will be useful for further studies by enzyme producers.

3.2.1 Primary screening or qualitative screening

A total of 151fungal isolates were collected and screened based on the formation of clear zones around the microbial colonies. Fifty two (52) isolates of them showed positive reactions to qualitative test of cellulase production as indicated by the clear zone formation in CMC agar media plates as shown in Table 2. Thirty nine (39) isolates showed positive reaction to xylanase production as indicated by clear zones in xylan agar media plates as shown in Table 3. The appearance of the clear zone around the colony after the addition of congo red solution was strong evidence for the secretion of cellulase and xylanase enzymes. Diameters of the colony and the clear zone were measured.

Table 2: Zone of hydrolysis by fungal isolates isolated for cellulase enzymes

Serial no.	Fungal isolates	Zone of hydrolysis (cm)	Serial no.	Fungal isolates	Zone of hydrolysis (cm)
1	30	11.50±0.02	27	8	2.53±0.04
2	19	11.10±0.01	28	71	2.45±0.01
3	4	9.33±0.01	29	55	2.30±0.01
4	133	8.91±0.02	30	23	2.25±0.02
5	49	8.85±0.03	31	90	2.22±0.02
6	87	8.51±0.03	32	56	2.06±0.03
7	1	8.42±0.01	33	5	2.00±0.02
8	81	8.25±0.04	34	45	1.97±0.04
9	89	8.02±0.02	35	59	1.83±0.03
10	145	7.69±0.02	36	134	1.78±0.02
11	34	7.51±0.01	37	74	1.71±0.02
12	126	7.20±0.02	38	78	1.68±0.01
13	85	6.13±0.02	39	80	1.61±0.01
14	142	5.82±0.03	40	129	1.57±0.02
15	95	5.79±0.01	41	79	1.54±0.03
16	15	5.50±0.01	42	77	1.46±0.04
17	151	4.79±0.04	43	144	1.40±0.02
18	131	4.60±0.02	44	138	1.39±0.02
19	120	4.51±0.03	45	38	1.37±0.03
20	48	4.22±0.02	46	62	1.26±0.02
21	128	4.16±0.01	47	35	1.24±0.04
22	2	4.08±0.02	48	132	1.22±0.02
23	81A	2.95±0.01	49	149	1.19±0.02
24	12	2.84±0.02	50	127	1.18±0.03
25	7	2.70±0.02	51	31	1.15±0.01
26	22	2.60±0.03	52	66	1.12±0.04

Table 3 Zone of hydrolysis by fungal isolates isolated for xylanase enzymes

Serial no.	Fungal isolates	Zone of hydrolysis (cm)	Serial no.	Fungal isolates	Zone of hydrolysis (cm)
1	30	11.31±0.04	21	62	2.05±0.01
2	126	8.07±0.01	22	79	2.02±0.03
3	95	7.30±0.02	23	138	1.96±0.02
4	12	7.12±0.02	24	7	1.85±0.02
5	142	6.50±0.01	25	81A	1.80±0.01
6	4	6.30±0.03	26	41	1.79±0.04
7	81	6.21±0.02	27	118	1.76±0.03
8	19	6.15±0.04	28	151	1.62±0.01
9	89	5.82±0.02	29	37	1.61±0.01
10	87	5.73±0.02	30	23	1.57±0.02
11	85	5.71±0.02	31	49	1.55±0.01

12	15	5.60±0.01	32	24	1.50±0.02
13	1	5.47±0.01	33	10	1.46±0.02
14	2	5.20±0.02	34	57	1.30±0.02
15	7	5.06±0.04	35	11	1.27±0.03
16	131	2.52±0.02	36	104	1.24±0.04
17	96	2.51±0.01	37	82	1.22±0.02
18	112	2.35±0.01	38	150	1.14±0.01
19	22	2.25±0.02	39	20	1.10±0.02
20	55	2.14±0.02			

Among fifty two and thirty nine fungal isolates which are positive for cellulose and xylan degradation respectively, only sixteen fungal isolates showed high cellulase activity (zone of hydrolysis ≥ 5cm) and fifteen fungal isolates showed high xylanase activity (zone of hydrolysis ≥ 5cm) as compared to other fungal isolates. Zone of hydrolysis by these fungal strains are shown in Fig. 1 (a-f) and Fig. 2 (a-f) respectively. The fungal cultures showing zone of clearance with diameter greater than 5.0 cm were further tested for the production of cellulase and xylanase in submerged fermentation (SmF). Plate screening method has previously been used for screening hydrolytic enzymes producing fungi and was reported to be suitable [14].

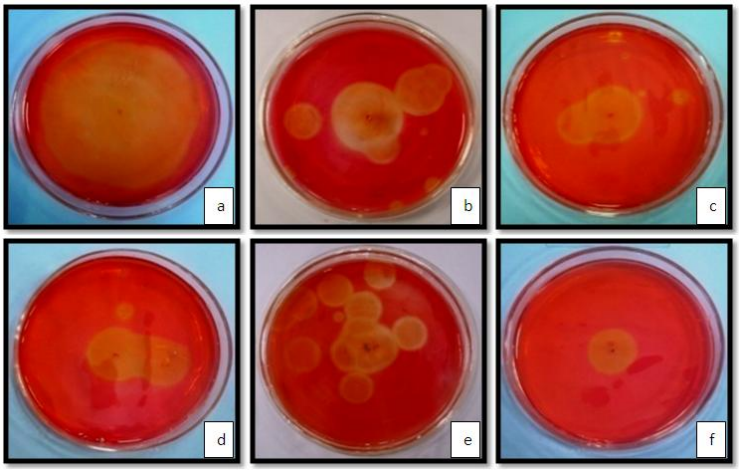

Figure 1: The zone of hydrolysis produced by fungal strains on mandel and reese agar medium containing CMC

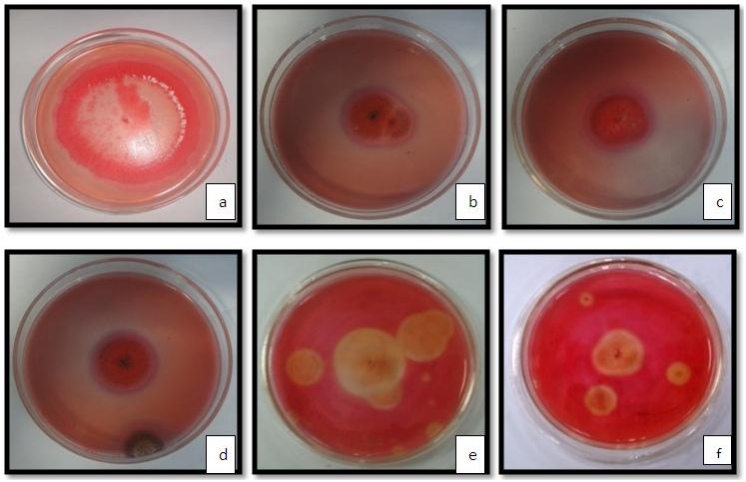

Figure 2: The zone of hydrolysis produced by fungal strains on mandel and reese agar medium containing xylan

3.2.2 Secondary screening

By observing the area of clear zone produced, the potent xylanase and cellulase enzymes producers were differentiated. However, hallows around the isolates on solid agar medium may sometimes be due to the presence of membrane bound hydrolyses which cause formation of the clearing when the substrates are being hydrolysed. Hence, plate assay agar screening method was confirmed under submerged conditions for cellulase and xylanase by using the standard procedures.

In this method, all the selected fungi from primary screening were secondary screened for extracellular cellulase and xylanase activity on Mandel and Reese medium containing vegetable waste as sole carbon source and incubated at 30°C under shaking conditions at 120 rpm for 3 to 6 days. The crude extract from media was harvested at the interval of 24 hrs up to 6 days. The enzyme quantity expected to increase with increase in fungal growth with period of incubation and then decline. This may be due to increase in concentration of certain toxic wastes and depletion of nutrients in fermentation media which leads to decreased fungal growth and enzymes production [15]. In the current experiment, the rate of cellulase and xylanase production increased with increase incubation time (Fig. 3 and Fig. 4).

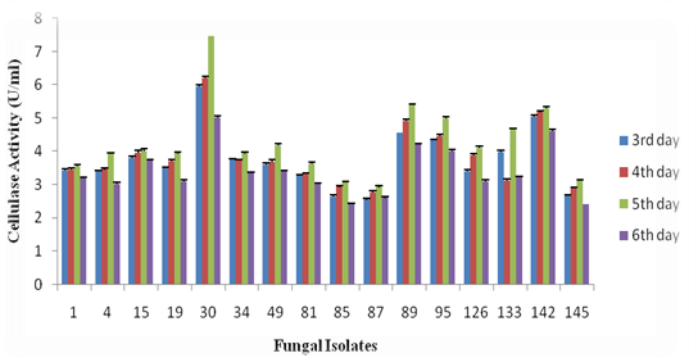

Figure 3 Secondary screening of fungal isolates for cellulase activity at different incubation days

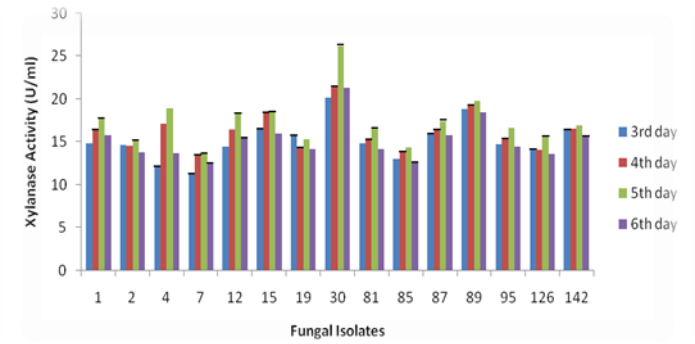

Figure 4 Secondary screening of fungal isolates for xylanase activity at different incubation days

The maximum amount of enzyme was recorded on 5th day for vegetable waste with maximum cellulase activity (7.46±0.02 U/ml) and xylanase activity (26.23±0.06 U/ml) was observed in case of fungal isolate numbered-30. Sun et al. [16] also recorded high enzyme activity on apple pomace by *Trichoderma* sp. at 120 h in SmF. Gautam et al. [17] also observed that the *Trichoderma* sp. is well known among the cellulolytic fungi for their potential to degrade organic municipal solid waste. Doolotkeldieva and Bobusheva [18] have been reported the soft-rot fungi, *Trichoderma viride* and *Trichoderma reesei* are the most extensively studied cellulolytic fungi. The present finding indicates that the fungal forms contain enzymes complexes for the effective hydrolysis of cellulose. The fungal strain selected after secondary screening was identified as *Trichoderma atroviride* by Institute of Microbial Technology, Chandigarh, India through nucleotide BLAST analysis which was based on 18S rRNA gene sequence.

IV. Conclusions

Fungi are known to produce cellulase and xylanases which are of high industrial importance. These enzymes are very essential for degradation of agricultural wastes, municipal solid wastes and other organic wastes. Present research is focused on the isolation and screening of cellulase and xylanase producing microorganisms. Total 151 fungal isolates were screened for these enzymes production by zone of clearing around the colony of microbes on agar plates. The selected fungal strains were examined cellulase and xylanase production in CMC and xylan broth media respectively. Among these microorganisms, *Trichoderma atroviride* showed most efficient cellulase and xylanase producer and showed maximum cellulase activity (7.46±0.02 U/ml) and xylanase activity (26.23±0.06 U/ml) at 5th day of incubation at 30°C.

Acknowledgement

The author gratefully acknowledges support by getting Senior Research Fellowship from the University Grant Commission (UGC) and lab facilities from Deptt. of Environmental Sciences, M.D.University, Rohtak.

References

[1]. M.A. Milala , A. Shugaba , A. Gidado , A.C. Ene, and J.A. Wafar, Studies on the Use of Agricultural Wastes for Cellulase Enzyme Production by *Aspegillus niger, Research Journal of Agricultural Biological Sciences, 1(4),* 2005, 325-328.

[2]. B.E. Dale, Biobased industrial products: bioprocessengineering when cost really count, Biotechnol. *Prog, 15*, 1999, 775-776.

[3]. M.L. Maki, M. Broere., K. Tin Leung, and Q. Wensheng, Characterization of some efficient cellulases producing bacteria isolated from paper mill sludges and organic fertilizers, *International Journal of Biochemistry and Molecular Biology, 2(2),* 2011, 146-154.

[4]. U. Holker, M. Hofer, and J. Lenz, Biotechnological advantages of laboratory scale solid state fermentation with fungi, *Appl Microbiol Biotechnol, 64*, 2004, 175-86.

[5]. H.Y. Fang, S.M. Changa, M.C. Hsieh, and T.J. Fang, Production, optimization growth conditions and properties of the xylanase from *Aspergillus carneus* M34, *J Mol Catal B Enzym , 49*, 2007, 36-42.

[6]. S.S. Dhiman, J. Sharma and B. Battan, Industrial Applications and future prospects of Microbial Xylanases: A Review, *BioResources,* 3(4), 2008, 1377- 1402.

[7]. S.D. Mansfield, C. Mooney, and J.N. Saddler, Substrate and enzyme characteristics that limit cellulose hydrolysis, *Biotechnological Progress 15*, 1999, 804-816.

[8]. A. Krisana, S. Rutchadaporn, G. Jarupan, E. Lily, T. Sutipa, and K. Kanyawim, Endo-1,4-β-xylanase from Aspergillus cf. niger BCC14405 isolated in Thailand: purification, characterization andgene isolation, *J. Biochem. Mol. Biol, 38*, 2005, 17-23.

[9]. S. Subramaniyan, and P. Prema, Biotechnology of microbial xylanases: Enzymology, molecular biology, and application, *Crit Rev Biotechnol, 2*, 2002, 33-64.

[10]. O. Garcia-Kirchner, M. Munoz-Aguilar, R. Perez-Villalva, and C. Huitro-Vargas, Mixed submerged fermentation with two filamentous fungi for cellulolytic and xylanolytic enzyme production, *Applied Biochemistry and Biotechnology, 98-100*, 2002, 1105-1114.

[11]. S.K. Ghatora, B.S. Chadia, A.K. Bhadan, and M.K. Bhat, Identification and characterisation of diverse xylanases from thermophilic and thermotolerant fungi, *Bioresources, 1(1),* 2011, 18-33.

[12]. G.L. Miller , Use of dinitrosalicyclic reagent for the determination of reducing sugars, *Anal Chemistry, 31(3),* 1959, 426-8.

[13]. L.R. Lynd, P.J. Weimer, W.H. Van Zyl, and I.S. Pretorius, Microbial cellulose utilization: fundamentals and biotechnology, *Microbiol. Mol. Biol. Rev., 66*, 2002, 506-577.

[14]. M.A. Abdel-Sater, and A.H.M. El–Said, Xylan-decomposing fungi and xylanolytic activity in agricultural and industrial wastes, *Int. J. Biodeterior.. Biodegrad,. 47*, 2002, 15-21.

[15]. R. Rajendran, R. Radhai, S.S.Karthik, and V. Rajalakshmi, Utilization of cellulosic biomass as a substrate for the production of bioethanol, *International Journal of Environmental Sciences, 5(4)*, 2015, 743-753.

[16]. H. Sun, G. Xiangyang, H. Zhikui, and P. Ming, Cellulase production by *Trichoderma sp.* on apple pomace under solid state fermentation, *Afr. J. Biotechnol, 9*, 2010, 163-166.

[17]. S.P. Gautam, P.S. Bundela, A.K. Pandey, M.K. Awasthi, and S. Sarsaiya, Screening of cellulolytic fungi for management of municipal solid waste, *J App Sci Environ Sanit (4),* 2010, 391-395.

[18]. T.D. Doolotkeldieva, and S.T. Bobusheva, Screening of wild-type fungal isolates for cellulolytic activity, *Microbiology Insights 4,* 2011, 1-10.