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Original Article

Isolation, identification and Screening of Xylanase producing fungi from Apple (*Pyrus malus* L.)

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Abstract: The objective of the present investigation was to isolate the fruit spoilage fungi from Apple *(Pyrus malus L.)* sold in twin cities of Hyderabad and to identify the strains that secrete maximum amount of xylanase. Recently xylanases have been explained for their use in many processing industries such as paper, pulp, food and textile industries etc. In all 12 fruit spoilage fungi were isolated, identified and screened based upon the formation of clearing zones on malt extract agar medium (MEA) enriched with xylan as the sole source of carbon. The fungal isolates were also tested for their potential to deteriorate the fruit by satisfying Koch's postulates (Pathogenicity test). Submerged fermentation was also carried out with the fungal isolates to identify the strain that could produce highest amount of xylanase at pH 6 and at temperature 28 ± 2°C. On screening, all the isolates could produce xylanase. However, the amount of enzyme production varied with the fungi. Growth was determined in terms of mycelial dry weight and soluble protein content was also determined which ranged between 90 to 200µg/ml. The pH change after the stipulated incubation period ranged from 5.5 to 7.0.

Keywords: Apple, Xylanase, Xylan, Malt Extract Agar, Screening, Submerged Fermentation.

Introduction

Xylan, a biopolymer after cellulose, is the most abundant renewable non-cellulosic polysaccharide present in wood, agricultural and several agro-industrial wastes. Studies reveal that xylan forms an interphase between lignin and other polysaccharides. It is mainly present in the secondary cell wall and covalently linked with lignin phenolic residues and other polysaccharides such as pectins and glucans. Hemicellulose (xylan), which is a constituent of primary and secondary cell walls of plant, includes xylans, xylanoglucans, mannans and hexoses, galactose and mannose. Xylanoglucan, a chain of β-1, 4-linked D-glucopyranose residues with terminal branches of α-1, 6-linked xylanopyranose, is a constituent of primary cell wall. Generally hemicelluloses are attached to cellulose microfibrils by hydrogen bonds and cross-links between cellulose microfibrils [1]. The formation of the cellulose-hemicellulose network probably gives rigidity to cell walls. At the initial stage of fruit ripening, breakdown of hemicellulose molecules results in the softening which disrupts the cellulose-hemicellulose network [2]. Biodegradation of xylan is a complex process that requires the coordination of several xylanolytic enzymes which hydrolyze xylan and arabinoxylan polymers. Xylanases are genetically single chain glycoproteins, ranging from 6–80 kDa, active between pH 4.5-6.5 and at temperature between 40 - 60°C. Xylanases from different sources differ in their requirements for temperature, pH, *etc*. for optimum functioning. The complete enzymatic hydrolysis of xylan into its constituent monosaccharides requires the synergistic action of a consortium of xylanolytic enzymes. This is due to the fact that xylans from different sources exhibit a significant variation in composition and structure. This enzyme group includes endo-β 1,4 xylanase (1,4-β-D xylan xylanohydrolase, EC 3.2.1.8), which attacks the main chain of xylans and β-D-xylosidase (1,4- β xylan xylanohydrolase, EC 3.2.1.37), which hydrolyze xylo-oligosaccharidesinto D-xylose, in addition to a variety of debranching enzymes, that is, α-L-arabino furanosidases, α-glucuronidases and acetyl esterases [3]. These enzymes have many applications in industrial point of view. Hence, maximum attention is being paid towards xylan degrading enzymes.

The hemicellulose fraction of the plant cell wall is reported to be degraded by hemicellulase (xylanase) to monomeric

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constituents. *Trichoderma* and *Aspergillus* species are reported to be efficient in the degradation of these substances by secreting xylanases [4]. Breakdown of hemicellulose is a crucial stage in fruit softening [5]. Hemicellulose undergoes depolymerization in the harvested apple fruit and is responsible for fruit softening. Fabiana *et al*. [6] have implicated that xylanases play a major role in tissue maceration and are responsible for disease cause. Therefore, it was felt necessary to investigate the xylanase producing capacity of the apple rot fungi.

Materials and Methods

Chemicals:

All chemicals used were of analytical grade and media components of highest purity grade. The microbiological media used were dehydrated media (Hi-Media, Mumbai). Production studies were carried out as batch cultures in 100ml Erlenmeyer flasks, containing 25ml of culture media. All the experiments were carried out independently in duplicates.

Substrate:

Birch wood xylan purchased from Sigma chemicals Co., USA was used as a substrate for xylanase production.

Isolation of fruit spoilage fungi:

Apple fruits were collected from twin cities (Hyderabad and Secundrabad). The areas of sample collection are Balanagar, Mehdipatnam, Sanathnagar, Lakdikapool, Manikonda, Ameerpet, Begumpet, Secundrabad, Mettiguda, Bharathnagar, Narayanguda, Kukatpally and Thirumalgiri. The diseased fruits were collected separately in polythene bags to avoid contamination. The symptoms were carefully noted; completely rotten fruits were avoided for isolation as they contained mostly secondary pathogens. If fruit bodies were present on the infected portions, slides were prepared by scraping the diseased portions. The fruit were surface sterilized with 0.1% mercuric chloride. Isolations were made from the juncture of healthy and diseased regions on the peel of the infected fruits. The diseased tissues were surface sterilized with 90% ethyl alcohol and transferred aseptically to Asthana and Hawker's medium slants. The pH of the medium was adjusted to 6 with the help of 0.1M HCl before sterilizing in an autoclave at 121^{o}C for 20 minutes. After 2 or 3 days the hyphal tips coming out of the infected tissues were transferred to fresh slants. The fungi were identified with the help of standard monographs [7, 8].

Pathogenicity tests:

Pathogenicity tests were conducted by scalpel injury method [9]. Surface sterilized healthy fruits were inoculated with respective fungi after inflicting a scalpel injury. Sufficient care was taken to maintain high humidity as well as to protect them from the attack of other organisms. At least six fruits were inoculated by the fungus and incubated at room temperature (28 $\pm$ 2^{o}C) for 7 days. The fungus was designated as a pathogen only after satisfying Koch's postulates. Whenever more than one organism was isolated from the single diseased lesion, the pathogenicity of each organism was confirmed separately.

Screening for xylanolytic activity on Malt Extract Agar medium (MEA):

All fungal isolates were screened by xylan-agar diffusion method for their abilities to produce extracellular xylanase during their growth on enriched Malt Extract Agar medium (MEA) containing xylan as the sole carbon source [10]. The inoculated plates were incubated for 5 days at 28 ± 2°C.Positive xylanolytic isolates were detected based on the clear zones of hydrolysis after flooding the plates with 0.1% aqueous Congo red followed by repeated washing with 1M NaCl [11]. Fungal strains, which produced distinct clearing zones around their colonies, were selected. The amount of xylanase produced was quantified under submerged fermentation condition.

Xylanase production under submerged fermentation:

For xylanase production under submerged conditions, Asthana and Hawker's medium supplemented with 1% birch wood xylan was used. For the inoculation of 25ml of culture broth, 5 discs each of 5.0mm in diameter were obtained by using a sterile cork borer from Asthana and Hawker's culture plate containing fungal lawn. Inoculated flasks were incubated at 28 ± 2°C under static conditions for 8 days. The culture medium was filtered using Whatman no.5 filter paper, the filtrate was centrifuged at 5000 rpm for 10 min. The clear supernatant was used as the crude extracellular enzyme source.

Quantitative assay for xylanase activity:

The amount of xylanase produced was measured by using 1% birch wood xylan as the substrate [12]. Xylanase activity was assayed in 3.0ml of a reaction mixture containing 1.0ml of crude extracellular enzyme source, 1ml of 1% birch wood xylan (prepared in 0.05 M Na-citrate buffer, pH 5.5) and 1ml of 0.05 M citrate buffer. The mixture was incubated at 55°C for 10 min. The reaction was stopped by the addition of 3.0ml of 3, 5-dinitrosalicylic acid (DNS) and the contents were boiled for 10 min [13]. After cooling, the color developed was read at 540 nm. The amount of reducing sugars liberated was quantified using xylose as standard. One unit of enzyme activity is defined as the amount of enzyme which releases 1 µmol of xylose in 1min under assay conditions [14].

Soluble protein assay:

Protein content of the culture supernatant was determined according to the method described by Lowry *et al.* [15] Using bovine serum albumin as standard.

pH determination:

Change in pH of the culture filtrate after 8 days of incubation was determined using pH paper.

Mycelial dry weight:

Mycelial biomass was collected on a pre-weighed Whatman filter paper no.5 dried to a constant weight at 60°C and reweighed. The difference in weight denoted the mycelial growth of fungus.

Results and Discussion

The present study mainly focused on the production of xylanase by the 12 fungi isolated from apple fruit. Intensive survey of wholesale and retail markets of Hyderabad was under taken at regular intervals during the month of June-November to isolate the fungal species from apple fruits. In all 9 fungal species representing 7 genera were recorded.

Identification of the fungal strains:

The fungal strains were stained by using lactophenol wet mount stain. They were identified on the basis of morphological, cultural and characteristic reproductive structures by using standard reference manuals [7, 8]. In all twelve fungi were identified. They are *Alternaria alternata, Alternaria sps, Aspergillus niger, A.nidulans, A. ochraceus, Aspergillus sps, Curvularia lunata, Curvularia sps, Fusarium oxysporum, Penicillium citrinum, Rhizopus stolonifer* and *Trichoderma* (Fig.1).

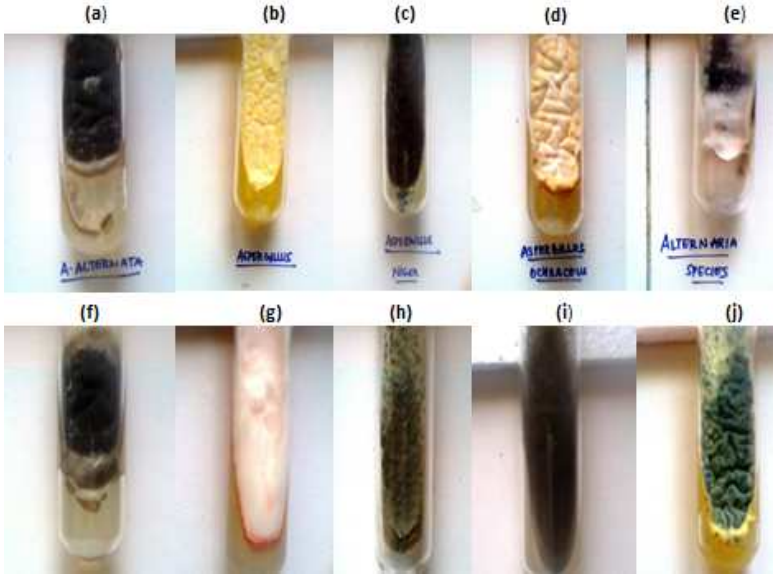


Fig.1: **Identified fungal strains on PDA**.
(a) *Alternaira alternata* ; (b) *Aspergillus sps*; (c) *Aspergillus niger* ; (d) *Aspergillus ochraceus* ;(e) *Alternaira sps* (f) *Curvularia lunata*; (g) *Fusarium oxysporum*; (h) *Trichoderma* ; (i) *Rhizopus stolonifer* ; (j) *Penicillium citrinum*

Pathogenicity test:

The healthy unripe fruits were inoculated with the isolates and incubated for 7 days. The organisms satisfying the Koch's postulates and designated as pathogen. All the isolates were tested for the pathogenicity test. Pathogenicity tests revealed that *C. lunata*, *F.oxysporum*, *R. stolonifer*, *A. niger* were able to spread rapidly in the fruit and the area of infection recorded for these fungi was maximum on 7th day of the incubation period (Table.1). The highest area of infection was given by *C. lunata* as it could spread rapidly in the fruit and caused significant damage to the fruit.

Table.1: Area of infection shown by fungal pathogens on Apple

Name of the Organism	Area of infection in Cm and Days of incubation		
	3rd	5th	7th
Alternaria alternata	0.20	0.50	0.95
Aspergillus sps	0.15	0.50	1.20
A. nidulans	0.10	0.30	0.80
A. niger	0.45	0.90	1.80
Curvularia lunata	0.56	2.10	3.00
F. oxysporum	0.40	0.90	2.00
Penicillium citrinum	Nil	Nil	Nil
Rhizopus stolonifer	0.60	1.00	1.90
Trichoderma	Nil	0.10	0.25

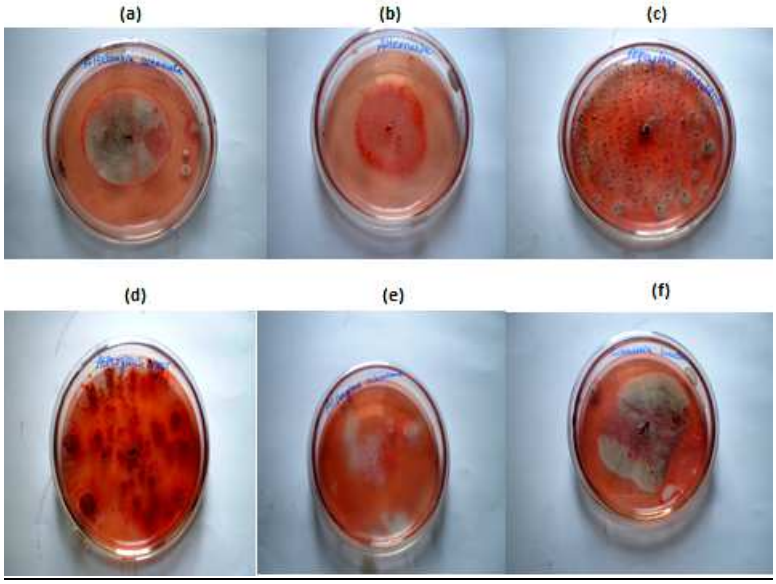
Screening for xylanolytic activity on malt extract agar medium:

The primary screening for the xylanolytic activity of all the fungal isolates was determined by taking the inoculum and seeded onto the malt extract agar medium. After 5 days of incubation, the plates were observed for the growth of the organism as well as the zone of hydrolysis. The results are depicted in the Table 2 and Fig.2. *C. lunata* showed the maximum zone of hydrolysis (5.6cm) followed by *A. alternaria* (5.0 cm) and *F. oxysporum* (4.2cm). *Trichoderma* showed (0.5 cm) minimum zone of hydrolysis followed by *Aspergillus nidulans* with a hydrolytic zone of 1.2cm. Quantitative xylanase was also done. From Table 2 it is clear that all the fungal isolates under study secreted xylanase at varied levels. Overall, maximum xylanase production was recorded in *C. lunata* (179.8U/ml) followed by *Penicillium citrinum* with an enzyme activity of 167.8 U/ml. On the other hand minimum activity was recorded in *Curvularia sps* and *Alternaria sps* with enzyme activities of 53.9 U/ml and 71.9 U/ml respectively. Four different *Aspergillus sps* isolated from apple, showed similar xylanase activity (107.9 U/ml).

Table.2: Assay of xylanase (qualitative and quantitative) by twelve fungi isolated from Apple (*Pyrus malus* L.)

S.No	Name of the organism	Qualitative assay	pH	Quantitative assay		Soluble Protein
		Hydrolysed zone (cm)		Mycelial dry weight (mg)	Xylanase activity (U/ml)	content (µg/ml)
1	*Alternaria sps*	4.5	7.0	194.0	71.9	90.0
2	*Alternaria alternata*	5.0	7.0	155.0	71.9	90.0
3	*Aspergillus nidulans*	1.2	7.0	53.0	107.9	156.0
4	*A.niger*	2.5	5.0	123.0	107.9	156.0
5	*A. ochraceus*	2.5	7.0	120.0	107.9	90.0
6	*Aspergillus sps*	3.3	7.0	166.0	107.9	156.0
7	*Curvularia lunata*	5.6	7.0	153.0	179.8	180.0
8	*Curvularia sps*	4.6	7.0	61.0	53.9	156.0
9	*Fusarium oxysporum*	4.2	7.0	186.0	89.9	90.0
10	*Penicillium citrinum*	2.8	7.0	148.0	167.8	90.0
11	*Rhizopus stolonifer*	3.0	5.5	38.0	89.9	114.0
12	*Trichoderma*	0.5	7.0	81.0	149.8	90.0

Enzyme production in microbes is associated with the growth phase [16]. So to confirm this mycelial dry weight of all the isolates was determined. In the present study the results depicted that the mycelial dry weight for all the fungal isolates was not related with enzyme production. For instance *Curvularia lunata* which showed the maximum enzyme activity could grow moderately as the mycelial biomass was less when compared to *Fusarium oxysporum* with low enzyme production (Table 2). Soluble protein content of the 12 fungal isolates was calculated, which showed their range from 90 to 200µg/ml. The pH changes at the end of incubation was very less with a range of 5.5-7.0.



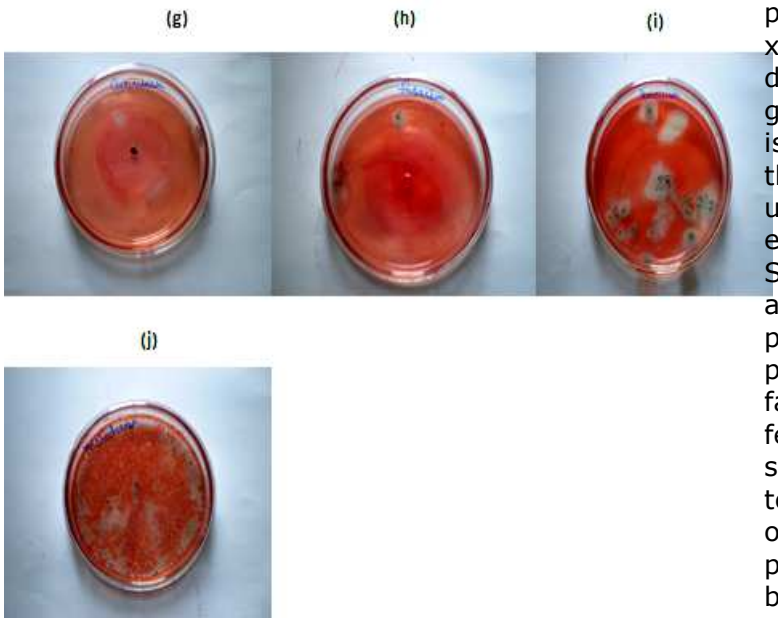


Fig.2: Screening for xylanolytic activity on Malt Extract Agar medium (hydrolysed zone in cm) produced by Fungi. (a) *Alternaira alternata*; (b) *Alternaira sps;* (c) *Aspergillus nidulans*; (d) *A. niger*; (e) *A. ochraceus* ; (f) *Curvularia lunata*; (g) *Curvularia*; (h) *Fusarium oxysporum*; (i) *Penicillium citrinum*; (j) *Trichoderma.*

Filamentous fungi are potent xylanase producers when compared to the large group of bacteria and yeast [17]. Therefore, an attempt has been made to focus on the isolation of fungi with xylanase producing capacity. The fungi which showed positive results for screening test on MEA for appearance of clear zones were further confirmed for enzyme production under submerged conditions using 1% xylan as the carbon source as described by Flannigan and Gilmour [18]. Tseng *et al*. [19] reported that some of the strains which were identified as potential xylanase producing microbes on MEA by screening method, did not show any enzyme activity in the liquid medium. In contrast, some strains, which were identified as negative, were able to produce high amounts of enzyme [11, 19]. Hence all the isolates screened on solid based medium were cultured in broth for the confirmation of xylanase production. This data is in agreement with Teather and Wood [11], Tseng *et al*. [19] and Sridevi and Charya [20], that the zone of hydrolysis is not an accurate method to decide the maximum enzyme production.

Conclusion

Research during the past few decades has been dedicated to enhance production, purification and characterization of microbial xylanases. But for commercial application detail knowledge of regulatory mechanisms governing enzyme production and functioning is required. Since application of xylanase in the commercial sector is widening, an understanding of its nature and properties for efficient and effective usage becomes crucial. Study of synergistic action of multiple forms and mechanism of action of xylanase makes it possible to use it for bio-bleaching of kraft pulp, paper pulp industry, bioprocessing of fabrics, waste paper recycling, ruminant's feed, baking and other applications. Further studies on optimization of cultural conditions to provide prospective information on optimization process for xylanase production, purification and characterization of xylanase by potent fungal strains are in progress.

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