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Optimization of Xylanase production by *Alternaria Alternata* isolated 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 under submerged conditions. Recently xylanases have been explained for their use in many processing industries such as paper, pulp, food and textile industries. In all 12 fruit spoilage fungi were isolated. *Alternaria alternata* was selected for further studies based on the pathogenicity test and initial screening by xylan - agar diffusion method for its ability to produce extracellular xylanase during its growth on enriched malt extract agar medium containing xylan as the sole source of carbon. The influence of various culture conditions including incubation period, synthetic media, initial pH, temperature, carbon sources and nitrogen sources on xylanase production by *A. alternata* was studied. Apart from birch wood xylan, xylose (174.0 U/ml) was the best inducer for the xylanase production. Xylanase production was maximum in medium B (medium A supplemented with 1 % fruit peel extract) with an enzyme activity of 102.0 U/ml. *Alternaria alternata* was more active in the production of xylanase when grown in Asthana and Hawker's medium at pH 7 (149.0 U/ml). Optimum xylanase activity was shown at temperature at 35°C (137.8 U/ml). On the 6th day of incubation period xylanase production was maximum with an enzyme activity of 203.8 U/ml. Among the nine different nitrogen sources selected to study their effect on xylanase production, Glycine was found to be the best nitrogen source for enzyme production as it showed maximum enzyme activity (185.0 U/ml).

Key words: Xylanase, *Alternaria alternata*, xylan, submerged fermentation.

1. INTRODUCTION

1.1. Xylan

Xylan, a biopolymer next to 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 (Fujino et al., 2000). 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 (Wakabayashi, 2000). Biodegradation of xylan is a complex process that requires the coordination of several xylanolytic enzymes which hydrolyze xylan and arabinoxylan polymers.

1.2. Xylanases

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-arabinofuranosidases, α-glucuronidases and acetyl esterases (Collin et al., 2005). 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 xylanase to monomeric constituents. *Trichoderma* and *Aspergillus* species are reported to be efficient in the degradation of these substances by secreting xylanases (Bajpai, 1997). Breakdown of hemicellulose is a crucial stage in fruit softening (Lohani et al., 2004). Hemicellulose undergoes depolymerization in the harvested apple fruit and is responsible for fruit softening. Fabiana et al. (2005) 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. Out of the various environmental factors temperature and pH play a significant role in the host-pathogen interaction. Temperature and moisture content of the post-harvest environment play an important role in promoting fungal advancement and decay (Fatima et al., 2006). Though the production of cellulolytic and pectolytic enzymes and their relation of pathogenicity and disease development has been studied by several investigators, very little information is available on the factors influencing on the production of cell wall dissolving enzymes by plant pathogens (Chande et al., 2007; Arotupin, 2007). Thus as there are no proper reports on efficient xylanase production by the fungus. *A. alternata* (isolated from apple fruit), an attempt has been made in the present study to provide prospective information on optimization process of xylanase production by *A. alternata* under submerged conditions.

2. MATERIALS AND METHODS

2.1. 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 100 ml Erlenmeyer flasks, containing 25 ml of culture media. All the experiments were carried out independently in duplicates.

2.2. Substrate

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

2.3. 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

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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 (Ellis, 1976; Raper and Fennell, 1965).

2.4. 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 (Nakamura et al., 1993). 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 (Teather and Wood 1982). Fungal strains, which produced distinct clearing zones around their colonies, were selected. The amount of xylanase produced was quantified under submerged fermentation condition.

2.5. 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 25 ml of culture broth, 5 discs each of 5.0 mm 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.

2.6. Quantitative assay for xylanase activity

The amount of xylanase produced was measured by using 1% birch wood xylan as the substrate (Bailey et al., 1992). Xylanase activity was assayed in 3.0 ml of a reaction mixture containing 1.0 ml of crude extracellular enzyme source, 1 ml of 1% birch wood xylan (prepared in 0.05 M Na-citrate buffer, pH 5.5) and 1 ml of 0.05 M citrate buffer. The mixture was incubated at 55°C for 10 min. The reaction was stopped by the addition of 3.0 ml of 3, 5- dinitrosalicylic acid (DNS) and the contents were boiled for 10 min (Miller, 1959). 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 1 min under assay conditions (Khan et al., 1986).

2.7. Soluble protein assay

Protein content of the culture supernatant was determined according to the method described by Lowry et al., (1951) using bovine serum albumin as standard.

2.8. pH determination

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

2.9. 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.

2.10. Organism

Alternaria alternata was employed for xylanase production based on the pathogenicity test and screening results. It is a potent phytopathogenic fungus well known for fruit-rots among various economically important fruits like banana, mango, grapes, apple and others. Asthana and Hawker's medium supplemented with 1% birch wood xylan was employed for production studies. 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 and incubated aerobically at (28± 2^{o}C).

2.11. Inoculum preparation

The fungus (*A. alternata*) was routinely grown and maintained on Asthana and Hawker's agar slants. They were subcultured from the old culture onto fresh agar slants. Five day old culture of the organism served as inoculum. The

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culture medium was filtered using Whatman no.5 filter paper, centrifuged at 5000 rpm for 10 min. The clear supernatant was used as the crude extracellular enzyme source.

2.12. Optimization of process parameters

The optimization of composition of medium and cultural conditions was carried out based on stepwise modification of the governing parameters for xylanase production. Production of xylanase on different synthetic media was carried out supplementing the basal media with 1% fruit peel and pulp extract. Cultivation of the organisms was also carried at different incubation periods, temperatures and pH. Effect of different carbon and nitrogen sources on xylanase production was also investigated.

2.13. Effect of incubation period

In this experiment the production of xylanase by *A. alternata* was carried along 8.0 days of incubation grown on Asthana and Hawkers medium supplemented with 1% xylan. One flask was harvested each day and the enzyme was extracted and estimated till the 8th day of incubation period was completed.

2.14. Effect of media

Substrates play a vital role in the growth of the organism, production of enzymes, secondary metabolites etc. The following synthetic media were selected to study the production of xylanase.

1. Asthana and Hawker's medium-A
2. Medium A+ 1 % fruit peel extract-B
3. Medium A+ 1 % fruit pulp extract-C
4. Xylose peptone medium- D

2.15. Effect of temperature

Effect of temperature on xylanase production by *A. alternata* was examined at various temperatures ranging from 20°C- 45°C. The flasks were incubated for 8 days and supernatant was used as a crude enzyme to calculate the xylanase activity and protein content.

2.16. Effect of pH

To determine the maximum enzyme production at particular pH, the medium was adjusted to different pH (3-8) prior to autoclaving and the organism was inoculated. The flasks were incubated for 8 days and supernatant was used for determining the xylanase activity.

2.17. Effect of carbon source

To check the effect of different carbon sources on xylanase production- glucose, sucrose, xylose, maltose, mannitol, starch and fructose were employed. Different carbon sources were added at a concentration of 1% to the media and incubated for 8 days and supernatant was used as a crude enzyme to calculate the xylanase activity and protein content.

2.18. Effect of nitrogen source

To know the effect of nitrogen source on xylanase production, the fermentation medium was supplemented with both organic and inorganic nitrogen sources. The nitrogen sources used were as asparagine, potassium nitrate, sodium nitrate, ammonium nitrate, glycine, ammonium chloride, ammonium sulphate and glutamic acid. The nitrogen source used at 1% level by replacing the prescribed nitrogen source of the fermentation medium and was incubated for 8 days and supernatant was used for determining the xylanase activity.

3. 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.

3.1. 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 (Ellis, 1976; Raper, 1965). In all twelve fungi were identified- *Alternaria alternata, Alternaria sps, Aspergillus niger, A.nidulans,*

Table 1

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

S.No	Name of the organism	Qualitative assay		Quantitative assay		
		Hydrolysed zone (cm)	pH	Mycelial dry weight (mg)	Xylanase activity (U/ml)	Soluble Protein 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

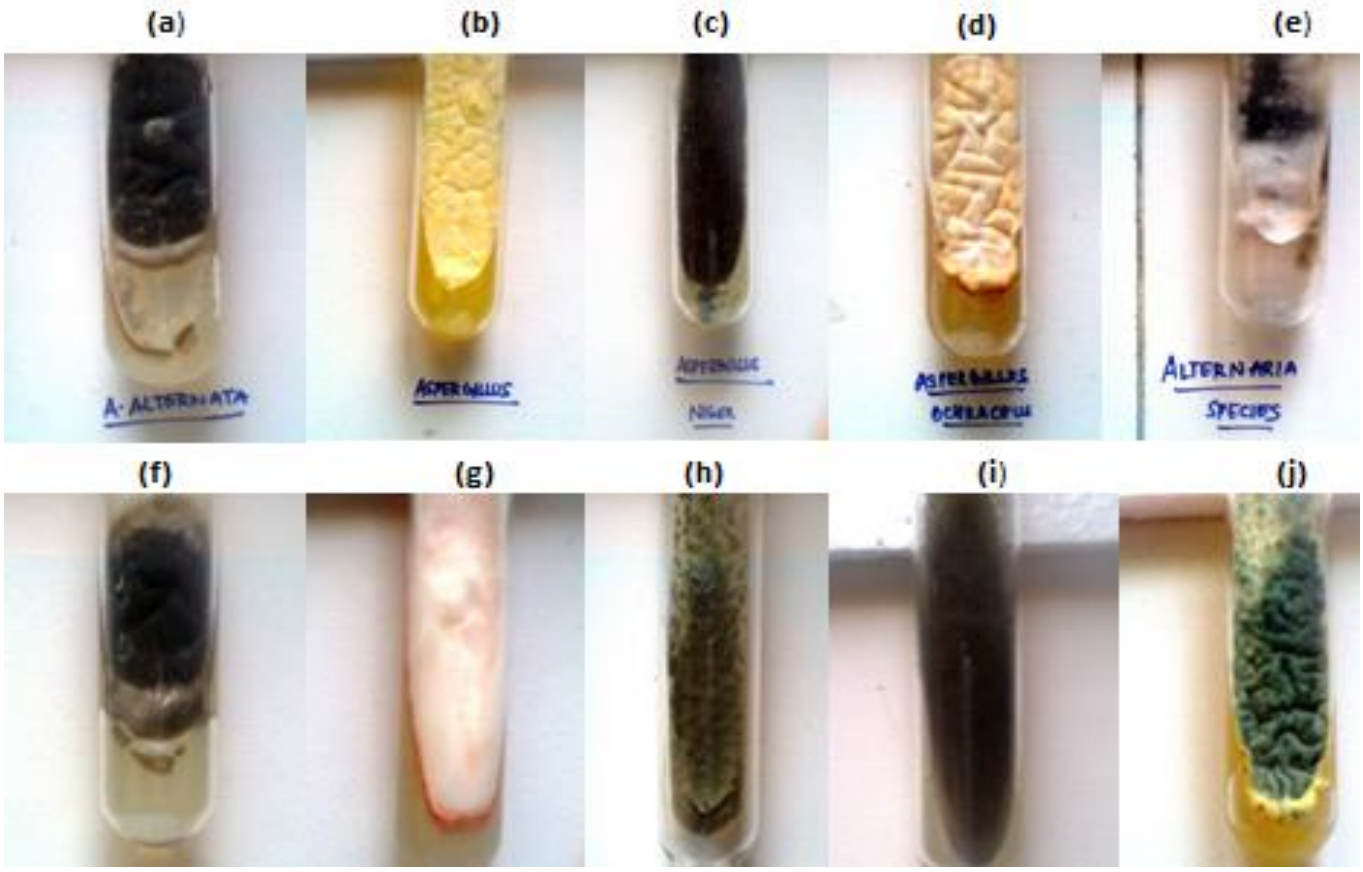

Figure 1

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*

A.ochraceus, Aspergillus sps, Curvularia lunata, Curvularia sps, Fusarium oxysporum, Penicillium citrinum, Rhizopus stolonifer and *Trichoderma* (Figure 1).

3.2. 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

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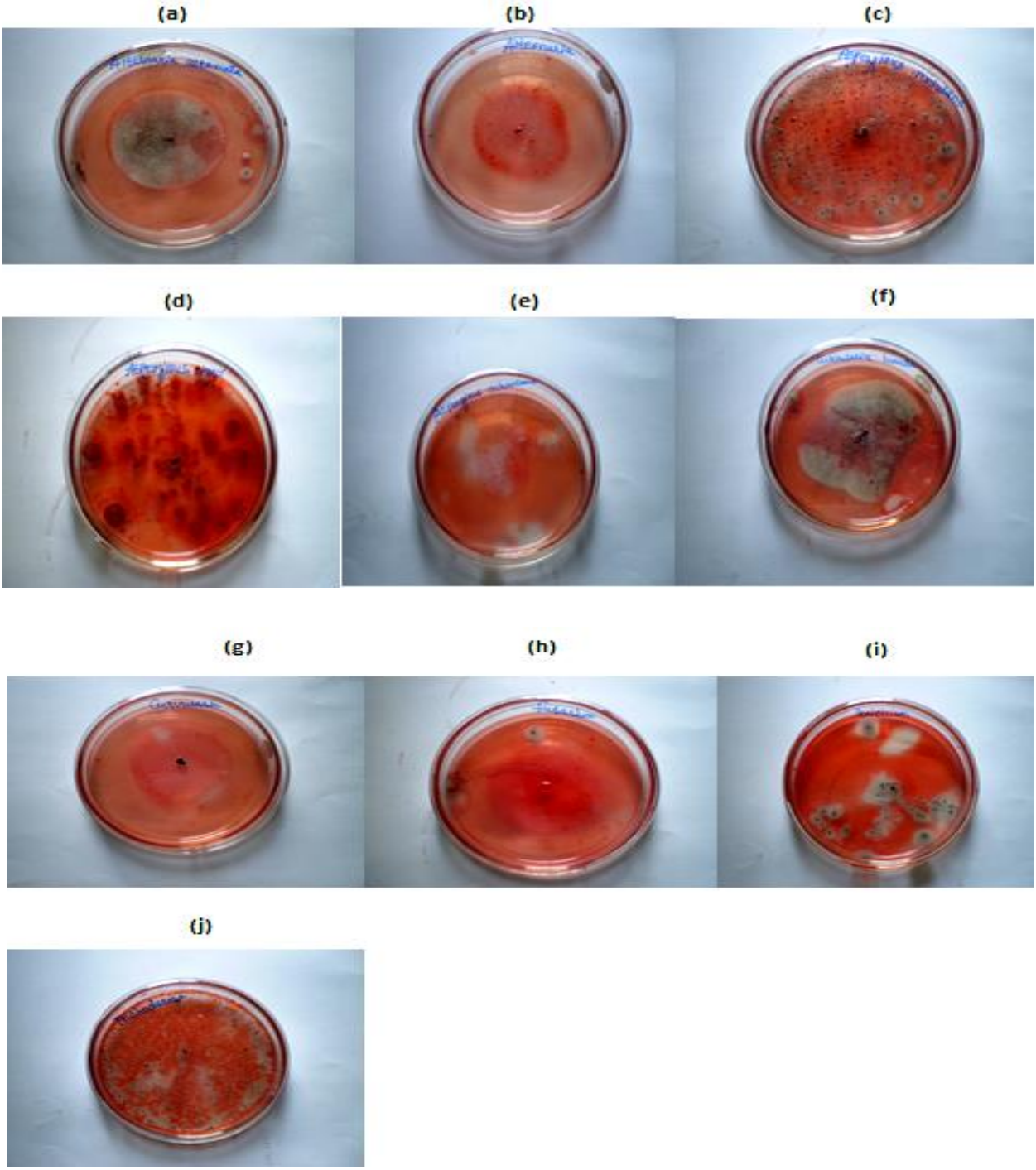

Figure 2

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.*

organism as well as the zone of hydrolysis (Figure 2). The results are depicted in the Table 1**.** *C. lunata* showed the maximum zone of hydrolysis (5.6 cm) followed by *A. alternaria* (5.0 cm) and *F.oxysporum* (4.2 cm). *Trichoderma* showed (0.5 cm) minimum zone of hydrolysis followed *by A. nidulans* with a hydrolytic zone of 1.2 cm. Quantitative xylanase was also done. From Table 1 it is clear that all the fungal isolates under study secreted xylanase at varied levels. Overall, maximum xylanase production was recorded in *C. lunata* (179.8 U/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

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Table 2

Effect of different incubation periods on xylanase production by *Alternaria alternata*

Days of incubation	Mycelial dry Weight (in mg)	Ph	Xylanase activity (U/ml)	Soluble Protein content (µg/ml)
Day 1	90.0	5.6	167.8	185.0
Day 2	110.0	6.0	174.0	214.0
Day 3	140.0	6.4	185.8	242.0
Day 4	120.0	6.8	174.0	285.0
Day 5	110.0	6.9	185.0	355.0
Day 6	120.0	7.0	203.0	399.0
Day 7	90.0	7.0	185.8	442.0
Day 8	210.0	7.0	179.8	471.0

Table 3

Production of xylanase on different synthetic media by *Alternaria alternata*

Medium	Mycelial dry Weight (in mg)	pH	Xylanase activity (U/ml)	Soluble Protein content (µg/ml)
Asthana and Hawker's medium (Medium A)	97.3	7.0	79.9	107.0
Medium A+ 1% fruit peel extract [Medium B]	94.1	6.5	102.0	135.0
Medium A+ 1% fruit pulp extract [Medium C]	94.6	6.7	95.0	164.0
Xylose –peptone [Medium D]	207.8	7.0	23.9	250.0

Alternaria sps with enzyme activities of 53.9 U/ml and 71.9 U/ml respectively. Out of four different *Aspergillus sps* isolated from apple, showed similar xylanase activity (107.9 U/ml). Enzyme production in microbes is associated with the growth phase (Tlecuitl-Beristain *et al.*, 2008). 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 1). Soluble protein content of the 12 fungal isolates was calculated, which showed their range from 90 to 200 µg/ml. The pH changes during 8 days of incubation was very less with a range of 5.5- 7.0. Filamentous fungi are potent xylanase producers when compared to the large group of bacteria and yeast (Wong et al., 1988). 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 (1980). Tseng et al. (2000) 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 (Teather and Wood, 1982; Tseng et al., 2000). 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 (1982), Tseng et al. (2000) and Sridevi and Charya (2011), that the zone of hydrolysis is not an accurate method to decide the maximum enzyme production.

3.3. Optimization of process parameters:- Effect of incubation period

In this experiment, the production of xylanase by *A.alternata* was studied along 8 days incubation when cultivated on Asthana and Hawker's medium with 1% xylan. The xylanase activity was determined after every 24hrs of incubation in order to determine the optimum incubation period for maximum production of xylanase. Table 2 reveals that the enzyme production however started after 24hrs of inoculation and showed maximum production at 6th day of incubation period (167.3 U/ml).A significant decrease of xylanase activities were seen from fifth day onwards. Hoda et al. (Hoda et al., 2012) have reported the effect of incubation periods on xylanase production by *A.niger* and *T.viride* on barley bran medium with higher enzyme production on second day. Our optimum incubation period for production of xylanase was at 5th day which is different when compared with other studies on different substrates.

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Table 4

Effect of different temperatures on xylanase production by *Alternaria alternata*

Temperature (°C)	Mycelial dry weight (in mg)	pH	Xylanase activity (U/ml)	Soluble Protein content (µg/ml)
20	116.0	6.8	84.0	107.0
25	128.0	6.9	125.8	142.0
30	107.0	7.0	128.2	100.0
35	119.0	7.0	137.8	142.0
40	44.0	6.9	59.9	100.0
45	60.0	7.0	59.9	100.0

Similarly Fadel (2001a) have also reported the effect of incubation on xylanase production by *T.harzianum* under solid state fermentation and suggested that maximum enzyme production depends on the nature of substrate, organism, additive nutrients and many other cultural conditions.

3.4. Effect of media

The effect of synthetic media on xylanase production by *A.alternata* was investigated. Substrate level is a vital factor and has a lot of influence on other culture fermentable factors. The suitable level depends on substrate type, substrate size, organism and enzyme studied (Fadel, 2001a). From the present investigation, it is clear that *A.alternata* was capable of producing xylanase but it exhibited great specificity for the substratum. Critical perusal of Table 3 reveals that *A.alternata* could produce maximum enzyme in medium A with 1% fruit peel extract 102.0 U/ml) followed by medium A supplemented with1% fruit pulp (95.0 U/ml) while medium A and D supported intermediate degree of enzyme production. Mycelial growth was maximum in medium B and C. Xylanase production was higher in the presence of xylose -peptone medium (medium D) probably as the medium provided both carbon and nitrogen sources which enhanced the enzyme production. The pH drift was towards alkaline side and the final pH was near neutral in all the media under study. Bhagavan Reddy (1986) also recorded varying degrees of xylanase production by three isolates of *Myrothecium roridum* with the medium containing different substrates**.** More or less similar results were obtained by using different substrates for production of xylanase by *Penicillium oxalicum* (Muthechilan et al., 2007).

3.5. Effect of temperature

All the forms of the life are greatly influenced by temperature. In fact the microorganisms are very sensitive to temperature since their temperature varies with that of environment. Temperature influences the rate of the chemical reaction and protein structure integrity thus affecting rate of enzymatic activity. At low temperature enzymes are not denatured, therefore, every 10°C rise in temperature results in rise of metabolic activity and growth of microorganisms. However, the enzymes have a range of thermal stability and beyond it, their denaturation takes place. Thus, high temperature kills the microorganism. Each microorganism shows characteristics temperature dependence and possess its own cardinal temperature i.e. minimal, maximum and optimal growth temperatures. The rate of enzymatic reaction is strongly influenced by temperature. As the temperature is raised, the reaction rate increases until the enzymes reach their maximum temperature, following these enzymes undergo conformational alteration resulting in a decrease or complete loss of activity caused by the increasing temperature. The production of xylanase was maximum at temperature near to ambient temperature (35°C) with an activity of 137.8 U/ml and the mycelial weight was also the maximum (Table 4). A lower enzyme activity was obtained with cultivation temperatures lower and higher than the optimum temperature. At 25°C and 30°C activity of xylanase was 125.8 U/ml and 128.2 U/ml respectively. The results obtained clearly indicate that the enzyme production corresponds closely to growth of the fungus and optimum temperature for Xylanase production is similar to the ambient temperature for the growth of the fungus. The results obtained was in agreement with those reported by Chirstakopoullos et al. (1999). Biswas et al. (1990) and Gupta et al. (2009) who also showed that the highest xylanase activities were obtained at optimum temperatures for growth of *Fusarium oxysporum*, *A.ochraceus* and *F.solani* F7 respectively.

3.6. Effect of pH

The pH of a solution reveals whether the solution is acidic, alkaline or neutral. A neutral solution contains pH equal to 7. The activity of microbial enzymes depends on the change present on the surface of the amino acids. Any change in

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Table 5
Effect of different pH on xylanase production by *Alternaria alternata*

pH	Mycelial dry weight (in mg)	pH	Xylanase activity (U/ml)	Soluble Protein content (µg/ml)
3	144.0	4.8	72.0	114.0
4	190.0	5.2	89.9	185.0
5	126.0	5.6	95.0	257.0
6	167.0	6.8	84.0	242.0

Table 6
Effect of different carbon sources on xylanase production by *Alternaria alternata*

Carbon source	Mycelial dry weight (in mg)	pH	Xylanase activity (U/ml)	Soluble Protein content (µg/ml)
Fructose	300.0	7.0	174.0	328.0
Glucose	270.0	6.8	131.8	285.0
Maltose	247.0	6.5	149.0	214.0
Mannitol	360.0	5.9	161.8	114.0
Starch	264.0	6.9	137.8	371.0
Sucrose	278.0	5.8	170.0	142.0
Xylose	89.0	7.1	174.0	185.0
Control	80.0	6.8	100.0	257.0

the environmental pH may either enhance the enzyme activity or inhibit its activity. Thus pH can dramatically affect the growth of microorganisms. Each species of microorganisms shows specific pH range for its growth. The initial pH influences enzymatic system and the transport of enzymes across cell membrane (Padmavathi and Kavya, 2011). Like other protein, enzymes are amphoteric molecules. The charge of the amino acids varies with the pH value of the environment according to their dissociation constant. A change in pH value might affect the charges and configuration of the active site and thereby change the activity, structural stability or solubility of the enzymes. Optimum enzyme activity was observed at pH 5.0 with an activity of 95.0 U/ml and the growth of the fungus was also maximum at the same pH value (Table 5)**.** However, xylanse activity decreased at low pH (3 and 6) and high pH range (above pH 7-8).This might be due to the fact that acidic and alkaline pH has inhibitory effect on the growth of *A.alternata* and enzyme production. Xylanases from different organisms show an optimum pH within a range of 4.0-7.0.Similar results were also reported by Hoda et al. (2012) who worked on the effect of different pH on xylanase production by *A.niger* with an optimum enzyme activity at pH 5.5.Kormelink et al. (1992) reported optimum xyalnase activity by *A.awamori* at pH 5.0, 5.5 and 6.0 respectively. However, certain xylanses from *Aspergillus kawaachii*, *Penicillium herque* and some other fungi including *Fusarium oxysporum* exhibited an optimum pH on the acidic side (pH 2.0-6.0) (Funaguma et al., 1991; Ito et al.,1992; Kulkarni et al., 1999).

3.7. Effect of supplemented carbon source

Carbon is the major structural and functional component of microbial cells and plays an important role in the nutrition in fungi. Carbon source is required for all biosynthesis leading to reproduction, product formation and cell maintenance. Production of primary metabolite by microorganisms is highly influenced by their growth, which is determined by the availability of the nutrients in the substrates. Many fungal species are able to thrive on different kinds of aliphatic hydrocarbons. Thus fungi are capable of using wide variety of carbon compounds but most of the fungi prefer simple sugars. A perusal of Table 6 indicates that *A.alternata* could produce maximum xylanase when xylose was used as the carbon source (174.0 U/ml), followed by sucrose (170.0 U/ml) when compared to the control medium. On the other hand sucrose followed by xylose could induce low enzyme production. Mannitol and maltose when supplemented as carbon sources supported only intermediate activity. Glucose was found to be poor substrate as it showed very less enzyme activity. Thus from the results obtained it is clear that there is no positive correlation between the vegetative growth and enzyme production. For instance fructose, sucrose and xylose were good in inducing the production of enzyme but showed less mycelial growth when compared to the other carbon sources understudy. Gupta et al. (2009) and Kalogeries et al. (1998) also reported an increment of 42% in xylanase production when xylose was used as carbon source in comparison with other carbon sources studied by them. Therefore, these findings suggest that size of carbon source in an important factor in xylanase production. Xylose has also been described as an effective inducer and carbon source for xylanase production by *Fusarium oxysporum* (Chirstakopoullos et al., 1999) and *T.lanuginosus* (Purkarthofer et al., 1993).

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Table 7
Effect of nitrogen sources on xylanase production by *Alternaria alternata*

Nitrogen source	Mycelial dry weight (in mg)	pH	Xylanase activity (U/ml)	Soluble Protein content (µg/ml)
Asparagine	67.0	7.0	128.2	142.0
Ammonium chloride	44.0	7.2	161.8	100.0
Ammonium nitrate	75.0	7.0	174.0	107.0
Ammonium sulphate	62.0	5.2	161.8	171.0
Glutamic acid	148.0	5.3	128.0	242.0
Glycine	117.0	5.0	185.0	187.0
Potassium nitrate	80.0	7.0	174.0	142.0
Sodium nitrate	226.0	7.0	155.8	171.0
Control	72.0	6.8	131.8	114.0

3.10. Effect of supplemented nitrogen sources

The effect of supplementation of different inorganic and organic nitrogen sources on xylanases production was evaluated. Fungi are reported to exhibit great specificity for nitrogen source present in the medium. Like carbon source, nitrogen is also used both for functional and structural purposes by fungi. The source of nitrogen has a profound influence on the metabolism of microorganisms. Literature is depleted with conflicting claims regarding the comparative superiority of a particular form or source of nitrogen over the other. The results (Table 7) indicated that the enzyme production was high in glycine (185.0 U/ml) when compared to the nitrogen sources studied. Asparagine and Glutamic acid were poor substrates as they showed very low enzyme activity but supported good mycelial growth of the fungus. Thus the results obtained also did not show any significant difference between organic and inorganic nitrogen source on the production of xylanase by *A.alternata*. The results obtained are in agreement with those reported by Ghanem et al. (2000). In contrary to this Gupta et al. (2009) reported ammonium sulphate as poor enzyme inducer by *F.solani* F7.

4. 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 purification and characterization of xylanase by potent fungal strains are in progress.

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