XYLANASE

The plant cell wall is approximately composed of 90% polysaccharides (McNeil *et al.,* 1984), and the major components are cellulose, lignin, hemicellulose and pectin. These components are also referred as lignocellulosic components (Shallom and Shallom, 2003; Badhan *et al.,* 2007). Lignocellulose is one of the most natural complex organic carbons that form the plant biomass. Depending on the ratio of these compounds, different agricultural wastes have different composition. These components can be isolated on the basis of their solubility in different solvents. However, occurrence of these polysaccharides differs between the primary and secondary cell wall, it also varies from species to species and the types of cells (Timmell, 1967).

Hemicellulose consists of several different sugar units and substituted side chains in the form of low molecular weight linear or branched polymers. This polymer is more soluble than cellulose with a Degree of Polymerization (D.P.) of less than 200. Structurally, hemicelluoses are much more complex than celluloses (Thomson, 1993). It includes a heterogenous group of cellulose like polysaccharides that are variable in composition. They also vary greatly in amount

and complexity in different cell types and in different plant species (Huisman *et al.,* 2000). Pentoses comprise of near about 75% monomers of hemicellulose. Hemicelluloses unite to form a big group of high molecular polysaccharides that are insoluble in water but soluble in alkaline solutions. Hemicelluloses are associated with celluloses, lignin and plays an important role as a structurally supportive building block of plant cell wall (Nakamura *et al.,* 2003; Dobrev *et al.,* 2007).

2.1 Type of Hemicelluloses:

Most hemicelluloses consist of heteroglycans containing different sugar residues (Dekker and Richards, 1976), and so there are different types of hemicelluloses. Different types of heterogylans include noncellulosic β-glucans, mannans, xyloglucans, arabinans etc.

Noncellulosic β-glucans are in the form of β-1, 3 and β-1, 4-linked glucopyranosyl residues (are also called mixed linked glucans), that is unbranched chains of β-1, 4-linked glucose are disrupted by β-1, 3 linkages in a ratio of 1:2 to 1:3 (Carpita, 1996). It is believed that these polysaccharides are specific to family Poaceae (McNeil *et al.,* 1984). Glucan (callose) is an another scarce but important component of plant cell walls whereas, callose may be present in small amounts, but may be accumulated in relation to injury in trees (Smith, 1993).

Mannans have a linear backbone of β-1, 4-linked mannose and are mainly water soluble. They also contain a less soluble minor fraction, referred as glucomannans. Glucomannans have a β-1, 4-linked backbone of glucose and mannose in a ratio of 3:1. Glactomannans are glucomannans with α-1, 6-linked galactopyranose side branches. The proportion of mannose, glucose, and galactose varies in different fractions. The secondary cell walls of hardwood contain a few percent of glucomannan whereas; galactomannan is the major hemicellulose in lignified cell

walls of softwood. Glucomannan and galactomannan have a backbone of approximately 150 sugar residues (Timmell, 1967).

Xyloglucans are made up of β-1, 4-linked glucopyranose chains (like cellulose), with xylosyl units added to the O-6 position of glucosyl units of the chain (Carpita and Gibit, 1993). Besides their role as structural support, these polymers are also involved in the control of cell wall elongation. Furthermore, they are believed to coat the cellulose microfibrils, while some are woven into the cellulose microfibrils and yet others are spanning the gap between two adjacent microfibrils (McNeil *et al.,* 1984; Pauly *et al.,* 1999; Rose and Bernett, 1999).

Xyloglucan is covalently linked to pectic polysaccharides and non-covalently bound to cellulose (Bauer *et al.,* 1973). They are present in higher quantities in the cell walls of dicots than in the monocots (Darvill *et al.,* 1978). The chain length of the xyloglucan backbone may vary from 300 to 3000 glucose units (Fry, 1989). However, in xyloglucan, the backbone is substituted with a number of side chains that alter the physical properties of the xyloglucan compared to cellulose (Vierhuis *et al.,* 2001).

Arabinans (arabans) are the other polymers of primary cell walls. They are present in nature wherever pectic substance occurs, but they are considered as hemicelluloses (Dekker and Richards, 1976; Coughlan *et al.,* 1993). The dominant constituents of these polymers are α-L-arabinofuranosyl residues (Aspinall and Cottrell, 1971; Siddiqui and Wood, 1974).

Xylans are among the most abundant biopolymers, after cellulose (Joseleau *et al.,* 1992) comprising approximately 1/3 of renewable carbon sources on the earth (Prade, 1996). Xylan constitutes about 20-40% of total plant biomass (Ninawe *et al.,* 2007) and represents an immense resource of biopolymers for practical applications accounting for 25-30% of the dry biomass of woody tissues in dicots

and lignified tissues of monocots. It may also occur up to 50% in some tissue of cereal grains (Moure *et al.,* 2006). Xylan and cellulose are strongly associated because of β-1, 4-linked xylan chain forms hydrogen bond with cellulose (Carpita, 1992).

2.1.1 Structural features of Xylan:

In plant cell walls, structure of xylans can differ strongly depending on its origin but it always contains a β-1, 4-linked xylose backbone (Wilkie, 1979). Schematic representation of xylan (Figure 4) lists different structures attached to xylan backbone which leds to large variety of xylan structures found in plants. Although, most xylans are branched structures but perusal of literature shows that some linear polysaccharides/xylans have also been reported (Montgomery *et al.,* 1956; Eda *et al.,* 1976). Cereal xylans contain large quantities of L-arabinose and are therefore often referred as arabinoxylans whereas; hardwood xylans are referred as glucuronoxylans due to the high amount of glucuronic acid attached to the backbone (Timmell, 1967).

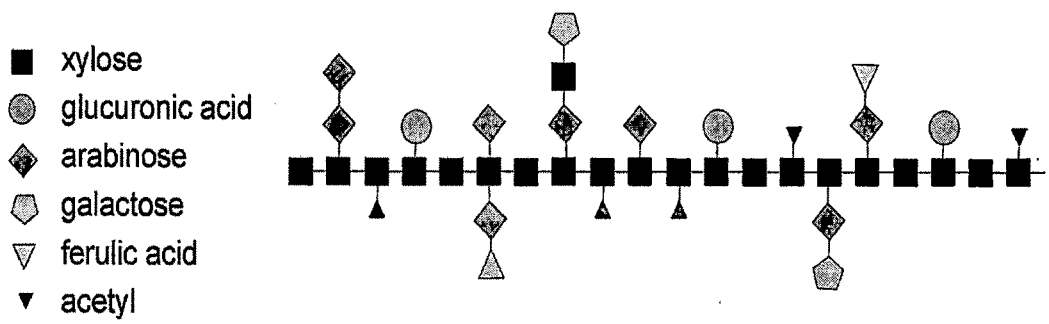

Figure 4: Schematic diagram of xylan molecule.

Arabinose is connected to the backbone of xylan via α-1, 2 or α-1, 3 linkages as single residues or as short side chains. These side chains can also contain xylose, β-1, 2-linked to arabinose, and galactose which can be either β-1, 5-linked to arabinose or β-1, 4-linked to xylose. Acetyl residues are attached to O-2 or O-3 of xylose in the backbone of xylan, but the degree of acetylation differs strongly

amongst xylans from different origins. Glucuronic acid and its 4-O-methyl ether are attached to the xylan backbone via a α-1, 2 linkage whereas; aromatic (feruloyl and *p*-coumaroyl) residues have so far only been found attached to O-5 of terminal arabinose residues. As a consequence of all these features, xylans form a very heterogeneous group of polysaccharides (Brillouet and Joseleau, 1987; Bajpai, 1997).

2.1.2 Enzymes required for hemicelluloses degradation:

Hemicellulases are the enzymes that break down hemicelluloses (Shallom and Shallom, 2003). These enzymes are classified according to the substrates on which they act and are collectively grouped as glycan hydrolases (EC 3.2.1). Although, the systems of hemicellulose degradation is analogous to that of non wood decaying Ascomycetes, it seems possible that evolutionary pressures in the tight molecular architecture of lignified cell walls could have altered enzyme sizes, shapes, and specificities (Kirk and Cullen, 1998). Complete degradation of heteropolysaccharides requires the action of several main chains and side chains cleaving enzyme xylanases. Enzymes which degrade hemicelluloses are hydrolytic in nature therefore; typically they are endo 1, 4-β-D-xylanases, endo 1, 4-β-D-mannanases, and endo 1, 4-β-D-galactanases. Compared to bacteria, xylan degrading enzyme system is well characterised in fungi (Polezeli *et al.,* 2005).

2.1.3 Classification of Hemicellulases:

On the basis of their mode of action, hemicellulases can be placed in three groups:

- Endoacting enzymes attacks polysaccharide chains internally, with little effect on short oligomers.
- Exoacting enzymes which operate via reducing or non-reducing termini of large or short chain substrates.
- A third group which includes acetylesterases and esterases that hydrolyse lignin glycoside bonds (Tenkanen and Poutanen, 1992).

On the basis of substrates, Dekker and Richards (1976) classified hemicellulases into following (Figure 5):

- L–arabinases: Enzyme that hydrolyse L–arabinans and are described as α-1, 3, 5-L-arabinan arabinanohydrolases. Most fungal arabinases are exo acting enzyme and many phytopathogenic fungi show constitutive L–arabinase activity (Fuches and Wouts, 1965).
- D–galactanases: They are specific for hydrolysing β-1, 3 and β-1, 4-D-galactopryanosyl linkages. These enzymes are almost entirely endoacting type.

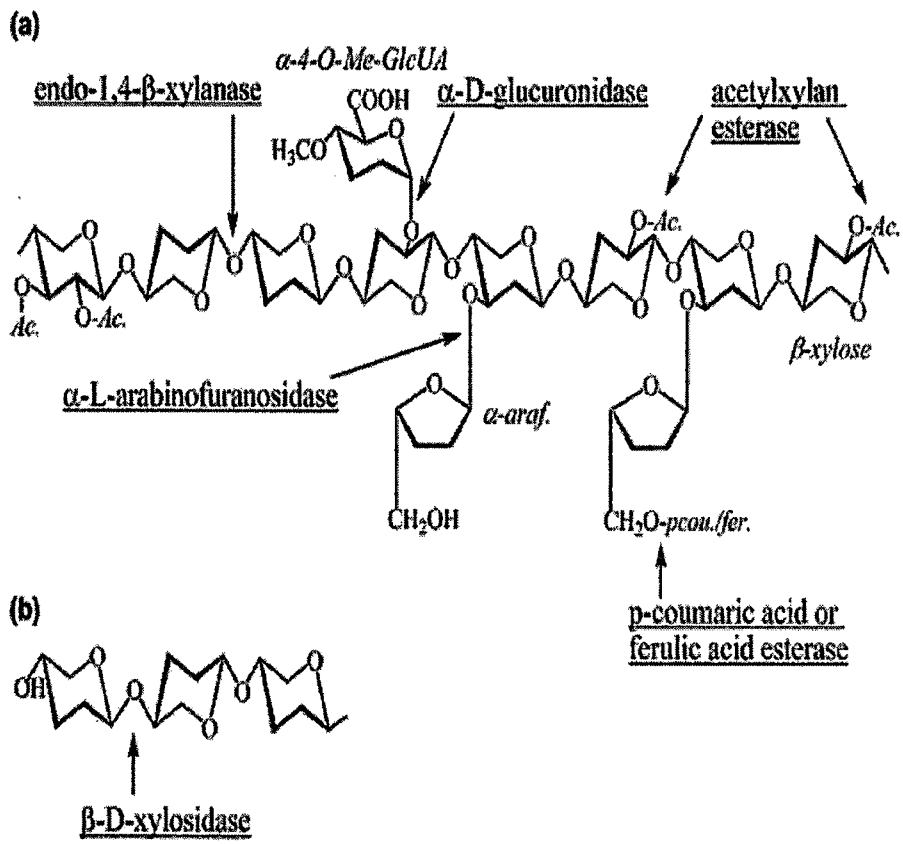

Figure 5: Mode of action of hemicellulases.

- D-mannases: They are groups of hemicellulases that hydrolyse β-1, 4-D-mannopyranosyl linkages of D–mannans and D–galacto–D–mannans. Various fungi are sources of these enzymes (Dekker and Richards, 1976).
- β- 1, 4-D-xylanases: They are capable of hydrolysing polysaccharides such as arabinoxylan, glucuroarabinoxylan, and glucuronoxylan (Coughlan and Hazelwood, 1993).

Xylanases are probably the most widely studied group of hemicellulases in bacteria and fungi due to their numerous biotechnological applications (Wong and Saddler, 1992; Viikari *et al.,* 1994). Hydrolysis of the characteristic backbone of hemicelluloses is brought about by β-1, 4-xylanases (1, 4-β-D-xylanohydrolases; EC 3.2.1.8) and 1, 4-β-D-xylosidases (1, 4-β-D-xylan xylohydrolase; EC 3.2.1.37). However, xylanase attack internal xylosidic linkages by random hydrolysis of xylan backbone and β-xylosidases releasing xylosyl residues by endwise attack of xylo-oligosacchrides (Wong *et al.,* 1988). The side groups present in xylan are removed by β-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.1), galactosidases and acetyl xylan esterases (EC 3.1.1.6). Degradation of xylan mainly requires two enzymes for the main chain cleavage. These enzymes are i) endo–β-1, 4-xylanase (EC 3.2.1.8) and ii) β-xylosidase (EC 3.2.1.37).

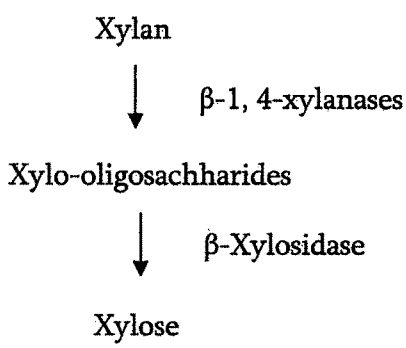

Figure 6: Mode of action of xylanases.

According to Zhang *et al.,* (2007) endo–β-1, 4-xylanases can catalyse the hydrolysis of backbone of xylan to produce xylo-oligosaccharides which can be converted to xylose by β-xylosidase (Figure 6).

2.1.4. Industrial applications of Xylanase:

In recent years, the biotechnological use of xylans and xylanases has grown remarkably (Bhat, 2000; Beg *et al.,* 2001; Subramaniyan and Prema, 2002; Techapun *et al.,* 2003; Sanghvi *et al.,* 2010). Therefore, end-products of xylan degradation are of considerable importance in commercial applications i.e. furfural and xylitol (Paraio *et al.,* 1998). Xylan can be converted to β-D-xylopyranosyl and its oligosaccharides via two types of hydrolysis: acid or enzymatic. Acid hydrolysis is often preferred because it is faster, but is always accompanied by the formation of toxic compounds that may hinder subsequent microbial fermentation. Furthermore, in the long run it can also lead to corrosion of the metallic equipment that comes in contact with the acid. Recently, some industries have shown interest in the development of efficient enzymatic processes to be used instead of acid hydrolysis in the treatment of material containing hemicellulose. Therefore, commercial xylanases are being industrially produced by some companies in Japan, Finland, Germany, Republic of Ireland, Denmark, Canada and the USA.

Xylanase in paper and pulp industries

The main industrial application of the xylanases is in bleaching of cellulose pulp. Use of these enzymes began in this sector during the last two decades, since peroxidases were applied to the degradation of lignin (Wong and Saddler, 1992; Viikari *et al.,* 1994; Tenkanen *et al.,* 1997; Araujo *et al,.* 1999; Bajpai, 1999; Christov *et al.,* 1999; Whitmire and Miti, 2002; Sandrim *et al.,* 2004). Some of such products are summarised in Table 2. At present, in many countries, including Brazil enzymatic hydrolysis is employed rather than chemical processes. The usual

method of enzymatic hydrolysis is known as the Kraft process, the name signifying strength or force in German. Three species of *Eucalyptus* (*E. grandis*, *E. saligna* and *E. urophylla*) are particularly favoured as a raw material in paper industries.

Product name	Application pH	Application Temperature(°C)	Supplier
Irgazyme 10	5-7	35-55	Gennencor
Igragazyme 40	6-8	35-70	Gennencor
Multifect XL	5-5.5	55-60	Gennencor
Cartazyme HS	3-5	30-50	Sandoz
Cartazyme HT	5-8	60-70	Sandoz
Ecopulp	5-6	50-55	Alco ltd.
VAI xylanase	5-6	55	Voest-Alphine

Table 2: Commercial xylanase preparations available for enzymatic bleaching of pulps. (Source Viikari *et al.*, 1994; Vicuna *et al.*, 1995; Polezeli *et al.*, 2005).

Xylanases in animal feed

Use of enzymes in production of animal feed is another important sector of agribusiness, with an annual world production exceeding 600 million tons and a turnover of >50 billion dollars. Xylanases are used in animal feed along with glucanases, pectinases, cellulases, proteases, amylases, phytases, galactosidases and lipases. These enzymes break down arabinoxylans from ingredients of the feed and reduce the viscosity of raw material (Twomey *et al.*, 2003).

Xylanase is added to feed containing maize and sorghum, both of which are low viscosity foods. Therefore, it helps to improve the digestion of nutrients in the initial part of the digestive tract, resulting in a better use of energy.

Xylanase in manufacture of bread, food and drinks

Like other hemicellulases, xylanases break down the hemicellulose in wheat flour, helping in the redistribution of water and leaving the dough softer and easier to knead. During the bread-baking process, xylanases delay crumb formation allowing the dough to grow. With the use of xylanases, there has been an increase in bread volumes with greater absorption of water and improved resistance to fermentation conditions (Maat *et al.,* 1992; Harbak and Thygesen, 2002; Camacho and Aguilar, 2003). A larger amount of arabino xylo-oligosaccharides in bread would be beneficial to health. In biscuit-making, xylanase is recommended for making cream crackers lighter and improving the texture, palatability and uniformity of the wafers. During the manufacturing of beer, the cellular wall of the barley is hydrolyzed releasing long chains of arabinoxylans which increases the beer's viscosity rendering it "muddy" in appearance. Thus, xylanases are used to hydrolyze arabinoxylans to lower oligosaccharides diminishing the beer's viscosity and consequently eliminating its muddy aspect (Debyser *et al.,* 1997; Dervilly *et al.,* 2002).

Pharmaceutical and chemical applications

Though xylanase and xylans are of little use in the pharmaceutical industry they are sometimes added in combination with a complex of enzymes (hemicellulases, proteases and others) as a dietary supplement or to treat poor digestion. Hydrolytic products of xylan, such as β-D-xylopyranosyl residues can be converted into combustible liquids (ethanol), solvents and artificial low calorie sweeteners.

A variety of commercial products containing xylitol, such as chewing gum can be found in the market. Although, the enzymatic hydrolysis of xylan is a promising method for obtaining β-D-xylopyranosyl units, at present commercial xylitol is produced on a large scale by chemical catalysis. This is considered a high cost process, mainly because the xylose has to be purified initially in several steps.

Looking to the applications of xylanase in industries for the production of various important products, extensive work has been initiated at international level. There is an urgent need of similar studies from our country. Therefore, present investigation has been carried out on isolation, production and purification of xylanase by solid state fermentation method. In the present investigation, for the production of xylanase, two strains *Trichoderma harzianum* and *Chrysosporium asperatum* were selected. Moreover, India has been in the list of developing countries where economy is mainly dependent on agriculture. With so much agro products, huge amounts of agricultural wastes are generated every year in India. Sometimes, these agricultural wastes are burnt by farmers as they are not even consumed by animals due to higher lignin content. Secretary enzymes of animals cannot digest this waste, thus animals are unable to metabolize completely these raw wastes. This results into a low energy food for animals. Therefore, these agricultural wastes are burnt away by farmers. Instead of burning, these agricultural wastes can be used for production of commercially important products such as enzymes, antibiotics, aroma compounds and also in production of biofuels. In respect to this, production of xylanase in the present investigation has been carried out using different agricultural waste by solid state fermentation. The aims of investigation were:

- Screening of wood rot fungi for production of xylanase.
- Production of xylanase by Solid State Fermentation using different agricultural wastes.
- Complete/Partial purification of xylanase
- To study the effect of various parameters on xylanase production.
- Characterisation of xylanase.
- Molecular characterisation of xylanase by SDS-PAGE and Native PAGE.

MATERIAL AND METHODS

2.2 General Procedures:

2.2.1 Chemicals

Birchwood xylan, Beechwood xylan and Oat spelt xylan were procured from Sigma Chemical Co., (USA). All other chemicals like carbon sources (sugars), nitrogen sources (yeast extract, malt extract, and soyabean meal) and other chemicals were obtained from Qualigens and SRL (India). All the chemicals used in the present study were of analytical grade unless otherwise stated.

2.2.2 Sterilisation

Media, solutions, and glass wares were sterilised by autoclaving at 121 °C, at 15 lbs pressure for 30 minutes. Sugars were sterilised at 121 °C, 15 lbs pressure for 10 minutes. Other sensitive chemicals for mutation studies i.e. ethidium bromide was filtered by Whatmann filter paper No.1. For all the experiments, distilled water was used.

For electrophoresis, all the chemicals viz. Acrylamide, Bisacrylamide, Tris buffer, DEAE-Sephadex were procured from Himedia (India). For protein precipitation, sterilised polypropylene centrifuge tubes were used. Purification of protein and molecular determination was carried out by using sterilised double distilled water. All the experiments were performed at room temperature (25-30 °C). Centrifugation was done by using REMI cooling centrifuge at 10,000 rpm at 4 °C whereas, dialysis membrane with cut off value of 12000-14000 Da was utilised for the partial purification of enzymes.

2.3 Fungal Isolate and Growth Conditions:

Near about 30 fruiting bodies of different wood rot fungi were collected from dead as well as infected living trees growing in different forests (*viz.* Girnar forest-Junagadh, Satpuda forest of Dediapada and Panchmahal, Pavagadh and Shivrajpur)

of Gujarat state. All these fruiting bodies were surface sterilised by 0.1% $HgCl_2$ followed by 70% ethanol for few seconds. It was then inoculated in different media as briefed in Table 3. For initial isolation of fungi, different mediums were used to optimize growth conditions. To prevent bacterial contamination every time all the mediums were supplemented with mixture of antibiotics of penicillin and chloramphenicol.

Sr. No.	Growth media used	pH
1	Potato dextrose agar	5.0-5.5
2	Malt extract agar	6.8-7.0
3	Malt extract glucose agar	5.8-6.0
4	Yeast extract agar	5.5-6.0
5	Saubrauds agar	5.8-6.0
6	Yeast extract glucose agar medium	5.5- 6.0
7	Czapex dox agar media	5.5-6.0

Table 3: Different growth media used for the isolation of fungi.

2.3.1 Establishment of pure culture and screening for xylanolytic enzyme

After optimisation of growth medium, pure cultures were established on suitable media and they were maintained in refrigerated conditions. All the pure cultures were subjected to on plate assay for xylanolytic activity as described by (Williams, 1983). All the cultures were inoculated on the optimised growth media supplemented with 1% xylan and zone of clearance was observed by flooding the plate with 0.1% Congo red solution. On the basis of zone of clearance, two strains giving best results were selected from thirty cultures for further studies. Both strains considered for further studies were identified from Agharkar Research

Institute, Pune by their morpho-taxonomic characteristics. Two strains identified were *Trichoderma harzianum* Rifai (Class Sordariomycetes; Hypocreales; Hypocreaceae) and *Chrysosporium asperatum* Carmichael (Class Euascomycetes; Onygenales; Onygenaceae). The cultures of both the strains were maintained in Saubrauds dextrose agar and Malt extract agar supplemented with 1% xylan respectively. Pure cultures were obtained after 7 days of inoculation and cultures were revived after every 15 days.

2.3.2 Solid substrates (Agricultural Wastes)

Six different agricultural wastes viz. wheat straw, rice straw, soya bean waste, pigeon pea hulls, corncobs and sugarcane bagasse were used for the production of xylanase enzyme. All these agricultural wastes were collected from local farms located around Vadodara, whereas the sugarcane bagasse was collected from the sugar industries near Vadodara. Initially, all these agricultural wastes were used as a sole source of substrate for production of xylanase. Later on, different combinations of agricultural wastes with different ratios (2–10%) were also used as a source of substrates for production of enzyme. In case of *Trichoderma harzianum,* wheat straw gave maximum production of xylanase. Therefore, different combinations of substrates with wheat straw were also checked whereas, *Chrysosporium asperatum* gave maximum production of xylanase with rice straw as a substrate. Various combinations of rice straw with different agricultural wastes as substrates were also checked for the production of xylanase.

2.3.3 Inoculum preparation

For the production of xylanase, inoculation was carried out by two different methods: i) direct inoculation of non activated fungal strain on growth media and ii) inoculation of activated fungal cultures. *Trichoderma harzianum* was activated in Czapex dox media whereas; *Chrysosporium asperatum* was activated by using Malt extract glucose media. Compositions of both media were enlisted in Table 4

and 5 respectively. Three plugs (2cm x 2cm) of one week old activated and non activated cultures were directly inoculated on production media.

Composition of both the media is as follow:

Ingredients	g/l
Sucrose	30
Sodium nitrate	3
K_2HPO_4	1
Magnesium sulphate	0.5
Potassium chloride	0.5
Ferrous sulphate	Trace
Distilled water	1000ml
pH	5.4

Table 4: Composition of Czapex dox media.

Ingredients	g/l
Malt extract	30.0
Glucose	10.0
Distilled water	1000ml
pH	5.5-6.0

Table 5: Composition of Malt extract media.

2.3.4 Production and harvesting of Enzyme

Trichoderma harzianum Rifai

The production media contained 5g of solid substrate moistened with distilled water. The moisture level was adjusted in ratio of 1:5 and the flasks were inoculated with three plugs (2cm x 2cm) of one week-old Czapex dox slant of the *Trichoderma harzianum* grown at 30 °C. Production medium inoculated with above said strain was incubated under static conditions at 30 °C and enzyme

production was checked after every 24 hours for 18 days. Enzyme was extracted in 50 ml of 0.1M Na-Acetate buffer on a rotary shaker at 250 rpm for 30 minutes. The content was filtered with Whatmann filter paper No.1 and the filtrate was then used as crude enzyme.

Chrysosporium asperatum Carmichael

The production media contained 5g of solid substrate moistened with Minimal salt medium. The moisture level of lignocellulosic substrate was adjusted to 1:5 (substrate: medium) with MS medium. The MS medium was composed of Peptone, 1g/l; Tween 80, 0.1% (v/v); $(NH_4)_2SO_4$, 1.4 g/l; KH_2PO_4, 2.0 g/l; Urea, 0.3 g/l; $CaCl_2$, 0.3 g/l; $MgSO_4$ $7H_2O$, 0.3 g/l; $FeSO_4$ $7H_2O$, 5.0 mg/l; $MnSO_4$ H_2O, 1.6 mg/l; $ZnSO_4$ $7H_2O$, 1.4 mg/l; $CoCl_2$, 2.0 mg/l; at pH 5. The flasks were inoculated with 1 ml of 10^6 spores suspensions from one week old malt extract agar plate of *Chrysosporium asperatum* grown at 30 °C. Inoculated production mediums were incubated under static conditions at 30 °C and enzyme production was checked after every 3 days till 24 days. Enzyme was extracted in 50 ml, 0.1M Na-Acetate buffer on a rotary shaker at 250 rpm for 30 minutes. The content was filtered with the Whatmann filter paper No.1 and the filtrate was then used as a crude enzyme source.

2.4. Study the effect of physio-chemical factor on xylanase production:

Spore suspension was prepared in 0.1% Tween 80 from one week-old grown culture of *Chrysosporium asperatum.* Inoculums with different concentrations such as 10^3, 10^4, and 10^6 spores/ml of solution were added in media to check production of xylanase. In second method, 1-5 plugs of culture size (2cm x 2cm) were added in production media for optimisation of xylanase production.

2.4.1 Effect of Carbon and Nitrogen supplements on xylanase production

For carbon source, glucose, maltose, lactose, sucrose, fructose and xylose were used in a ratio of 1:1 to 1:5 (carbon: substrate). Organic nitrogen sources such as peptone, malt extract, beef extract, yeast extract were supplemented in media

whereas, inorganic nitrogen sources such as sodium nitrate, ammonium sulphate, urea were supplemented in the production media to check the effect of their presence on enzyme production.

2.4.2 Moistening agents

Moistening agents in different ratio ranging from 1:1 to 1:8 were used to optimise the maximum production of xylanase. Different moistening agents such as distilled water, tap water, MS medium, modified MS medium, Toyamas salt solution, Basal salt solution, modified Basal salt solution etc. were used. Composition of different moistening agents was briefed in Table 6 as given below:

Sr. No.	Moistening Agents	Composition
1	MS media	Peptone, 1g/l; Tween 80, 0.1% (v/v); $(NH_4)_2SO_4$, 1.4 g/l; KH_2PO_4, 2.0 g/l; urea, 0.3 g/l; $CaCl_2$, 0.3 g/l; $MgSO_4$ $7H_2O$, 0.3 g/l; $FeSO_4$ $7H_2O$, 5.0 mg/l; $MnSO_4$ H_2O, 1.6 mg/l; $ZnSO_4$ $7H_2O$, 1.4 mg/l; $CoCl_2$, 2.0 mg/l; at pH 5.0.
2	Modified MS media	Peptone, 1g/l; Tween 80, 0.1% (v/v); $(NH_4)_2SO_4$, 0.5 g/l; KH_2PO_4, 3.5 g/l; urea, 0.5 g/l; $CaCl_2$, 0.1 g/l; $MgSO_4$ $7H_2O$, 0.1 g/l; $FeSO_4$ $7H_2O$, 5.0 mg/l; $MnSO_4$ H_2O, 1.6 mg/l; $ZnSO_4$ $7H_2O$, 1.4 mg/l; $CoCl_2$, 2.0 mg/l; at pH 5.0.
3	Modified Toyamas salt solution	$(NH_4)_2SO_4$, 10.0 g/l; KH_2PO_4, 3.0 g/l; urea, 0.3 g/l; $CaCl_2$, 1.0 g/l; $MgSO_4$ $7H_2O$, 0.5 g/l (w/v) and Tween-80, 0.1% (v/v) at pH 5.0).
4	Basal salt solution	Nacl 5.0 g, $(NH_4)_2SO_4$, 0.5 g/l; KH_2PO_4, 3.5 g/l; urea, 0.5 g/l; $CaCl_2$, 0.1 g/l; $MgSO_4$ $7H_2O$, 0.1 g/l; $FeSO_4$ $7H_2O$, 5.0 mg/l; $MnSO_4$ H_2O, 1.6 mg/l; $ZnSO_4$ $7H_2O$, 1.4 mg/l; $CoCl_2$, 2.0 mg/l; at pH 7.0.
5	Modified Basal salt solution	$(NH_4)_2SO_4$, 0.1g/l; KH_2PO_4, 1.25 g/l; urea, 0.25 g/l; $CaCl_2$, 0.5 g/l; $MgSO_4$ $7H_2O$, 0.1 g/l; $FeSO_4$ $7H_2O$, 2.0 mg/l; $MnSO_4$ H_2O, 1.2 mg/l; $ZnSO_4$ $7H_2O$, 1.0 mg/l; $CoCl_2$, 2.0 mg/l; at pH 7.0.

Table 6: Media composition of different moistening agents.

2.4.3. Pretreatment of substrate

In order to study the influence of pretreatment of substrate on xylanase production, rice straw were soaked in 0.1M HCl, 0.1M NaOH, 0.1M Hydrofloric acid and then thoroughly washed until the straw became neutralized completely. Complete removal of pretreatment chemical fraction was confirmed by checking the pH. Thereafter, the straw was dried overnight in oven at 60 °C temperature. For physical treatment, straw was treated in water bath for 1 hour, dried over night and used.

2.4.4. Mutation studies (for *C. asperatum*)

Mutation study was undertaken in order to examine the effect of different mutagens on production of enzyme. Mutation was induced by successive stages of physical (UV-irradiation), chemical mutagenesis (ethidium bromide) and by mixed mutagenesis on the wild type strain. In mixed mutagenesis, wild type strain was first exposed to UV radiation followed by chemical mutagenesis. Same experiment was also followed in reverse order of the reaction. For physical mutagenesis, spore suspension of wild type strain was both prepared in sterile saline water or in solution with Tween 80 (0.1%) and exposed to UV-irradiation for various time intervals ranging from 30 seconds to 10 minutes. For chemical mutagenesis, spore suspension of strain was treated with ethidium bromide 10, 50, 100, 150, 200, 300 mg/ml for 0–30 minutes. The best mutant was obtained following 10 minutes of treatment of chemical treatment. Qualitative detection of xylanase was done by zone of clearance on Malt extract agar + xylan agar plates using Congo red as indicator stain (Williams, 1983). Quantitative estimation of xylanase activity from various selected mutants was performed by Dinitro Salicylic Reagent (DNSA) method as described by Miller (1959).

2.5 Parameters of Xylanase activity:

Preparation of Reagents for xylanase activity

Na-Acetate buffer pH 5.0 (for 100 ml): Na-Acetate 50 ml of 0.1M and 50 ml of 0.1M acetic acid were mixed and pH 5 was adjusted by adding acetic acid in solution.

Na-Acetate buffer pH 4.5 (for 100ml): Na-Acetate 10 ml of 0.1M and 90 ml of 0.1M acetic acid were mixed and pH 4.5 was adjusted by adding acetic acid in solution.

Substrate: 1% Birchwood xylan was prepared by dissolving 1g of Birchwood xylan in 100 ml of Na- Acetate buffer.

Ingredients	g/l
DNSA	10
Na-K- Tartarate	300
NaoH	20
Distilled water	1000 ml

Table 7: Composition of DNSA.

Enzyme Assay:

The reaction mixture contained 1% of 1000 µl xylan, 900 µl of 0.1M Na-Acetate buffer, and 100 µl of enzyme. The release of reducing sugars in 30 minutes at 30 °C, pH 5.0 (0.1M Na-Acetate buffer) was measured as xylose equivalents using the dinitrosalisylic acid method as described by Miller (1959). Composition of DNSA is given in Table 7. One unit of enzyme activity (U) is defined as the amount of enzyme liberating 1 µmol of xylose per minutes. All the sets were performed in triplicates and the standard error was reported.

Optimisation of enzyme activity:

Birchwood xylan used in assay buffer system for the enzyme–substrate reaction was supplemented at six different concentrations as: 0.5, 1, 1.5, 2, 2.5 and 3%. To check optimum temperature for xylanase activity, enzymes were incubated with xylan in 0.1M Na-Acetate buffer, pH 5 as substrate at 16, 30, 37, 40, 50, 60, 70 and 80 °C. For thermostability studies, enzyme was kept at 70 °C and assayed for xylanase activity after every 5 minutes. Enzyme activity was also checked individually at different pH values of 2, 3, 4, 4.5, 5, 6 and 7.

2.5.1 Partial purification and molecular weight determination (*T. harzianum* and *C. asperatum*)

Crude xylanase was partially purified by adding ammonium sulphate in crude broth. For different percent saturation, ammonium sulphate was added by following ammonium sulphate precipitation table as given by Dawson *et al.*, (1969). A detail of the precipitation table is as below:

%	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767
10		28	57	86	118	137	190	183	216	251	288	326	365	406	449	494	540	592	640	694
15			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657
20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
33							12	43	74	107	142	177	214	252	292	333	378	426	472	522
35								31	63	94	129	164	200	238	278	319	364	411	457	506
40									31	63	97	132	168	205	245	285	328	375	420	469
45										32	65	99	134	171	210	250	293	339	383	431
50											33	66	101	137	176	214	256	302	345	392
55												33	67	103	141	179	220	264	307	353
60													34	69	105	143	183	227	269	314
65														34	70	107	147	190	232	275
70															35	72	110	153	194	237
75																36	74	115	155	198
80																	38	77	117	157
85																		39	77	118
90																			38	77
95																				39

Table 8: Ammonium sulphate precipitation table.

Precipitation was done by using REMI cooling centrifuge (at 4 °C). After centrifugation, enzyme pellets were dissolved in minimum quantity of Na-Acetate buffer (pH 5.0). All saturated fractions were checked for xylanase activity and fractions having maximum activity were subjected to dialysis. Dialysis of enzyme was done with column chromatography membrane having cut off value (12000-14000 Da) against Na-Acetate buffer with pH 5.0 for *T. harzianum* and pH 4.5 for *C. asperatum*. Dialysed enzyme was collected and stored for further characterization. Optimisation of enzyme activity with respect to temperature, pH, substrate concentration, incubation time and effect of metal ions was studied. Molecular characterization of enzyme was done using SDS PAGE and xylanase activity was determined by staining the gel with Congo red dye.

2.5.2 Purification of xylanase enzyme by Column chromatography (*C. asperatum*)

Purification of xylanase was carried out by using DEAE-Sephadex G-75 column. Prior to purification, the column was subjected to activation and equilibration.

Activation and equilibration of resin:

DEAE Sephadex G-75, resin was initially activated by treating it with HCl for 30 minutes. After activation, it was washed with distilled water till neutral pH was obtained. After subsequent washing with distilled water, resin was immersed in 1M NaOH solution for 15 minutes. Thereafter, it was washed again with distilled water till the pH became neutral. Once the neutral pH was obtained, resin was kept in Na-Acetate buffer overnight for equilibration. Column was washed with the same buffer and loaded with dialysed enzyme for 20-25 minutes for proper binding of enzyme in column. Subsequently, an enzyme was eluted with a linear gradient of NaCl (0-1M). Fractions of 5.0 ml were collected at a flow rate of 90 ml/h. Different fractions of an enzyme were collected and absorbance at 280nm was recorded. The fraction having maximum absorbance was preserved in cooling condition. Column was washed with distilled water till neutral pH was recorded. This eluted fraction was used further for all studies.

2.6 Parameters studied for Xylanase activity:

2.6.1 Effect of substrate concentration

Six different concentrations like 0.5%, 1%, 1.5%, 2%, 2.5% and 3% of xylan was used for checking effect of substrate concentration on enzyme activity.

2.6.2 Effect of pH on xylanase activity

Na-Acetate buffer with pH ranging from 2.0 to 6.0 was used to check the effect of pH on xylanase activity. Different buffers such as phosphate buffer and citrate buffer were also checked for adaptability of these enzymes for xylanase activity. Phosphate buffer with pH range of 4.0 to 7.5 was used while citrate buffer with pH range 5.0 to 7.0 was used for the xylanase activity.

2.6.3 Effect of temperature on xylanase activity

The activity of the xylanase was measured with a temperature range from 10-60 °C. The enzyme was incubated for 25 minutes with xylan in 0.1M Na-Acetate

buffer, as a substrate at above mentioned temperature and the enzyme activity was checked individually at different temperatures.

2.6.4 Effect of metal ions on enzyme activity

To study the effect of metal ions on xylanase activity, reaction mixture consisting of 1 ml xylan substrate, 0.1 ml of dialyzed enzyme and 1 ml of different metal ions (1mM) such as Mn^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+} were incubated at 37 °C for 30 minutes and estimated quantitatively by following DNSA method (Miller, 1959).

2.6.5 Molecular weight determination

Electrophoresis was done using Medox electrophoresis unit (Bioneeds Instruments, Ahmedabad). Gels with 15×10 cm were employed in experiments. In all experiments, 12% gels were employed to check single band of purified enzyme. Electrophoresis of partially purified enzyme and purified enzyme was performed as described by Laemmli (1970). Composition of 12% resolving gel and stacking gel is given in Table 9 and 10.

Composition	Resolving (7ml)
30% Polyacrylamide	2.8
1.5 M Tris Buffer (pH 8.8)	1.75
10% SDS	0.07
10% APS(Ammonium per sulphate)	0.07
TEMED	0.0028
Distilled water	2.13

Table 9: Composition of 12% resolving gel.

Chemicals	Stacking (4ml)
30% Polyacrylamide	0.68
1.5M Tris Buffer (pH 6.8)	0.5
10% SDS	0.04
10% APS (Ammonium per sulphate)	0.04
TEMED	0.004
Distilled water	2.72

Table 10: Composition of stacking gel.

Chemicals	Per (ml)
Bromophenol Blue	0.02mg
Glycerol	0.2
β-mercaptoethanol	0.2
Tris Buffer (pH 6.8)	0

Table 11: Composition of Treatment buffer (5X).

Enzyme was treated with treatment buffer for 3-5 minutes in boiling water bath. After cooling, 15-20 µl (approx.) treated enzymes were loaded in gel. Running buffer used for electrophoresis is glycine SDS buffer. Composition of treatment buffer and glycine-SDS buffer is briefed in Table 11 and 12.

Chemicals	g/l
Tris base	15.1
Glycine	94
SDS	10%
Distilled water	1000

Table 12: Composition of glycine SDS buffer (5X).

After electrophoresis, gel was stained with Commassie Brilliant Blue R-250 (CBB) dye solution. Composition of CBB dye solution is briefed as below (Table 13).

Chemicals	g/100ml
Commassie Brilliant Blue R-250	0.005
Methanol	30
Acetic acid	10
Distilled water	60

Table 13: Composition of CBB staining solution.

Gel was stained overnight with CBB and it was then de-stained with same solution without CBB. Nearly 7-8 wash were given to de-stain the gel. Band was observed and photographs were taken by using Sony digital camera DSC T10 with 8 megapixel value. After photography these gels were preserved in 0.1% acetic acid solution.

Native gels:

For enzyme activity, staining gel was made of similar composition with SDS as exception. In place of SDS in gel, substrate 1% xylan was added to resolving gel. In running buffer, SDS was not been added. Both, SDS-PAGE and Native gels were run at constant voltage of 80V.

Staining of native gels:

Native gels were stained with 0.1% Congo red solution for half an hour. After staining, gel was washed with 1M NaCl solution till zone of clearance was observed. Zone of clearance was photographed by using Sony digital camera with 8 megapixels. Gel was preserved in distilled water solution.

RESULTS AND DISCUSSION

Part I- For *Trichoderma harzianum* Rifai

Part II- For *Chrysosporium asperatum* Carmichael

2.7T Xylanase production using solid substrates:

Various agro-industrial residues *viz.* wheat straw, rice straw, corn cobs, sugar cane bagasse and soya bean waste were used for Solid State Fermentation (SSF) at 30 °C using distilled water as the moistening agent in the proportion of 1:5 (substrate: liquid). In wheat straw, (5g) maximum xylanase yield (146 IU/ml) was obtained in 12 days of cultivation (Table 14T). On the other hand in corncobs, rice straw, sugarcane bagasse and soya bean meal there is relatively decrease in enzyme activity (Table 14T).

Substrates	Xylanase Activity (IU/ml)
Rice Straw	135±1.12
Soyabean Waste	112±0.81
Wheat Straw	146±0.55
Corn cobs	137±0.98
Sugarcane baggase	98±0.74

Table 14T: Effect of different substrates on xylanase production.

Similar results are also noted by Gupta *et al.,* (2009). According to him, increasing concentration of substrate led to decrease in the enzyme activity due to fact that high concentration of substrate led to increase in viscosity, which influence medium components and oxygen transfer. When wheat straw was used, there was a significant difference in xylanase titres, this fact may be attributed to its hemicellulose nature and favourable degradability (Sonia *et al.,* 2005; Sanghvi *et al.,* 2010). Wheat straw has been known for being ideally suitable for xylanase production in *Thermoascus aurantiacus*, *Penicillium canescens* and *Penicillium citrinum* cultures (Kalogeris *et al.,* 1998; Bakri *et al.,* 2008; Nair *et al.,* 2008). In the present study also maximum xylanase production is reported when wheat straw was used as a substrate.

2.7T1 Effect of incubation time on xylanase production and activity

The xylanase activity was determined after 24 hours of incubation for 18 days in order to determine the optimum incubation period for the maximum production of xylanase. The enzyme production however, started after 48 hours of inoculation of fungal inoculum and showed maximum xylanase production (146 IU/ml) on 12th day of incubation period at 30 °C. In some fungi, high xylanase production has been shown to be linked strictly to cellulase production due to time course or incubation period (Haltrich *et al.,* 1996; Christakopoullos *et al.,* 1999; Kang *et al.,* 2004). In the present investigation, enzyme production increased gradually from 48 hours till 12th day thereafter increased incubation period resulted in decrease in the enzyme production. The declined enzyme production may be associated with the susceptible portion of xylan molecules, which was rapidly digested and only crystalline portion was left behind which cannot be used by the organism for the production of an enzyme (Roose, 1963; Jing *et al.,* 1998). To check the optimum incubation time for enzyme–substrate reaction, mixture of enzyme substrate was taken at different intervals and the maximum activity (146 IU/ml) was reported after 30 minutes of incubation (Table 15T).

Incubation time (mins)	Xylanase Activity (IU/ml)
5	35±0.21
10	55±0.57
15	59±0.54
20	95±0.79
25	125±0.87
30	146±1.10
35	138±0.44
40	115±0.25

Table 15T: Activity of xylanase-substrate reaction at different incubation time.

2.7T2 Influence of temperature and pH

The culture was able to produce xylanase with distilled water as moistening agent at temperature range between 20-37 °C. Maximum xylanase activity (146 IU/ml)

was reported after 12th day of incubation period at 30 °C. Further increase in temperature, i.e. above 40 °C not only inhibited the fungal growth but also hampered xylanase production. Our results indicate that production of an enzyme is closely related with the growth of fungus; as optimum temperature for xylanase production is similar to optimum temperature required for the growth of *Trichoderma harzianum*. Similar results on the highest xylanase titres in fungal systems have already been reported by earlier workers and it is considered to happen generally at temperatures that are optimum for growth of cultures in SSF (Biswas *et al.,* 1990; Christakopoullos *et al.,* 1999). Temperature optima of dialysed enzymes were done and maximum activity was obtained at 30 °C (Table 16T).

Temperature	Xylanase Activity (IU/ml)
16	89±1.6
30	146±0.85
37	139±0.62
40	130±0.24
50	125±1.10
60	135±1.8
70	140±2.3
80	85±0.58

Table 16T: Effect of temperature on xylanase activity.

Most of the fungi are able to grow in a wide variety of pH ranging from 5.0–8.0 (Paredes *et al.,* 1998). In the present study, initial pH of the medium was adjusted to variable range by adding 0.1N HCl and production of an enzyme was tested at wide range of pH starting from 3 to 7. Xylanase production was found to be the maximum at pH 5.0 with xylanase activity (146 IU/ml). There is no marked difference in xylanase titres at pH 3 and 4 but the activity was declined from pH 6 onwards. Shah and Dutta (2005) reported similar observations, that the highest xylanase titres in fungal systems are usually found at pH 5.0 for growth of cultures in SSF. Not only crude enzyme but dialysed enzyme also showed maximum activity at pH 5.0 (Figure 7).

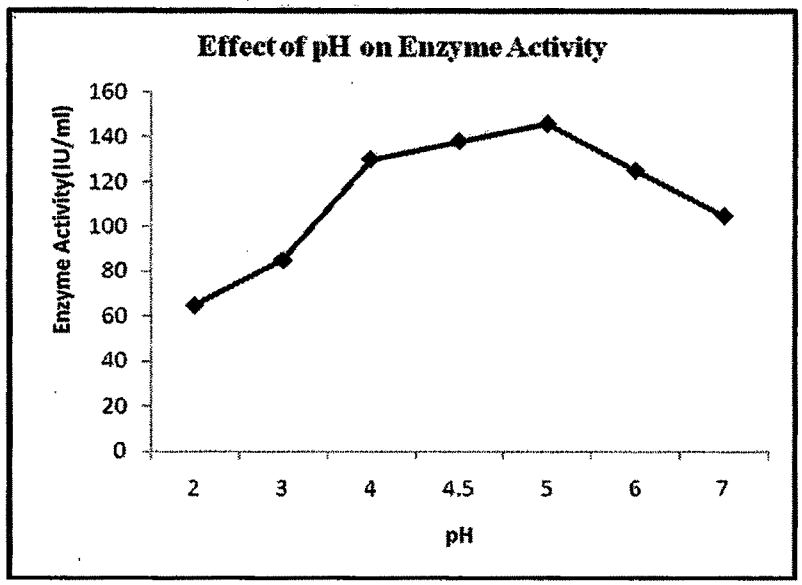

Figure 7: Effect of pH on xylanase activity.

2.7T3 Influence of moistening agents and moisture level

Moistening agents and moisture level plays an important role in the process of SSF. Shah and Dutta (2005) reported that xylanase, cellulase and protein production were sensitive to the composition of the moistening agent. In the present study, all media with different moistening agents supported enzyme production but the maximum xylanase production (146 IU/ml) was recorded when distilled water was used as a moistening agents (Table 17T).

Moistening Agent	Incubation (Days)	Xylanase Activity(IU/ml)
d/w	12	146±0.65
MS medium	12	135±0.90
Basal salt solution	12	120±1.57
Modified MS medium	12	130±0.52

Table 17T: Effect of different moistening agents on xylanase activity.

In addition to distilled water, other moistening agents used were tap water, MS medium, modified MS medium and Basal salt solution. There are reports that mineral salt solution is better inducer for high production of xylanase enzyme (Reese *et al.,* 1969; Haq *et al.,* 1993; Shah and Dutta, 2005). In the present study,

use of distilled water as moistening agent gave better results as compared to that of mineral solution. Similar results are also documented by Butt *et al.,* (2002) that distilled water is a better moistening agent for production of xylanase.

Moisture content in SSF is a crucial factor, which determines the success of the enzyme production in SSF (Ramesh and Lonsane, 1990). A higher than optimum moisture level causes decreased porosity, alteration in particle structure, gummy texture, lower oxygen transfer and enhancement of the aerial mycelia (Raimbault and Alazard, 1980). Moreover, it leads to conglomeration of the substrate or sticking of particles to the wall of reactor which ultimately results into substrate to become more prominent to bacterial contamination (Lonsane *et al.,* 1985). Xylanase production was optimized by adjusting the initial moisture content of SSF from 1:1 to 1:6 (wheat straw: distilled water), corresponding 50-86% moisture level. Increase in moisture level enhances xylanase production up to 80%, while higher to that concentration does not increase xylanase production due to increase in protein level.

Moisture Level	Incubation (Days)	Xylanase Activity (IU/ml)
1:1	12	52±1.0
1:2	12	64±1.6
1:3	12	125±1.4
1:4	12	139±0.87
1:5	12	146±0.5
1:6	12	127±0.7

Table 18T: Effect of moisture level on xylanase production.

Maximum activity (146 IU/ml) was obtained with moisture levels adjusted to 1:5 (Table 18T). Bakri *et al.,* (2008) has reported optimal initial moisture content for xylanolytic enzyme production by *Cochliodomus sativus* was 80% using wheat straw as substrate.

2.7T4 Influence of Carbon and nitrogen sources

The enzyme production by microorganism mainly depends on growth conditions and nutrient availability to them. Therefore, it is expected that improving nutritional value of wheat straw by supplementation of carbon will also improve growth of fungus and subsequently enzyme production. Flask containing media were supplemented with different carbon sources (glucose, xylose, maltose, lactose, fructose, and sucrose) in ratio of 1:5 (carbon source: substrate). Flask supplemented with xylose shows the maximum activity (156 IU/ml) which is slightly more than the media without any carbon source (Table 19T).

Carbon Sources	Xylanase Activity (IU/ml)
Glucose	140±0.15
Maltose	135±1.5
Xylose	160±0.85
Fructose	138±2.6
Lactose	136±1.4
Sucrose	142±0.79

Table 19T: Effect of carbon sources on production of enzyme.

Maximum activity (156 IU/ml) was obtained using xylose, while flask supplemented with maltose shows minimum production of an enzyme (135 IU/ml). Xylose has been described as an effective inducer and carbon source for xylanase production in several microorganisms including *A. pullulans* (Priem *et al.,* 1991), *T. lanuginosus* (Purkarthofer *et al.,* 1993), *F. oxysporium* (Chirstakopoullos *et al.,* 1999) *F. solani* (Gupta *et al.,* 2009). Xylanase with minimal cellulases can be produced by using a low nitrogen-to-carbon ratio (Gerber *et al.,* 1997). In the present study, effect of both organic (yeast extract, malt extract, beef extract, peptone) and inorganic nitrogen sources (urea,

ammonium sulphate, sodium nitrate, ammonium nitrate) on production of xylanase has been studied. Xylanase activity (141 IU/ml) was evident when yeast extract was added. These results are in agreement with those reported in the literature where fungi were found to produce higher xylanase activities on organic nitrogen sources (Purkarthofer *et al.,* 1993; Lemos *et al.,* 2001; Bakri *et al.,* 2008; Yang *et al.,* 2006).

2.7T5 Pre-treatment of substrates

Several structural and compositional factors affect fermentability of lignocellulosic materials, which include cellulose protection by lignin, hemicellulose sheathing, degree of hemicellulose acetylation, cellulose crystallinity etc. These barriers differ in relative significance, depending on the substrate material (Teh-An Hsu, 1996). The alteration of the substrate using pre-treatment techniques leads to a change in physical nature of lignin increases the available surface area and pore sizes, partial depolymerization of hemicelluloses which enhances availability of the substrate (Shah and Dutta, 2005).

In the present work, wheat straw was treated with mild acid, mild alkali as well as hot water steam. None of the pre-treatments used in the study were found to be advantageous for the increase in production of xylanases. It was found that when growth of fungi decreases it ultimately leads to decrease in enzyme production. This decrease could also be caused by formation of toxic inhibitors (acids and phenolics derived from carbohydrates and lignins) during chemical pre-treatment (Teh-An Hsu, 1996; Sanghvi *et al.,* 2010).

2.7T6 Influence of metal ions

Referring to the potential use of the xylanase enzyme in pulp and paper industry, some metal ions occurring in pulp industries were included in the present investigation. As shown in Table 20T, metal ions Ca^{2+} and Zn^{2+} slightly enhanced xylanase activity. There is no significant effect of other metal ions on enzyme

activity. Similar experimental results are reported for *T. harzianum* (Seyis and Nilufer, 2005) for *Aspergillus terreus* (Ghanem *et al.,* 2000) and for *T. lanuginosus* (Cesar and Mirsa, 1996).

Metal Ions	Xylanase Activity (IU/ml)
Mn^{2+}	139±0.3
Zn^{2+}	149±1.4
Cu^{2+}	143±2.3
Ca^{2+}	152±0.24
K^{2+}	140±2.1
Mg^{2+}	138±1.8

Table 20T: Effect of metal ions on enzyme activity.

These studies have showed that Ca^{2+} enhanced xylanase activities while Mn^{2+} and Zn^{2+} ions also had positive effect on xylanase activity (Cesar and Mirsa, 1996; Castro *et al.,* 1997).

2.7T7 Influence of different combinations and particle size of substrate

Wheat straws of different particle sizes were tested in order to determine their effects on xylanase production. It was apparent that particle size affected the enzyme production. The highest titre of (146 IU/ml) xylanase was produced by the wheat straw of particle size 0.45-0.5 mm whereas, lower activities were produced on the wheat straw of other sizes. Our study is in agreement with Yang *et al.,* (2006), where the maximum production was obtained at mesh size of 0.35-0.4 mm. These findings also confirmed that straw particle size and carbon source plays crucial role in xylanase production (Kalogeris *et al.,* 1998). Different combinations of substrate have also been taken into consideration to check production profile of xylanase. Ratio of 1:1, 1:2, and 1:3 was taken and *vice versa* of that has also been

considered in the present study. Maximum enzyme production (146 IU/ml) was found in the combination of 1:1 (wheat straw: rice straw).

2.7T8 Partial purification of enzyme, molecular weight determination

The ammonium sulphate fractionation (20–80% saturation) of crude xylanase yielded 76.5% of the enzyme activity. Gawande and Kamat (1999) reported that xylanases from two *Aspergillus* sp. were concentrated from 30-80% ammonium sulphate saturation with 62% and 67% yield. SDS-PAGE and native PAGE analysis shows homogenous nature in band pattern (mw 29000 Da, Figure 8) of purified xylanase. Kulkarni *et al.*, (1999) reported that microbial xylanase are single subunit proteins within range of 8-145 Kda. Gupta *et al.*, (2009) reported xylanase from *F. solani* F7 89 Kda. Sardar *et al.*, (2000) reported 24 Kda xylanase from *A. niger.*

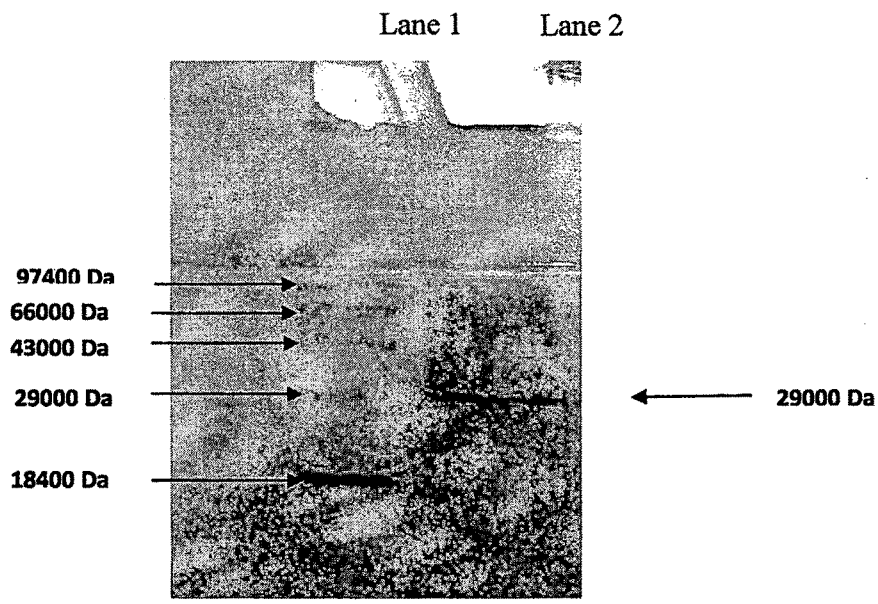

Figure 8: SDS PAGE of partially purified xylanase enzyme.
Lane 1: Ladder Medium Range (Bangalore Gene Pvt.Ltd. Cat. No. RPMW-M 106005).
Lane 2: Partially purified Enzyme (29,000 Da).

2.7T9 Thermostability of xylanase

Utilization of enzymes in industrial processes often encounters the problem of thermal inactivation of the enzymes (Shah and Dutta, 2005). In the present study, xylanase retained nearly 90% activity at 65 °C for 30 minutes at pH 5.0, while at

70 °C it showed 75% activity at pH 5.0 for 30 minutes. Thermo stability has been reported for xylanase from fungi, *Thermomyces lanuginosus*, *Paecilomyces themophila* (Li *et al.*, 2005; Yang *et al.*, 2006).

2.7T10 Storage stability of xylanase

For industrial applications, enzyme stored at different temperatures i. e. at room temperature, in deep freeze and cooling conditions has been done in earlier studies (Shah and Dutta, 2005). Enzyme retained its 95% of activity at 4-5 °C after storage for a month. Enzyme retains its 75% activity when stored at room temperature for 3 days. In the present study also enzyme was kept in freezing condition for 3 weeks and residues semisolid lyophilized enzyme was checked for the activity and it was found to retain nearly 70% of activity. Thus, the isolate *Trichoderma harzianum* was found to be an active producer of xylanases with negligible level of cellulase and can be cultivated on inexpensive substrate like wheat straw under SSF.

2.7T11 Properties of xylanase

Xylanase from *Trichoderma harzianum* shows maximum activity at pH 5.0 and temperature 30 °C. Enzyme activity was found maximum at 30 °C; however it is thermostable in nature and retain its 75% of activity at 70 °C. Activity was enhanced with addition of Ca^{2+} ions and has positive effect on production of enzyme in comparison of Zn^{2+} and Mn^{2+}. Addition of different detergents exhibits no effect on enzyme activity.

2.8C Effect of solid substrates and combinations on xylanase production:

Various agro-industrial residues *viz.* rice straw, wheat straw, pigeon peas hulls, sugar cane bagasse and soya bean waste were used for SSF at 30 °C using MS medium as a moistening agent in the proportion of 1:5 (substrate: liquid). Maximum xylanase yield i.e. 158.85±0.2 IU/ml was obtained after 15 days of cultivation on rice straw (Figure 9), whereas in corncobs, wheat straw, sugarcane bagasse and soya bean meal, there was a decrease in production of enzyme was observed (Figure 9).

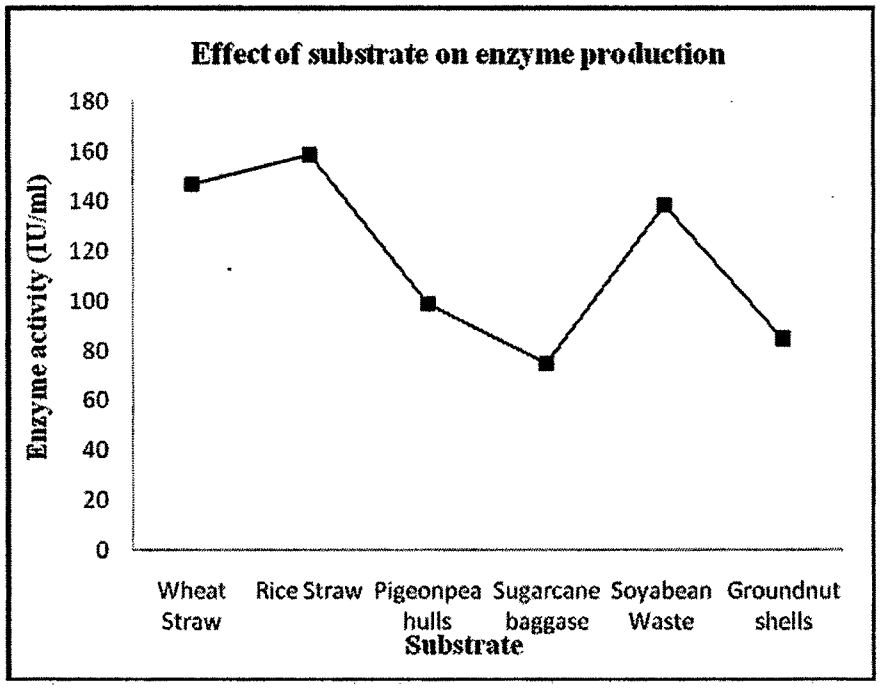

Figure 9: Effect of substrate on production of xylanase enzyme.

Ancharida (1999) found that rice straw is the best inducer for xylanase production. On the other hand, Fadel and Fouda (1993) reported that among alkali-treated agricultural wastes tested; rice straw was the best substrate for xylanase production using different species i.e. *Penicillium funiculosum.* Colina et al., (2003) also reported that rice straw was the best substrate for xylanase activity in

Trichoderma reesei. In the present study, the maximum xylanase activity (158.85±0.2 IU/ml) was obtained using rice straw. However, increasing concentration of substrate more than 5g leads to decrease in the enzyme activity due to fact that high concentration of substrate leads to increase in viscosity, which influences the medium components and oxygen transfer (Gupta *et al.,* 2009; Sanghvi *et al.,* 2010). Similar results are also reported in our earlier study (Sanghvi *et al.,* 2010) that increase in substrate concentration decreased the xylanase production when wheat straw was used as a substrate for *Trichoderma harzianum.*

In the present investigation, combinations of different substrates were also checked for production of xylanase. Available literature indicates that wheat straw is ideally suitable for xylanase production in cultures of other fungal species such as *Thermoascus aurantiacus, Penicillium canescens* and *Penicillium citrinum* cultures (Kalogeris *et al.,* 1998; Butt *et al.,* 2002; Colina *et al.,* 2003). Therefore, different combinations of rice straw with wheat straw have also been tried out in the present study to evaluate production of xylanase from *C. asperatum.* Xylanase activity increased to (165.15±0.75 IU/ml) when combination of rice straw: wheat straw (2:1) was used. Wang *et al.,* (2003) reported that combinations of rice bran: wheat bran (3:2) gave 9.6 U/ml yield of β-xylanase.

Different combinations of Rice Straw: Wheat Straw	Xylanase activity(IU/ml)
1:1	157.26±0.2
1:2	155.14±0.3
2:1	165.15±0.75
3:1	152.00±0.15

Table 21C: Effect of different combinations (rice straw: wheat straw) on enzyme production.

It is evident from the (Table 21C), that increasing or decreasing concentrations from aforementioned combinations will relatively decrease enzyme activity.

2.8C1 Effect of incubation time on xylanase production and activity

The production of xylanase was assessed after 3 days of incubation up to 24 days in order to determine the optimum incubation period for the maximum production of xylanase. The enzyme production however, started after 2 days of inoculation and maximum activity (158.85±0.2 IU/ml) was recorded on 15th day of incubation period at 30 °C (Table 22C).

Incubation Time (Days)	Enzyme Activity(IU/ml)
3	65±0.2
6	89.98±0.15
9	95.85±0.23
12	115.68±0.14
15	158.85±0.2
18	147.65±0.19
21	75.64±0.14
24	66.16±0.12

Table 22C: Effect of incubation time (days) on xylanase production.

Available literature indicates that in some fungi, high xylanase production has been shown to be linked strictly with cellulase production due to time course or incubation period (Haltrich *et al.*, 1996; Christakopoullos *et al.*, 1999; Kang *et al.*, 2004). Further increase in incubation period, resulted in decrease in enzyme production, which may be due to the susceptible portion of xylan molecules can be rapidly digested and only crystalline portion was left behind which cannot be used by the organism for the production of enzyme (Roose, 1963; Jing *et al.*, 1998). Optimisation of incubation time in terms of enzyme activity at different time intervals was measured and maximum activity (158.85±0.32 IU/ml) was found after 30 minutes of incubation.

2.8C2 Influence of temperature and pH

The culture was able to produce xylanase with MS medium as moistening agent at the temperature range of 20-45 °C, showing maximum activity (158.85±0.15 IU/ml) after 15th day of incubation period at 30 °C. Generally, production of enzyme is closely related to the growth of fungus as optimum temperature for any enzyme production is similar to optimum temperature for growth of fungus. Similar observations (i.e. highest xylanase titres in fungal systems) have already been reported at temperatures which are optimum for growth of cultures in SSF (Biswas *et al.,* 1990; Christakopoullos *et al.,* 1999).

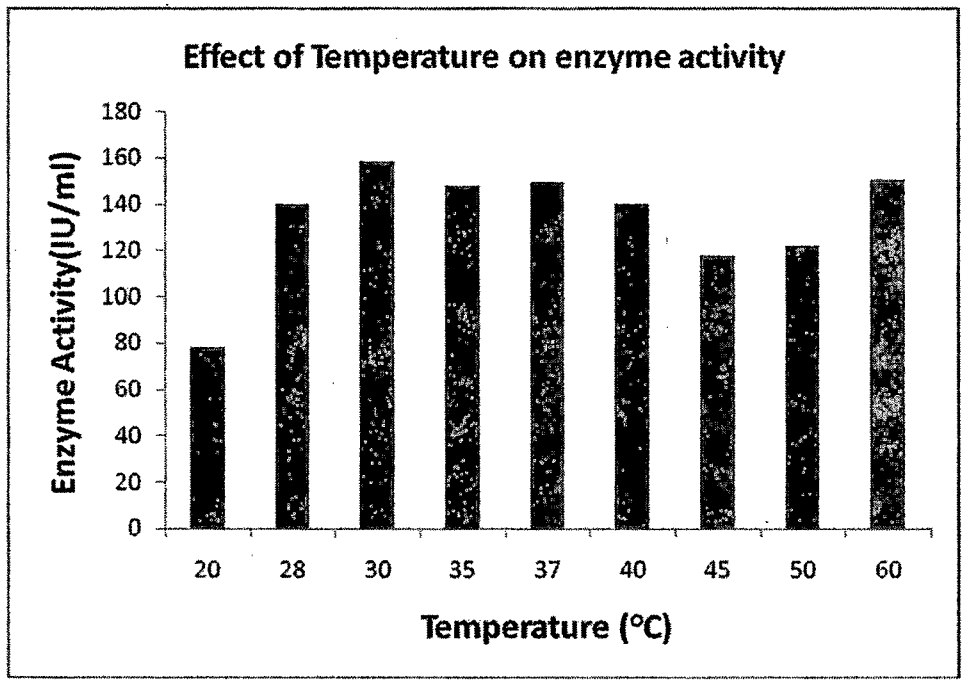

Figure 10: Effect of temperature on xylanase activity.

Purified enzyme was been kept at different temperatures for its optimization and peak activity was recorded at 30 °C (Figure 10), however it retained its 85% activity at 60 °C after incubation of 30 minutes.

Most of the fungi are able to grow in a wide range of pH starting from 5.0-8.0 (Paredes, 1998). To check the effect of pH on xylanase production, variable pH range of medium was adjusted by adding 0.1N HCl. Production of xylanase was

found to be the highest at pH 4.5 with peak xylanase activity of 158.85±0.25 IU/ml. Similarly, purified enzyme was also tested at different pH range starting from 3 to 7 for the optimization of pH.

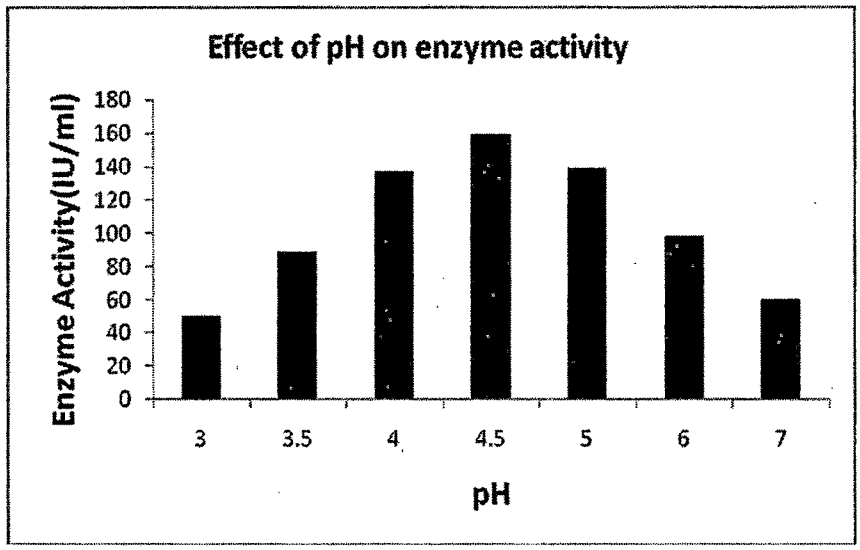

Figure 11: Effect of pH on xylanase activity.

There is no marked difference in xylanase titres at pH 3 and 4 but activity starts decreasing from pH 5 and after pH 6, it shows significant decrease in activity (Figure 11).

2.8C3 Effect of inoculum level on xylanase production

To define the suitable level of inoculums size for xylanase production, various levels of inoculum 1 ml of (10^1–10^6) were used. The time taken to achieve maximum xylanase production by using the inoculum size of 10^3, 10^4 and 10^6 spores/ml was 9, 12 and 15 days respectively. It was observed that maximum xylanase was found at 15th day of fermentation with inoculum size of 1×10^6 spores/ml. Raimbault and Alazard (1980) reported that maximum enzyme production and declination were achieved much faster due to the rapid degradation of substrate as a consequence of rapid growth.

2.8C4 Influence of moistening agents and moisture level

As reported by Shah and Dutta (2005), in the present study also media with different moistening agents supported enzyme production but the maximum xylanase production (158.85±0.15 IU/ml) was recorded when MS medium was used as a moistening agents (Table 23C).

Moistening Agent	Incubation (Days)	Xylanase Activity(IU/ml)
d/w	15	125±0.25
MS medium	15	158.85±0.15
Basal salt solution	15	95±0.3
Tap Water	15	85±0.11
Modified MS medium	15	147.28±0.14
Toyama's salt solution	15	156.30±0.1

Table 23C: Effect of different moistening agents on production of xylanase.

In addition to MS medium, various other moistening agents such as distilled water, tap water, MS medium, Basal salt solution, modified MS medium, Toyama's salt solution, etc. were used as moistening agents. Butt *et al.*, (2002) reported that use of distilled water as moistening agent gave better results as compared to that of mineral solution for production of xylanase. However, results of present study indicated that mineral salt solution was better inducer for higher production of xylanase compared to that of other moistening agents in case of *Chrysosporium asperatum*. This might be due to the fact that the salts present in MS medium supports growth of *C. asperatum* which ultimately supports the xylanase production. Similar results were also reported by the earlier workers (Reese *et al.*, 1969; Haq *et al.*, 1993).

Moisture content in SSF is a crucial factor for the production of any enzyme in SSF (Ramesh and Lonsane, 1990). According to Raimbault and Alazard (1980), increase in moisture content enhances enzyme production only to a certain extent and a

higher than optimum moisture level does not enhance enzyme production. Moisture content in the SSF exceeding the optimum level causes decreased porosity, alteration in particle structure, gummy texture, lower oxygen transfer and enhancement of the aerial mycelia (Raimbault and Alazard, 1980; Sanghvi *et al.*, 2010). Moreover, it leads to conglomeration of the substrate or sticking of particles to the wall of reactor. This feature ultimately results into much prominence of the substrate for bacterial contamination (Lonsane *et al.*, 1985). Xylanase production was optimized by adjusting the initial moisture content of SSF from 1:1 to 1:6 (rice straw: MS medium), corresponding 50-86% moisture level.

Moisture Level	Incubation (Days)	Xylanase Activity(IU/ml)
1:1	15	52±0.15
1:2	15	85±0.1
1:3	15	125±0.2
1:4	15	139±0.2
1:5	15	158.85±0.1
1:6	15	147±0.19

Table 24C: Effect of moisture level on xylanase production.

Increase in moisture level enhances xylanase production up to 1:5 (80%), while higher to that concentration does not enhance xylanase production due to increase in protein level. Peak activity (158.85±0.1 IU/ml) was obtained with moisture levels adjusted 1:5 (Table 24C). Maximum xylanase production was reported by Ancaharida (1999) when moisture content of rice straw was adjusted to 75-80%.

2.8C5 Influence of carbon and nitrogen sources

The enzyme production by microorganism mainly depends on growth conditions and nutrient available to them. Therefore, it is anticipated that improving nutritional value of rice straw by supplementing various carbon sources will also improve *Chrysosporium asperatum* growth which should subsequently enhance

an enzyme production. Therefore, flask containing media were supplemented with different carbon sources (glucose, xylose, maltose, lactose, fructose, and sucrose) in ratio of 1:5 (carbon: substrate). Among them flask supplemented with xylose showed maximum enzyme production (170±0.16 IU/ml) as compared to other carbon sources supplemented (Figure 12). When compared with the media lacking any carbon source, it showed improved production of xylanase (Figure 12). Xylose has been described as an effective inducer and good carbon source to enhance xylanase production in other fungal species *Fusarium solani* (Gupta *et al.*, 2009), *Fusarium oxysporium* (Christakopoullos *et al.*, 1999) which belongs to same family.

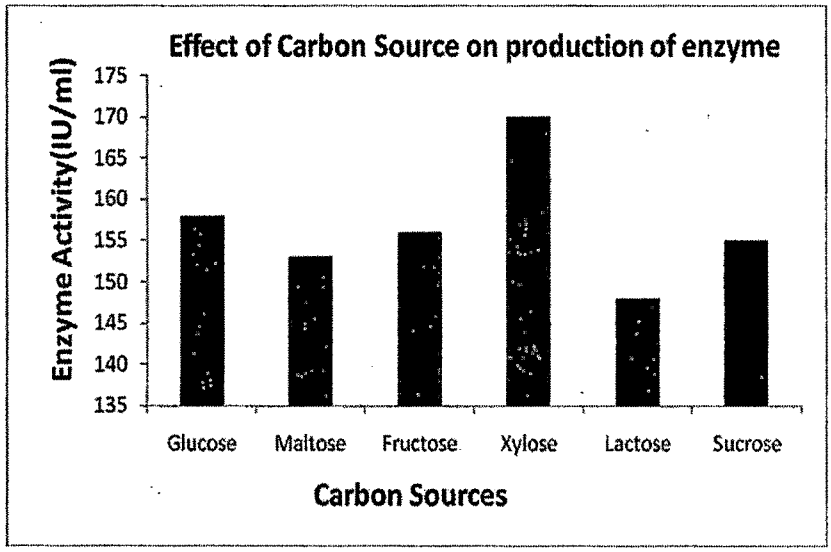

Figure 12: Effect of carbon source on production of enzyme.

Other carbon sources showed no much significant variation in the improvement on the production of xylanase. Production of an enzyme was relatively similar to the flasks that were lacking supplementation of any carbon sources. This may be attributed to the fact that different organisms have different uptake and binding affinity towards different carbon sources (Sanghvi *et al.*, 2010).

Perusal of literature indicates that nitrogen sources have a dramatic effect on the production of xylanolytic enzyme. Shah and Dutta (2005) concluded that the degree of selectivity for the production of a particular enzyme may be regulated by the carbon to nitrogen ratio. Xylanase with minimal cellulases can be produced by using a low nitrogen-to-carbon ratio (Gerber *et al.*, 1997). In the present study, effect of both organic (yeast extract, malt extract, beef extract, peptone) and inorganic nitrogen sources (urea, ammonium sulphate, sodium nitrate, ammonium nitrate) on the production of xylanase was investigated. Xylanase peak (152.5±0.3 IU/ml) was evident when malt extract was added in media. Results recorded in the present investigation are in agreement with those reported in the literature where fungi were found to produce higher xylanase on organic nitrogen sources (Lemos *et al.*, 2001).

2.8C6 Pre-treatment of substrates

Several structural and compositional factors are said to affect the fermentation ability of lignocellulosic materials. These factors include cellulose protection by lignin, hemicellulose sheathing, degree of hemicellulose acetylation, cellulose crystallinity etc. Depending on the substrate material, these barriers differ in relative significance (Teh-An Hsu, 1996). The alteration of the substrate using pretreatment techniques leads to a change in physical nature of lignin, increases in the available surface area, pore sizes, partial depolymerization of hemicellulose, decrystallization of cellulose and deacetylation of hemicellulose, which enhance the availability of the substrate (Shah and Dutta, 2005).

In the present work, rice straw was treated with mild acid, mild alkali as well as with hot water steam. None of the pretreatments used in the study were found to be advantageous for the increase in production of xylanases. On the contrary, it was noticed that fungal growth was decreased on media which eventually led to decreased enzyme production. This decrease in xylanase production could be

caused by formation of toxic inhibitors (acids and phenolics derived from carbohydrates and lignins) during chemical pre-treatment (Teh-An Hsu, 1996).

2.8C7 Influence of particle size of substrate

In solid culture, the particle size of the substrate determines the void space which is occupied by air (oxygen). Since the rate of oxygen transfer into the void space affects growth, the substrate should contain particles of suitable sizes to enhance mass transfer (Pandey, 1992). In the present study, rice straws of different particle sizes were tested in order to determine their effects on xylanase production. It was apparent that particle size affects the enzyme production. The highest titre of xylanase was produced by the rice straw of particle size that was greater than 3 mm whereas; it was lowest on the rice straw of other sizes. However, Ancharida (1999) reported that rice straw of 4mm particle size showed enhanced production of xylanase. In the present study, particle size less than 3mm showed less xylanase production. Lower production of xylanase with particle size less than 3mm might be due to clumping of substrate which leads to low aeration that is resulting in poor growth of fungi which consequently leading to less xylanase production. Kalogeris *et al.,* (1998) had also reported that substrate particle size plays an important role in xylanase production.

2.8C8 Mutation studies

The mutant of *C. asperatum* was developed by using physical and chemical mutagenesis and the mutants were selected initially on the basis of zone of clearance on xylan agar plates. Several potent mutants thus selected were evaluated for their xylanase production ability using xylan as the carbon source. Comparisons of xylanase production ability of various potent mutants obtained through physical (UV) and chemical mutagenesis (ethidium bromide) are shown in (Table 25C). It was observed that xylanase activity of mutant *C. asperatum* increases as compared to wild type strain in case of UV mutagenesis, while in chemical mutagenesis (ethidium bromide treated) xylanase activity decreases.

Sr.No.	Mutagenic agents	Strains of *C.asperatum*	Xylanase activity (IU/ml)
1	None	KSR-1	158.85±0.25
2	UV	KSR-1.1	175.23±0.1
3	Et Br	KSR-1.2	134.12±0.1
4	UV+ Et Br	KSR-1.3	163.0±0.16

Table 25C: Effect of mutagenesis on production of xylanase.

It was also noted that mutated strain showed higher thermostability of the xylanase enzyme as compared to that of wild type one. Singh *et al.,* (1995) reported that increased xylanase production up to two to three fold in *Fusarium oxysporium* when mutated with UV irradiation and N-methyl-N-0-nitrosoguanidine (NTG) treatment. In the present study, also increased xylanase production was observed only with the culture mutated by physical mutagenesis.

2.8C9 Purification and molecular weight determination of xylanase

The purification of xylanase from a culture of *C. asperatum* is summarized in Table 26C. It was essential to undertake all the purification steps at 4 °C in order to ensure the purity of the final product of xylanase. About 90% of proteins in the crude extract were filtered/seperated out in the 80% saturation by ammonium sulphate.

Fraction	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Purification (fold)
Crude broth	300	643.87	2.14	1.0
Ammonium sulphate precipitated fraction	60	230.07	3.83	1.78
DEAE-Sephadex	35	158.8	4.53	2.11

Table 26C: Purification profile of xylanase.

From the elution profile of the column chromatography and SDS PAGE, purified xylanase appeared to be homogeneous (Figure 13). There was only one band detected in the denatured gel at mw 45000 Da., showing purified xylanase enzyme. Kulkarni *et al.,* (1999) reported that microbial xylanases are single subunit proteins within range of 8-145 Kda.

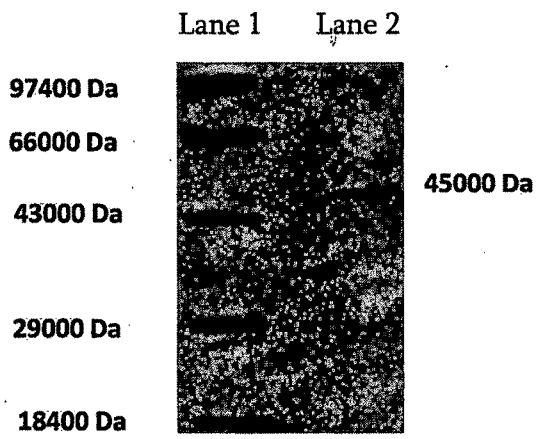

Figure 13: SDS Page of purified xylanase from *Chrysosporium asperatum.*

Lane 1: Ladder Medium Range (Bangalore Gene Pvt. Ltd. Cat. No. RPMW-M 106005)
Lane 2: Purified enzyme (45000 Da).

2.8C10 Influence of metal ions

Xylanase is one of the potential enzymes that are used in the pulp and paper industry. It has been found that some metal ions occurring in pulp play pivotal role in the enhancement of xylanase production (Castro *et al.,* 1997; Ghanem *et al.,* 2000; Seyis and Nilufer, 2005). Therefore, effect of these metal ions on the production of xylanase was included in the present investigation. As shown in Figure 14, metal ions particularly Ca^{2+} slightly enhanced xylanase productions.

There was no significant effect of other metal ions on enzyme activity except Ca^{2+}. Similar experimental results are also reported for *Tricoderma harzianum* (Seyis and Nilufer, 2005), and for *Aspergillus terreus* (Ghanem *et al.,* 2000). These studies have showed that Ca^{2+} enhanced xylanase activities while Mn^{2+} and Zn^{2+} ions also

had positive effect on xylanase activity (Castro *et al.,* 1997). Our results are reconfirmed with earlier reports that only Ca^{2+} ions had influenced the xylanase production while Mn^{2+} and Zn^{2+} ions showed minor effect on the production of enzyme.

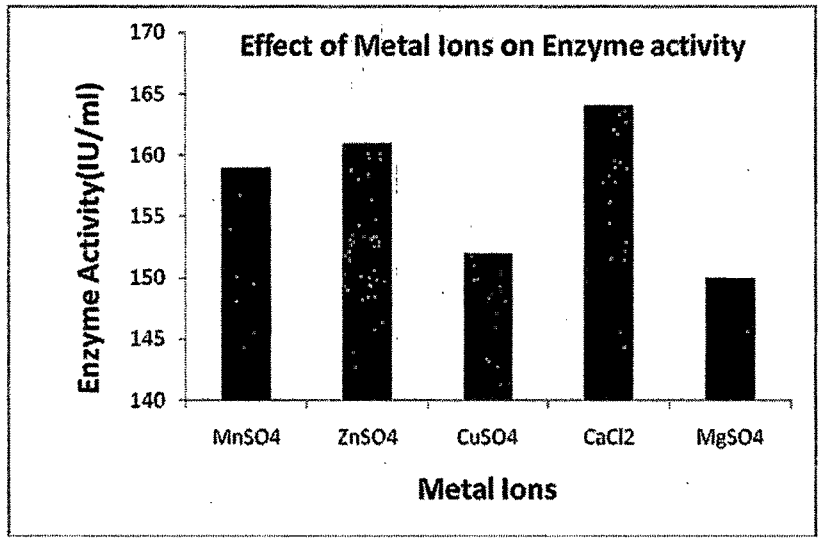

Figure 14: Effect of metal ions on xylanase activity.

2.8C11 Thermal stability

Use of enzyme in industrial application generally encounters problem of thermal inactivation. In the present investigation, complete inactivation of enzyme was recorded at 75 °C in wild type strain. Xylanase retained its 85% activity at 60 °C after incubation for 35 minutes. It was then declined gradually with the increase in temperature above 60 °C. However, O'Fagain (1995) reported that thermostability of proteins can be increased by several methods such as random mutation, chemical mutations or by use of additives. In present investigation also mutated strain retained 75% of its activity at 70 °C up to 30 minutes whereas, at 80 °C enzyme retained only 25% activity after incubation of 30 minutes. As compared to wild type strain of *Chrysosporium asperatum*, xylanase produced by mutated strain was found to be relatively more stable even at 70 °C. Therefore, mutated strain of *C. asperatum* might be more suitable for industrial application as it requires more thermal stability.

2.8C12 Storage stability

Shah and Dutta (2005) carried out various experiments on enzyme storage for industrial applications. In this context, earlier workers stored the enzyme at different temperatures such as room temperature, in deep freeze and cooling conditions. In their case, enzyme retained its 65% of activity at 4-5 °C after storage for a month whereas; it retained its 75% activity when stored at room temperature for 3 days. In the present study also xylanase enzyme was kept in freezing condition for 4 weeks and residual semisolid lyophilized enzyme was checked for activity. In our study, it was found that enzyme retained nearly 70% activity after 4 weeks storage. Looking to all the experimental conditions, it may be inferred that the isolate of *Chrysosporium asperatum* are active producers of xylanases with negligible level of cellulase. Thus, it can be cultivated on inexpensive substrate like rice straw under SSF for higher xylanase production.

2.8C13 Properties of xylanase from *C. asperatum*

Screening of several wood rot fungi for the production of xylanase showed that *C. asperatum* is a good producer, thus it was identified by morpho-taxonomic characters. Production of xylanase was carried out by solid state fermentation using different substrates, among them rice straw substrate gave maximal production of xylanase. Further, purification and charcterisation of xylanase was carried out. Purification of xylanase was done using DEAE-Sephadex G-75. Molecular mass was determined by denaturing gel electrophoresis technique and it was found to be 45000 Da. with 2.11 fold purification. Enzyme retained about 85% activity at 60 °C for 30minutes in case of wild types strains, while in mutated strains; enzyme retained 75% of its activity at 70 °C for 30 minutes. Results of this study indicate that *C. asperatum* is one of the potential producers of thermostable xylanase as compared to *Phanerochaete chrysosporium* a member of the same family Onygenaceae.