J. Basic. Appl. Sci. Res., 1(12)3002-3011, 2011

ISSN 2090-4304
Journal of Basic and Applied Scientific Research
www.textroad.com

Protein Analysis of Fungal Thermostable Xylanases Produced Under Optimal Submerged Fermentation Conditions

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ABSTRACT

Xylanases are a major group of enzymes, mostly produced from microbial fermentation processes, and have wide industrial and biotechnological applications. The production cost of xylanase is the major factor limiting its use, thus indicating the need for low cost production systems for market of this enzyme. This research has mainly focused on four strains of thermophilic fungi viz. *Sporotrichum thermophile*, *Chaetomium thermophile*, *Humicola grisea* and *Torula thermophila*. Four thermophilic fungi were screened for their production of xylanolytic enzymes in soluble and lignocellulosic insoluble substrate (Kallar grass, xylan, glucose, cellobiose and wheat bran). Kallar grass used as single substrate for enzyme production, it was noticed that the higher enzyme activity (4.8Unit/ml) was produced by *H. grisea* where as the (lower 1.3 unit/ml) was produced by *C. thermophile*. However when supplemented with 0.5 % xylan the enzyme activity increased to a level of 1.2,2.9,1.2 and 2.2 fold for *S. thermophile*,*C. thermophile*, *N. grisea* and *T.thermophila* respectively. The carbon source combination consisting of Kallar grass plus xylan plus glucose were found to be the best for production of xylanases from these fungus in order of *H.grises* > *C. thermophila* > *T.thermophila* > *S. thermophila*. The maximum enzyme activity for *S. thermophile* and *C. thermophile* was marked at pH 6. that of *H. grisea* and *T. thermophila* was comparable at pH 5 and 6 respectively. Enzyme activity values for *S. thermophile* ,*C.thermophile*,*H. grisea* and *T. thermophila* were recorded as 4.0,6.7,7 and 5.7 respectively. The optimum pH for xylanases produced from four various fungi is 6. The optimum temperature for xylanases assay produced from the various species of fungi was found to be 70 ºC. The xylanase enzyme produced from *C, thermophile* showed a high thermostability (56 at 70ºC) when compared to others (40-55 % at 70ºC). Also *C. thermophile* xylanase showed high protein content (2.4 mg/ml) compared to other fungi (1.7-2 mg/ml). *C. thermophile* samples showing 7-8 bands whereas ,*H. grisea*,*S.thermophile* and *T.thermophila* samples resulted in 5,4 and 2 bands respectively. It is recommended that *H. grises* ,*C.thermophile*,*T.thermophila* and *S. thermophile* can be exploited for production of thermostable xylanases using kallar grass plus xylan plus glucose in the medium as carbon source. The four crude enzymes produced in this study a potential to be a candidate for the application and feed and food industry.

KEY WORDS: Thermostable enzymes, Fungal xylanase, Polyacrylamide gel electrophoresis (PAGE), Lignocellulosic substrate, Protein profile, Thermostability.

INTRODUCTION

Xylan, the second most abundant polysaccharide and a major component in plant cell wall consists of β-1, 4-linked xylopyranosyl residues. The plant cell wall is a composite material in which cellulose, hemicellulose (mainly xylan) and lignin are closely associated **(Puls, 1997; Bissoon *et al.*, 2002).** Three major constituents of wood are cellulose (35-50%), hemicellulose (20-30%)- a group of carbohydrates in which xylan forms the major class- and lignin (20-30%). Xylan is a heteropolysaccharide containing substituent groups of acetyl, 4-O-methyl-D-glucuronosyl and α-arabinofuranosyl residues linked to the backbone of β-1,4,-linked xylopyranose units and has binding properties mediated by covalent and non-covalent interactions with lignin, cellulose and other polymers. Lignin is bound to xylans by an ester linkage to 4-O-methyl-D-glucuronic acid residues. The complex structure of xylan needs different enzymes for its complete hydrolysis. Endo-1,4-β-xylanases(1,4-β-D-xylanxylanohydrolase, E.C.3.2.1.8) depolymerise xylan by the random hydrolysis of xylan backbone and 1,4-β-D-xylosidases (1,4,β-D-xylan xylohydrolase E.C.3.2.1.37) split off small oligosaccharides. The side groups present in xylan are liberated by α-L-arabinofuranosidase, α-D-glucuronidase, galactosidase and acetyl xylan esterase.

Organic wastes from agricultural residues comprise cellulose, hemicellulose and lignin in an average ratio of 4:3:3, with hemicellulose being the second most abundant fraction available in nature **(Taiz and Honigman, 1976; Abdel-Sater and El-Said,2001)**. Xylans, the major portion of the hemicellulose of plant cell walls, are hetero-polymers consisting principally of xylose and arabinose **(Biely,1985; Coughlan and Hazlewood,1993)** and is

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amenable to degradation by different microorganisms, including bacteria, yeasts, and fungi. Several types of enzymes appear to be involved in the degradation of native xylan. Endoxylanases, which attack the linear polyxylose chain in a manner analogous to that of endoglucanase on cellulose, are the most important and have accordingly received the most attention **(Sunna and Antranikian,1997)**. β-xylosidase hydrolyzes xylobiose and oligosaccharides to complete the conversion of xylan to xylose and probably relieves the end product inhibition of endoxylanase activity **(Srinivasan and Rele,1999; Subramaniyan and Prema,2000; Chávez *et al*.,2006).**

This activity appears to be extracellular in fungi but cell-associated in bacteria, although relatively few of these enzymes have been studied in detail **(Esteghlalian *et al*., 2008)**. Other enzymes play an important role in the removal of side groups from polymeric xylan to create more sites for subsequent enzymatic hydrolysis and possibly contribute to lignin solubilization by attacking the covalent bonds responsible for the integrity of the lignocarbohydrate matrix **(Bachmann and McCarthy,1991; Contreras *et al*.,2008).** The choice of an appropriate substrate is of great importance for the successful production of xylanases.

The substrate not only serves as carbon and energy source, but also provides the necessary inducing compounds for the organism, preferentially for an extended period of time, for an increased overall productivity of the fermentation process **(Duff and Murrayh,1996; Haltrich *et al*.,1996).** Purified xylans are frequently used for small-scale experiments and are considered as excellent substrates not only because of high yields of xylanase obtained, but also because they cause a selective induction of xylanase with little or no concomitant cellulase activities **(Biswas *et al*.,1990; Gilbert *et al*.,1992).**

However the high cost of xylan and other good substrates has limited their applications for larger-scale production processes, hence, the need to search for cheaper alternatives.

Several inexpensive substrates, mainly insoluble lignocellulosic material, such as barley husk, corn cobs, sugarcane bagasse have been used **(Camassola and Dillon,2009).** Although poorly investigated, the use of soluble sugars for the production of xylanases has been shown to circumvent problems associated with the use of high concentrations of insoluble substrates, such as media viscosity and difficulties with agitation. In addition, xylose, which can be easily obtained from the xylan portion of lignocellulosic material, has been described as an effective inducer of xylanase activity in several organisms **(Haltrich *et al*.,1996)**. Biotechnological applications of xylanases have broadened markedly since they are now widely employed as supplements in paper manufacturing, animal feeds, biobleaching of pulp and paper, and in the production of bioethanol **(Beg *et al*.,2000; Subramaniyan and Prema,2000; Techapun *et al*.,2003)**. The most important industrial application of xylanase is in the pre-bleaching of Kraft pulp. This allows for a lower consumption of chemicals during the bleaching process, and also results in a brighter product than can be achieved without the enzymatic treatment **(Viikari *et al*.,1994; Ninawe and Kuhad,2006).** Most of the commercial xylanase is produced by microbial fermentation process.

The production cost of xylanase is the major factor preventing its use, thus indicating the need for low cost production systems for market of this enzyme. Therefore, the main objective of this study is to investigate the potential of cheap raw substrates such as Kallar grass and wheat bran for the production of xylanases by four thermophilic fungi. Effect of temperatures, pH, carbon sources, thermostability and pH stability of the four xylanases produced by these organisms on the raw substrates, protein profile was also investigated to determine their potential industrial applications.

MATERIALS AND METHODS

I-Fungal strains:

The pure fungi used in this study consisted of *Sporotrichum thermophile*, *Chaetomium thermophile*, *Humicola grisea* and *Torula thermophila* which obtained from National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan. The fungi were grown on potato dextrose agar slants at 45ºC until sporeulation(5 days) and then were maintained at 4ºC until used. The inoculum was prepared by adding 4 ml of sterile distilled water to an agar slant and adjusting the spore suspension to $1x10^6$ spores per ml. Four the previously mentioned fungal strains were employed to select suitable fungi for xylanase maximum production.

II-Fungal growth medium:

Growth medium was made from the ingredient obtained from Sigma company according to the method of **Eggins and Pugh (1962)** as shown in the following (%):Yeast extract,0.05;L-Asparagine,0.05;$MgSO_4$,2.0; KH_2PO_4,10.0;$(NH_4)_2SO_4$,5.0 and $CaCl_2$,1.0.The distilled water supplemented to 100 ml. The pH of the medium was adjusted to 5 using 0.1 N HCl.

III-Carbon source:

The main carbon source was used in the present study " Kallar" grass which was grinded into pieces of about 0.5 mm. To optimize the substrate combination for production of maximum enzyme further enrichment with xylan, glucose, cellebiose and wheat bran was done in the following manner : 1% Kallar grass (KaG); 1% KaG + 0.5 % xylan, 1% KaG + 0.5 % xylan + 0.25% glucose; 1% KaG+ 0.5 xylan+ 0.25% cellebiose; 1% KaG + 0.5 xylan+ 0.5 wheat bran.

The culture flasks containing 100 ml of each substrate were plugged with cotton, covered with aluminum foil and autoclaved to 121 ºC for 10 minutes.

IV-Fungal inoculum preparation:

For preparation of inoculum from each fungi to a conical flask (500 ml capacity containing 100 ml cooled and presterilized medium at 121ºC for 10 minutes) one loopful each of pure slant was inoculated in inoculating hood. Each flask containing inoculum in growth medium was kept in orbital flask at 100 rpm for 24 hours at 45 ºC.

V-Propagation of organism on different carbon sources to optimize production of xylanase:

In a conical flask (500 ml capacity already containing 100 ml) cooled presterilized growth medium supplemented with different carbon source as described earlier, 10 ml of inoculum was introduced and kept in orbital shaker at 100 rpm for 6 days at 45ºC.

VI-Separation of xylanase:

After propagation the culture mixture was centrifuged using Beckman (Model J 2-21) centrifuge for 10 minutes at 10.000 rpm. The supernatant was further filtered through Buchner funnel under vacuum to get a clear filtrate. The filtrate was made contamination free by the addition of 0.02 % sodium azide.

VII-Enzyme evaluation by spectrophotometer:

The enzyme filtrate obtained was tested for the enzyme activity (at different pH and temperature). Protein content and molecular weight.

VIII-Xylanase activity: the activity of the enzyme tested according to **Miller (1959).**

IX-Reagents:

1- **Citrate buffer (pH:5):** To 25 ml 0.1 % citric acid 25 ml of 0.2 % of NaH_2PO_4 was mixed and the pH was adjusted to 5 with further addition of 0.2 % NaH_2PO_4.
2- **Xylan sugar (1%):** xylan (0.5 g) was dissolved in distilled water and made the volume to 50 ml.
3- **Dinitrosalicylate reagent (DNS):** this reagent was prepared by mixing (182 g of Na K tartarate , 10 g of NaOH,10 g of 3- dinitrosalicylic acid, 2 g of phenol, 0.5 g of Na_2SO_5 in water and made the volume to one liter.
4- **Assay:** in a test tube containing 1 ml of citrate buffer and 0.5 ml of xylan solution a volume of 0.5 ml of diluted enzyme (Enzyme : water = 1:4 v/v) was added. The mixture was incubated for 15 minutes at 50 ºC in orbital shaker at 100 rpm. After expiry of time 3 ml of dinitrosalicylate reagent (DNS) was added to test tube then boiled for 5 minutes according to **Miller (1959)** method. A control was run –parallel as above but the enzyme was added after addition of DNS reagent. The absorbance of the mixture in each test tube was taken with help of computational spectrophotometer (Model 4-3210, Hitachi, Japan) at 550nm. A zero point absorbance was adjusted by a blank reagent containing equal volume of DNS and water.

Calculation of enzyme activity:

The factor was derived from slope of standard curve (Fig.1) made by taking absorbance at 550 nm after reacting with DNS reagent with different concentration of xylose.

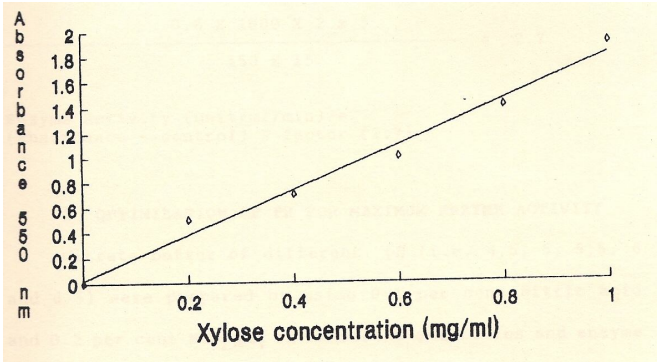

Fig. (1): Standard curve xylose concentration for enzyme activity in unit /ml.

Factor= slope x 1000 x vol of sample x dilution / mol.wt of xylose x time (min).

0.6 x 1000 x 2 x 5 / 150 x 15 = 2.7

Enzyme activity (Unit /ml/ min) = (absorbance – control) x factor (2.7).

Optimization of pH for maximum enzyme activity:

Citrate buffer of different pH (4.5,5,5.5,6 and 6.5) were prepared by using 0.1 % citric acid and 0.2 % NaH_2PO_4 in different quantities and enzyme activity was tested by using the assay described earlier to select optimum pH.

Optimum temperature for maximum enzyme activity:

After adding 1 ml citrate buffer (pH 5) and xylan and enzyme in each test tube it was incubated at different temperature (50,60 and 70ºC) for 15 minutes. The enzyme activity was tested with help of spectrophotometer at 550 nm using micro-cuvette tube after addition of DNS reagent and boiling for 5 minutes with a view to select optimum temperature.

Thermostability of enzyme:

The enzyme (10 ml each) in test tube obtained from different substrates combination for each species of fungi were incubated at 50, 60 and 70 ºC for 24 hours in orbital shaker at 100 rpm. Enzyme was tested for its activity by the same procedure described earlier to select thermostability of enzyme.

Determination of protein:

The protein in enzyme was determined by **Bradford method (1976).**

Enzyme evolution by gel electrophoresis

Gel electrophoresis of enzyme was carried out by the method of **Laemmli(1970)** using 7.5 % polyacrylamide gel.

RESULTS AND DISCUSSION

Xylanases have great potential in various industrial processes, including the manufacture of bread, food and drinks, improvement of nutritional properties of agricultural silage and grain feed, for processing plant fibers in the textile industry, in pharmaceutical and chemical applications, and in the cellulose pulp and paper manufacturing processes **(Beg *et al*.,2001; Kaur *et al*.,2011).** Xylan biodegradation is performed by xylanolytic complex, which is primarily produced by fungi and bacteria.

The production of microbial xylanases has attracted great interest due to their potential application in chemical, pharmaceutical and food industries. Xylanases preparations free of cellulose activity are of particular interest for the pre-treatment of paper pulps to decrease the xylan content and , therefore, reduce the dependence on chlorine used for bleaching in the brightening process **(Gilbert *et al*.,1993).** The production of enzyme by using xylan rich substrate mainly depends on the selection of a suitable strain from an appropriate habitat. Fungal systems have been mainly used for enzyme production **(Ghosh *et al*.,1991; Imai *et al*.,1994; Crabb and Mitchinsan,1997; Irshad *et al*.,2011).** Extensive work has been going on in many laboratories to select a proper organism for production of concentrated xylanases and efficient inducer for large-scale enzyme production particularly using biomass wastes in microbial fermentation.

This study was conducted to produce xylan hydrolyzing enzymes from different species of thermophilic fungi when grown on five different combinations of insoluble and soluble carbon substrates. The enzymes obtained from different sources of fungi were characterized for their enzyme activity, optimum pH and temperature. the results relating to these parameters have been presented and discussed here:

I-Effect of different combination of carbon source on enzyme production:

Different carbon sources were used to find economical and potent inducer of xylanase using the four thermophilic fungi. By conducting screening of various carbon sources it is realistic to identify highly efficient inducer of enzyme which lead to substantial increase in yield.

Kallar grass, a straw (salt tolerant) lignocellulosic material consisting of 30 % hemicelluloses **(Latif *et al*., 1988)** was mainly employed as a substrate for fungi in this study. Other carbon sources like xylan, glucose, cellebiose and wheat bran were also incorporated in the liquid growth culture medium to see their enrichment effect of the production of xylanse in the form of enzyme activity. Data on the enzyme activity were presented in Table (1). Kallar grass is a natural source of xylanase inducer **(Latif,1990)**. Kallar grass used as single substrate for enzyme production, it was noticed that the higher enzyme activity (4.8 Unit / ml) was produced by *H. grisea* where as the (lower 1.3 unit/ml) was produced by *C. thermophile*. However when supplemented with 0.5 % xylan the enzyme activity increased to a level of 1.2,2.9,1.2 and 2.2 fold for *S. thermophile*,*C. thermophile*, *N. grisea* and *T.thermophila* respectively.

The enzyme activities further increased when 0.25 % glucose was added. The level of increase was 1.5,5,1.5 and 3.4 fold for *S. thermophile*, *C. thermophile*, *H. grisea* and *T. thermophila* respectively. Thus an increase of 0.75% carbon source (in the form of 0.5% xylan plus 0.25 % glucose) had a marked effect on enzyme induction. Substituting glucose by cellebiose could not induce xylanase to similar level except in *H. grises* whereas addition of wheat bran resulted in moderate effect on these enzyme production. It is concluded that the carbon source combination consisting of Kallar grass plus xylan plus glucose were found to be the best for production of xylanases from these fungus in order of *H.grises* > *C. thermophila* > *T.thermophila* > *S. thermophila*.

Kamble and Jadhav (2011) use of purified xylan as a substrate for enhanced production of xylanase is uneconomical and, therefore, the use of agro residues is cost effective method. In the present study agro-residues such as Wheat bran, Rice bran, Apple pomace and substrates Birchwood and Oat spelt xylan were supplemented as sole carbon sources for xylanase production in the production medium.

Table (1): Xylanase activity of various fungi when grown on different carbon sources at 45ºC in submerged culture.

Carbon source	Enzyme activity (unit /ml)			
	Species of fungi			
	Sporotorichum thermophile	*Chaetomium thermophile*	*Humicola grisea*	*Torula thermophila*
Kallar grass (1%)	2.5	1.3	4.8	1.6
KG(1%) + Xylan (0.5%)	3	3.8	5.7	3.7
KG (1%)+Xylan(0.5%)+ glucose (0.25%)	3.7	6.6	7.0	5.7
KG (1%)+ xylan (0.5%)+ Cellebiose (0.25%)	2.7	5	7.0	4.4
KG(1%)+ xylan(0.5%)+ Wheat bran (0.5%)	2.2	4.9	4.1	3.3

Optimum pH for maximizing enzyme activity:

Initial pH influences many enzymatic systems and the transport of several species of enzymes across the cell membrane. In view of the fact that enzymes are proteins, the ionic character of the amino and catalytic property of the enzyme in strikingly affected. Fermentation at lower and higher pH proved to be detrimental, perhaps because of the inactivation of the enzyme system. Each microorganism thereby holds a certain pH range for its optimal growth and activity **(Poorna and Prema,2007)**.

High stability at wide range of pH and high temperature is required for the enzyme in animal feed. The enzymes produced from the four species of fungi were assayed at different pH values of citrate buffer to find optimum pH. The maximum enzyme activity for *S. thermophile* and *C. thermophile* was marked at pH 6 . that of *H. grisea* and *T. thermophila* was comparable at pH 5 and 6 respectively as shown in table (2). In Table (2) enzyme activity values for *S. thermophile* ,*C.thermophile*,*H. grisea* and *T. thermophila* were recorded as 4.0,6.7,7 and 5.7 respectively.

It was concluded that the optimum pH for xylanases produced from four various fungi is 6. Maximum activity was observed at pH 6 by the investigated four thermophilic fungi used for production of xylanases. This is very important from practical point of view as pH control would not be critical as observed with many other enzyme preparations. High stability at wide range of pH and high temperature is required for the enzyme is animal feed. **Khonzue *et al*., (2011)** found that the optimal pH and pH stability of crude xylanases enzymes from *Thermomyces lanuginosus* THKU-56 were at 7 and in the range of pH 6 to 9 respectively. The fungal included in this study produced xylanases to a varied extent. **Ghatora *et al*.,(2006)** identify and characterize of diverse xylanases from thermophilic and thermotolerant fungi.

Table (2): Xylanase activity of various fungi at 50ºC when assayed at different pH.

pH	Enzyme activity (unit /ml)			
	Species of fungi			
	Sporotorichum thermophile	*Chaetomium thermophile*	*Humicola grisea*	*Torula thermophila*
4.5	3.2	3.3	6.8	3.2
5	3.7	4.6	7.0	5.6
5.5	3.9	5.3	6.9	5.9
6	4.0	6.7	6.9	5.7
6.5	3.7	5.6	5.8	5.4

Optimization of temperature for maximum enzyme activity:

Temperature is one of the important parameters that determine the success of the optimization system. Xylanase production has been shown to be influenced by pH and temperature in several species of thermophilic fungi. An Alkalo-thermophilic *Bacillus halodurans* MTCC 9512 have been reported for maximum xylanase activity at the pH 9.5 and temperature 55^{o}C in submerged condition using xylan as carbon source **(Garg *et al*.,2009).**

The xylanases produced from *S. thermophile*, *C. thermophile*, *N. grisea* and *T. thermophile* were assayed at different temperatures so as to select optimum temperature. For *S. thermophile* as shown in table (3) the values of enzyme activity recorded are 3.7,4.5 and 5.4 at 50,60 and 70 ºC respectively. Maximal enzyme activity was found at 70 ºC.

A decrease in xylanase titer was obtained with cultivation temperatures either below or above the temperature optima. The decreased yield at low temperatures was possibly due to lower transport of substrates across the cells. On proceeding towards optimum temperature of enzyme production, increased kinetic energy of reacting molecules increase the reaction rate. At higher temperature , thermal denaturation of enzymes of the metabolic pathway occurs, which increase the maintenance energy requirement of cellular growth, thereby resulting in poorer production of the metabolites and even loss of enzyme activity signifying that the end –point of fermentation should be carefully controlled. It also has been stated that microorganisms synthesize only a reduced number of proteins essential for growth and other physiological processes under conditions of high temperatures **(Gawande and Kamat,1999).**

Table (3): Xylanase activity of four thermophilic fungi when assayed at different temperature 50ºC for 15 min.

Temperature (ºC)	Xylanase activity (Unit/ml)			
	S.thermophile	*C.thermophile*	*H.grises*	*T.thermophila*
50	3.7	6.6	7.0	5.7
60	4.5	6.9	7.8	6.1
70	5.4	7.2	8.4	7.2

The optimum temperature for xylanases assay produced from the various species of fungi was found to be 70 ºC and this is in accordance with those of earlier worker conducted on *C. thermophile* **(Ganju *et al*., 1989)**, and *Myceliopthera thermophila* **(Zamost *et al*., 1991)** whose reported the optimum temperature for xylanases produced from these species was 70ºC. Contradictory to this study reported by **Tan *et al*.,(1987)** whose reported the optimum temperature for xylanase from *Thermoascus aurantiacus* was 78ºC.

Effect of different combination of carbon source on extracellular protein:

Requirements for efficient xylanase production differ from one fungal strain to another as far as carbon source requirement is concerned. Just providing nutrient to the microorganisms could not be a sufficient criterion for choosing a substrate; oxygen transfer and heat dispersion are equally important.

All the four fungal species produced the maximum extracellular protein when a substrate comprising Kallar grass plus xylan plus glucose was employed with values of 1.6 for *S.thermophile*, 2.4 for *C. thermophile*, 2.6 for *H.grisea* and 1.7 for *T. thermophila*. Analysis of variance studies, made on the data thus obtained indicated that the amount of extracellular proteins produced by different species using carbon sources as well as by each species separately on various carbon sources simultaneously mutually differed significantly ($P<0.5$) , more over the interaction between species as well as carbon sources was significant too. This necessitates further the pairwise analysis of the data for which DMR test was used. Significant difference were noticed in treatment means using five different carbon source except carbon source combination 1 and 2 which mutually differed non significantly. Similarly DMR test was applied to the data regarding species as the source of variation and paired differences between all the four species used were found non- significant between species,1,2,3 except 4 show significant difference with others (Table 4). It was noticed that the maximum extracellular protein was corresponded to the activity produced from a carbon sources combinations of kallar grass plus xylan plus glucose. These result agreed well with those of **Gilbert *et al*.,(1992)** who stated that there is correspondence between enzyme activity produced from different carbon source and protein content (Table 5).

Kallar grass is a suitable support and carrier because of its porosity and low cost. Kallar grass contains a blend of soluble sugar vital for the initiation of growth and replication of the microorganisms. Moreover, it provides a large surface area and efficient aeration by remaining loose even under the moist conditions during the fermentation.

Table (4): Extracellular protein produced by various fungi when grown on different carbon sources.

Carbon source	Protein content (mg /ml)			
	Species of fungi			
	Sporotorichum thermophile	*Chaetomium thermophile*	*Humicola grisea*	*Torula thermophila*
Kallar grass (1%)	1.4	1.8	1.3	1.3
KG(1%) + Xylan (0.5%)	1.5	1.8	1.6	1.4
KG (1%)+Xylan(0.5%)+ glucose (0.25%)	1.6	2.4	2.0	1.7
KG (1%)+ xylan (0.5%)+ Cellibiose (0.25%)	1.4	2.0	1.9	1.7
KG(1%)+ xylan(0.5%)+ Wheat bran (0.5%)	1.4	1.9	1.3	1.3

Table (5): Analysis of variance with respect to data shown in table (4).

S.O.V.	df	SS	MS	F-Value
Carbon source	4	1.28	0.321	20.05
Species	3	1.97	0.657	41.06
Carbon source x species	12	0.46	0.038	2.38
Error	20	0.016		
Total	39			

Duncan's Multiple Range Test for carbon source					
Carbon source	1	2	3	4	5
Mean	1.46 (C)	1.59(C)	1.93(A)	1.75(B)	1.46(C)
For fungal species	1	2	3	4	
Mean	1.47 (B)	2.01(A)	1.59(B)	1.47(B)	

SDS –PAGE Gel

SDS-PAGE gel was run in order to determine the pattern of bands based on molecular size marker (Lane 1 in figure 2). In general the silver staining , since very sensitive could not be handled properly and also because of the nature of the samples obtained from different carbon sources a lot of smearing effect was obtained rather than clear bands.

So it's concluded that no sharp bands obtained for the standard used, however , it become difficult to determine molecular weight.

Characterization of xylanases using zymogram technique:

The use of activity staining to detect xylanase bands in non- denaturing polyacrylamide gels is illustrated in Fig.3-6. This technique enabled to detect various level of activity bands when protein samples were obtained from different carbon combination for these fungi as explained in figure 7.

As shown in figure (3) protein stain of *S. thermophile* from " Kallar grass" (Lane 1) showed streaks specially at the onset of resolving gel. This can be due to nature of substrate (Kallar grass). However, when used with other substrate the streak effect was reduced (Lane 2-5). All lanes showed 9-12 bands. Zymogram analysis illustrate a discrete band as marked in figure 3 which is faint in lane 6.

The protein sample of *C. thermophile* showed streaking . Thus silver staining did not show clear bands, the possibility could be experimental error in silver staining procedure or running the gel high voltage (200 V) may have affected this since the protein sample contained. Polysaccharide and phenolic compounds due to nature of carbon source along with it, however, considering the activity staining sharp bands were observed for *C. thermophile* (Fig.4).

For *H. grises* fig.5 discrete activity bands were obtained in each lane except for the bottom band did not appearing in lane 1. The bands in the middle seems to be showing true most active fragment and thus major xylanase component, the protein stain could not be developed well.

Similarly in case of *T.thermophila* as illustrated in fig. 6 protein gel does not show discrete bands however, two bands in lane 5,6 and 10.

Fig.(2): SDS-PAGE of xylanases protein samples of *S. thermophile* lane 1-5 , *C. thermophile* , lane 6-10, *H. grises* lane 11-15 and *T. thermophila* , Lane 16-20. Lanes 1,6,11,16: Kallar grass (1%).Lanes 2,7,12,17 : Kallar grass (1%)+ xylan (0.5%) + Glucose (0.25%).Lane 3,8,13,18: Kallar grass (1%)+ xylan (0.5%)+ Glucose (0.25%).Lane 4,9,14,19: Kallar grass (1%)+ xylan (0.5%)+ Cellobiose (0.25%).Lanes 5,10,15,20: Kallar grass (1%)+ xylan (0.5%)+ Wheat bran (0.5%).

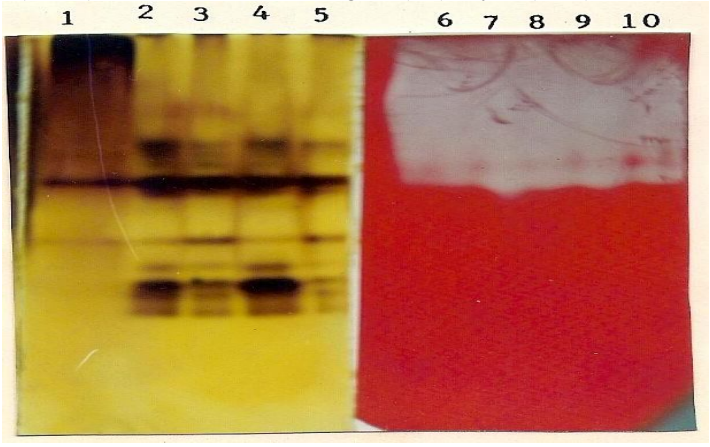

Fig.(3): Side by side xylanase zymogram of *S. thermophile* of protein sample run on native gel (7.5%). Lane 1-5 protein samples and 6-10 zymogram. Lanes 1 and 6: Kallar grass (1%).Lanes 2 and 7 : Kallar grass (1%)+ xylan (0.5%) + Glucose (0.25%).Lane 3 and 8: Kallar grass (1%)+ xylan (0.5%)+ Glucose (0.25%).Lane 4 and 9: Kallar grass (1%)+ xylan (0.5%)+ Cellobiose (0.25%).Lanes 5 and 10: Kallar grass (1%)+ xylan (0.5%)+ Wheat bran (0.5%).

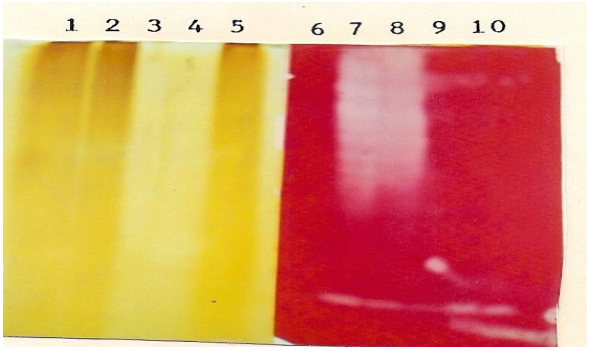

Figure (4): Side by side xylanase zymogram of *C.thermophile* protein samples run on native gel (7.5%). Lanes 1-5 protein samples and 6-10 zymogram. Follow legend as in Fig.(4).

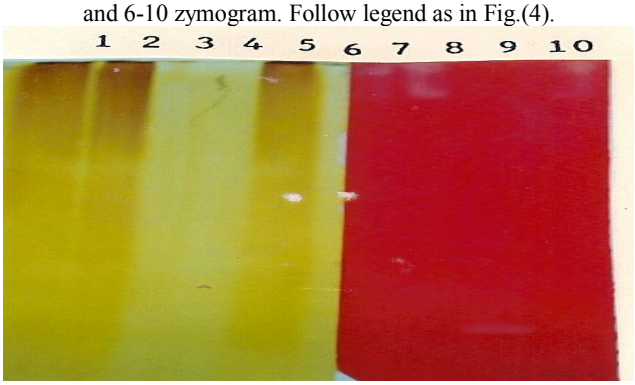

Figure (5): Side by side xylanase zymogram of *H. grises* protein samples run on native gel (7.5%). Lanes 1-5 protein samples and 6-10 zymogram. Follow legend as in Fig.(4).

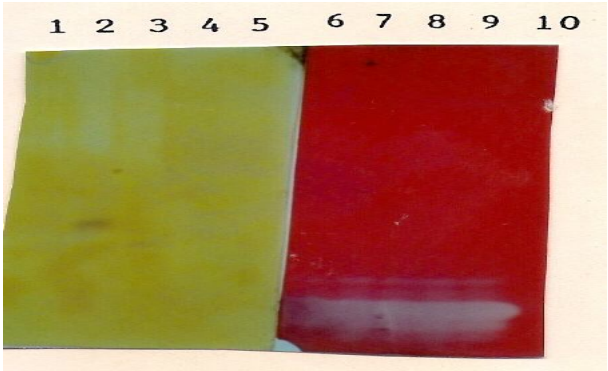

Figure (6): Side by side xylanase zymogram of *T.thermophile* protein samples run on native gel (7.5%). Lanes 1-5 protein samples and 6-10 zymogram. Follow legend as in Fig.(6).

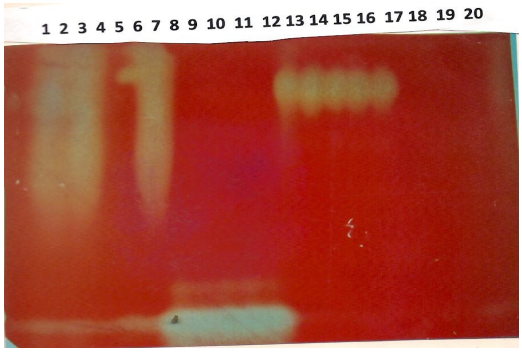

Figure (7): Side by side xylanase zymogram of *C.thermophile* lanes 1-5 , *H. grises* Lanes 6-10, *S.thermophile* 11-15 and *T. thermophile* Lanes 16-20.

Conclusion

This paper was conducted to optimize xylanases production conditions by four thermophilic fungi namely *Sporotrichum thermophile*, *Chaetomium thermophile*, *Humicola grisea* and *Torula thermophila* using Kallar grass with xylane,glucose , cellibiose and wheat bran as carbon sources singly and combination as a substrate. Kallar grass plus xylan plus glucose was found to be the best substrate for four thermostable xylanases production. H. grisea gives the maximum activity compared to others. For enzymes assay the optimum pH was 6 and the optimum temperature was 70ºC. *C.thermophile* showed a high thermostability when compared to others and protein content compared to other fungi. Xylanase zymogram on SDS-PAGE showed that, *C. thermophile* samples contained 7-8 bands whereas ,*H. grisea*, *S. thermophile* and *T. thermophila* samples resulted in 5,3,4 and 2 bands respectively. It is recommended that *H. grises* ,*C.thermophile,T.thermophila* and *S. thermophile* can be exploited for production of thermostable xylanases using kallar grass plus xylan plus glucose in the medium as carbon source.

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