Cloning and Expression of *Chaetomium Thermophilum* Xylanase 11-A gene in *Pichia pastoris*

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By

Saiqa Andleeb

National Institute For Biotechnology & Genetic Engineering
(NIBGE), Faisalabad
&
Quaid-i-Azam University, Islamabad, Pakistan

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Dedicated to:
That lonely pilgrim who bows his head on the wall of Kabba and pray "May God give all happiness to my lovely parents and save my dreams"

Certificate

This dissertation submitted by Ms Saiqa Andleeb, is accepted in its present form by the Department of Biological Sciences, Quaid-i-Azam University, Islamabad, as satisfying the thesis requirements for the degree of Master of Philosophy in Biotechnology.

Internal Examiner

Dr. Farooq Latif

External Examiner

Dr. Azra Khanum

Chairman

Dr. Yusuf Zafar

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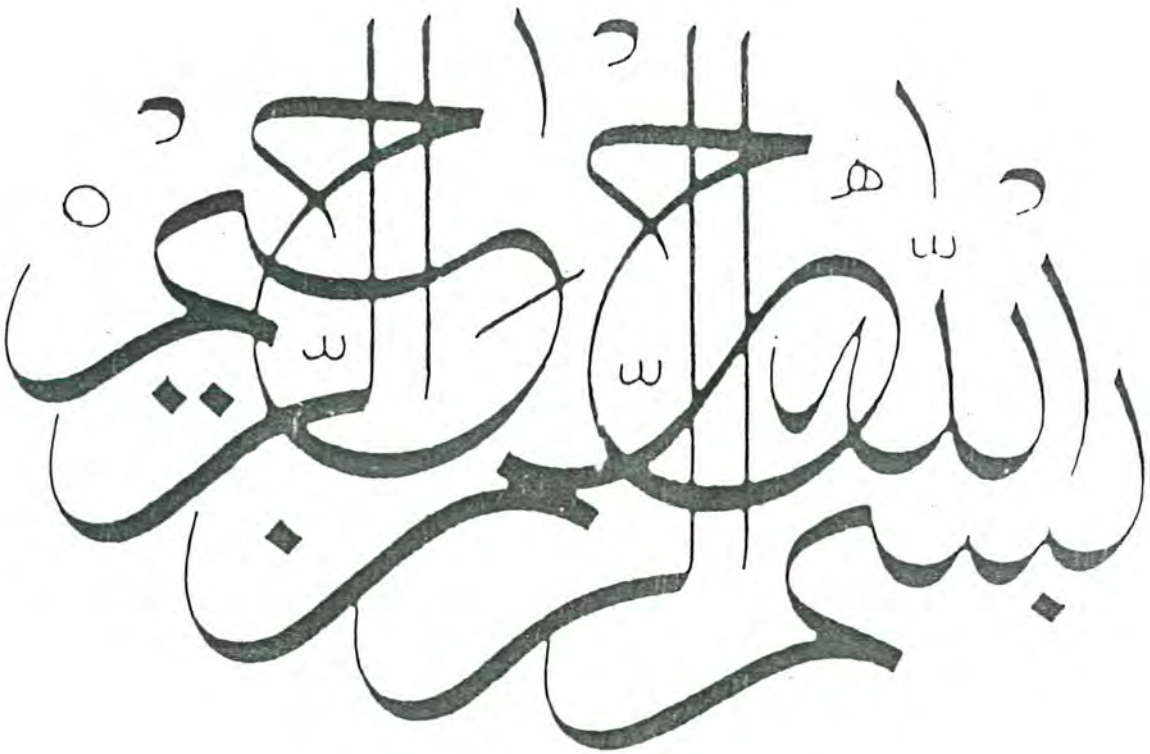

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Saiqa Andleeb

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List of abbreviations

Abbreviations	Full text
°C	Degree centigrade
µl	Micro liter
µg	Micro gram
bp	Base pair
ddd H_2O	Double distilled deionized water
DNA	Deoxy ribonucleic acid
dNTPs	Deoxy riboucleotide triphosphate s
EDTA	Ethylenediamine tetra acetic acid
gm	Gram
kb	Kilo base
M	Mole
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Milli liter
mM	Mili Mole
mm	Milli meter
N	Normal
NaCl	Sodium chloride
PCR	Polymerase chain reaction
rpm	Revolutions per minutes
Taq	*thermous aquatiqus*
U	Unit
V	Volt
UV	Ultra violet
SDS	Sodium dodecyl sulphate
Rn	Reaction
LB	Luria-Bertini
$CaCl_2$	Calcium chloride
IPTG	isopropyl-thio-β-D-galactoside
X-Gal	5-bromo-4-chlor-3-indolyl-β-D-galactopyranoside
PEG	Poly ethylene glycol
O.D	Optical density
DNS	Dinitrosalicylate
D	Dalton
pI	Isoelectric point
PAGE	Polyacrylamide gel electrophoresis
DS	Dubose salt
NMR	Nuclear magnetic resonance

B	Biotin
MGM	Minimal glycerol medium
PMSF	Phenyl methyl sulphonyl fluoride
HPLC	High performance liquid chromatography
BSA	Bovine serum albumin
TEMED	N,N,N',N'- Tetra methyl diamine
AP	Alkaline phosphate
TAE	Tris acetate EDTA
APS	Ammonium persulphate
YPD	Yeast peptone dextrose medium
P	Primer
YNB	Yeast nitrogen base
GY	Glycerol

Abstract

The thermophilic fungi have considerable importance because of the thermostable nature of their enzymes, which is mostly an industrial requirement, such as in textile denim and biofinishing, pulp and paper and poultry feed pelleting, etc. Among the various thermophilic fungi, Chaetomium thermophile has a potential source of xylanase and cellulase enzymes, both required in the treatment of fiber in the poultry feed.

In spite of the fact that a number of microorganisms are good producers of xylanase, still the titer of the enzyme need to be enhanced by using DNA recombinant technology. Efforts were made to fulfill the requirement of the industries. For enhanced enzyme production we construct a prokaryotic and eukaryotic expression cassette that can be cloned under specific strong promoters and enhancer elements to get maximum gene expression.

In the present study prokaryotic expression system i.e. pET and eukaryotic expression system Pichia pastoris was used to express xylanase gene. In prokaryotic and eukaryotic expression system E. coli BL21 strain and Pichia pastoris GS115 strain were used as model organisms and pET 32a(+) and pPIC3.5k vectors, respectively were used. In these expression systems the xylanase gene was induced by using 1 mM IPTG, 1 mM lactose and 100% methanol up to final concentration of 30%, respectively.

In case of prokaryotic expression system the confirmed PCR target xylanase gene fragment from pSSZ810(a) approx. 810 bp was ligated into pET 32a(+) vector and transformed into BL21 strain of E.coli for expression. The recombinant clone pSSZ810(b) resulted in maximum xylanase activity of 6.02 U/mL in the presence of 1 mM lactose inducer and 2% xylose as a carbon energy source. The maximum xylanase

activity in case of 1 mM lactose inducer and 2% xylose was observed after incubation of 10 mins at 40 °C whereas in case of 1mM IPTG inducer the maximum activity 4.62 U/mL was observed after incubation of 2 hrs at 40 °C. Xylose formed was also detected by HPLC analysis, which showed larger peak in case of 1 mM lactose as compared to 1 mM IPTG inducer. SDS-PAGE and western blot analysis showed approx. 43 kDa molecular weight of xylanase along with fusion protein of pET 32a(+) vector. With and without fusion protein the xylanase activity was 4.0 and 0.387 U/mL, respectively as compared to control. The activity of total protein of Ecoli BL21 transformed with pSSZ810(b) was observed in the presence of 1mM IPTG and 1 mM lactose supplemented with 2% xylose and 100mg/ml. In case of 1mM IPTG inducer the maximum and minimum activity was 0.66 and 0.37 mg/ml whereas in case of 1 mM lactose inducer was 0.6 and 0.35 mg/ml, respectively.

In case of yeast expression system the xylanase gene from pSSZ810(b) was ligated into Pichia pastoris pPIC3.5k vector. Confirmed recombinanat clone pSSZ810(c) was transformed into the genome of P.pastoris GS115 strain through electroporation. Transformants were selected on YPD (yeast peptone dextrose medium) plates containing antibiotic geneticin (100 mg/mL) up to final concentration of 0.75 mg/mL. The maximum xylanase activity of 2.04 U/ml was observed in the presence of 100% methanol as inducer and after incubation of 2 hrs at 50 °C as compared to control. HPLC analysis represents larger peak of xylose as compared to prokaryotic expression cassette. SDS-PAGE indicates approx. 28 kDa protein.

Chapter No. 1

Introduction and Review of literature

Chapter 1

Introduction and review of literature

1: Introduction:

Organic wastes from renewable forest and agriculture residues comprise cellulose, hemi-cellulose and lignin in an average ratio of 4:3:3 (Brauns and Brauns, 1960); the exact percentage of these three components vary from source to source (Sitton *et al*, 1979). Uses of these renewable resources have been reported by Detroy *et al.* (1981, Phillps (1985) and Lynd *et al.* (1991).

Hemi-cellulose is the second most abundant fraction available in nature. The monomers of various hemicelluloses are useful in the production of different antibiotics, alcohols, animal feed, chemicals and fuels (Hopwood, 1981; Thompson, 1983). Various microorganisms are actively involved in the degradation of hemi-celluloses. The presence of microorganisms that degrade hemi-cellulose, particularly xylan, which is 80 % of the constituent of hemi-cellulose, was reported over 100 years ago by Hoppe-Seyler (1889), who described a gas production process using wood xylan suspension and river mud microbes.

Hemi-cellulose refers to plant cell wall polysaccharides. Hemi-celluloses are usually named according to main sugar residues in the back bone e.g. xylan, glucomannan, galactan and glucans. Xylan is one of the most abundant plant structural polysaccharides and is 80% of hemi-cellulose. The main chain of xylan is composed of β-1-4 linked β-xylopyranose residues, which are decorated with acetyl, arabino-furanosyl, and 4-methyl-O-glucuronyl side chains. The presence of β-1-4 linkages between two adjacent xylose residues in xylan was reported by Whistler (1950), Aspinall (1959) and Chanda *et al.* (1950). The presence of β-1-4 linkages was demonstrated by

Jayme and Satree (1942) and Whistler (1950). Xylan is covalently and non-covalently attached to cellulose, pectin, lignin and other polysaccharides to maintain cell wall integrity (Hori *et al.*, 1985).

The enzymes which breakdown hemi-celluloses are referred to as hemi-cellulases: they are defined and classified according to the substrates on which they act. β-xylanases cleave the β-1-4 -xylopyranosyl linkages of xylans. The backbone xylose polymer is hydrolyzed by β-1-4-xylanases (xylanase), whereas the side chains are removed by the action of arabino-furanosidases, α-glucuronidases and acetyl xylan esterases. Based on amino acid sequence similarities among the catalytic domains, xylanases have been grouped into glycoside hydrolase families F/10 and G/11 (Kuno *et al.* 1998).

1.1: Occurrence of xylanase enzyme:

Xylanases are widely distributed. They occur in both prokaryotes and eukaryotes, including protozoa, insects, snails and germinating plant seeds. (Taiz and Honigman, 1976). Amongst the prokaryotes, bacteria and cyanobacteria from marine environments produce xylanases (Dekker, 1985). Extra-cellular and intracellular xylanases from various bacterial and fungal sources have been studied extensively. Intracellular xylanases occur in rumen bacteria and protozoa (Dekker and Richards, 1976).

Enzymes that degrade xylan have many industrial applications such as nutritional improvement of foods (e.g. clarification of juices; making bread fluffier; separation of wheat or other cereal gluten from starch), increasing animal feed digestibility (Alam *et al.* 1994), biobleaching of pulp in the pulp and paper industry, biopulping (Christov and Prior, 1996), nutritional improvement of lignocellulosic food stock, production of ethanol, methane and other products and in processing of food (Wong and Saddler, 1992).

Xylanases have gained increasing attention as supplements in the poultry feed to enhance digestibility of fibrous material (4-5%). Addition of exogenous enzymes in

poultry feeds is now a common practice in Western Europe, America and Australia (Rotter, 1990). Multinational companies are supplying these enzymes to feed companies in Pakistan, which are becoming more and more adaptive to the advantages carried out by the addition of these enzymes to feed mix. However, import of these enzymes is at the cost of heavy foreign exchange. In the developed countries nature of problems encountered in the feed industry are different than that of the under developed countries. The agriculturally self-sufficient countries can use cereal grains and other vegetable meals including the soya bean meal according to requirements of feed. However, on the contrary, in countries like Pakistan there is a shortage of cereals and every now and then we have to import expensive soya bean meal and other value added feed ingredients.

One of the major sources of economic gains in Pakistan today is the progress in the poultry feed industry. In order to improve the economics, edible cereals like wheat, corn and rice must be partially replaced (20-40%) with the seed metals rich in proteins however, high in non-starch polysaccharides (high in fiber). This may increase in fiber content to 5-15% by the use of sunflower oil meal, cottonseed meal, rice polishing and soybean meal, however, the treatment with enzyme will alleviate the excessive use of chicks. High fiber material or polysaccharides such as cellulose, hemi-cellulose, pentosans and oligosaccharides adversely affect the digestive processes when present in sufficient dietary concentrations. These are known to be as anti-nutritive factors and only addition of supplemental enzyme preparation can improve nutrient utilization and growth of young poultry (MacNab and Smithard, 1992).

1.2: Filamentous fungi:

The fungi constitute a most fascinating group of organisms exhibiting great diversity in form, structure, habit, life history and mode of nutritional and mycelial tropic stage adequately distinguishing the fungi as separate kingdom (Hawksworth *et al.*, 1983). Fungi are the most common industrial sources for hemi-cellulases such as glucanases, xylanases, galactanases, mannases, galactomanases and pentosanases.

Prasertsan *et al.* (1992) investigated commercial enzymes from three fungi using crude enzyme solutions including xylanase.

Filamentous fungi are widely known for their capacity to secrete large amounts of a variety of hydrolytic enzymes such as alpha-amylases, proteases and amyloglucosidases and various plant cell wall degrading enzymes such as cellulases, hemi-cellulases and pectinases.

Filamentous fungi produce organic acids, antibiotics, numerous industrial enzymes, food additives and condiments and also used as food (mushroom). *Aspergillus niger* and *Trichoderma ressei* produce xylanase (Hamlyn, 1998) enzyme that have applications in poultry feed industry and also in paper and pulp industry.

The members of the fungal genus *Aspergillus* are commonly used for the production of polysaccharide-degrading enzymes. This genus produces a wide spectrum of cell wall-degrading enzymes, allowing not only complete degradation of the polysaccharides but also tailored modifications by using specific enzymes purified from these fungi (Ronald *et al.*, 2001).

1.3: Thermophilic fungi:

The study of extreme environments has considerable biotechnological potential. For example, the two thermophilic species of bacteria, *Thermus aquaticus* and *Thermococcus litoralis* are used as sources of the enzyme DNA polymerase, for the polymerase chain reaction (PCR). Similarly, the thermophilic fungi have considerable importance because of the thermostable nature of their enzymes, which is mostly an industrial requirement, such as feed pelleting in poultry industry, cellulase treatment of garments in textile (denim and biofinishing) and bioleaching of pulp in the pulp and paper industry.

Thermophilic fungi are a small assemblage in mycota that have a minimum temperature of growth at or above 20°C and a maximum temperature of growth extending up to 60 to 62°C. As the only representatives of eukaryotic organisms that can grow at temperatures above 45°C, the thermophilic fungi are valuable experimental systems for investigations of mechanisms that allow growth at moderately high temperature yet limit their growth beyond 60 to 62°C. Some species have the ability to grow at ambient temperatures if cultures are initiated with germinated spores or mycelial inoculum or if a nutritionally rich medium is used. Some extracellular enzymes from thermophilic fungi are being produced commercially, and a few others have commercial prospects. Genes of thermophilic fungi encoding lipase, protease, xylanase, and cellulase have been cloned and over expressed in heterologous fungi (Maheshwari *et al.*, 2000).

At NIBGE there is a large collection of thermophilic fungi (Latif *et al.*, 1995), which have potential application in the poultry feed industry and paper and pulp industry. *Chaetomium thermophile* shows large amounts of extra cellular cellulase and xylanase activity when grown on cellulosic or lignocellulosic substrates as carbon sources (Latif *et al.*, 1994).

1.4: Chaetomium thermophile:

The production of thermo-stable cellulases and xylanases from thermophilic fungi is important for their use in industry as these processes involve high temperature (Yu *et al.*, 1987). Thermophilic fungi are members of true fungi eumycetes with temperature maximum between 50-60 °C. *Chaetomium thermophilum* is a thermophilic fungus that produces thermo stable xylanase (Latif *et al.*, 1994). *Chaetomium thermophilum* belongs to the kingdom Fungi and phylum Ascomycota. *Chaetomium* is a filamentous fungus frequently found in soil, air and plant debris. The major inducible endoxylanase secreted by these fungi is Xyn11- A. Enhanced enzyme production can be achieved by isolation, characterization, cloning and expression of the genes under specific strong promoters and enhancer elements. This strategy has been found to be very efficient as compared to traditional methods. Gene cloning is recently being employed to study the structure and

function of number of enzymes and proteins (Bergquist *et al.*, 2002). The genetically engineered organisms may be grown on agro industrial wastes for the production of enzymes, which may not only increase the enzyme production but also may reduce environmental pollution and will be more economical.

1.5: Xylanase enzyme:

The xylanases that have been structurally characterized to date can be classified into the glycoside hydrolase families 10 and 11, corresponding to former families F and G, respectively (Henrissat & Davies, 1997). Family 10 enzymes have an $(\alpha/\beta)_8$ barrel fold with a molecular mass of approximately 35 kDa. Family 11 xylanases are somewhat smaller, approximately 20 kDa, and their fold contains a α-helix and two β-sheets packed against each other, forming a so-called β-sandwich. Due to the industrial applications of xylanase, both xylanase families are well studied.

The overall structure of xylanase from *C. thermophilum* (CTX) was dominated by one α-helix and two strongly twisted β-sheets, which were packed against each other. This is the protein fold of family 11 xylanases. According to Törrönen *et al.* (1994), the shape of the molecule resembles a right hand: two β-sheets and the α-helix form fingers and a palm, a long loop between the B7 and B8 strands forms a thumb, and a loop between the B6 and B9 strands forms a cord (Hakulinen *et al.*, 2003).

The two families of xylanases also differ in patterns of cleavage of various heteroxylans. For example, members of the F/10 family of xylanases cleave arabinoxylans at the β-1, 4-linkages at the non-reducing ends of arabino-furanose-branched xylo-pyranoses while members of the G/11 family do not.

The protein can be of a foreign source. However, the promoter for the gene must come from the host that will be producing the protein. A promoter is the segment of DNA located immediately in front of each gene. The promoter regulates when, how

much and how often the gene is transcribed. While the simplicity of *E. coli* makes it a desirable host for production of a foreign protein, it also has its disadvantages as a host cell. *E. coli* is a prokaryote. Like all prokaryotes, *E. coli* does not have any of the membrane bound organelles found in eukaryotes. In eukaryotes a protein is often modified after it is initially produced. Some of the best-studied modifications occur in different organelles, such as the endoplasmic reticulum or the Golgi apparatus. These modifications, in many cases, are necessary to convert the promoter to a functional form. These so-called, post-translational modifications, often involve addition of different forms of glycolation. Any eukaryotic protein can be mass translated in *E. coli*, but many are not quite finished and hence, they are nonfunctional. *E. coli* will give you the same primary structure, as occurs when that protein is initially produced in its own cell type. Due to different problems other eukaryotic cells are considered such as mammalian, insect and yeast cells. They have been studied as suitable replacements for *E. coli*. Of the three, yeast cells are the most desirable. They combine the ease of genetic manipulation and rapid growth characteristics of prokaryotic organism with the sub cellular machinery for performing post-translational protein modification of eukaryotic cells (Cregg *et al.*, 1993).

Many foreign proteins have been successfully mass produced in the yeast *Saccharomyces cerevisiae* but problem is that many of the secreted proteins of *S. cerevisiae* are not found free in the medium rather in the periplasmic space. This leads to problems with purification and further decreases product yield (Buckholz *et al.*, 1991). Due to this problem several other species of yeast have been analyzed. *Pichia pastoris* is used as eukaryotic expression system for recombinant proteins. It is similar to the *S. cerevisiae* but one of the drawbacks with *Saccharomyces cerevisiae* was that it did not have a strong inducible promoter. *Pichia pastoris* has a strong inducible promoter. This inducible promoter is related to the fact that *Pichia pastoris* is methylotrophic yeast. The first step in the utilization of methanol is the oxidation of methanol to formaldehyde and hydrogen peroxide (Ledeboer *et al.*, 1985). This step is catalyzed by the enzyme alcohol oxidase. The expression of this gene is tightly regulated. When the yeast is grown on glucose or ethanol, alcohol oxidase is not detectable in the cells. However, when the

yeast is grown on methanol, alcohol oxidase can make up to thirty-five percent of the total cellular protein. The control of the amount of alcohol oxidase is largely transcriptional (Cregg *et al.*, 1985).

There are two alcohol oxidase genes: AOX1 and AOX2. The protein coding regions of the genes are largely homologous, 92 percent and 97 percent at the nucleotide and amino acid sequence levels respectively (Ohi *et al.*, 1994). The promoters share very little homology. No mRNA of the two genes is detectable when the yeast is grown in glycerol. The promoter region for AOX2 has a repressor region the leads to the inhibition of gene expression, and an activation region that leads to the enhancement of gene expression. The AOX1 gene promoter probably has a similar mechanism (Ohi *et al.*, 1994).

Expression of the AOX 1 gene is controlled at the level of transcription. The regulation of the AOX 1 gene is a two step process; a repression/depression mechanism plus an induction mechanism (e.g. Gal 1 gene in *Saccharomyces* (Johnston, 1987). Growth on glucose represses transcription, even in the presence of inducer methanol. For this reason, growth on glycerol is recommended for the optimal induction with methanol. Growth on glucose only (depression) is not sufficient to generate even minute levels of expression from the AOX1 gene. The inducer, methanol, is necessary for even detectable levels of AOX1 expression (Ellis *et al.*, 1985; Koutz *et al.*, 1989; Tschopp *et al.*, 1987a).

To achieve optimal conditions for growth and protein synthesis, it has proven necessary to keep the methanol level in the media within a narrow range. This is due to the cytotoxic effects of methanol as well as to the intracellular formation of formaldehyde, the first metabolite after the oxidation of methanol. In order to avoid monitoring the methanol concentration during growth in shake flask cultures, we established a simple, yet efficient feeding strategy for methanol plus glucose that both optimizes cell growth and heterologous protein production and allows the cultivation of numerous clones in parallel without expensive equipment. A significant increase in cell

density and protein yield compared to the widely used standard feeding protocol was observed.

P. pastoris has been adapted to the procedures of the "protein structure factory" with respect to downscaling and parallelisation of cloning and transformation procedures as well as detection of protein expression in a high throughput manner.

Pichia pastoris is methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. Methanol is converted into formaldehyde, this reaction generates hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism takes place in the specialized cell organelle, called peroxisome. Alcohol oxidase has poor affinity for oxygen and *Pichia pastoris* compensates by generating large amount of the enzyme. The promoter regulating the production of alcohol oxidase is the one used to derive heterologous protein expression in *Pichia*.

In the present studies efforts were made to clone the xylanase gene in yeast model system. In this context the expression model system of *Pichia pastoris* is selected (Invitrogen, USA). *Pichia pastoris* has many advantages of higher eukaryotic expression systems such as protein processing, protein folding, and post-translational modification. It is faster, easier, and less expensive to use than other eukaryotic expression systems such as baculovirus or mammalian tissue culture, and generally gives higher expression levels.

2: Review of literature:

Enzymes that degrade xylan have many industrial applications, including the conversion of lignocellulosic material to fuels and chemicals (Coughlan *et al.*, 1993) and the processing of hemi-cellulose to paper (Buchert *et al.*, 1992; Eriksson *et al.*, 1990; Viikari *et al.*, 1994). During the process of pulp bleaching, xylanases are used instead of chlorine to increase the extractability of lignin for the production of high quality paper (Daneault *et al.*, 1994). The use of xylanase to either replace or reduce the amount of chlorine used in pulp bleaching would have a strong positive effect on the environmental impact of the process.

The filamentous fungus *Aspergillus niger* is being used increasingly as a host for the production of heterologous proteins (van den Hondel *et al.*, 1991a; Jeenes *et al.*, 1991). The yeast *Saccharomyces cerevisiae* can neither utilize nor degrade xylan (Jeffries, 1983), but it possesses a number of attributes that render it an attractive host for the expression and production of β-xylanases (Romanose *et al.*, 1992). It is a unicellular fungus, which provides posttranslational processing such endoproteolytic cleavage and glycosylation. *S. cerevisiae* normally secretes few proteins in low abundance, so that a secreted heterologus protein is produced separate from the majority of yeast proteins. Furthermore it is generally regarded as safe (GRAS), which allows its use in the food industry.

Aspergillus kawachii is a common fungus used in the fermentation of *shochu* and this strain makes a large quantity of citric acid and simultaneously produces many interesting acid-stable enzymes including several xylanases and cellulases. Xylanase A encoded by *xyn A* gene which is a major component of the xylanase family of *A. kawachii* was also studied (Ito *et al.*, 1991).

Trichoderma reesei is a filamentous mesophilic fungus that is well known for its cellulolytic and xylanolytic enzymatic activities (Penttila *et al.*, 1990). The two inducible

endo-xylanases secreted by this fungus are Xyn1 and Xyn2 (Torronen *et al.*, 1992). They are both relatively small protein molecules with molecular masses of 19 and 21 kDa.

Several thermophilic fungi produce xylanolytic enzymes. Majority of them secrete endoxylanases and β-xylosidases, which are glycoproteins. Xylanases from thermophilic fungi do not appear to be different from their mesophilic counterparts in their pH stability, molecular weights, amino acid composition and sequence, isoelectric points and occurrence as multiple enzymes. The carbohydrate moiety usually associated with these enzymes appears to play a significant role in stabilizing conformational structure of the protein at elevated temperatures. Xylanases of thermophiles are generally inducible and have almost similar regulatory mechanisms as in mesophiles. Since some thermophilic fungi produce all the components of xylanolytic complex more efficiently and rapidly than mesophiles, they make better candidates for commercial exploitation (Archana *et al.*, 1999).

Humicola lanuginosa was shown to produce cellulase free xylanase, and it was found to be useful in biobleaching of eucalyptus Kraft pulp. The xylanase of *Sporotrichum dimorphosum* also served to reduce lignin content from unbleached softwood and hardwood Kraft pulps.

Humicola lanuginosus is a thermophilic fungus that produces xylanase without any accompanying cellulase activity (Sinner *et al.*, 1991). The production of cellulose free endoxylanase by thermophilic fungus, *Thermomyces lanuginosus* was investigated in semi-solid fermentation and liquid fermentation. Corncob, xylan and xylose were the best inducers for endoxylanase production.

Xylanases from the thermophilic fungi *Thermoascus aurantiacus* (Tan *et al.*, 1987; Khandke *et al.*, 1989), *Thermomyces lanuginosus* (*Humicola lanuginosa*) (Kitpreechavanich *et al.*, 1984; Bennett *et al.*, 1998; Anand *et al.*, 1990; Gomes *et al* .,1993; Purkathofer *et al.*, 1993; Cesar and Mrsa 1996), *Talaromyces byssochlamydoides* (Yoshioka *et al.*, 1981), *Humicola grisea* var. *thermoidea* (Monti *et al.*, 1991; de

Almeida *et al.*, 1995), *Talaromyces emersoni* (Tuohy *et al.*, 1993), *Chaetomium thermophile* var. *coprophile* (Ganju *et al.*, 1989) and *Humicola insolens* (Düsterhöft *et al.*, 1997) have been studied. These studies have shown that xylanases are co-induced in response to xylan or natural substrates containing hemi-cellulose or even by pure cellulose (Ganju *et al.*, 1989). X ylanases are commonly induced with cellulases and secreted into the medium. Geographical isolates of the same thermophilic fungus may differ in enzyme productivity and in structural and biochemical properties of xylanases (Kitpreechavanich *et al.*, 1984; Bennett *et al.*, 1998; Cesar & Mrša 1996). Some thermophilic fungi produce multiple forms of xylanases that differ in molecular size, stability, adsorption or activity on insoluble substrates (Tuohy *et al.*, 1993; Düsterhöft *et al.*, 1997). They are generally single-chain glycoproteins, ranging from 6 to 80 kDa, active between pH 4.5 to 6.5, and at temperatures between 55 to 65° C.

Pichia pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. Methanol is converted into formaldehyde using molecular oxygen by the enzyme alcohol oxidase. In addition to formaldehyde, this reaction generates hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism takes place in the specialized cell organelle, called peroxisome. Alcohol oxidase has poor affinity for oxygen and *Pichia pastoris* compensates by generating large amount of the enzyme. The promoter regulating the production of alcohol oxidase is the one used to derive heterologous protein expression in *Pichia*.

Two genes in *Pichia pastoris* code for alcohol oxidase namely *AOX1* and *AOX2*. Expression of the *AOX1* gene is tightly regulated and induced by methanol to very high levels, typically >30% of the total soluble protein in cells grown on methanol as compared to *AOX2*. This slow growth on methanol allows isolation of Mut^s strains (*aox1*) (Cregg *et al.*, 1989; Koutz *et al.*, 1989).

Expression of the *AOX1* gene is controlled at the level of transcription. The regulation of the *AOX1* gene is a two-step process; a repression/depression mechanism plus an induction mechanism (e.g. *GAL1* gene in *Saccharomyces* (Johnston, 1987)).

Growth on glucose represses transcription, even in the presence of inducer methanol. For this reason, growth on glycerol is recommended for the optimal induction with methanol. Growth on glucose only (depression) is not sufficient to generate even minute levels of expression from the *AOX1* gene. The inducer, methanol, is necessary for even detectable levels of *AOX1* expression (Ellis *et al.*, 1985; Koutz *et al.*, 1989; Tschopp *et al.*, 1987a).

The different enzymes for methanol metabolism are compartmentalized in peroxisomes. The proliferation of peroxisomes is a reflection of environmental conditions. When the cells are grown on glucose very few peroxisomes are present. When grown on methanol, peroxisomes may take up to 80 percent of the total cell volume because the alcohol oxidase promoter is tightly regulated and is a strong promoter. The production of foreign protein can be repressed until the culture is saturated with colonies, and then the production of the foreign protein can begin with the derepression and induction of the gene. In addition to being able to regulate the production of the protein very tightly, the post- translational modifications made by *P. pastoris* are more suitable for use in humans.

The structure of carbohydrate added to secrete proteins is known to be very organism specific. Many proteins secreted from *S. cerevisiae* have been demonstrated to be antigenic when introduced into mammals thus the use of glycoprotein products synthesized by yeast for therapeutic purposes has been avoided. A comparison of a *S. cerevisiae* protein secreted from *S. cerevisiae* and *P. pastoris* has shown distinct differences between N-linked oligosaccharide structures added to proteins secreted from this yeast. The majority of the N-linked oligosaccharide chains are high mannose. However, the length of the carbohydrate chains is much shorter in *P. pastoris*. Even the longest chains of protein produced in *P. pastoris* contained only approximately thirty mannose residues, which is significantly shorter than the 50 to 150 mannose residue chains typically found on *S. cerevisiae* glycoproteins. The second major significant difference between the glycolation by *S. cerevisiae* and *P. pastoris* is that glycans from *P. pastoris* do not have alpha 1,3-linked mannose residues that are characteristic of *S. cerevisiae* (Cregg *et al.*, 1993). The enzyme that makes alpha 1,3-linkages is alpha 1,3

mannosyl transferase and it is undetectable in *P. pastoris*. It is significant, because the alpha 1,3-linkages on *S. cerevisiae* glycans are primarily responsible for the highly antigenic nature of glycoproteins used for therapeutic products (Cregg *et al.*, 1993).

The isolation and purification of a foreign protein product is done by growing *P. pastoris* on a simple mineral media and does not secrete high amounts of endogenous protein. Therefore the heterologous protein secreted into the culture is relatively pure and purification is easier to accomplish (Faber *et al.*, 1995). Secretion of the foreign protein is accomplished by recombining a signal sequence in front of the desired foreign gene when it is inserted into the host DNA.

hMR-1 (Homo Myofibrillogenesis Regulator 1, AF417001) is a novel homo gene. The former studies revealed that hMR-1 is a transmembrane protein which shows protein interaction with sarcomeric proteins like myomesin I, myosin regulatory light chain, alpha-enolase and some cell regulator proteins such as eukaryotic translation initiation factor3 subunit 5 (eIF3S5) etc. In this work, the cloning of the homologous gene of hMR-1 from mouse C57BL/6J and its expression is done by using *Pichia pastoris* yeast system. Two pairs of primers were synthesized according to the hMR-1 gene homologous sequence on mouse genome chromosome 1. The mouse MR-1 gene (mMR-1) was cloned by PCR following the first round RT-PCR from mouse C57BL/6J spleen total RNA (Li *et al.*, 2005). Sequence analysis verified that mMR-1 gene and amino acids sequence showed 90.4% and 90.1% identity with hMR-1, respectively (Li *et al.*, 2005). The mMR-1 *Pichia pastoris* expression vector pPIC9-mMR-1 was constructed by fusion of the flanking mMR-1 ORF in the pPIC9 plasmid (Li *et al.*, 2005). After linearization of pPIC9-mMR-1 with Sal I, the 8.5kb DNA fragment was transformed into *Pichia pastoris* GS115 strain by electroporation. GS115/Mut+ pPIC9-mMR-1 transformants were selected on minimal methanol medium. Integration of mMR-1 gene into the yeast genome in the recombinants was verified by PCR from the transformants total DNA. The mMR-1 protein was expressed by induction under the concentration of 0.5 % methanol. The specific induced protein of 25 kD molecular mass in SDS-PAGE was confirmed to be the mMR-1 protein by Western blot using hMR-1 polyclonal

antibody. The expression level of this recombinant mMR-1 protein was about 50 mg/L (Li *et al.*, 2005)

The porcine alpha interferon gene was inserted into the *Pichia pastoris* expression vector of pPICZalphaA which contains AOX I promoter and alpha-factor signal sequence. The recombinant plasmid was transformed into host cell *E. coli* JM109 and then was extracted for analysis of restriction enzymes. It was confirmed that heterogeneous gene spliced into vector pPICZalphaA was IFNalpha gene. The recombinant plasmid of pPICZalphaA-IFNalpha was linearnized by *Sac*I and transformed into KM71 by electroporation. SDS-PAGE and Western blot analysis showed that IFNalpha product was observed in the supernatants with a little larger molecular weight size than the natural IFNalpha. The rIFN gene has the same antigenicity as natural one. The expressed rIFN accumulated up to about 0.45 mg/mL. The cytokine activity of the supernatants was verified by WISH/VSV system, which was about 2.1x10(4) IU/mL (Hung *et al.*, 2005).

Recent developments with respect to the *Pichia* expression system have had an impact on not only the expression levels that can be achieved, but also the bioactivity of various heterologous proteins. The recent developments, as well as strategies for reducing proteolytic degradation of the expressed recombinant protein at cultivation, cellular and protein levels (Macauley-Patrick, 2005). The problems associated with post-translational modifications performed on recombinant proteins by *P. pastoris* were discussed, including the effects on bioactivity and function of these proteins, and some engineering strategies for minimizing unwanted glycosylations.

In the past the methylotrophic yeast *Pichia pastoris* has developed into a highly successful system for the production of a variety of heterologous proteins. The increasing popularity of this particular expression system can be attributed to several factors, most importantly: (1) the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris* and their similarity to those of *Saccharomyces cerevisiae*, one of the most well-characterized experimental systems in modern biology;

(2) the ability of *P. pastoris* to produce foreign proteins at high levels, either intracellularly or extracellularly; (3) the capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing; and (4) the availability of the expression system as a commercially available kit (Cereghino *et al.*, 2000)

The use of the methylotrophic yeast, *Pichia pastoris*, as a cellular host for the expression of recombinant proteins has become increasing popular in recent times. *P. pastoris* is easier to genetically manipulate and culture than mammalian cells and can be grown to high cell densities. Equally important, *P. pastoris* is also a eukaryote, and thereby provides the potential for producing soluble, correctly folded recombinant proteins that have undergone all the post-translational modifications required for functionality. A further benefit of the *P. pastoris* system is that strong promoters are available to drive the expression of a foreign gene(s) of interest, thus enabling production of large amounts of the target protein(s) with relative technical ease and at a lower cost than most other eukaryotic systems. Daly and Hearn (2005) studied the different features and developments under the influences of *P. pastoris* strain selection; the choice of expression vectors and promoters; procedures for the transformation and integration of the vectors into the *P. pastoris* genome; the consequences of rare codon usage and truncated transcripts; and techniques employed to achieve multi-copy integration numbers. The impact of the alcohol oxidase (AOX) pathways in terms of the mut+ and mut(s) phenotypes, intracellular expression and folding pathways was examined.

The yeast *Pichia pastoris* is a convenient production system that enables expression of heterologous proteins in high amounts. As a fermentation method, shaking flasks are very popular because of their simplicity of handling and their low cost. The comparison of the expression level of the enzyme acetyl cholinesterase in a transformed strain of *P. pastoris* grown in different flasks, presenting various designs but all with the same volume. A several-thousand-fold difference appeared in the expression levels; and the results could not be explained by differences between the flasks in the oxygenation

of the medium. The data showed that the flask designing is an important factor to consider for optimizing fermentation processes (Villatte *et al.*, 2001).

Heterologous expression in *Pichia pastoris* has many of the advantages of eukaryotic expression, proper folding and disulfide bond formation, glycosylation, and secretion. Contrary to other eukaryotic systems, protein production from *P. pastoris* occurs in simple minimal defined media making this system attractive for production of labeled proteins for NMR analysis. *P. pastoris* is therefore the expression system of choice for NMR of proteins that cannot be refolded from inclusion bodies or that require post-translational modifications for depends critically on growth conditions, and attainment of high cell densities by fermentation has been shown to improve protein yields by 10-100-fold. Unfortunately, the cost of the isotopically enriched fermentation media components, particularly $^{15}NH_4OH$, is prohibitively high proper folding or function. The yield of expressed proteins from *P. pastoris*. The fermentation methods that allow for both ^{15}N-labeling from $(^{15}NH_4)2SO_4$ and ^{13}C-labeling from ^{13}C-glucose or ^{13}C-glycerol of proteins produced in *Pichia pastoris*. Expression of an 83 amino acid fragment of thrombomodulin with two N-linked glycosylation sites shows that fermentation is more cost effective than shake flask growth for isotopic enrichment (Wood & Komives, 1999).

Pick ford and O'Leary, (2004) described that the methylotrophic yeast *Pichia pastoris* is now an established expression system for the production of recombinant protein for nuclear magnetic resonance (NMR) studies. It is capable of expressing high levels of heterologous proteins and possesses the ability to perform many of the posttranslational modifications of higher eukaryotes. Different efficient methods for the production of uniformly ^{13}C, ^{15}N-labeled proteins in shake flasks and of uniformly ^{13}C, ^{15}N-labeled and ^{2}H, ^{13}C, ^{15}N-labeled proteins in fermenters were described and also provide details of two chromatographic procedures, cation exchange and concanavalin A lectin affinity, that have proved useful in purifying *P. pastoris*-expressed proteins for NMR studies.

The *Pichia pastoris* heterologous gene expression system has been utilized to produce attractive levels of a variety of intracellular and extracellular proteins of interest. Recent advances had improved its utility that include: (1) methods for the construction of *P. pastoris* strains with multiple copies of AOX1-promoter-driven expression cassettes; (2) mixed-feed culture strategies for high foreign protein volumetric productivity rates; (3) methods to reduce proteolysis of some products in high cell-density culture media; (4) tested procedures for purification of secreted products; and (5) detailed information on the structures of N-linked oligosaccharides on *P. pastoris* secreted proteins (Cregg *et al.*, 1993).

The gene coding for agglutinin from *Galanthus nivalis* (GNA) was expressed in, and secreted by, the methylotrophic yeast, *Pichia pastoris*. Transformants of *P. pastoris* were selected and a process to produce and purify gram quantities of recombinant GNA was developed. GNA was secreted at approximately 80 mg l^{-1} at the 200 L scale and was purified to 95% homogeneity using hydrophobic interaction chromatography. The recombinant protein was similar to the protein synthesized in plant with respect to structure and biological activity (Baumgartner *et al.*, 2003).

Bottner and Lang (2004) processed that the methylotrophic yeast *Pichia pastoris* had become a powerful host for the heterologous expression of proteins. To serve the increasing demand for clones expressing different cDNAs, they developed a cultivation and induction protocol amenable to automation to increase the number of clones screened for protein expression. Therefore cDNAs were cloned for intracellular expression. The resulting fusion proteins carry affinity tags (6*HIS and StrepII, respectively) at the N- and C-terminus for the immunological detection and chromatographic purification of full-length proteins. Expression was controlled by the tightly regulated and highly inducible alcohol oxidase 1 (AOX1) promoter. The screening procedure was based on a culture volume of 2 mL in a 24-well format. Lysis of the cells occurred via chemical lysis without mechanical disruption. Using the optimized feeding and induction protocol, they were now able to screen for and identify

expression clones that produce heterologous protein with a yield of 2 mg per L culture volume or higher.

Cregg *et al.*, 2000 isolated the methylotrophic yeast *Pichia pastoris* which was now one of the standard tools used in molecular biology for the generation of recombinant protein. *P. pastoris* had demonstrated its most powerful success as a large-scale (fermentation) recombinant protein production tool. What began more than 20 years ago as a program to convert abundant methanol to a protein source for animal feed had been developed into what is today two important biological tools: a model eukaryote used in cell biology research and a recombinant protein production system. To date well over 200 heterologous proteins had been expressed in *P. pastoris*. Significant advances in the development of new strains and vectors, improved techniques, and the commercial availability of these tools coupled with a better understanding of the biology of *Pichia* species had led to this microbe's value and power in commercial and research labs alike (Cregg *et al.*, 2000).

In order to provide proteins for the 'protein structure factory', a structural genomic initiative, they were working on the high-throughput expression of human proteins. Therefore, cDNAs were cloned for intracellular expression. The resulting fusion proteins carried affinity tags (6*HIS and StrepII, respectively) at the N- and C-terminus for the immunological detection and chromatographic purification of full-length proteins. Expression was controlled by the tightly regulated and highly inducible alcoholoxidase 1 (AOX1) promoter. They had developed cultivation and induction protocol amendable to automation to increase the number of clones screened for protein expression. The screening procedure was based on a culture volume of 2 ml in a 24-well format. Lysis of the cells occurred via a chemical lysis without mechanical disruption. Using the optimized feeding and induction protocol, they were now able to screen for and identify expression clones which produce heterologous protein with a yield of 5 mg $L^{(-1)}$ culture volume or higher (Boettner *et al.*, 2002).

It was observed that a secreted recombinant protein ovine interferon-tau (r-oIFN-tau) was producrd in *Pichia pastoris* during fermentation. The protein was degraded increasingly after 48 h of induction and the rate of degradation increased towards the end of fermentation at 72 h, when the fermentation was stopped. Proteases, whose primary source was the vacuoles, was found in increasing levels in the cytoplasm and in the fermentation broth after 48 h of induction and reached maximal values when the batch was completed at 72 h. Protease levels at various cell fractions as well as in the culture supernatant were lower when glycerol was used as the carbon source instead of methanol. It can be concluded that methanol metabolism along with cell lysis towards the end of fermentation contributes to increased proteolytic activity and eventual degradation of recombinant protein (Sinha *et al.*, 1992).

Thaumatin is an intensely sweet-tasting protein that was secreted by the methylotrophic yeast *Pichia pastoris*. The mature thaumatin II gene was directly cloned from Taq polymerase-amplified PCR products by using TA cloning methods and fused the pPIC9K expression vector that contains *Saccharomyces cerevisiae* prepro alpha-mating factor secretion signal. Several additional amino acid residues were introduced at both the N- and C-terminal ends by genetic modification to investigate the role of the terminal end region for elicitation of sweetness in the thaumatin molecule. The secondary and tertiary structures of purified recombinant thaumatin were almost identical to those of the plant thaumatin molecule. Recombinant thaumatin II elicited a sweet taste as native plant thaumatin II; its threshold value of sweetness to humans was around 50 nM, which is the same as that of plant thaumatin II. Masuda *et al.*, (2004) demonstrated that the functional expression of thaumatin II was attained by *Pichia pastoris* systems and that the N- and C-terminal regions of the thaumatin II molecule do not -play an important role in eliciting the sweet taste of thaumatin.

Numerous heterologous proteins have been produced at greater than gram per liter levels in the methylotrophic yeast, *Pichia pastoris* by using the methanol oxidase promoter. The factors that influence protein production in that system included are copy number of the expression cassette, site and mode of chromosomal integration of the

expression cassette, mRNA 5'- and 3'-untranslated regions (UTR), translational start codon (AUG) context, A+T composition of cDNA, transcriptional and translational blocks, nature of secretion signal, endogenous protease activity, host strain physiology, media and growth conditions, and fermentation parameters. All these factors should be considered in designing an optimal production system. The inherent ability of *P. pastoris* to convert the zymogen (pro-enzyme) form of matrix metalloproteinases (MMP) into active mature forms (which tend to self-degrade, and in some instances also cause damage to cells), largely limits the use of this system for the production of MMP. However, this problem can be partly alleviated by co-expression of tissue inhibitor of MMP (TIMP-1) (Sreekrishna *et al.*, 1997).

3: Objectives:

The main objectives in case of prokaryotic expression system *E. coli* are:

1. Cloning of intron-less gene fragment Xyn 11-A in pET 32a(+) expression vector.
2. Transformation of pSSZ810(b) recombinant vector in the *E. coli* BL21 strain for xylanase expression.
3. Confirmation of xylanase gene expression through various methods such as, Bradford analysis and xylanase assay in the presence of carbon sources and IPTG/lactose (1 mM) inducers.
4. Effect of different carbon sources on xylanase production.
5. Effect of xylose as a cabon energy source for xylanase activity.
6. Analysis of xylanase protein by different methods i.e. SDS-PAGE and western blotting techniques.
7. Detection of xylanase protein through HPLC analysis.
8. Confirmation of xylanase activity on 1 % xylan LB agar plates.

In case of yeast expression system *Pichia pastoris* main objectives are:

1. Cloning of intron-less gene fragment Xyn 11-A from pSSZ810(b) into ppic3.5k vector of *Pichia pastoris*.
2. Transformation of pSSZ810(c) recombinant vector in the *Pichia pastoris* GS115 strain for xylanase expression.
3. Confirmation of xylanase gene expression through various methods such as, Bradford analysis and xylanase assay in the presence of 100 % methanol inducer.
4. Analysis of xylanase protein by different methods i.e. SDS-PAGE and SDS-PAGE silver staining.
5. Detection of xylanase protein through HPLC analysis.

Chapter No.2

Materials and Methods

Chapter 2

MATERIALS AND METHODS

2.1: Background

In a previous study a xylanase (Xyn 11-A) gene was isolated from *Chaetomium thermophile* strain NIBGE-1. Fresh slants and petri plates were prepared by using the Eggins and Pugh (1962) agar medium (appendix 1). A set of primers was designed based on the reported sequence from Genebank nucleotide sequence database accession no. AJ508931. Specific primers Xyn 11-A (F) and Xyn 11-A (R) are shown in (appendix 2) were designed to amplify an 860 bp DNA fragment. This fragment was cloned into T/A cloning vector pTZ57R (MBI Fermentas) (appendix 28). The cloned gene was sequenced from Microsynth GmbH, Switzerland. The sequence was submitted to Genebank and assigned accession no. AY366479 (Khan et al., 2004). Sequencing and characterization of gene information revealed a 35 bp intron with two exons in the isolated gene fragment. The gene sequence AY366479 was compared with other xylanase genes from other organisms using online software. Intron was removed by amplifying the insert along with vector backbone except intron by using primers P_3 and P_4 as shown in (appendix 2). This gene was named as Xyn 11-A and cloned in TA vector pTZ57R. The construct was named as pSSZ810(a).

2.2: Cloning and expression of xylanase gene Xyn 11-A in prokaryotic and eukaryotic expression systems

In this study Xyn 11-A was expressed in both prokaryotic and eukaryotic expression systems. For expression in prokaryotic system the gene was cloned in pET 32a(+) vector (appendix 29) and for expression in eukaryotic system the gene was cloned in pPIC 3.5k vector. For expression in prokaryotic system *Escherichia coli* (*E. coli*)

strain BL21 was used while for eukaryotic expression, *Pichia pastoris* (methylotrophic yeast) strain GS115 was used as a model organism.

2.2.1: Expression of Xyn 11-A gene in prokaryotic expression system

2.2.2: Cloning of Xyn 11-A gene into pET vector (pET 32 a(+))

The cloned Xyn 11-A gene from pSSZ810(a) was removed by *Hin*d III and *E.co*RI restriction enzymes. The digestion was run on a 0.8 % agarose gel (appendix 3) and restricted fragment of expected size was eluted from the gel by DNA extraction kit (MBI Fermentas) following protocol as described in appendix 4. Elution was confirmed by running 2 µl of gel extracted DNA on an agarose gel 0.8 % (appendix 3). The purified fragment of 810 bp was ligated into pET expression vector at *Hin*d III and *E.co*RI sites. The resultant vector was named as pSSZ810(b). Heat shock competent cells of *E. coli* 10b were prepared (appendix 5) and used to transform pSSZ810(b) following protocol as described in appendix 6. Transformed cells were selected on Luria Bertani (LB) medium (appendix 7) supplemented with ampicillin (100mg/ml).

2.2.3: Confirmation of Xyn11-A into pET expression vector:

Few colonies were picked randomly from LB plates and cultured in LB liquid medium (appendix 7) supplemented with ampicillin (100 mg/ml). Plasmid was extracted by miniprep method from the overnight culture (appendix 8). The construct pSSZ810 (b) was confirmed through digestion with different combinations of restriction enzymes i:e *Hin*dIII, *Eco*RI, *Xho*I and *Xba*I (appendix 9).

2.2.4: Expression of xylanase gene in prokaryotic system:

The construct pSSZ810 (b) was transformed into *E. coli* BL21 competent cells through heat shock method (appendix 6). Transformants were selected on LB agar medium (appendix 7) supplemented with ampicillin.

2.2.5: PCR confirmation of transformants:

Colonies were picked from the plate and used for PCR directly by using specific set of primers (appendix 2). Composition of the reaction mixture and PCR profile is shown in (appendix 10). PCR was confirmed by running 25 µl sample out of 50 µl on a 0.8 % agarose gel (appendix 3).

2.2.6: Confirmation of xylanase gene expression:

Expression of xylanase was confirmed through enzyme assay, SDS-PAGE and western blotting techniques. The colonies were cultured in LB liquid media (appendix 7) supplemented with ampicillin (100 mg/ml) and 34 mg/ml chloramphenicol. Two ml of overnight culture was transfered to 50 ml LB broth medium (appendix 7) with ampicillin and chloramphenicol and grown for further 3 hours at 37° C. After three hours growth, 70 µl of (1 mM) IPTG was added which act as inducer. The culture was equally divided into 9 test tubes. The cells were incubated at 37° C in a shaker and allowed to grow for 2.5 hours. Samples were taken after each 0.5 hours. Cells were pelleted down and given freeze thaw treatment. Cell lysate was dissolved in 1 ml citrate buffer and sonicated for complete lysis. The lysate was centrifuged at 13,500 rpm and supernatant was used for further analysis.

2.2.6.1: Quantification and assay for xylanase activity

The quantity of xylanase protein was estimated by Bradford analysis and assay for xylanase activity was determined by DNS (dinitrosalicylis acid) are given below:

i) Bradford analysis

100 µl of sample and 1 ml of Bradford reagent (appendix 11) was added in a test tube. The absorbance was taken at 595 nm with spectrophotometer. BSA solution of 1 mg/ml strength was used to make different protein concentrations. These were further diluted to a final volume of 100 µl as shown in Table 1. The standard curve of BSA protein was shown in (graph 1).

Table.1. Concentration of BSA standard curve for protein estimation:

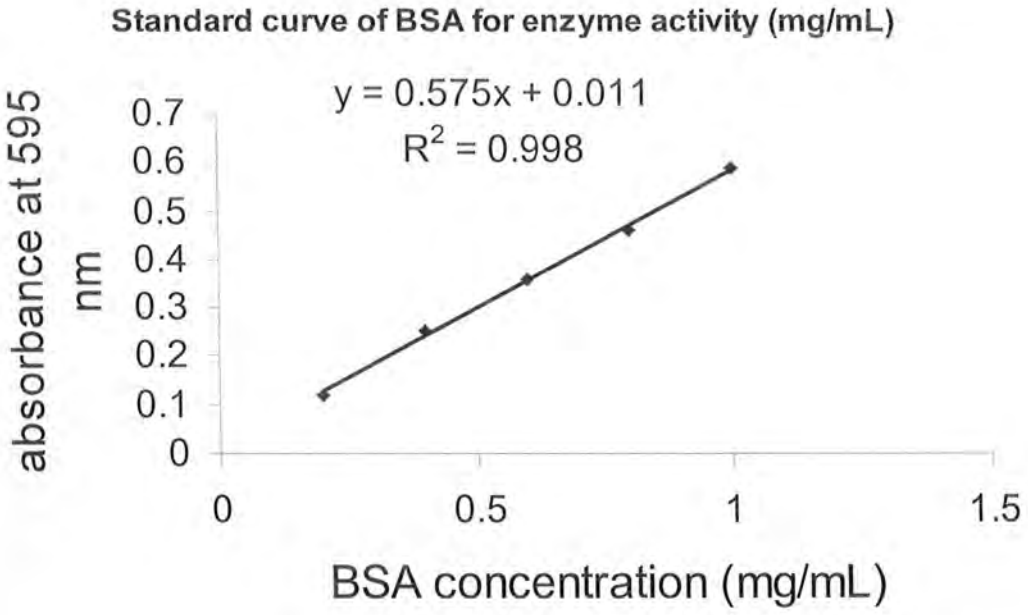
S.No.	Concentration of BSA solution (mg/ml)	volume of BSA solution (µl)	Distilled water (µl)	Total volume (µl)	Absorbance at 595m
1	0.2	20	80	100	0.12
2	0.4	40	60	100	0.25
3	0.6	60	40	100	0.36
4	0.8	80	20	100	0.46
5	1.0	100	0	100	0.59

Quantity of protein was determined from standard curve made from different known concentration of bovine serum albumin (BSA) after treating with Bradford reagent by following formula:

Protein in mg/ml = factor x absorbance of sample

Whereas,

Factor = slope x dilution x volume of sample



ii) 'Xylanase activity:

The activity of xylanase was determined by the method described by Tuncer *et al.*, (1999) against oat spelt xylan.

a) Reagents

1 ml citrate phosphate buffer and dinitrosalicylis acid (DNS) reagents were prepared as shown in (appendix 12).

b) Substrate

1% substrate was prepared by dissolving 1g of oat spelt xylan in 100 mL of distilled water.

c) Assay

1 ml citrate phosphate buffer (appendix 12), 0.5 ml of xylan and 0.5 ml of sample (enzyme) were added in a test tube. The mixture was incubated at 40°C for 2 hrs in orbital shaker at 60 rpm. After the incubation, 3 ml of dinitrosalicylis acid (DNS) reagent (appendix 12) was added and then mixture was placed in boiling water bath for 5 minutes. Reaction was stopped by immediate cooling in water.

A control was run parallel as above but the sample (enzyme) was added after addition of DNS reagent. The absorbance of the mixture in each test tube was taken with the help of spectrophotometer at 550 nm.

A zero point absorbance was adjusted by blank containing 2 ml of distilled water and 3 ml of DNS reagent.

Different concentrations of xylose were made from 0.1% xylose and diluted to a final volume of 2 ml with citrate phosphate buffer as shown in Table 2. The absorbance was checked at 550 nm, standard curve was plotted against different xylose concentrations (mg/ml) as shown in graph 2 to get linear regression equation.

Table.2. Concentration of 0.1% xylose for the standard curve of enzyme activity:

S. No.	volume of xylose solution (ml)	H2O (ml)	Citrate phosphate buffer (ml)	Total volume (ml)	DNS reagent (ml)	Absorbance (ml)
1	0.1	o.4	1.5	2	3	0.392
2	0.2	0.3	1.5	2	3	0.810
3	0.3	0.2	1.5	2	3	1.290
4	0.4	0.1	1.5	2	3	1.770
5	0.5	0.0	1.5	2	3	2.250

Calculation of xylanase activity:

Factor was calculated from the slope of the standard curve (graph 2). The enzyme activity was calculated by multiplying the factor with actual absorbance of sample after substracting the absorbance of control.

Enzyme activity = (absorbance of sample – control) x factor

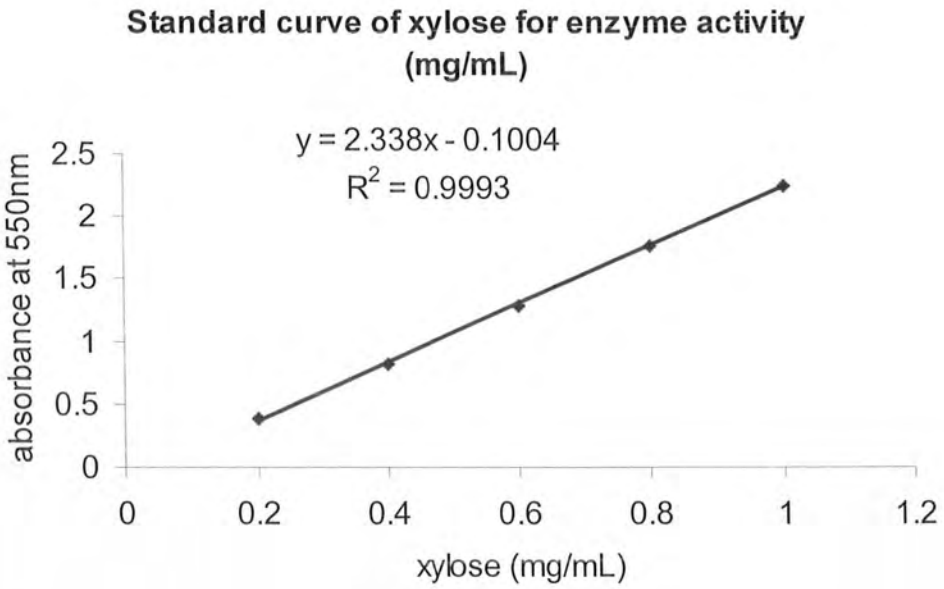
Whereas factor = $\frac{\text{X (concentration of sample x 2 (per ml) x dilution}}{\text{Mol. Wt of xylose x time of incubation (minute)}}$

$$X \text{ (concentration of sample)} = \frac{y + b}{m}$$

where, b = intercept

m = slope

A = absorption



2.2.6.2: Xylanase activity without fusion protein:

Purified protein was digested with enterokinase enzyme (appendix 13), sample was prepared for DNS method as given above.

2.2.7: Effect of different soluble carbon substrates on the induction of xylanase enzyme:

Different soluble carbon substrates were used at 0.5% and 1% level to induce the xylanase gene expression in LB media (appendix 7) and Dubose Salt (DS) media (appendix 14). These carbon sources were xylose, glucose, cellubiose and lactose.

Inoculum preparation for induction of xylanase gene by using different carbon substrates:

Colonies were picked and cultured in the test tubes containing 3 ml DS broth media (appendix 14) and LB broth media (appendix 7) with ampicillin antibiotic and xylose, glucose, cellubiose and lactose at 0.5% and 1% were used as a carbon source. Next day 1 ml culture was transferred to 2 ml LB broth media (appendix 7) and DS broth media (appendix 14) with ampicillin antibiotic, respectively and grown for 3 hours on a shaker at 37 °C. After three hours growth, 4.7 µl of (1 mM) IPTG was added that act as inducer. Cultures were further grown on a shaker at 37 °C for one and half an hour and for 7 hours, respectively. Medium containing growth was centrifuged at 13,500 rpm for 5 minutes. Samples were prepared for enzyme assay as above.

2.2.8: 2% xylose as energy source in xylanase activity

Inoculum preparation with (1 mM) IPTG and (1 mM) lactose inducer

Colonies were grown in DS broth media (appendix 14) substituted with ampicillin and 2% xylose as a carbon source. The expression of xylanase was induced with (1mM) IPTG and (1mM) lactose inducers separately. Samples were prepared for enzyme assay as above. The concentration of xylose was determined by both DNS method as given above and free xylose by HPLC analysis as given below.

2.2.9: HPLC analysis (High performance liquid chromatography):

The concentrations of monomeric sugars (soluble monosaccharides) and cellobiose, total sugars (monosaccharides and oligosaccharides), as well as carbohydrate degradation products and sugar alcohols can be determined using this procedure. Monomeric sugars are quantified by HPLC with refractive index detection.

HPLC system equipped with refractive index detector and the following columns:

- Biorad Aminex HPX-87H column (or equivalent) with corresponding guard column.
- Prepared 0.001 N sulphuric acid for use as a HPLC mobile phase. In a 1L volumetric flask, added 27.8 µl concentrated sulphuric acid and bring to volume with HPLC grade water. Filtered through a 0.2 µm filter and degas before use. If concentrated sulphuric acid is not available, standardized 10 N sulphuric acid may also be used. 0.2 ml of 10 N sulphuric acid in a 2 L volumetric flask, brought to volume with HPLC grade water will also produce 0.001 N sulphuric acid.
- Prepared a series of calibration standards containing the compounds that are to be quantified. Use a four point calibration. If standards are prepared outside of the suggested ranges, the new range for these calibration curves must be validated.
- Prepared the sample(s) for HPLC analysis by passing it through a 0.2 µm filter into an autosampler vial.

Analyzed the calibration standards, and samples by HPLC using a Biorad Aminex HPX-87H column.

HPLC conditions:

- Sample volume: 20 µl, dependent on sample concentration and detector limits
- Mobile phase: 0.001 N sulfuric acid, 0.2 µm filtered and degassed
- Flow rate: 0.6 ml / minute
- Column temperature: 45 – 65 °C
- Detector: refractive index
- Run time: 12 minutes

2.2.10: Analysis of xylanase Xyn-11 A gene expression

i. SDS-PAGE (Sodium Dodecyle Sulphate Poly Acrylamide Gel Electrophoresis):

Preparation of sample for SDS-PAGE:

Picked single colony and cultured in LB liquid media (appendix 7) supplemented with ampicillin (100 mg/ml) and 34 mg/ml chloramphenicol. Next day 1 ml of overnight culture was transfered to 2 ml LB broth medium (appendix 7) with ampicillin and chloramphenicol and grown for further 3 hours at 37 °C. After three hours growth, 4.7 µl of (1 m M) IPTG was added which act as inducer. The culture was equally divided into 3 test tubes. The cells were incubated at 37 °C in a shaker and allowed to grow for 2.5 hours. Samples were taken out one by one at different time periods, pelleted down the cells and frozen for half an hour, thawed the cells and dissolved pellet in 100 µl protein extraction reagent (Bug Buster). Thawed the cells and sonicated for 20 seconds with the interval of 30 seconds and placed on ice for 30 seconds, this process was repeated for 2-3 times, then added 5 µl SDS-PAGE loading dye. Placed at 4 °C until ready to assay. Before assay boiled protein sample at 100 °C for 10 minutes, then loaded on SDS-PAGE gel for analysis.

Preparation of SDS-PAGE:

Electrophoresis was performed as described by Laemmli et al., (1970) using a discontinuous buffer system, for the analysis and separation of proteins. Reagents and solution preparation was shown in (appendix 15).

Gel preparation

Proteins were resolved on SDS-polyacrylamide gel. The gel was prepared as follows. Glass plates were assembled by keeping spacers (1 mm thickness) between the sides and bottom and holding them with clamps. The resolving gel was prepared as shown in (appendix 16). The resolving gel solution was poured inside the assembled glass plates in vertical position, leaving 1-2 inches empty at the top, to form a smooth upper layer of gel. N-butanol was layered at the top of the gel, and the gel was allowed to polymerize for about 45 minutes. The stacking gel was prepared as shown in (appendix 16). After polymerization of resolving gel, N- butanol was removed with the help of tissue paper from the top of the resolving gel, and washed the gel with distilled water.

Dried with tissue paper and stacking gel was poured on the top of resolving gel and a comb was inserted in it. Allowed the gel to polymerize at room temperature.

Electrophoresis:

Bottom spacer was removed and glass plates with polymerized gel were fixed in electrophoresis chamber before starting electrophoresis. Comb was picked out and wells were washed with running buffer (1 X Tris Glycine buffer) 4-5 times with the help of Hemilton syringe. Leakage was checked by adding buffer in upper part of the gel. The lower buffer chamber was filled with running buffer so that the bottom of the gel was dipped in it. About 10 µl of 10 kDa protein size marker (MBI Fermentas) was loaded as standard in a well and 10 – 40 µl of each of the samples was loaded with the help of Hemilton syringe in different wells. Gel was run at a current of 30-40 volts for overnight. When dye reached the bottom of the gel, electric supply was disconnected and gel was removed from glass plates for staining.

Staining of gel with Coomassie Brilliant Blue:

Gel was placed in a tray along with staining solution and with constant agitation on shaking bath. Duration of staining was 4-6 hours to overnight.

Destaining:

After staining, the gel was shifted to destaining solution. It was destained with constant agitation until the background became transparent and protein bands became visible in the form of blue colored bands.

ii: Western blot analysis:

Protein expression was analyzed by western blotting. 15 µg of protein from both transformed and non-transformed cells was run on 15% SDS-PAGE gels. The protein was run quickly on SDS- polyacrylamide gel. Western blot analysis was done by using polyclonal antibodies raised against xylanase protein. The different buffers used in western blots are given in (appendix 17).

Nitrocellulose membrane was wetted in water and then equilibrated for 15 minutes in transfer buffer. Gel was also equilibrated in transfer buffer for 15 minutes, especially if transfer buffer contain MeOH which will cause the polyacrylamide gel to shrink slightly. Transfer "sandwich" was assembled. During sandwich assemblance the follwing points were kept in mind.

- Avoid bubbles which will block transfer of proteins.
- Re-wet layers in transfer buffer.
- Assemble by laying layers down beginning near the center and lowering the edges.
- Roll pipette or test tube across sandwich to expel trapped air bubbles.
- Trim layers to size of gel to prevent "short- circuiting" which will reduce transfer efficiency.
- Use enough Whatmann 3MM filter paper to make sandwich tight in cassette.
- Lower cassette into electrophoresis tank (wet electroblotting system) or between electrodes.

Protein were transferred at lower voltage i.e. 50 volts or in the cold room because swelling may occur during transfer due to increase in temperature. Lower acrylamide concentrations were used to better transfer of higher molecular weight proteins.

Different steps in western blot:

I. Transfer proteins to nitrocellulose.
II. Orient and mark blot.
III. Block non-specific binding sites by using BSA.
IV. Bind primary antibody at cold room for 2-3 hours.
V. Three times washing after each 15 minutes.
VI. Bind secondary antibody at cold room for 2-3 hours.
VII. Three times washing after each 15 minutes.
VIII. Detection using the AP substrate.

2.2.11: Selection of transformants and non-transformants on 1% xylan LB agar plates

Inoculum preparation with (1 m M) IPTG and (1 m M) lactose inducers:

Colonies were picked and cultured in the test tubes containing 3 ml LB broth media (appendix 7) supplemented with ampicillin. Next day 1 ml culture was transferred to 2 ml LB broth media (appendix 7) with ampicillin antibiotic and grown for 3 hours on a shaker at 37° C. After three hours growth, 4.7 µl of (1 m M) IPTG was added that act as inducer. Cultures were further grown on a shaker at 37° C for two and half an hour. Medium containing growth was centrifuged at 13,500 rpm for 5 minutes. Supernatant was discarded and pellet was spread on LB agar medium (appendix 7) containing antibiotics ampicillin, streptomycin, kanamycin, tetracycline and 1% xylan. Samples were incubated on LB agar medium (appendix 7) containing ampicillin and 1% xylan. Plates were incubated at 45 °C for 2 days, and then kept for 24 hours at 50 °C in order to observe the size of clearing zones.

2.3: Expression of Xyn11-A gene in eukaryotic expression system

Pichia pastoris was used for expression of xylanase Xyn 11-A gene as given below.

2.3.1: Cloning of xylanase gene in pPIC3.5K:

The recombinant vector pSSZ810(b) and pPIC3.5K (appendix 30) was digested with *Eco*RI and *Not*I restriction enzymes (appendix 18), placed at 37°C for 1 hour. Digestion was run on 0.5% agarose gel (appendix 3). The targeted fragment approx. 810 bp and pPIC 3.5k were eluted from the gel by using extraction Kit (MBI, Fermentas) as described in appendix 3. Eluted fragments were ligated (appendix 19) and transformed into heat shocked competent cells of *E. coli* TOP10F′ (appendix 6).

2.3.2: Confirmation of xylanase gene in pPIC3.5K vector:

Colonies were selected randomly from over night grown LB agar medium (appendix 7) containing ampicillin (100 mg/ml). Plasmid extraction was done by miniprep method (appendix 8). Restriction/digestion analysis was carried out by using *Eco*RI, *Not*I and *Kpn*I enzymes (appendix 20).

2.4: Expression of xylanase gene Xyn11- A in *Pichia pastoris* strain GS115:

Construct pSSZ810(c) plasmid was isolated from TOP10'F *E. coli* strain by high purity Midiprep protocol (appendix 21). pSSZ810(c) was linearized with *Not*I restriction enzyme for integration in the genomic DNA of *Pichia pastoris* (appendix 22).

2.4.1: Competent cell preparation of Pichia pastoris GS115 strain:

Picked single colony of *Pichia pastoris* GS115 strain from YPD solid media plate and cultured into 5 ml YPD liquid media (appendix 23) without antibiotic, incubated at 30 °C on an orbital shaker at (120-150 rpm) upto the time when OD_{600} (1.0) or density reached upto 10^6 cells/ml. Electrocompetent cells were prepared by electrochemical method which is given in (appendix 24).

2.4.2: Electroporation mediated transformation of pSSZ810(c) into Pichia pastoris GS115 strain:

The electroporation apparatus was adjusted to 2.0 kvolts. Electroporation cuvettes with a gap of 2 mm were used in this experiment. Transferred the electrocompetent cells GS115 (80 µl as removed from the -70°C freezer and thawed on ice) and pSSZ810(C) (0.1µg linearized) in the cuvettes and electric shock was given at 2.0 kvolts. Then immediately 1ml 1M sorbitol was added and placed the cuvettes on shaker at 30°C for 2 hour. After 2 hour the medium was spread on the YPD agar plates (appendix 23) containing antibiotic geneticin (0.75 mg/ml) and transferred these plates to incubator at 30 °C until colonies appeared.

2.4.3: Selection of transformants on Yeast peptone dextrose (YPD) medium containing different concentration of antibiotic geneticin:

Different concentration of geneticin YPD agar media (appendix 23) plates were prepared for the selection of transformants. These concentrations were 0.25, 0.5, 0.75, 1.0 and 1.25 mg/ml, respectively. The method for YPD media plates containing antibiotic geneticin is given in (appendix 23).

2.4.4: PCR confirmation of Pichia pastoris transformant:

Colonies were picked from the YPD agar medium (appendix 23) containing 0.75 mg/ml geneticin plate and cultured into YPD liquid medium (appendix 23) without antibiotic, incubated at 30 °C till the time OD_{600} reaches upto (1.0). The protocol used for PCR confirmation was given as follow:

i. Placed 10µl of *pichia pastoris* culture into 1.5 ml microcentrifuge tube.

ii. Added 5µl zymolyse enzyme and incubated at 30°C for 10 minutes.

iii. The sample was frozen at -70 °C for 10 minutes.

iv. Reaction mixture was prepared as given on next page

	1Rx	
PCR buffer	5.0µl	75.0µl
$MgCl_2$	4.0µl	60.0µl
dNTPs	1.0 µl	15.0 µl
P_1 (F)	1.0 µl	15.0 µl
P_2 (R)	1.0 µl	15.0 µl
Taq polymerase	0.4 µl	6.0 µl
Yeast culture	5.0 µl	-------
ddd H_2O	34.6 µl	519.0µl

positive control = pSSZ810(c) plasmid dilution.

Negative control= water control

Denaturation = 94°C for 5 min

Annealing = 60°C for 1 min

Extention = 72°C for 1 min

Extention = 72°C for 10 min

No. of cycles = 35

Analyzed a 25 µl out of 50 µl amplified reaction by 0.8% agarose gel electrophoresis (appendix 3).

2.4.5: Optimization of Pichia pastoris growth for xylanase assay

The optimized growth conditions and expression of protein in *Pichia pastoris* was given as follows:

I. *Pichia pastoris* transformants having pSSZ810(c) were picked from 0.75 mg/ml geneticin YPD solid media (appendix 23) plates along with non-transformant GS115 as a control and cultured into YPD broth media (appendix 23) without antibiotic at 30 °C (OD_{600} = 1.0).

II. Harvested the cells by centrifugation at 3000 rpm for 5 minute at room temperature.

III. Discarded supernatant and resuspended cell pellet into 25 ml minimal glycerol media MGM (appendix 25) in a 100 ml flask. Placed at 28-30 °C in a shaking incubator (150 – 200 rpm) until growth was reaches log phase. Once the cells are in log phase, they can be induced for xylanase expression. Add 0.02% 10 X Dextrose (appendix 26).

IV. Take 1 ml culture before each induction of 100 % methanol to a final concentration of 30 µl in 25 ml MG medium (appendix 25).

V. Induced culture was collected at different time intervals i.e. 24, 48, 72, 96 and 120 hrs, respectively. Transfered 1 ml of expression culture into 1.5 microcentrifuge tubes. These samples were used to analyze expression levels and determine the optimal time from post-induction to harvest.

VI. Centrifuged cells at 13,500 rpm at room temperature for 2-3 minutes. Collected both pellet and supernatant in 1.5 microcentrifuge tubes.

For intracellular and secreted expression, both supernatant and pellet was stored at -70 °C until ready for protein assay.

2.4.6: Analysis for xylanase Xyn11- A gene in Pichia pastoris system

i) SDS-PAGE:

Preparation of samples for SDS-PAGE:

a) ***Preparation of cell pellets***

I. Thawed cells pellets quickly and placed on ice.

II. Dissolved pellet in 1 ml distilled water, add 100 µl Breaking buffer (appendix 27).
III. Added an equal volume of acid washed glass beads (size 0.5mm).
IV. Vortex for 30 seconds, then incubated on ice for 30 seconds. Repeated for several times.
V. Centrifuged at 13,500 rpm for 10 minutes.
VI. Transferred clear supernatant to a fresh 1.5 microcentrifuge tube, added 50 µl SDS-PAGE loading dye.
VII. Boiled for 10 minutes at 100°C in a dry bath.
VIII. Loaded 10-20 µl sample per well into SDS-PAGE gel and remaining were stored at -20°C for used in future.

Preparation of SDS-PAGE

Preparation of SDS-PAGE:

Electrophoresis was performed as described by Laemmli et al., (1970) using a discontinuous buffer system, for the analysis and separation of proteins. Reagents and solution preparation was shown in (appendix 15).

Gel preparation

Proteins were resolved on SDS-polyacrylamide gel. The gel was prepared as follows. Glass plates were assembled by keeping spacers (1 mm thickness) between the sides and bottom and holding them with clamps. The resolving gel was prepared as shown in (appendix 16). The resolving gel solution was poured inside the assembled glass plates in vertical position, leaving 1-2 inches empty at the top, to form a smooth upper layer of gel. N-butanol was layered at the top of the gel, and the gel was allowed to polymerize for about 45 minutes. The stacking gel was prepared as shown in (appendix 16). After polymerization of resolving gel, N- butanol was removed with the help of tissue paper from the top of the resolving gel, and washed the gel with distilled water. Dried with tissue paper and stacking gel was poured on the top of resolving gel and a comb was inserted in it. Allowed the gel to polymerize at room temperature.

Electrophoresis:

Bottom spacer was removed and glass plates with polymerized gel were fixed in electrophoresis chamber before starting electrophoresis. Comb was picked out and wells were washed with running buffer (1 X Tris Glycine buffer) 4-5 times with the help of Hemilton syringe. Leakage was checked by adding buffer in upper part of the gel. The lower buffer chamber was filled with running buffer so that the bottom of the gel was dipped in it. About 10 µl of 10 kDa protein size marker (MBI Fermentas) was loaded as standard in a well and 10 – 40 µl of each of the samples was loaded with the help of Hemilton syringe in different wells. Gel was run at a current of 30-40 volts for overnight. When dye reached the bottom of the gel, electric supply was disconnected and gel was removed from glass plates for staining.

Staining of gel with Coomassie Brilliant Blue:

Gel was placed in a tray along with staining solution and with constant agitation on shaking bath. Duration of staining was 4-6 hours to overnight.

Destaining:

After staining, the gel was shifted to destaining solution. It was destained with constant agitation until the background became transparent and protein bands became visible in the form of blue colored bands.

Silver staining of SDS-PAGE of xylanase Xyn-11 A protein

The process of silver staining of SDS-PAGE of xylanase Xyn 11-A protein in *Pichia pastoris* is given below:

I. Gel was placed into the fixative enhancer solution with gentle agitation, fixed the gel for 1 hr or may be overnight.
II. Distilled water was added after removing fixative enhancer solution. SDS-PAGE gel was washed upto 2-3 times.
III. Oxidizer was added and agitated the gel for 5 minutes.
IV. SDS-PAGE gel was washed with distilled water after every 15 minutes. Washing may be 5-6 times until light yellow color appeared.

V. Silver reagent was added and agitated for 20 minutes.
VI. SDS-PAGE gel was quickly rinsed by distilled water, washing may be 2-3 times.
VII. Developer solution was added to enhance the bands and slowly agitated until bands appeared.
VIII. SDS-PAGE gel was rinsed with water upto 2-3 times.

ii) Bradford analysis

Transformants were optimized at maximum OD_{600} (1.0) as given above. Samples were prepared as follows.

i. Thawed cells quickly and placed on ice.
ii. Added 5µl zymolyase enzyme and incubated at 30 °C for 10 minutes.
iii. Freezed the sample at -70 °C for 10 minutes.
iv. Sonicated for complete cell lysis, centrifuged at 13,500 rpm for 2 minutes.
v. Supernatant was collected for Bradford analysis as given above (page no 39 & 40).

The standard curve and quantitation of protein was given as above (page no 26 & 27).

iii) DNS (Dinitrosalicylis acid)

Sample preparation was similar as given in Bradford method and xylanase assay for expression of xylanase gene in eukaryotic system were similar as given above in case of prokaryotic expression (page no. 27 & 28). The standard curve and calculation of xylanase activity was given as above in Table 2 and graph 2 (page no. 28 & 29).

2.4.7: HPLC analysis:

For HPLC analysis the sample was prepared as given above protocol (page no. 8).

Chapter No.3

Results

Chapter 3

RESULTS

The present study describes the expression of *Chaetomium thermophile* Xyn 11-A gene in both prokaryotic (*E. coli*) and eukaryotic (*Pichia pastoris*) systems. The results obtained are discussed as under.

2.1: Cloning and expression of xylanase gene Xyn 11-A in prokaryotic expression system

The PCR amplified 810 bp intron-less fragment using specific primers was cloned into T/A cloning vector pTZ57R. The resultant recombinant clone pSSZ810(a), as shown in Figure 1, was confirmed through digestion as shown in Figure 2. After digestion with *Eco*RI and *Hin*dIII, the xylanase gene of approx. 810 bp was cloned into the pET expression vector, and finally transformed into *E. coli* strain BL21 for bacterial expression.

2.1.1: Confirmation of cloning of PCR amplified fragment without intron into pET vector

To confirm the cloning of intron-less Xyn11-A gene in pSSZ810(b), *Eco*RI and *Hin*dIII restriction enzymes were used. *Eco*RI and *Hin*dIII are present upstream and downstream of the xylanase gene, respectively as shown in Figure 3. Upon digestion with these enzymes approx. 810 bp size fragment should be produced along with the vector back bone of 5.4 kb as shown in figure 4. To further confirm the cloning of intron-less fragment in the pET 32a(+) vector, it was digested with the *Hin*dIII and *Eco*R1. Upon digestion with *Hin*dIII and *Eco*R1, it produced approx. 900 bp fragment as shown in Figure 4, lane 1 & 2. Similarly, it was also digested with *Hin*dIII and *Xho*1 which produced a correct sized fragment of approx. 600 bp as shown in figure 2, lane 3. Confirmation of pSSZ810(b) was also made with *Hin*dIII and *Xba*1 restriction enzymes

which produced approx. 800 and 600 bp fragment representing as shown in Figure 2, lanes 7 and 8.

2.1.2: Confirmation of transformation of E. coli with pSSZ810(b)

After confirmation of recombinant clone pSSZ810(b) through combination of different restriction enzymes, pSSZ810(b) and pET 32a(+) vector was transformed into *E. coli* BL21 strain by heat shock transformation method. The target gene from pSSZ810(b) was confirmed through PCR analysis by using set of Xyn 11-A specific primers P_1 and P_2, respectively (appendix 2). The amplification of 810 bp fragment, as shown in Figure. 5, confirmed the transformation of recombinant vector in *E. coli* strain BL21.

2.1.3: Confirmation of xylanase gene expression

Expression of xylanase in prokaryotic system *E. coli* was confirmed through Bradford analysis, xylanase assay, SDS-PAGE and Western blotting techniques.

i: Bradford method

The activity of total protein of *E. coli* strain BL21 transformed with pSSZ810(b) was induced by the addition of IPTG (1 mM) and lactose (1 mM) in DS broth media containing ampicillin (100 mg/mL) and 2% xylose as carbon energy source. The xylanase protein in mg/mL was determined by Bradford method as shown in Table 3. In case of IPTG (1mM) inducer the maximum and minimum activity of protein was 0.66 and 0.37 mg/mL, respectively as compared to control (0.18 mg/mL). Similarly, in case of lactose (1mM) inducer, the maximum and minimum activity of protein was 0.60 mg/mL and 0.35 mg/mL, respectively. The intracellular protein from expression of recombinant vector pSSZ810(b) was 0.66 as shown in Table 3.

ii) xylanase activity

The estimation of the xylanase gene expression in the form of fusion protein was carried out using xylanase assay. The xylanase gene expression in *E. coli* strain BL21 was induced by IPTG (1mM) inducer in LB broth media as indicated in Table 4 and

shown in Figure 8. The activity of xylanase by DNS method was obtained in U/mL. The maximum activity of xylanase gene U/mL was obtained after incubation of 2 hours at 40 °C. The maximum and minimum production of xylanase was 4.6 and 3.99 U/mL, respectively as compared to control 4.19 and 3.91 U/mL, respectively as shown in figure. 6. Figure 6, lanel shows dark color due to production of free xylose sugar after incubation with xylanase enzyme as compared to enzyme control (shown in lane 4).

Table 3: Estimation of total protein produced by *E. coli* in BL21 transformed with pSSZ810(b)

Time of incubation *	Protein concentration (mg/mL) **									
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5 (control)	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10 (control)
At zero hrs	0.44	------	------	------	0.18	0.60	------	------	------	0.50
After 4 hrs	------	0.66	------	-----	------	------	0.55	------	------	------
After 8 hrs	------	------	0.37	------	------	------	------	0.35	------	------
After 12 hrs	------	------	------	0.55	------	------	------	------	0.46	------

* Induction with IPTG (1 mM) and lactose (1 mM)

** **Sample 1-4:** induced by 1 mM IPTG + DS + pSSZ810(b) + 2% xylose; **sample 5:** induced by 1 mM IPTG + DS + pET 32a(+) + 2% xylose; **sample 6-9:** induced by 1 mM lactose + DS media + pSSZ810(b) + 2% x ylose; **sample 10:** induced by 1 mM lactose + DS media + pET32a(+) + 2% xylose.

Table 4: Xylanase activity in cell lysate of *E. coli* (BL21) harboring the pET expression vector with intron-less xylanase fragment

Time of induction*	Xylanase activity U/mL **		
	Sample 1	Sample 2	Sample 3 (control)
At zero hr	3.19	3.74	3.44

After 30 mins	4.62	3.40	3.81
After 60 mins	4.03	3.80	4.19
After 90 mins	3.95	3.99	3.92
After 120 mins	4.19	3.94	3.75

* Induction with IPTG (1 mM)
** **Sample 1 & 2:** pSSZ810(b)
Sample 3: pET 32a(+)

2.1.4: Xylanase activity after digestion with Enterokinase restriction enzyme

The expression of recombinant clone pSSZ810(b) in BL21 after digestion with enterokinase enzyme was obtained by using xylanase assay. The activity of digested and undigested fusion protein is indicated in Table 5 and shown in Figure 9. The maximum activity of xylanase gene was observed after 2 hours of incubation at 40 °C. The maximum and minimum production of digested protein was 0.38 and 0.34 U/mL, respectively as compared to control (0.22 U/mL) whereas the maximum and minimum activity of undigested protein was 4.0 and 3.48 U/mL, respectively. pSSZ810(b) digested with enterokinase enzyme shows less color (as shown in figure 7, lane 4 and 5) as compared to undigested pSSZ810(b) (lane 6 and 7). The controls having digested and undigested pET 32a(+) are shown in lanes 1 and 2.

Table 5: Xylanase production in digested and undigested fusion protein after digestion with enterokinase enzyme in the pET 32a(+) expression vector

Time of incubation	Xylanase activity U/ml					
	Digested samples*			Undigested samples**		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
2 hrs	0.387	0.345	0.221	3.48	4.0	0.779

* **Sample 1 & 2:** digested pSSZ810(b)
Sample 3: digested pET 32a(+)

** **Sample 1 & 2:** undigested pSSZ810(b)

Sample 3: undigested pET 32a(+)

2.1.5: Effect of different carbon source on xylanase production

Effect of different carbon sources was observed on the xylanase gene expression, which was carried out through xylanase assay. The xylanase activity in *E. coli* BL21 strain in DS liquid media containing ampicillin (100 mg/mL) and different carbon sources i.e. xylose, glucose, cellobiose and lactose, respectively was carried out after 1.5 and 7 hours, respectively as indicated in Table 6 and shown in Figure 10. The maximum activity of xylanase U/mL in the presence of carbon sources was obtained after incubation of 10 min at 40 °C. The maximum xylanase activity after 1.5 hrs, observed in the presence of 1 % xylose, was 2.8 U/mL whereas minimum activity, observed in the presence of 1 % cellubiose. On the other hand the maximum activity of xylanase was observed after 7 hrs in the presence of 0.5 % lactose of 1.708 U/mL whereas minimum activity was observed in the presence of 1 % lactose as shown in Table 6 and shown in Figure 10.

Table 6: Xylanase activity in cell lysate of *E. coli* (BL21) in the presence of different carbon sources

***E. coli* strain transformed with vector**	**Medium used**	**Carbon source**	**Enzyme activity U/mL**	
			After one and half an hour	**After 7 hrs**
pSSZ810(b)	DS*	0.5 % xylose	1.375	0.575
pSSZ810(b)	DS	0.5 % glucose	-0.454	1.139
pSSZ810(b)	DS	0.5 % cellubiose	0.515	1.539
pSSZ810(b)	DS	0.5 % lactose	0.860	1.708
pSSZ810(b)	DS	Without carbon source	2.787	0.236
pSSZ810(b)	DS	1 % xylose	2.302	0133

pSSZ810(b)	DS	1 % glucose	1.363	1.369
pSSZ810(b)	DS	1 % cellubiose	-0.136	-1.424
pSSZ810(b)	DS	1 % lactose	0.412	-5.853
pSSZ810(b)	LB	Without carbon source	2.696	-0.983

* **DS:** Dubose Salt medium

** **LB:** Luria Bertani medium

2.1.6: Effect of xylose and lactose as energy/carbon sources with and without IPTG inducer

The maximum activity of xylanase was observed in the presence of 1% lactose and 1% xylose, respectively and DS broth media that was induced by IPTG (1 mM), as shown in Table 7 and Figure 11. The maximum activity of xylanase (U/mL) in the presence of carbon sources was obtained after incubation of 10 min at 40 °C.

In case of induction the maximum activity of 0.830 U/mL was observed in the presence of 1 % xylose whereas mimimum activity of 0.218 U/mL was obtained in the presence of 1 % lactose. In case where no carbon source was used, the xylanase activity was 0.848 U/mL. Table 7 also shows that the activity in LB media without carbon source was 0.466 U/mL.

Table 7: Xylanase productivity in the presence of lactose and xylose with IPTG inducer

E. coli strain transformed with vector	Medium used	Carbon source	Enzyme activity U/mL
pSSZ810(b)	DS*	1 % lactose	0.218
pSSZ810(b)	DS	1 % xylose	0.830
pSSZ810(b)	DS	Without carbon source	0.848
pET 32a(+) control	DS	Without carbon source	0.569
pSSZ810(b)	LB**	Without carbon source	0.466
pET 32a(+) control	LB	Without carbon source	0.060

* **DS:** Dubose Salt medium

** **LB:** Luria Bertani medium

In case where no induction was done, the maximum activity, observed in the presence of 1 % lactose, was 3.09 U/mL whereas mimimum activity, obtained in the presence of 1 % xylose, was 2.59 U/mL. In case where no carbon source was used, the xylanase activity was 0.266 U/mL as compared to control (0.133 U/mL). Similarly, the activity of xylanase in LB media without carbon source was 0.248 U/mL as compared to control (0.157 U/mL) as shown in Table 8 and Figure 12.

Table 8: Xylanase productivity in the presence of lactose and xylose without IPTG inducer

E. coli strain transformed with vector	Medium used	Carbon source	Enzyme activity U/mL
pSSZ810(b)	DS*	1 % lactose	3.09
pSSZ810(b)	DS	1 % xylose	2.59
pSSZ810(b)	DS	Without carbon source	0.266
pET 32a(+) control	DS	Without carbon source	0.133
pSSZ810(b)	LB**	Without carbon source	0.248
pET 32a(+) control	LB	Without carbon source	0.157

* **DS:** Dubose Salt medium

** **LB:** Luria Bertani medium

2.1.7: Effect of xylose (as a carbon source) on xylanase activity

The activity of xylanase gene in *E. coli* strain B L21 having r ecombinant clone pSSZ810(b) in the presence of 30% xylose, upto final concentration of 2%, was obtained as determined by xylanase assay. The maximum activity of xylanase (U/mL) in the presence of carbon sources was obtained after incubation of 10 min at 40 °C. The production of xylanase was induced by IPTG (1 mM) and lactose (1 mM) inducers, respectively. The maximum and minimum activity of xylanase in the presence of (1 mM) lactose was 6.02 and 1.4 U/mL, respectively whereas in the presence of IPTG it was 1.0 and 0.05 U/mL as indicated in Table 9 and shown in Figure 13. Similarly, the production of xylanase in *E. coli* strain BL21 having pSSZ810(b), in DS media having 2 % xylose and lactose (1 mM), was 0.42 U/mL as compared to control (*E. coli* having

pET 32a(+) in DS media having 2 % xylose and IPTG (1mM)) (0.30 U/mL) as indicated in Table 9 and shown in Figure 13.

Table 9: Xylanase activity in the presence of 30% xylose and IPTG/lactose inducers

Time of incubation *	Protein concentration (mg/mL) **									
	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S10
At zero hrs	1.04	------	------	------	0.30	1.43	------	------	------	0.42
4 hrs	------	0.30	------	-----	------	------	2.89	------	------	------
8 hrs	------	------	0.05	------	------	------	------	6.02	------	------
12 hrs	------	------	------	0.60	------	------	------	------	2.06	------

* Induction with IPTG (1 mM) and lactose (1 mM)

** **Sample 1-4:** induced by 1 mM IPTG + DS + pSSZ810(b) + 2% xylose; **sample 5:** induced by 1 mM IPTG + DS + pET 32a(+) + 2% xylose; **sample 6-9:** induced by 1 mM lactose + DS media + pSSZ810(b) + 2% x ylose; **sample 10:** induced by 1 mM lactose + DS media + pSSZ810(b) + 2% xylose.

2.1.8: Selection of transformants on 1% xylan LB agar plates

Inoculum with 1 m M IPTG and 1 mM lactose inducers

Xylanase activity of *E. coli* strain BL21 transformed with pSSZ810(b) along with control (*E. coli* having pET 32a(+)), induced by IPTG (1 mM) and lactose (1 mM) inducers, respectively was observed on 1 % xylan LB agar plates as shown in Figures 14 and 15. These results showed maximum clearance of xylan plates by bacterial cells transformed with pSSZ810(b) while no clearance zone was obtained in the control.

Moreover, higher xylanase activity was obtained when lactose was used as an inducer as shown in Figure 15.

2.1.9: HPLC analysis xylan hydrolysis

HPLC analysis was carried out to determine the effect of recombinant enzyme on xylan hydrolysis to xylose. The hydrolysis was carried out at 40 °C for 10 minutes. The pattern of free xylose sugar released from enzyme is shown in Figure 16 along with standard concentration of 1% xylose. The maximum peak of xylose was observed when lactose (1 mM) was used as inducer as shown in Figure 16 (j, k, l and m) as compared to IPTG (1 mM) as shown in Figure 16 (d, e, f and g), respectively along with pET 32a(+) and pSSZ810(b) controls.

2.1.10: Analysis of xylanase protein by different methods

i) SDS-PAGE analysis for xylanase protein induced by IPTG inducer

After transformation and confirmation of pSSZ810(b) and pET in *E. coli* strain BL21, the samples were processed and analysed on SDS-PAGE. The intensity of desirable protein (approx. 43 kDa) increased with increase in time after IPTG induction of 30, 60, 90, and 120 minutes, respectively as shown in Figure 17. The maximum production of desirable protein was observed after 90 and 120 min, respectively as compared to control pET 32a(+).

ii) SDS-PAGE analysis for xylanase protein induced by IPTG/lactose inducers

The expression of targeted gene was induced by the addition of IPTG (1 mM) and lactose (1 mM) inducers, respectively in DS broth media containing ampicillin and 2 % xylose as energy/carbon source. These samples were processed and analyzed on SDS-PAGE. The intensity of desirable protein approx. 43 kDa increased with increase in time after IPTG and lactose induction of zero, 4, 8 and 12 hrs, respectively as shown in Figure 18. Maximum activity of pSSZ810(b) having xylanase protein was observed in case of lactose as compared to IPTG as indicated in Figure 18.

iii: Western blotting of xylanase gene in prokaryotic expression system

To demonstrate the expression of xylanase protein in a prokaryotic system (*E. coli* strain BL21), western analysis was done. The protein was quickly run through SDS-polyacrylamide gel. Western blot analysis was done by using polyclonal antibodies raised against fusion protein regions which were available in lab. The western blot of fused Xyn 11-A protein is shown in Figure 19. A protein of approx. 43 kDa was detected in all the samples, while the antibodies did not detect any protein in the controls.

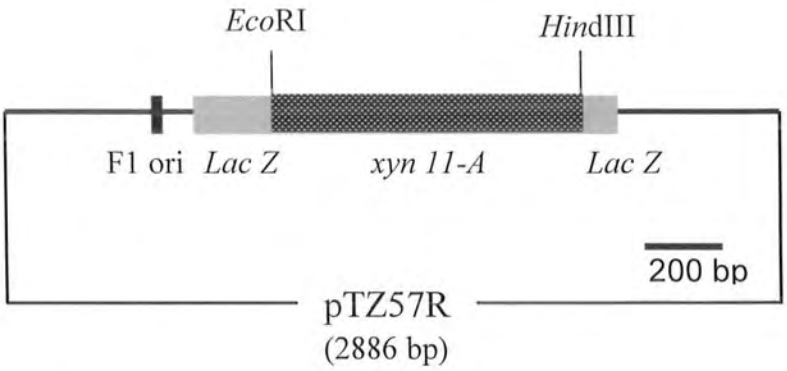


Fig: 1. Recombinant clone pSSZ810(a) with *Eco*RI and *Hin*dIII.

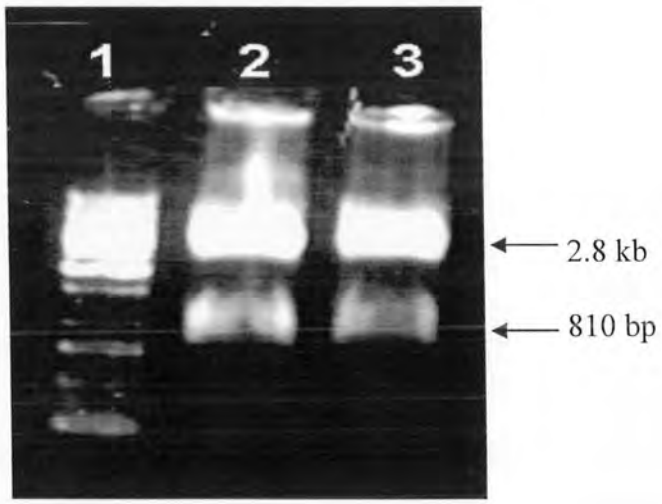


Fig: 2. Confirmation of cloning of xylanase gene in pTZ57R through restriction/digestion with *EcoRI* and *Hin*d III. Lane.2 and 3: pTZ57R digested with *EcoRI*.Lane. 1: represent 1Kb DNA ladder.

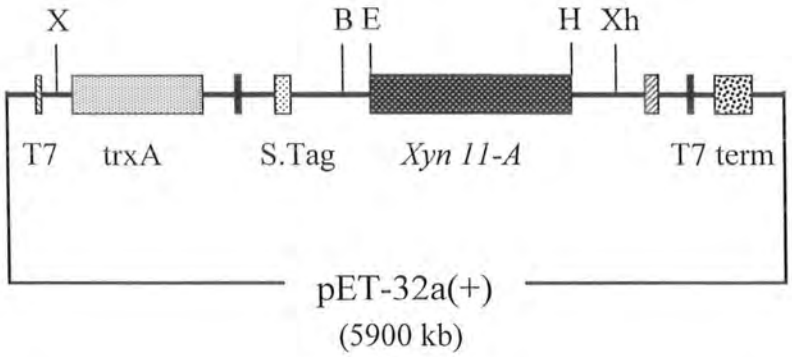


Fig: 3. Recombinant clone pSSZ810(b) with restriction sites. E: *Eco*RI; H: *Hin*dIII; B: *Bam*HI; X: *Xba*I; Xh: *Xho*I; T7: T7 promoter; T7 term: T7 terminater

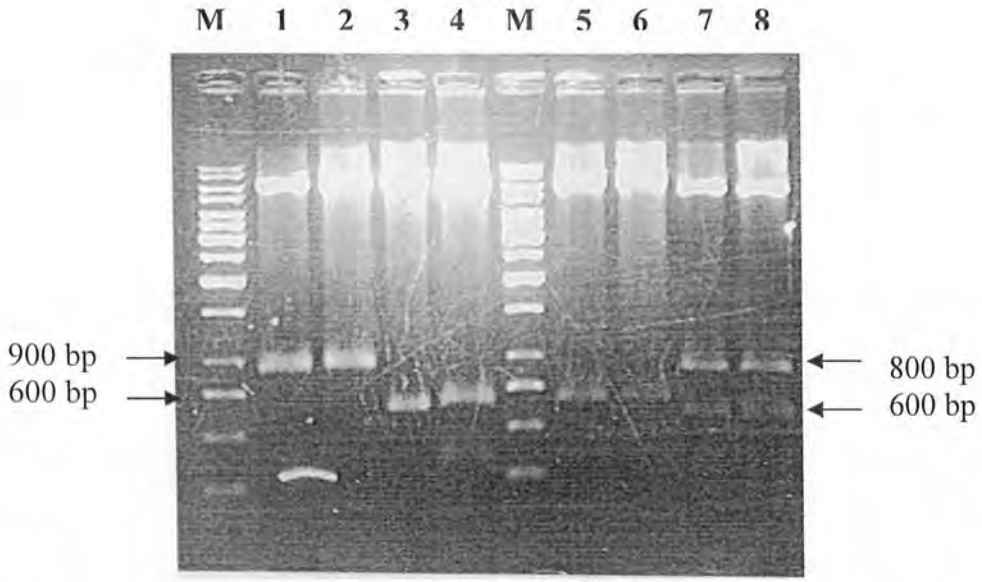


Fig: 4. Confirmation of cloning of xylanase gene in pET 32a(+) through restriction/digestion with combination of enzymes. Lane 1 and 2: pET 32a(+) digestion with *Hin*dIII and *Eco*R1, Lane 3: pET 32a(+) digestion with *Hin*dIII and *Xho*1, Lane 7 and 8: pET 32a(+) digestion with *Hin*dIII and *Xba*1, M: 1 Kb DNA ladder.

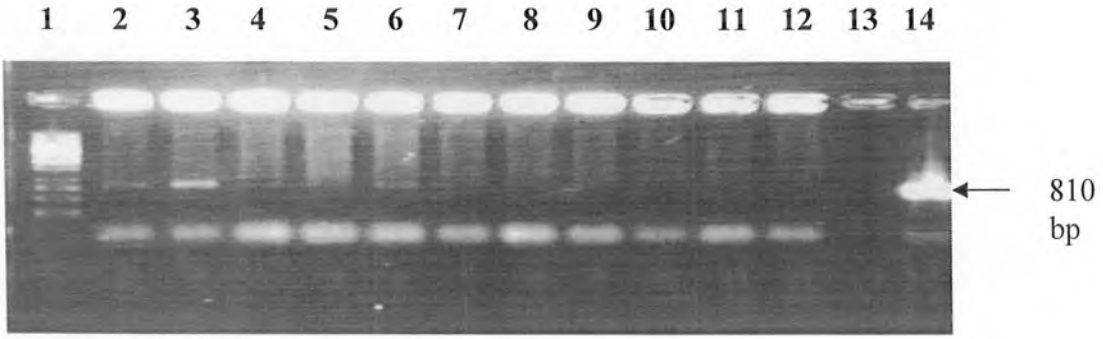


Fig: 5. Agarose gel electrophoresis of pSSZ810(b) in *E. coli* BL21 strain through PCR analysis using xylanase specific primer (appendix 2). Lane 1: 1 kb DNA ladder, Lanes 2- 9: selected transformed BL21 colonies containing pSSZ810(b), Lanes 10 – 12: transformed BL21 colonies containing pET 32a(+), Lane 13: water control, Lane 14: positive control.

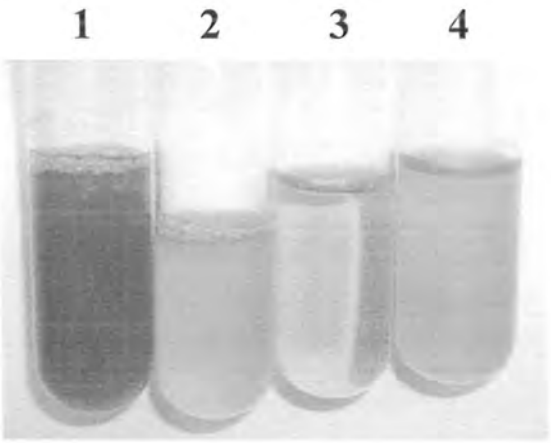


Fig: 6. Xylanase enzyme assay to measure the xylanase activity of the recombinant clone.
Tube 1: E. coli having pSSZ810(b)
Tube 2: Substrate control
Tube 3: Water control (blank)
Tube 4: Enzyme control

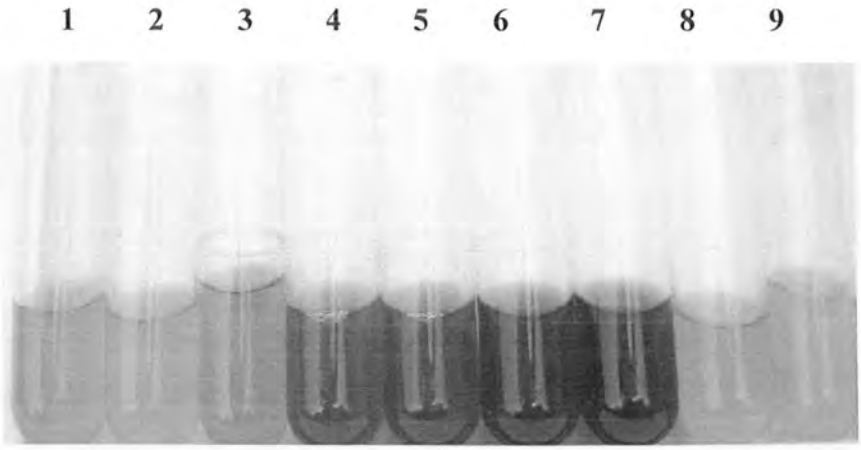


Fig: 7. Xylanase enzyme assay to estimate the xylanase activity of the recombinant clone pSSZ810(b) after digestion with enterokinase enzyme.
Lane 1: undigested pET 32a(+) control
Lane 2: digested pET 32a(+) control
Lane 3: Enzyme control
Lane 4 and 5: Digested pSSZ810(b)
Lane 6 and 7: Undigested pSSZ810(b)
Lane 8: Water control (blank)
Lane 9: Substrate control

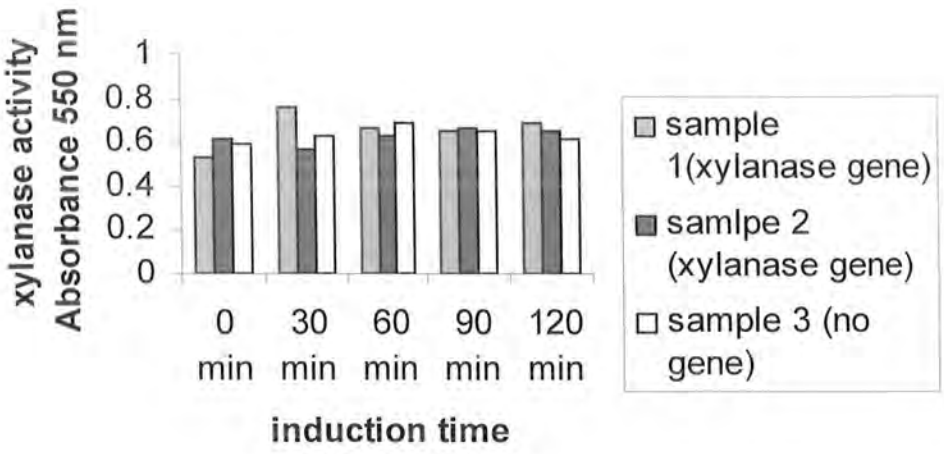
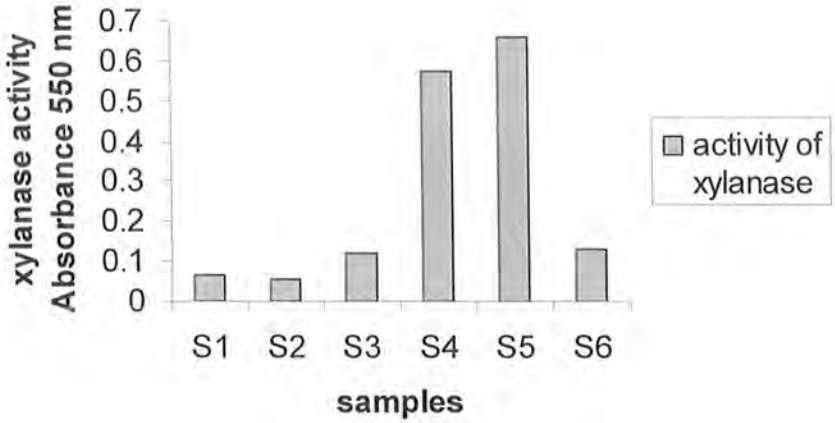
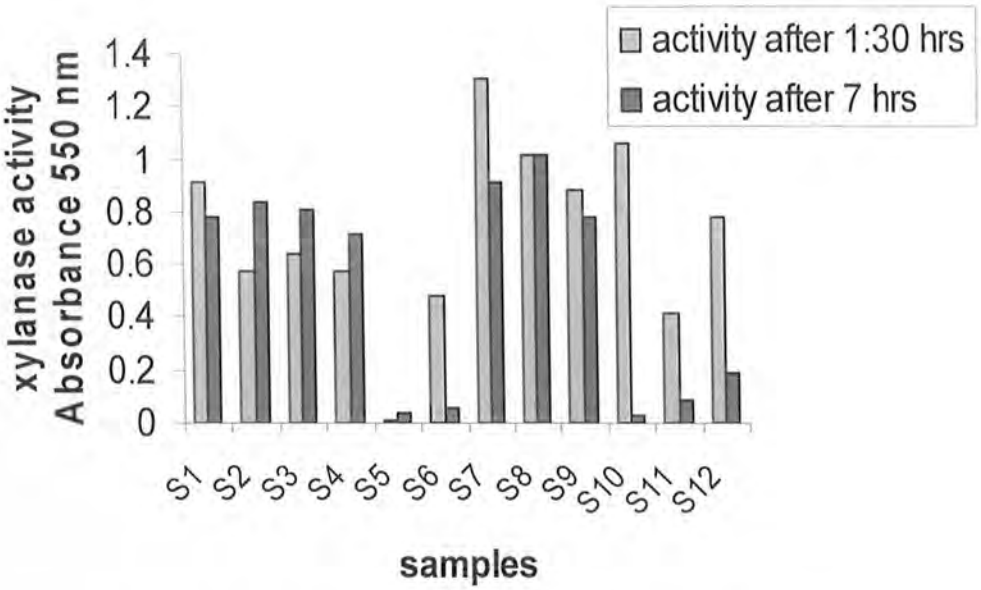


Fig: 8. Xylanase activity in the cell lysate of *E. coli* (BL21) harboring the pET 32a(+) expression vector with intron-less xylanase fragment.



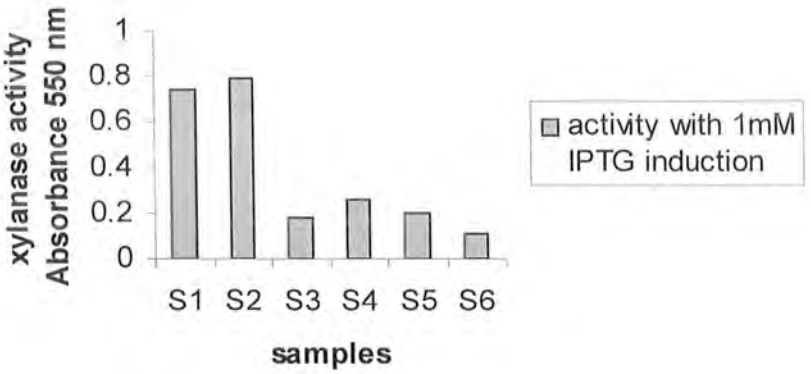
Sample 1: (digested fusion protein)
Sample 2: (digested fusion protein)
Sample 3: (digested pET) control
Sample 4: (without carbon source + DS media + pET)
Sample 5: (LB media + xylanase gene)
Sample 6: (LB media + pET)

Fig: 9. Xylanase enzyme assay to estimate the xylanase activity of the recombinant clone pSSZ810(b) after digestion with enterokinase enzyme.



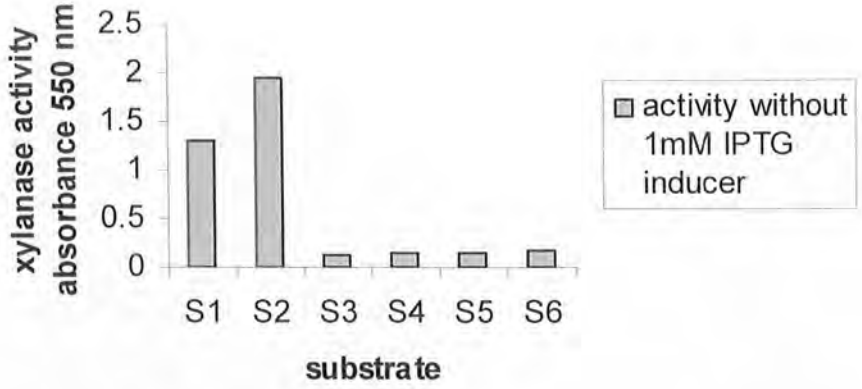
Sample 1: (0.5% xylose + DS media + xylanase gene)
Sample 2: (0.5% glucose + DS media + xylanase gene)
Sample 3: (0.5% celubiose + DS media + xylanase gene)
Sample 4: (0.5% lactose + DS media + xylanase gene)
Sample 5: (without carbon source + DS media + pET)
Sample 6: (without carbon source + DS media + xylanase gene)
Sample 7: (1% xylose + DS media + xylanase gene)
Sample 8: (1% glucose + DS media + xylanase gene)
Sample 9: (1% cellubiose + DS media + xylanase gene)
Sample 10: (1% lactose + DS media + xylanase gene)
Sample 11: (LB media + pET),
Sample 12: (LB media + xylanase gene).

Fig: 10. Xylanase activity in cell lysate of *E. coli* (BL21) in the presence of different carbon sources.



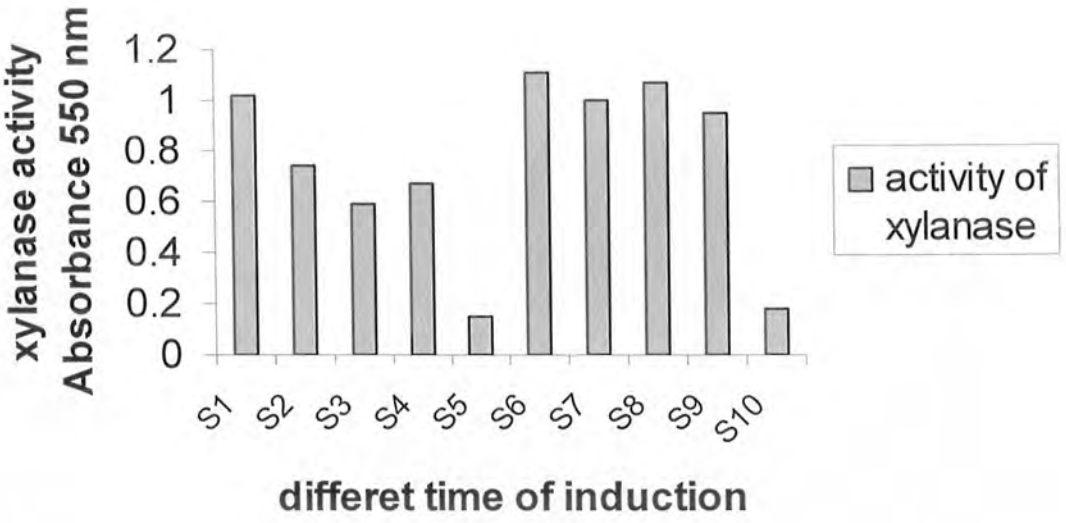
Sample 1: (1% lactose + DS media + xylanase gene)
Sample 2: (1% xylose + DS media + xylanase gene)
Sample 3: (without carbon source + DS media + xylanase gene)
Sample 4: (without carbon source + DS media + pET)
Sample 5: (LB media + xylanase gene)
Sample 6: (LB media + pET)

Fig: 11. Xylanase production in the presence of lactose and with IPTG inducer.



Sample 1: (1% lactose + DS media + xylanase gene)
Sample 2: (1% xylose + DS media + xylanase gene)
Sample 3: (without carbon source + DS media + xylanase gene)
Sample 4: (without carbon source + DS media + pET)
Sample 5: (LB media + xylanase gene)
Sample 6: (LB media + pET)

Fig: 12. Xylanase productivity in the presence of lactose and without IPTG inducer.



Sample 1-4: (induced by 1 m M IPTG + DS + xylanase gene + 2 % xylose); **Sample 5:** (induced by 1 m M IPTG + DS + pET + 2 % xylose); **Sample 6-9**: (induced by 1 m M lactose + DS media + xylanase gene + 2 % xylose), **Sample 10**: (induced by 1 m M lactose + DS media + xylanase gene + 2 % xylose).

Fig: 13. Xylanase activity in the presence of 30% xylose with IPTG/lactose (1 mM) inducer.

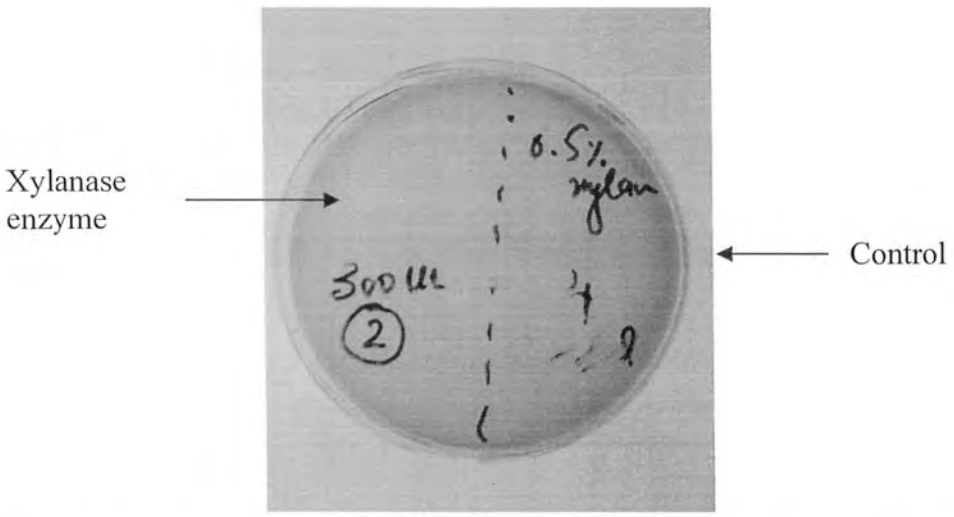


Fig: 14. Xylanase activity of recombinant *E. coli* strain BL21 on 1 % xylan LB agar plates in the presence of 1 mM IPTG inducer.

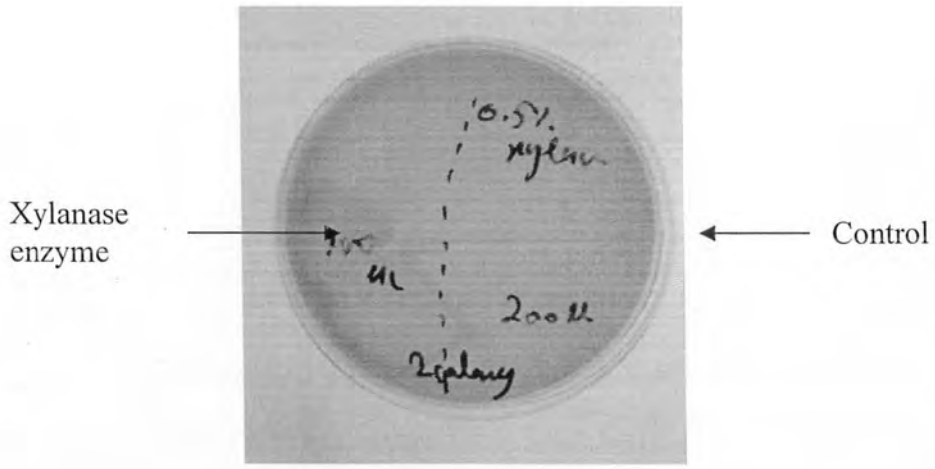
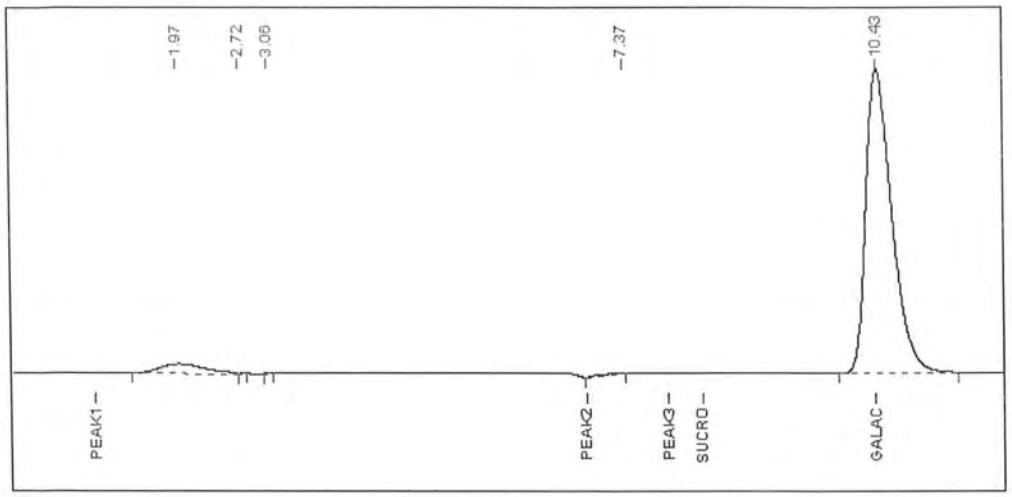
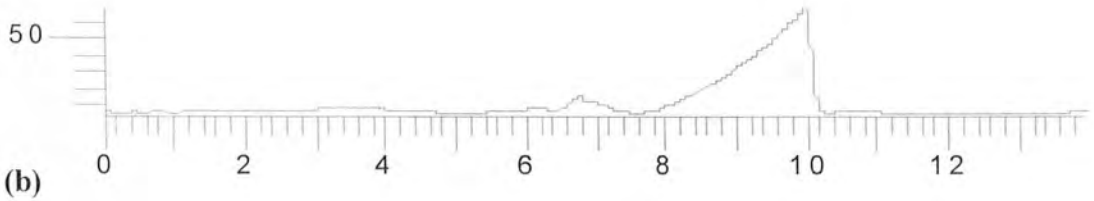


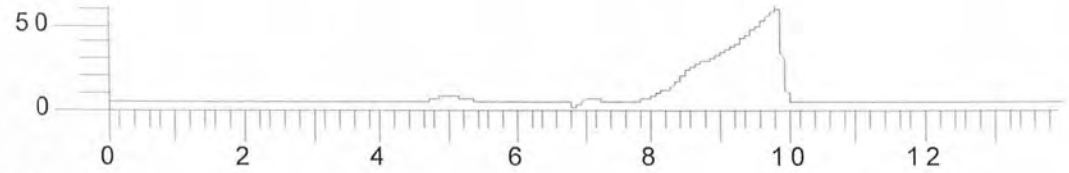
Fig: 15. Xylanase activity of recombinant *E. coli* strain BL21 on 1 % xylan LB agar plates in the presence of 1 mM lactose inducer.



(a)



(b)



(c)

Fig: 16. HPLC analysis for xylanase detection.
(a) Standard 1% xylose
(b) Substrate control
(c) Enzyme control.

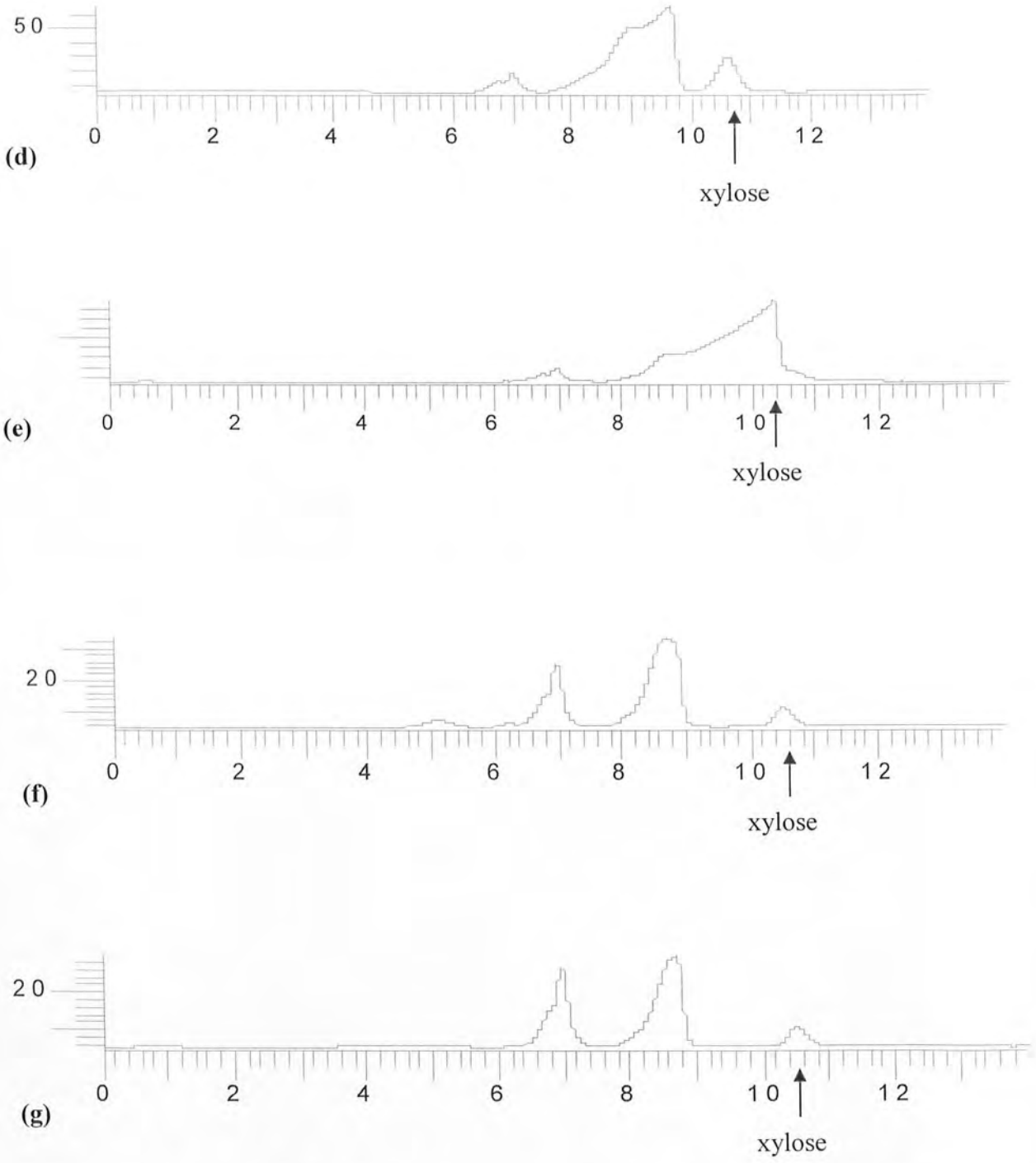
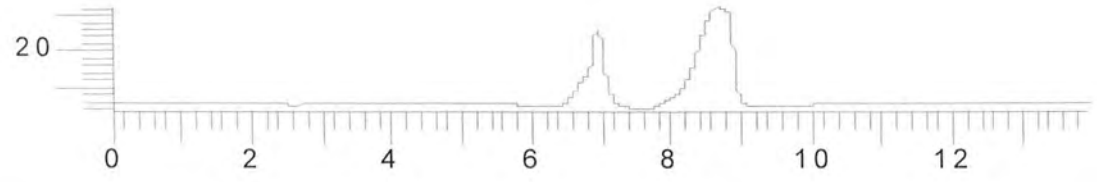
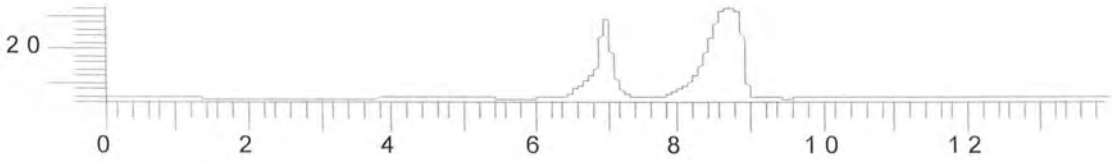


Fig: 16. HPLC analysis for protein detection. Samples induced by 1 mM IPTG are represented as (d), (e), (f) and (g).



(h)



(i)

Fig: 16. HPLC analysis for protein purification.

(h) pET 32a(+) control

(i) pSSZ810(b) control

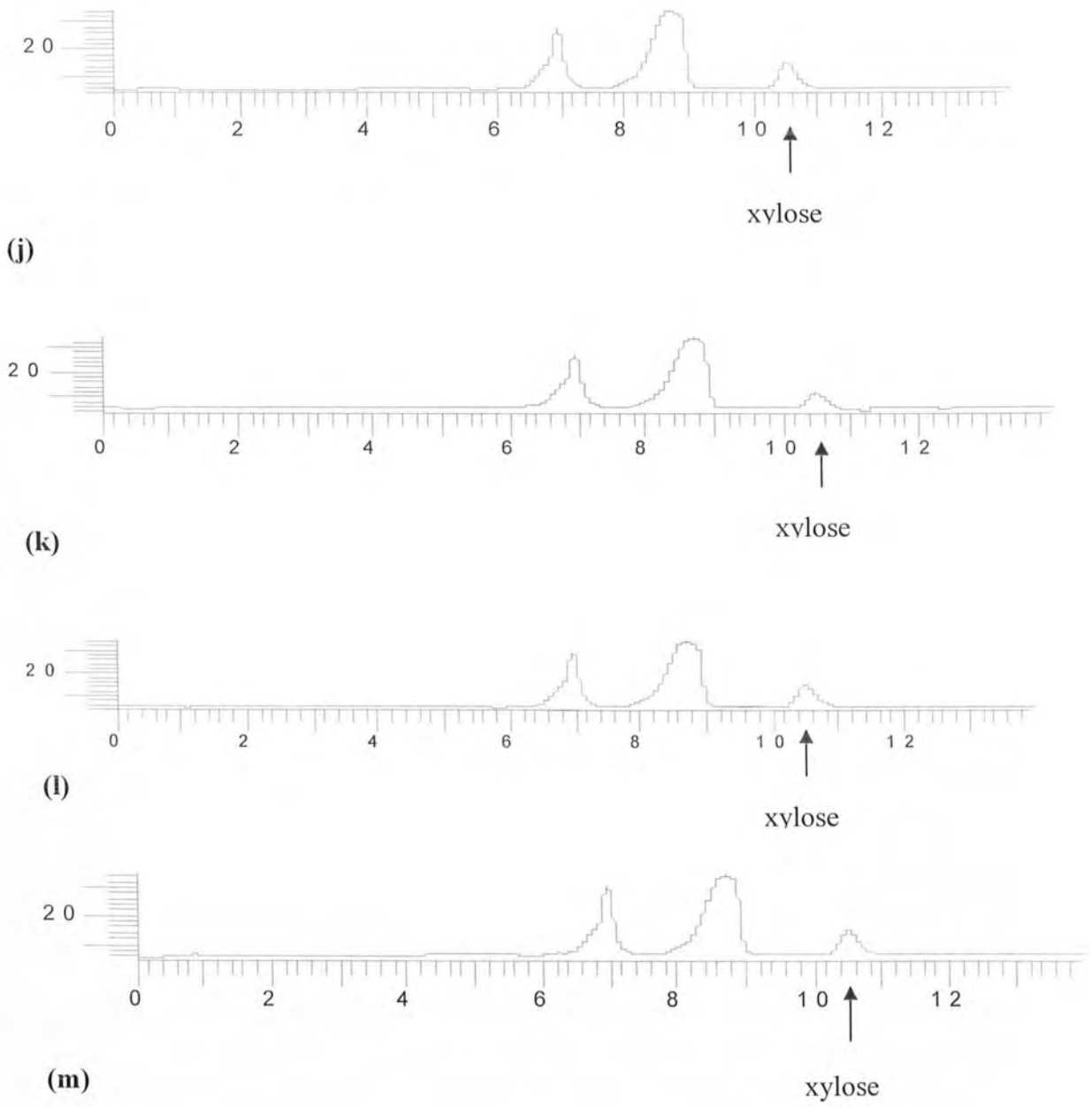


Fig: 16. HPLC analysis for protein detection. Samples induced by 1 mM lactose are represented as (j), (k), (l) and (m).

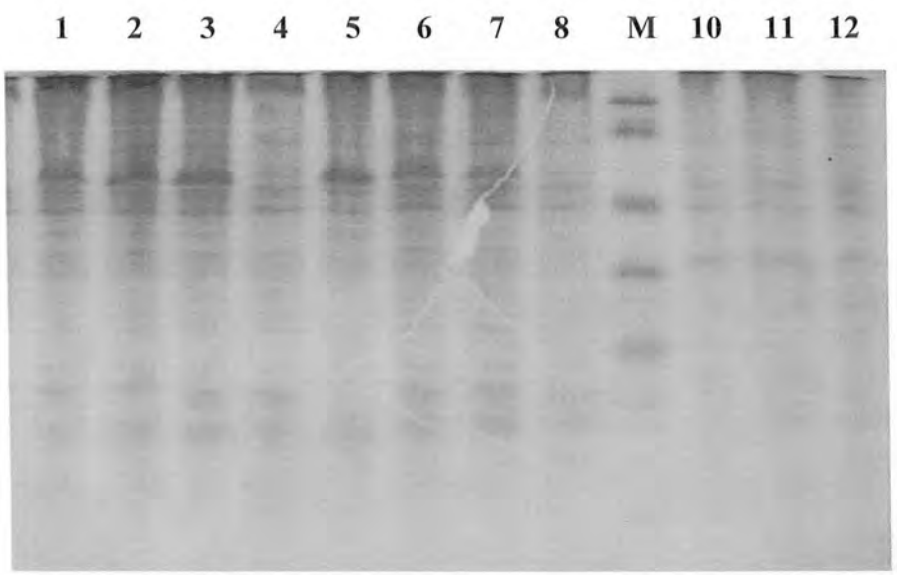


Fig:17. SDS-PAGE analysis of total protein isolated from bacterial strain BL21 transformed with pSSZ810(b) and pET 32a(+). Lanes 1-8: pSSZ810(b) transformed BL21. Lane 9: protein marker, Lanes 10-12: pET 32a(+) transformed BL21From right to left in Lanes 12, 8 and 4: protein induced after zero minute, Lanes 11, 7 and 3: protein induced after 30 minutes, Lanes 10, 6 and 2: protein induced after 60 minutes, Lanes 5 and 1: protein induced after 90 minutes. M: represent prestained protein marker #SM0441 118 k Da (MBI Fermentas).

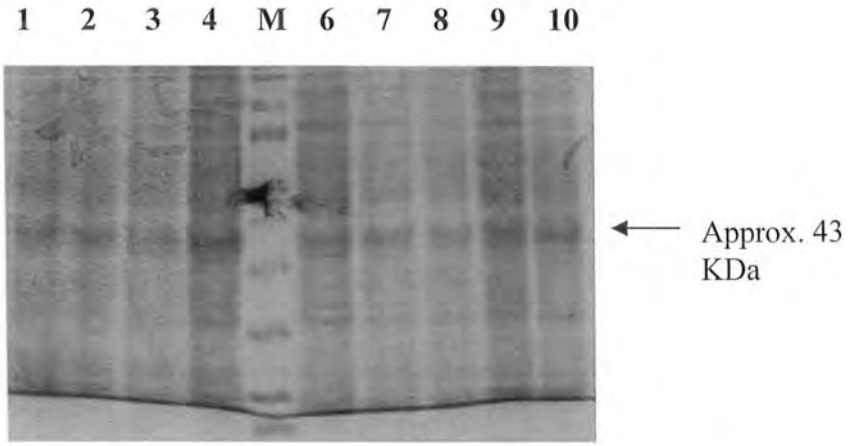


Fig: 18. SDS-PAGE of xylanase gene pSSZ810(b) vector transformed in *E.coli* BL21 strain. From left to right Lane 1 and 2: Sample 1 (induced by 1 mM IPTG + DS + pSSZ810(b) + 2 % xylose), Lane 3: Sample 2 (induced by 1 mM IPTG + DS + pET + 2 % xylose), Lane 4 and 6: Sample 5 and 3 (induced by 1 mM lactose + DS media + pSSZ810(b) + 2 % xylose), Lane 5: M (represents prestained protein marker (MBI Fermentas), Lane 7 and 8: Sample 6 and 7 (induced by 1 mM lactose + DS media + pSSZ810(b) + 2 % xylose), Lane 9 and 10: sample 8 and 10 (induced by 1 mM lactose + DS media + pSSZ810(b) + 2 % xylose)

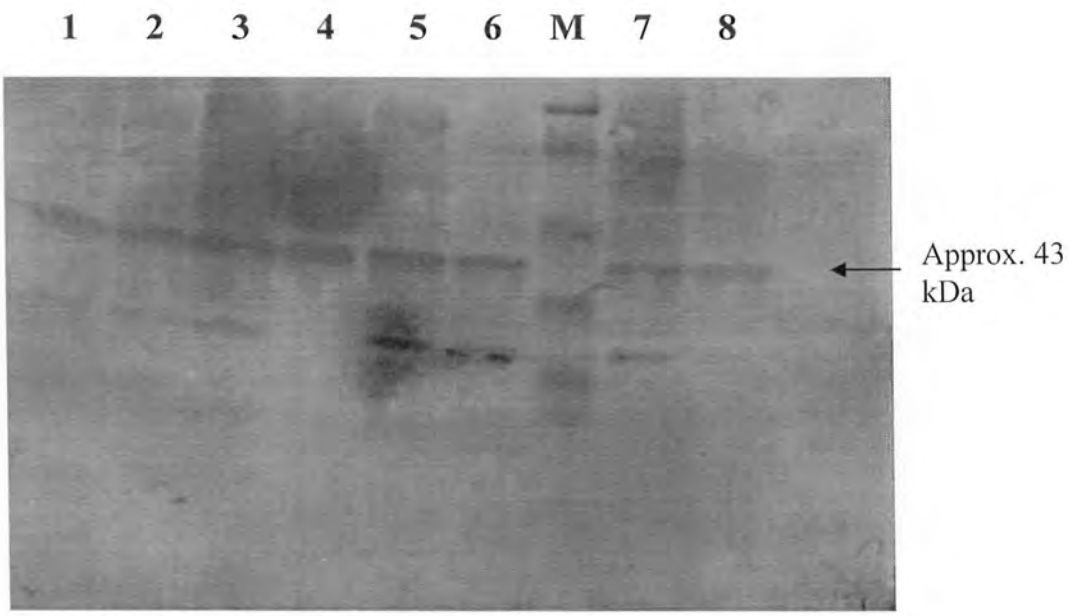


Fig: 19. Western blotting of xylanase gene pSSZ810(b) vector transformed in *E. coli* BL21 strain. From left to right Lane 1, 2 and 3: Sample 1-4 (induced by 1 mM IPTG + DS + pSSZ810(b) + 2 % xylose), Lane 4: Sample 5 (induced by 1 mM IPTG + DS + pET 32a(+) + 2 % xylose), Lane 5 and 6: Sample 7 & 8 (induced by 1 mM lactose + DS media + pSSZ810(b) + 2 % xylose), Lane 7 and 8: Sample 9 & 10 (induced by 1 mM lactose + DS media + pSSZ810(b) + 2 % xylose), M: represents prestained protein marker #SM0441 118 k Da (MBI Fermentas).

2.2: Cloning of xylanase gene Xyn 11-A gene into *Pichia pastoris* vector pPIC3.5K (yeast expression system)

2.1.1: Cloning of xylanase gene in pPIC3.5K

Xylanase gene from pSSZ810(b) was digested with *Eco*RI and *Not*I enzymes and cloned at the same sites in pPIC3.5K. The resultant vector was named as pSSZ810(c) as shown in Figure 23. pSSZ810(b) and pPIC3.5k were digested with *Eco*RI and *Not*I as shown in Figure 20. when pSSZ810(b) was digested with *Eco*RI and *Not*I, it should produced 810 bp fragment from the vector backbone of pET 32a(+) vector whereas pPIC3.5k produced a linear fragment of approx. 9000 bp as shown in (Fig: 20). Figure 20 indicates, *Eco*RI and *Not*I are unique restriction sites of pPIC3.5k in multiple cloning site whereas in pSSZ810(b) located on upstream and downstream of xylanase gene. Both fragments were eluted from agarose gel as shown in Figure 21. Figure 22 shows the elution of the target fragments of approx. 810 and 9000 bp, respectively when running on 0.5 % agarose gel electrophoresis and ligated at 16 °C.

2.2.2: Confirmation of clone pSSZ810(c) in E. coli TOP10F' strain

Ligation product was transformed into *E. coli* TOP10F' strain by heat shock method. The recombinant clone pSSZ810(c) was confirmed through digestion analysis. Upon digestion with *Eco*RI and *Not*I, recombinant clone pSSZ810(c) produced approx. 810 bp fragment along with vector backbone of pPIC3.5K of 9000 bp as shown in Figure 23. Xylanase gene has an internal *Kpn*I restriction site. Another *Kpn*I site is located in the vector backbone as shown in Figure 24. Therefore upon digestion with *Kpn*I pSSZ810(c) should produced two fragments of sizes 500 and 300 bp, respectively. When pSSZ810(c) was digested with *Kpn*I it produced two fragments of the expected sizes (i.e. 500 and 300 bp) as shown in Figure 24.

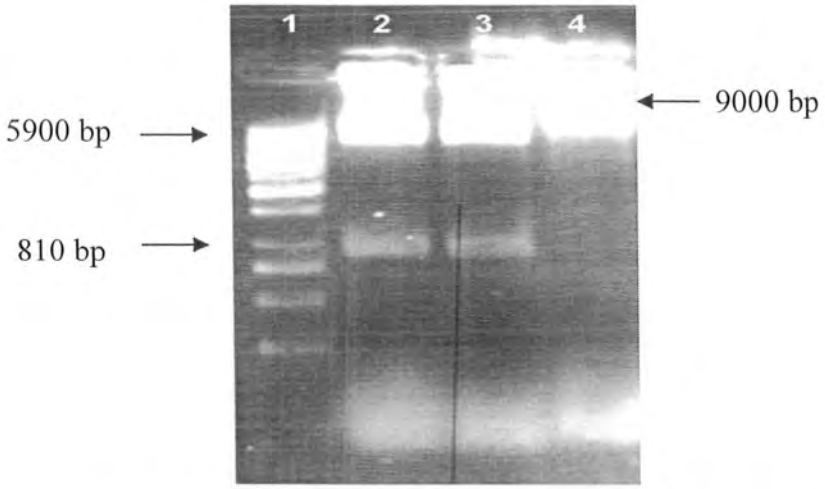


Fig: 20. pPIC3.5K and pSSZ810(b) were digested with *Eco*RI and *Not*I.

Lane 1: represents 1 kb DNA ladder,
Lane 2 & 3: represents pSSZ810(b) digestion
Lane 4: represents linearized pPIC3.5K

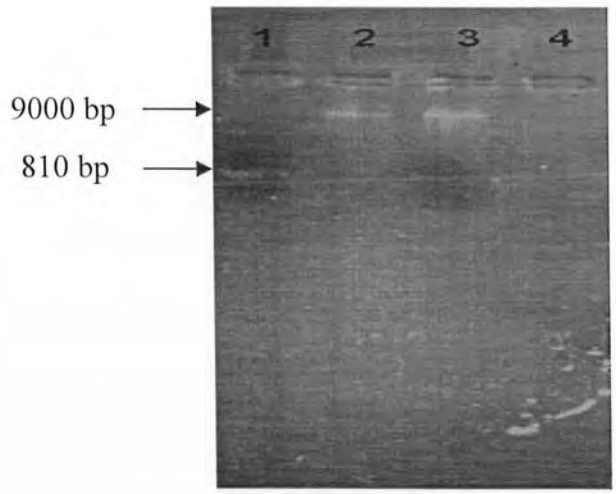


Fig: 21. pPIC3.5k and pSSZ810(b) eluted from 0.5% agarose gel.

Lane 1: represents eluted fragment,
Lane 2 & 3: represents eluted pPIC3.5k.

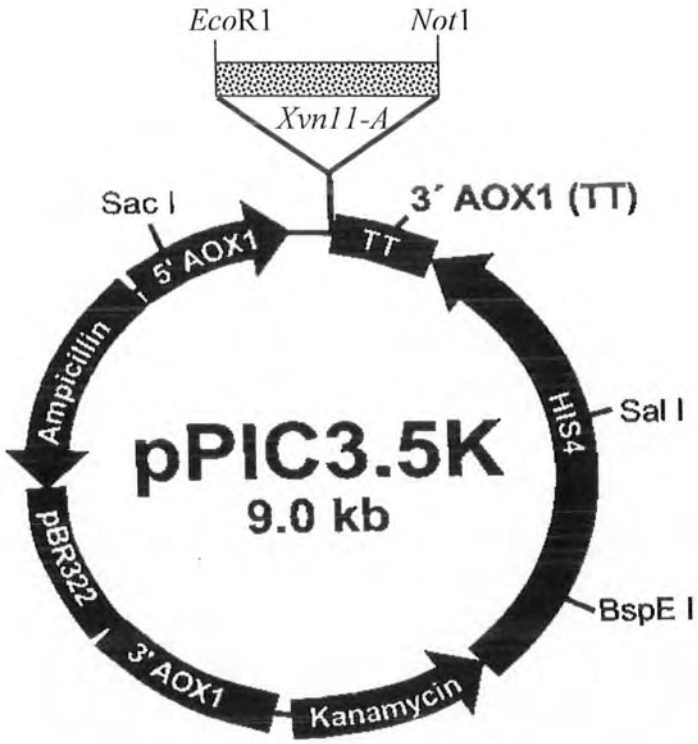


Fig: 22. Recombinanat clone pSSZ810(c) having *Eco*RI and *Not*I restriction sites.

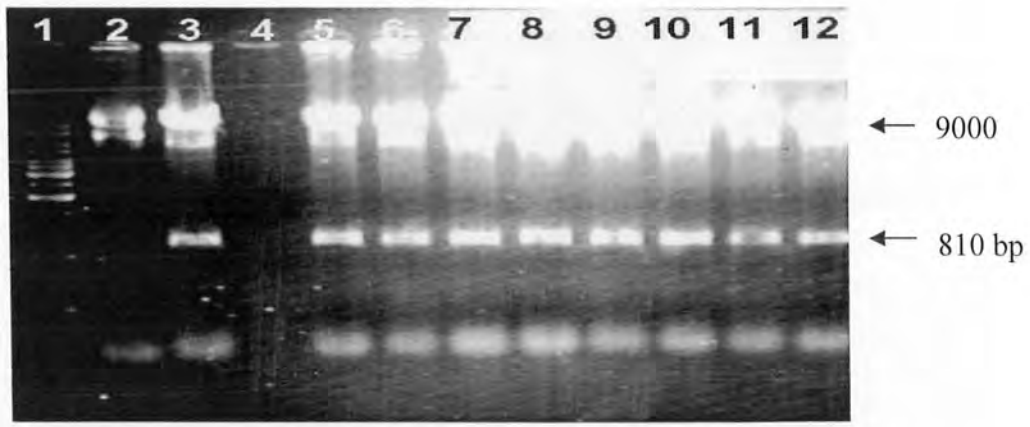


Fig: 23. pSSZ810(c) was confirmed through restriction/digestion with *Eco*RI and *Not*I restriction enzymes.

Lane 1: represents 1 kb DNA ladder,
Lane 2- 12: selected colonies.

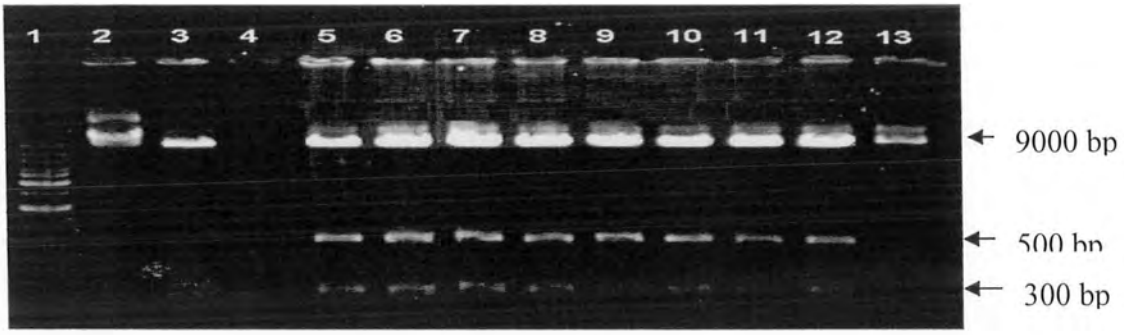


Fig: 24. Confirmation of pSSZ810(c) with digestion through *Kpn* I enzyme.
Lane 1 represents 1 kb DNA ladder,

2.3: Cloning and expression of xylanase gene Xyn 11-A gene in *Pichia pastoris* (yeast expression system) GS115 strain

2.3.1: Transformation of xylanase gene pSSZ810(c) in Pichia pastoris GS115 strain

pSSZ810(c) plasmid was isolated from TOP10F′ *E. coli* strain and linearized with *Not I* restriction enzyme as shown in figure 19. The linearized fragment was treated with phenol: chloroform for purification. The linearized fragment was transformed into *P. pastoris* strain GS115 through electroporation. Concentration of geneticin was optimized for the selection of transformants from 30, 50, 70, 90, 110, and 130 µg/ml as shown in Figure 28. Suitable concentration of geneticin antibiotic for selection of transformants was found to be 0.75 mg/mL. The transformants were grown on YPD agar media plates containing 0.75mg/ml geneticin antibiotic as shown in Figure 29.

2.3.2: Confirmation of the transformants in Pichia pastoris GS115 strain

The integration of linearized fragment pSSZ810(c) having xylanase gene in the genome of *Pichia pastoris* was confirmed through PCR amplification by using xylanase specific primers (appendix 2). Figure 26 shows the agarose gel electrophoresis of amplified 810 bp fragment confirming the transformants of *P. pastoris* with pSSZ810(c).

2.3.3: Confirmation of xylanase gene expression

The expression of xylanase gene in *Pichia pastoris* GS115 strain was confirmed through Bradford analysis, xylanase assay and SDS-polyacrylamide method, respectively.

i) Bradford analysis

The expressed protein of *Pichia pastoris* GS115 strain carrying xylanase gene in pSSZ810(c) was induced by the addition of 100 % methanol up to a final concentration of 0.5 % in YPD liquid media without antibiotic geneticin. The activity of expressed protein in mg/mL was determined by Bradford method is shown in Table 10. The maximum and minimum activity of protein after 48 and 96 hrs of induction were 0.394 and 0.015 mg/mL, respectively. The production of protein in recombinant vector

pSSZ810(c) in GS115 strain at different times of induction with 100 % methanol (i.e after 24, 48, and 96 hrs of growth, respectively) is shown in Table 10.

Table 10: Bradford analysis of xylanase gene pSSZ810(c) in *Pichia pastoris* GS115 strain

Time of induction *	Protein concentration (mg/mL) **			
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**
At zero hr	0.234	0.159	0.177	0.196
After 24 hrs	0.339	0.202	0.165	0.220
After 48 hrs	0.394	0.195	0.174	0.304
After 96 hrs	0.244	0.163	0.015	0.24

* 100 % methanol inducer

** **Sample 1-3:** transformants having pSSZ810(c) xylanase gene

Sample 4: non-transformant *Pichia pastoris* GS115 strain.

ii) Xylanase assay in Pichia pastoris GS115 strain

The production of the xylanase protein by recombinant *P. pastoris* having pSSZ810(c) was determined by using xylanase assay. The xylanase activity in *Pichia pastoris* GS115 strain was induced by 100 % methanol up to a final concentration of 0.5 % in Minimal Glycerol Media (MGM) broth as indicated in Table 11 and shown in Figure 27. The activity of xylanase by DNS method was obtained in U/mL. The maximum activity of xylanase gene (U/mL) was obtained after incubation of 2 hours at 50 °C. The maximum and minimum production of xylanase was 2.04 and 0.006 U/mL as compared to control 0.484 and 0.06 U/mL, respectively. Control only had *Pichia pastoris* GS115 strain.

Table 11: Xylanase activity in *Pichia pastoris* GS115 strain

Time of induction *	Xylanase activity (U/mL) **			
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**
At zero hr	0.769	0.242	0.5	0.06
After 24 hrs	0.424	0.632	0.006	0.484
After 48 hrs	2.04	0.014	0.151	0.242
After 96 hrs	0.06	0.175	0.133	0.436

* 100 % methanol inducer

** **Sample 1-3:** transformants having pSSZ810(c) xylanase gene

Sample 4: non-transformant *Pichia pastoris* GS115 strain.

2.3.4: HPLC analysis of xylanase protein

HPLC analysis was carried out to check the products formed from xylanase assay products by hydrolysis. The hydrolysis was carried out at 50 °C for 2 hrs. The pattern of free xylose sugar released from enzyme is shown in Figure 32) along with standard concentration of 1% xylose. The peak of free xylose was observed in *Pichia pastoris* transformants having recombinant vector pSSZ810(c) as compared to control (non-transformant).

2.3.5: Xylanase gene in Pichia pastoris GS115 strain by SDS-PAGE

The expression of targeted gene in *Pichia pastoris* transformants having recombinant vector pSSZ810(c) was induced by the addition of 100 % methanol in Minimal Glycerol broth media without geneticin. The samples were collected at different times of induction i.e after 24, 48, 72 and 96 hrs respectively. These samples were processed and analyzed on SDS-PAGE and analyzed by comassive blue staining. The intensity of desirable protein approx. 28 kDa was increased with increase in time after 100 % methanol up to a final concentration of 0.5 % induction as shown in Figure 30. The maximum activity of pSSZ810(b) having xylanase protein was observed in lane

12 at after 96 hrs of induction period whereas there is no band was observed in case of non-transformant *Pichia pastoris* GS115. Figure 31 represents SDS-PAGE silver staining of xylanase protein from *P. pastoris* GS115 strain having recombinant vector pSSZ810(c). The intensity of desired protein 28 kDa is shown in Figure 31 lane 1 and 2.

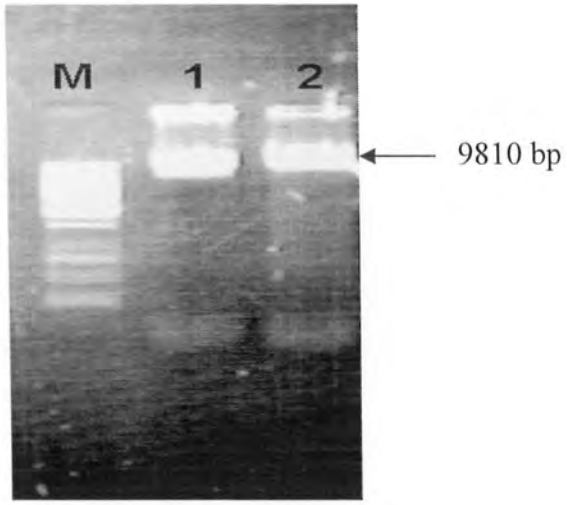


Fig: 25. pSSZ810(c) was linearized with *Not*I restriction enzyme.
Lane 1: represents pSSZ810(c) digested with *Not*I
Lane 2: represents pPIC3.5K vector digested with *Not*I
M: represents 1 kb DNA ladder.

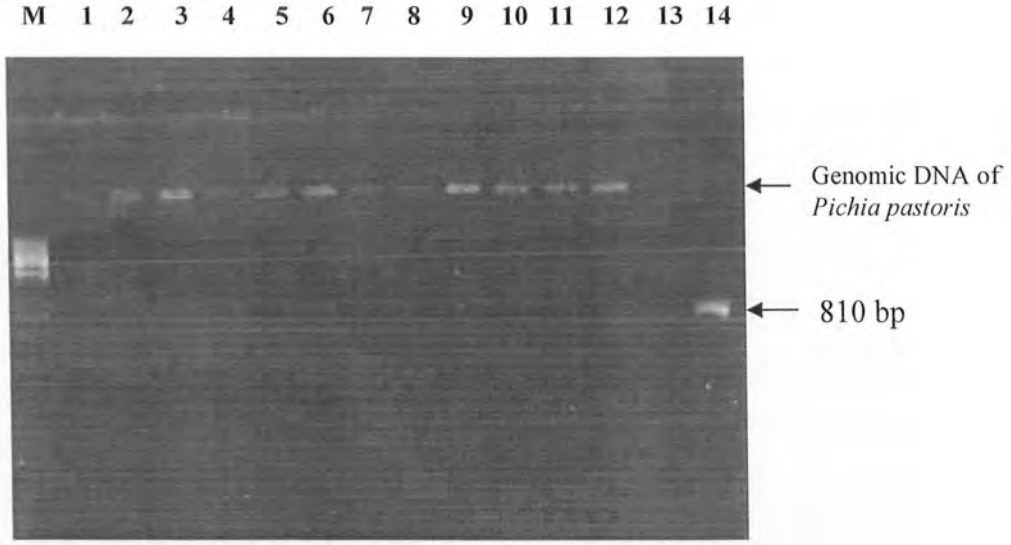


Fig: 26. Agarose gel electrophoresis of integration of PCR amplified fragments from *Pichia pastoris* DNA.

Lane 1: 1 kb DNA ladder marker
Lane 2-12: *P. pastoris* DNA isolated from selected colonies
Lane 13: negative control (water control)
Lane 14: positive control having xylanase gene

Fig: 27. Xylanase production by *Pichia pastoris* strain GS115 at different time periods:

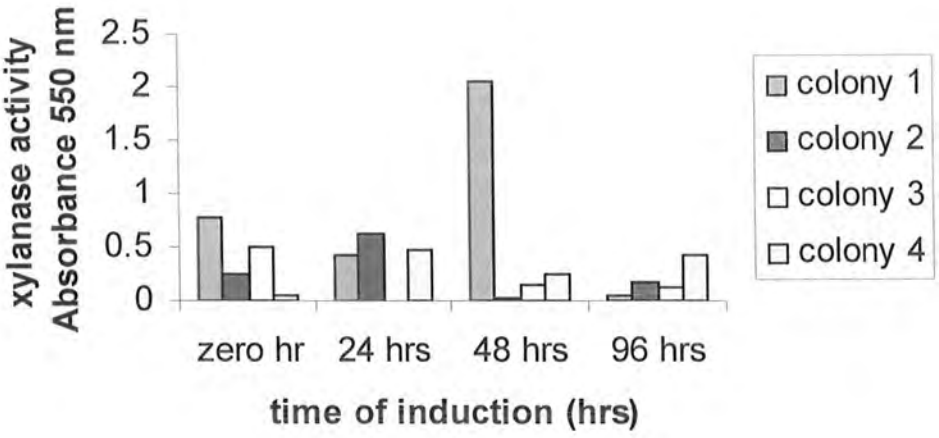


Fig: 27. production of xylanase at different time period:

Colony 1: having pSSZ810(c) at zero, 24, 48 and 96 hrs.
Colony 2: having pSSZ810(c) at zero, 24, 48 and 96 hrs.
Colony 3: having pSSZ810(c) at zero, 24, 48 and 96 hrs.
Colony 4: without pSSZ810(c) at zero, 24, 48 and 96 hrs.

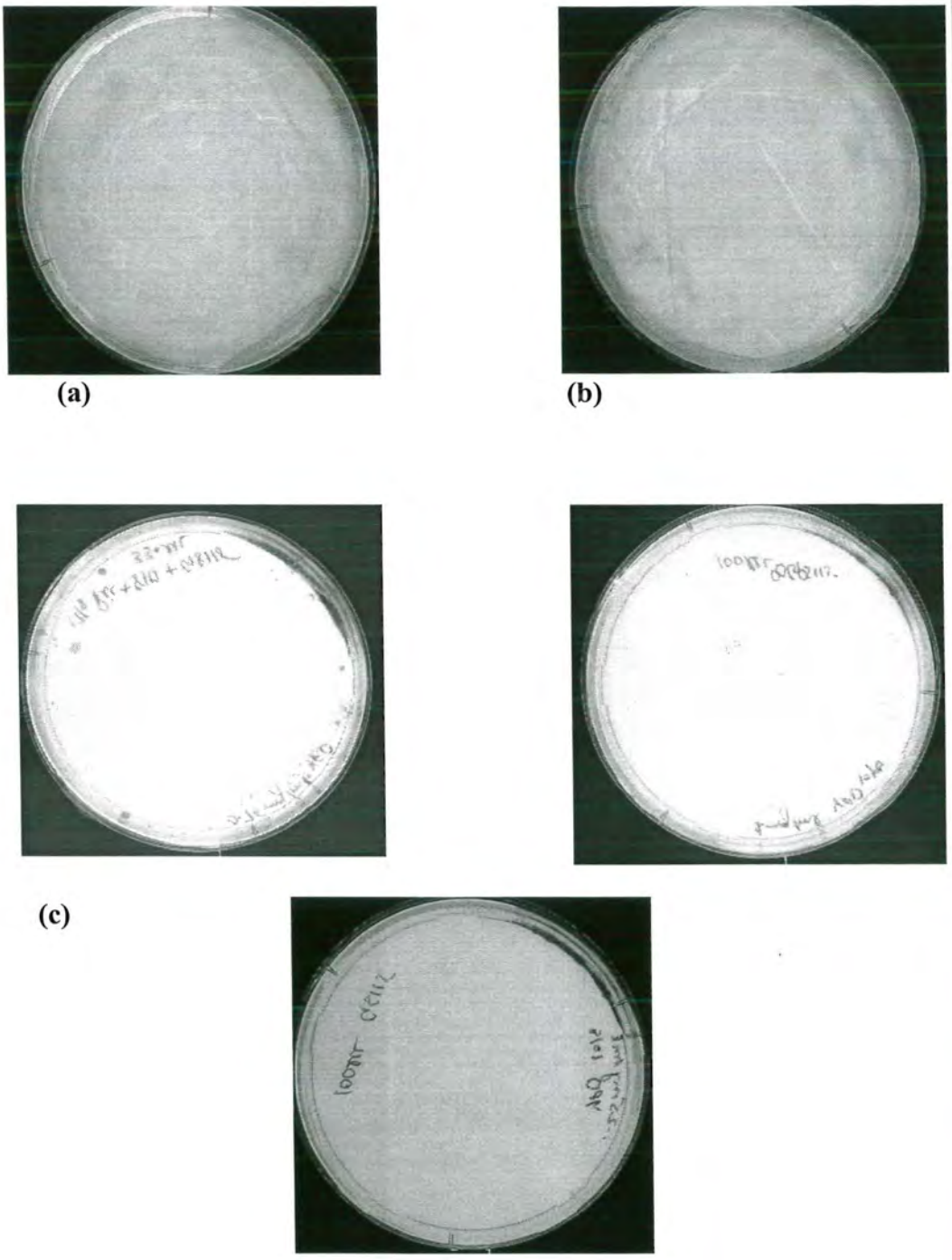
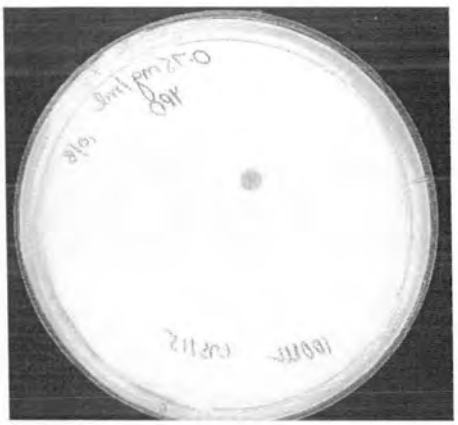


Fig: 28. Optimization of antibiotic geneticin for selection of *Pichia pastoris* transformants. (a): 0.25 mg/mL; (b): 0.5 mg/mL; (c): o.75 mg/mL; (d): 1.0 mg/mL; (e): 1.25 mg/mL.



(a)

(b)

(c)

Fig: 29. Selection of transformants on YPD agar media supplemented with 0.75 mg/mL geneticin.

(a) Represents colony of *Pichia pastoris* strain GS115,
(b) and (c) represents transformants having pSSZ810(c).

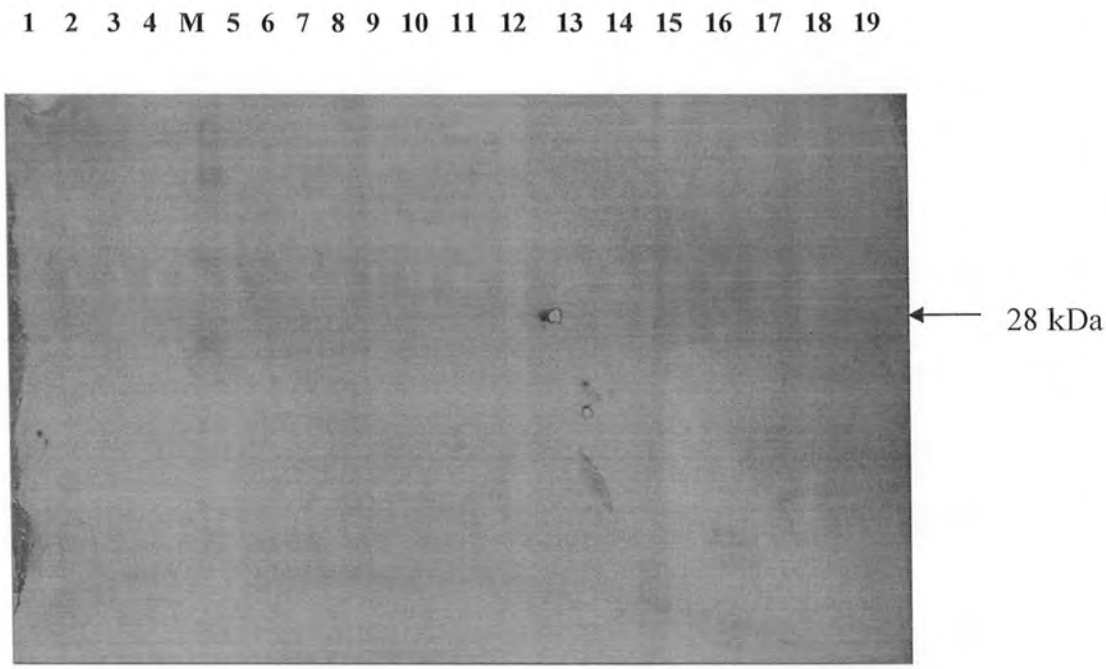


Fig: 30. SDS-PAGE analysis for detection of xylanase protein from *Pichia pastoris* strain GS115 having pSSZ810(c). Lane 1-4: Represent non-transformant GS115. Lane 5-19: Represent transformants having pSSZ810(c) M: represents prestained protein marker 118 k Da (MBI Fermentas).

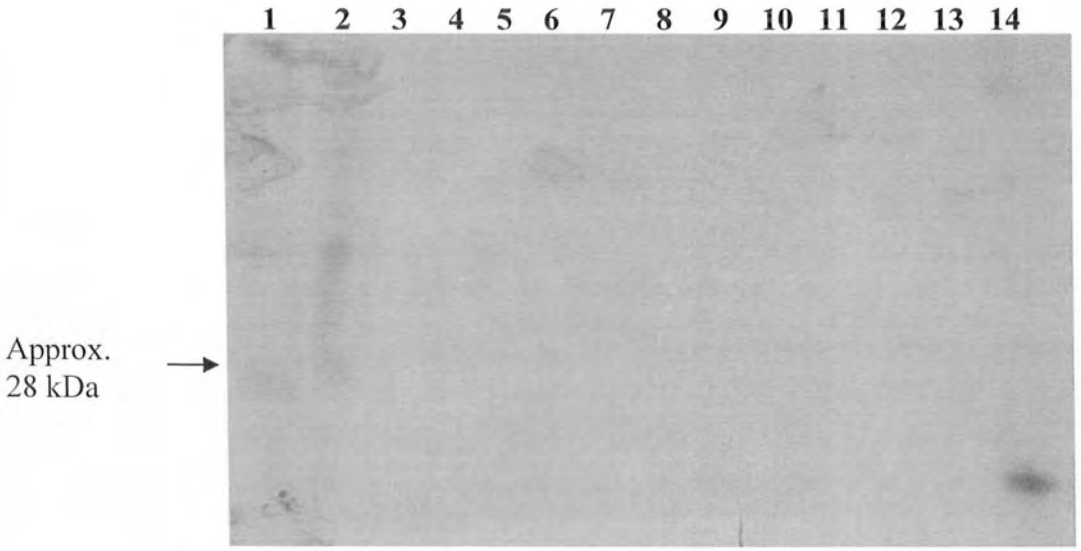
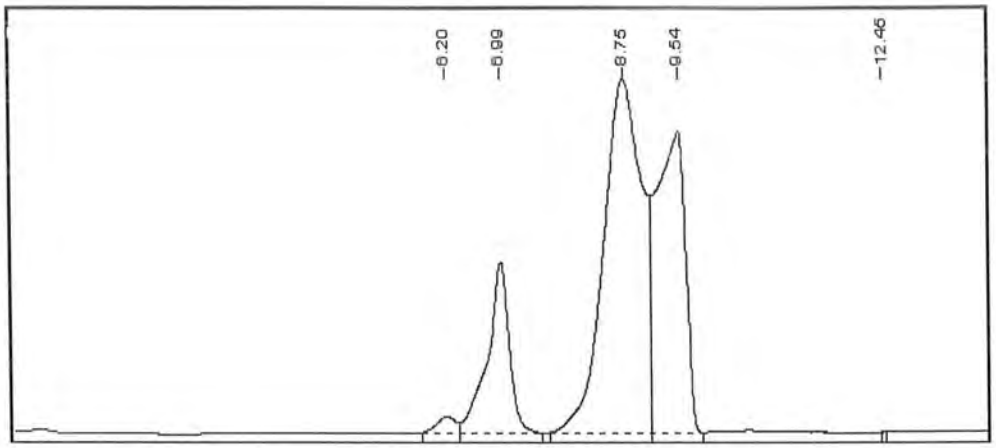
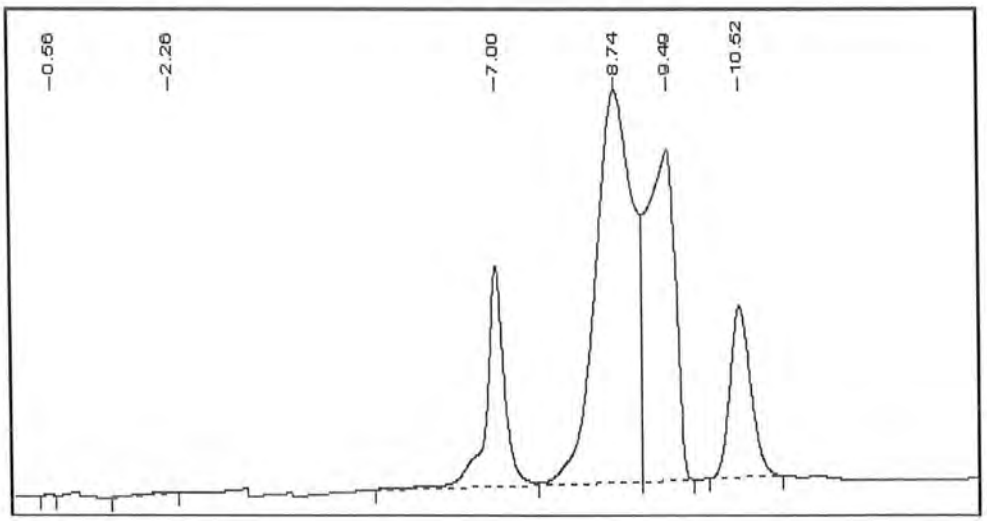


Fig: 31. SDS-PAGE silver staining analysis for detection of xylanase protein from *Pichia pastoris* strain GS115 having pSSZ810(c). From left to right, Lane 1- 14: Represents transformants of *Pichia pastoris*, M: Represents prestained protein marker 118 k Da.



(a)



(b)

Fig: 32. HPLC analysis of xylanase gene pSSZ810(c) detection in *Pichia pastoris* GS115 strain:

(a): Non-transformant (GS115 strain)
(b): Transformant (GS115 having xylanase gene pSSZ810(c))

Chapter No.4

Discussion

Chapter 4

Discussion

The objective of the present study was to express xylanase gene (xyn11-A) isolated from *Chaetomium thermophile* in heterologous systems such as *E. coli* and *Pichia pastoris*.

At NIBGE there is a large collection of thermophilic fungi (Latif *et al.* 1995), which have potential application in the poultry feed, textile, detergent and paper and pulp industry. *Chaetomium thermophile* shows large amounts of extra-cellular cellulase and xylanase activity when grown on cellulosic or lignocellulosic substrates as carbon sources (Latif *et al.* 1994). Xylanases have been grouped into glycoside hydrolase families F/10 and G/11 (Kuno *et al.* 1998), corresponding to families F and G, respectively (Henrissat, 1993). Family 11 xylanases have smaller size approximately 20 kDa and their fold contains a α- helix and two β- sheets packed against each other, forming so called β sandwich.

In case of *Chaetomium thermophile,* xylanase production needs improvements for commercial point of view. Other heterologous systems, suitable for industrial applications, were used for expression of xylanase. Different genes of thermophilic fungi encoding lipase, protease, xylanase and cellulase have been cloned and over-expressed in heterologous fungi (Maheswari *et al.*, 2000).

In the present study, prokaryotic (*E. coli* strain BL21 having pET32a(+) vector) as well as eukaryotic (*Pichia pastoris* strain GS115 having pPIC3.5K vector) expression systems were used as model organisms for expression of xylanase gene. A number of pET vectors, which are active in bacteria were originally constructed by Studier and colleagues. Two general types of pET vectors are available: transcription vectors and translation vectors. (Studier and Moffatt 1986). Transcription vectors are designed for expression of target genes that already carry their own prokaryotic ribosome binding site

and AUG start codon. (Kozak, 1984) Translation vectors contain the highly efficient ribosome binding site from the phage T7 major capsid protein and are used for the expression of target genes without their own ribosomal binding site. The choice of a pET vector for expression of a heterologous gene usually involves a combination of factors. For examples (a) The application intended for the expressed protein (b) Specific information known about the expressed protein (c) Cloning strategy. Applications for proteins expressed in pET vectors vary widely. For example, analytical amounts of a target protein may be needed for activity studies, screening and characterizing mutants, screening for ligand interactions, and antigen (for antibodies) preparation. Large amounts of active protein may be required for structural studies, activity, use as a reagent, or affinity matrix preparation.

Heterologous expression in *Pichia pastoris* has many of the advantages of eukaryotic expression, proper folding and disulfide bond formation, glycosylation, and secretion. The *Pichia pastoris* heterologous gene expression system has been utilized to produce attractive levels of a variety of intracellular and extracellular proteins of interest. Recent advances had improved its utility that include: (1) methods for the construction of *P. pastoris* strains with multiple copies of AOX1-promoter-driven expression cassettes; (2) mixed-feed culture strategies for high foreign protein volumetric productivity rates; (3) methods to reduce proteolysis of some products in high cell-density culture media; (4) tested procedures for purification of secreted products; and (5) detailed information on the structures of N-linked oligosaccharides on *P. pastoris* secreted proteins (Cregg *et al.*, 1993).

The increasing popularity of this particular expression system can be attributed to several factors, most importantly: (1) the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris* and their similarity to those of *Saccharomyces cerevisiae*, one of the most well-characterized experimental systems in modern biology; (2) the ability of *P. pastoris* to produce foreign proteins at high levels, either intracellularly or extracellularly; (3) the capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and

proteolytic processing; and (4) the availability of the expression system as a commercially available kit (Cereghino *et al.*, 2000).

A further benefit of the *P. pastoris* system is that strong promoters are available to drive the expression of a foreign gene(s) of interest, thus enabling production of large amounts of the target protein(s) with relative technical ease and at a lower cost than most other eukaryotic systems. Daly and Hearn (2005) studied the different features and developments under the influences of *P. pastoris* strain selection; the choice of expression vectors and promoters; procedures for the transformation and integration of the vectors into the *P. pastoris* genome; the consequences of rare codon usage and truncated transcripts; and techniques employed to achieve multi-copy integration numbers. The impact of the alcohol oxidase (AOX) pathways in terms of the mut+ and mut(s) phenotypes, intracellular expression and folding pathways was examined.

Li *et al.* (2005) demonstrated the expression of mMR-1 protein in *Pichia pastoris* by induction under the concentration of 0.5 % methanol. The expression level of this recombinant mMR-1 protein was about 50 mg/L.

In the present study maximum activity of *Chaetomium thermophilum* xylanase gene expression was observed after incubation of 2 hrs at 40 °C in the presence of 1 mM IPTG and 1 mM lactose inducers in prokaryotic expression system (*E. coli)* and eukaryotic system (*Pichia pastoris)* in the presence of 100% methanol inducer and after incubation of 2 hrs at 50 °C. The maximum activity was observed in case of *Pichia pastoris* as shown in HPLC analysis.

It was observed that a secreted recombinant protein ovine interferon-tau (r-oIFN-tau) was produced in *Pichia pastoris* during fermentation. The protein was degraded increasingly after 48 h of induction and the rate of degradation increased towards the end of fermentation at 72 h, when the fermentation was stopped. Proteases, whose primary source was the vacuoles, was found in increasing levels in the cytoplasm and in the fermentation broth after 48 h of induction and reached maximal values when the batch

was completed at 72 h. Protease levels at various cell fractions as well as in the culture supernatant were lower when glycerol was used as the carbon source instead of methanol. It can be concluded that methanol metabolism along with cell lysis towards the end of fermentation contributes to increased proteolytic activity and eventual degradation of recombinant protein. In our case the protein was degraded after 96 hrs of induction, and degradation kept on increasing till 120 hrs of induction.

Xylanase protein is also detected by HPLC analysis in both prokaryotic and eukaryotic expression systems. Eukaryotic expression system represents greater peak as compared to prokaryotic system (*E. coli)* because *Pichia pastoris* was developed for high level expression of recombinant protein. *Pichia pastoris* has many of the advantages of higher eukaryotic systems such as protein processing, protein folding and posttranslational modification. It is easily to manipulate as *E.coli* or *Saccharomyces cerevisiae*. It is faster, easier and less expensive to use than other eukaryotic expression systems such as baculovirus or mammalian tissue culture, and generally gives higher expression levels. *Pichia* is very useful as a protein expression system.

The maximum xylanase activity was observed in the presence of 100% methanol inducer and after incubation of 2 hrs at 50 °C is lower as compared 2 mg/L to previous studies (Bottner and Land, 2004).

4.1: CONCLUSION:

Although *Chaetomium thermophile* produces xylanase enzyme but due to its slow growing nature and likelihood of producing many toxic compounds makes it unfit for industrial application. On the other hand model systems like *E. coli* and *Pichia pastoris* are fast growing, easy to handle and above all do not produce toxic compounds. So these organisms can be easily used for industrial application. Although the expression of xylanase protein reported in the present study is much lower than the reported potential of these organisms for expression of heterologous proteins but it can be enhanced by further modifications and studies.

4.2: FUTURE PROSPECTS:

- Production of protein at high scale level on bioreactors is relatively easy and can be upscale up to any level.
- Production of protein will be pure and can be maximized by multiple copy gene integrations.
- Western blotting, Dot blotting and southern analyses will be used for better analysis of xylanase Xyn 11-A in *Pichia pastoris*.

Chapter No.5

References

Chapter 5

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Chapter No.6

Appendices

APPENDICES

Appendix# 1

Composition of the growth medium

The growth medium used in the research work was according to the method of Eggins and Paugh (1962) as follows:

Components	**Quantity g/L**
Yeast extract	0.5
L. asparagines	0.5
$MgSO_4$	0.2
KH_2PO_4	1.0
$(NH_4)_2SO_4$	0.5
$CaCl_2$	0.1
KCl	0.5

Additionally according to the requirement following ingredients can be added.

Ball mill cellulose (to maintain spores)	2 %
Agar (for solid medium)	2.5 %
Glucose (for growth medium)	1.0 %
Rose Bengal	30 µg/mL

Adjust pH = 5.0 and autoclave for 20 minutes.

Appendix# 2

Primers

Xyn 11-A (F): 5´- GGC GAT AGC TAG CAT GGT CAA CTT CTC AAC TCTC -3´(34 mers)

Xyn 11-A (R): 5´- GGA AGG GCC CGC ACT GCA TGC TTG TTA GC -3´

Xyn 1 (P_1): 5´- GCA TGG TCA ACT TCT CAA CTC) 21 mers

Xyn 3 (P_3): (AGA CTC GAG TCG AAC CCC GGT ATC GAC) 27 mers

Xyn (P_4): (CTG CTC GAG GCG CTG GAA ATG TTT TGT TGG) 30 mers.

Appendix#3

Agarose Gel Electrophoresis

DNA fragments were separated by electrophoresis on 1 % (w/v) agarose gels in 0.5 X TAE buffer containing ethidium bromide (10 mg/ml). Fragment sizes were estimated by comparison with Fermentas 1kb DNA ladder. Fermentas 6 X DNA loading dye was used.

50X Tris-acetate EDTA buffer (TAE)

1. Tris base	242 gm
2. Glacial acetic acid	57.1 ml
3. 0.5 M EDTA (pH 8.0)	100 ml

Make up the final volume with distilled water to 1000 ml.

6X Gel Loading Buffer

1. Bromophenol blue	0.25% (w/v)
2. Xylene cyanol FF.	0.25% (w/v)
3. Glycerol	30.0% (v/v)

Dissolve in distilled water.

Appendix # 4

RAPID GEL EXTRACTION PROTOCOL

NOTE:

Perform all centrifugation at room temperature.

Before beginning preheats an aliquot of TE to 65 to 70 °C. Equilibrate a water bath or heat block to 50 °C. Verify that ethanol has been added to wash Buffer (L2).

1. GEL SLICE EXCISION

Cut the area of gel containing the DNA fragment using a clean, sharp blade. Minimize the amount of surrounding agarose excised with the fragment.

2. GEL SLICES WEIGHING

Weigh the gel slice.

a. For $\leq 2\%$ agarose gel, place up to 400 mg of gel into a 1.5 ml polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 30 µl of Gel solubilization Buffer (L1) for every 10 mg of gel.

b. For $\geq 2\%$ agarose gel, place up to 400 mg of gel into a 5 ml polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 60 µl of Gel solubilization Buffer (L1) for every 10 mg of gel.

3. GEL SOLUBILIZATION

Incubate at 50 °C for $\geq$ 15 min. Mix every 3 min to ensure gel dissolution. After gel slice appear dissolved, incubate for 5 min longer.

4. CARTRIDGE LOADING

Place a spin cartridge into a 2 ml wash tube. Pipette the mixture from step 3 into the spin cartridge. Centrifuge the mixture in a microcentrifuge at > 12,000 rpm for 1 min. Discard the flow through.

Note: Load no more than 400 mg agarose per cartridge.

5. (OPTIONAL CARTRIDGE WASH)

Place the spin cartridge back into the 2 ml wash tube. Add 500 µl Gel Solublization Buffer (L1) to the spin cartridge. Incubate at room temperature for 1 min, then centrifuge at > 12,000 rpm for 1 min. Discard the flow through.

6. CARTRIDGE WASH

Place the spin cartridge back into the 2 ml wash tube. Add 700 µl of Wash Buffer (L2) (containing ethanol) to the spin cartridge and incubate for 5 min at room temperature. Centrifuge at > 12,000 rpm for 1 min. Discard the flow through. Centrifuge again for 1 min to remove residual wash buffer.

7. DNA ELUTION

Place the spin cartridge into a 1.5 ml recovery tube (supplied). Add 50 µl of warm TE Buffer (TE) directly to the center of the spin cartridge. Incubate for 1 min at room temperature, then centrifuge at > 12,000 rpm for 2 min.

Appendix# 5

Competent cell formation method

Chemical method

1. A single colony was picked from culture plate and inoculated in 25 mL LB medium (appendix 7), the flask was incubated at 37 °C with shaking overnight.
2. 1 mL was transferred in 250 mL LB medium (appendix 7) and shake at 37 °C until, the optimal density reached up to 0.4-0.5.
3. The culture was divided in two 50 mL falcon tubes.
4. Centrifuged cells at 5000 rpm for 5 minute at 4°C.
5. Supernatant was discarded and pellet was resuspended very gently in 5 mL $MgCl_2$.
6. Centrifuged at 3000 rpm for 5 minutes at 4°C.
7. Supernatant was discarded and pellet was gently resuspended in 5 mL of $CaCl_2$.
8. Keeping the tubes in ice for 30 minutes, with gentle shaking after every 5 minutes.
9. Centrifuged at 3000 rpm for 5 minutes at 4°C.
10. Supernatant was discarded and resuspended pellet in 2 mL of $CaCl_2$ and 0.7 mL of 100 % glycerol.

11. Dispensed 200 µL in 1.5mL eppendorf tubes. Immediately cells were immersed in liquid nitrogen and were stored at – 70 °C.

Note:

Whole process was done on ice and in laminar flow.

Appendix # 6

Heat shock transformation method

E. coli 10b was used for making heat shock competent cells, following the procedure modified by Cohen *et al.* 1972. Competent cells were stored at -70°C for use in future.

1. For bacterial transformation the competent cells were allowed to thaw on ice.
2. Added 5 µl of ligation reaction in 200 µL heat shocked competent cells.
3. Heat shock of 42°C was given for one and half minutes (Sambrook *et al.*1989).
4. Immediately transferred these cells on ice for 10 minutes.
5. 800 µl of LB medium was added without antibiotic and incubated the culture at 37°C for 1 hour.
6. This culture was spread on LB agar plates (appendix 7) containing ampicillin (100 mg/mL) and placed at 37°C for overnight.

Appendix # 7

LB (LURIA-BERTANI) MEDIUM

For 500 mL medium

Components	**Quatity**
1.0% Tryptone	5 g
0.5% Yeast extract	2.5 g
0.5% NaCl	2.5 g
1.5% Agar	7 g
1% Xylan (in case xylan plates)	4 g

Adjust pH to 7-7.2 and autoclave.

Appendix # 8

PLASMID ISOLATION FROM *E. coli*

ALKALINE LYSIS METHOD

Mini Prep Solutions

Solution I (Suspension buffer)

Tris (pH 7.4-7.6)	50 mM
EDTA	1 mM
RNase	100 µg/ml

Solution II (Denaturation soln.)

NaOH	0.2 N
SDS	1 %

Solution III (Neutralization soln.)

Potassium acetate	3 M
Glacial acetic acid	11.5ml/100ml
(pH 4.8-5.0)	

Mini Prep

Following protocol was used for the isolation of plasmid DNA from E. coli.

1. A single *E. coli* colony was cultured in 3 ml liquid LB medium containing ampicillin or kanamycin and grown overnight at 37 °C.
2. The E. coli culture was centrifuged in 1.5 ml Eppendorf tube at 14000 rpm for 1 minute.
3. The supernatant was discarded and the pellet was allowed to dry on tissue paper.
4. 200 ml of solution I (Appendix 8) was added to Eppendorf tube and the pellet was suspended in the solution with the help of vortex.
5. 200 ml of solution II (Appendix 8) was added to Eppendorf tube and mixed well by inverting gently and incubate for 5 minutes at room temperature.

6. 200 ml of solution III (Appendix 8) was added to Eppendorf tube mixed well and incubate for 5 minutes at room temperature then centrifuged at 14000 rpm for 5 minutes.
7. The supernatant was taken in fresh Eppendorf tube and two volume of 100% ethanol were added.
8. Eppendorf tube was kept at -20 °C for 20 minutes and then centrifuged at 14000 rpm for 10 minutes.
9. The supernatant was discarded and the pellet was washed with 70% ethanol and pellet was air dried.
10. 20 ml of sterile distilled water was added to the pellet to dissolve DNA and was stored at -20 °C.

    The plasmid concentration and quality were detected by 1% agarose gel using stranded DNA markers.

Appendix # 9

Digestion of pSSZ810(b)

Digestion reaction 1:

	1Rx (µl)
DNA	4.0
HIND III	2.0
EcoRI	1.0
Buffer (y+ tango)	4.0
RNAase	2.0
d_3H_2O	7.0
	20.0

Digestion reaction 2:

1Rx (µl)

DNA	4.0
HIND III	1.0
Xho1	1.0
Buffer (R+)	2.0
RNAase	2.0
d_3H_2O	10.0
	20.0

Digestion reaction 3:

	1Rx (µl)
DNA	4.0
HIND III	1.0
Xba1	1.0
Buffer (y+ tango)	2.0
RNAase	2.0
d_3H_2O	10.0
	20.0

This digestion was placed at 37°C in an incubator for 2-3 hrs, run on (0.8%) agarose gel.

Appendix # 10

pSSZ810(b) PCR profile

positive control = gene of interest.

Negative control= water control

Denaturation = 94˚C for 5 min

Annealing = 60˚C for 1 min

Extention = 72˚C for 1 min

Extention = 72˚C for 10 min

Hold temperature = 22˚C

No. of cycles = 35

Appendix # 11

Bradford reagent

Dissolved 100 mg of coomassie brilliant blue G-250 in 50 ml of 95 % ethanol. Then added 100 ml of 85% concentrated phosphoric acid and the volume was made to 1 liter. It was filtered through whatman filter paper No. 1. The Bradford reagent was stored at 4°C.

Appendix # 12

1) Citrate phosphate buffer (pH = 5)

24.3 mL 0.1M citric acid and 25.7 mL disodium hydrogen phosphate (Na2HPO4) were mixed with magnetic stirrer and pH was adjusted to 5 by citric acid or Na2HPO4.After pH adjusting the volume was made to 100 mL distilled water.

2) Preparation of DNS (Dinitrosalicylic Acid) solution

Different ingredients were used for the preparation of DNS. These were as follows:

i.	distilled water	1416 mL
ii.	3-4, Dinitrosalicylic acid	10.6g
iii.	NaOH	19.5g

The above ingredients were dissolved and gently heated in water bath at about 80 C until a clear solution was obtained. Then the following chemicals were added:

i.	Rochelle salt	19.5g
ii.	Phenol(melted at 60 C)	7.5mL
iii.	Sodium metabisulfate	8.3g

After dissolving all the above ingredients, the solution was filtered through a large coarse sintered glass filter and stored at room temperature in an amber colored bottle to avoid photo-oxidation. It was stable for 6 months.

Appendix # 13

Digestion of fusion protein with enterokinase enzyme

	1Rx (µl)
Protein	20.0
Buffer	3.0
Ekmax	3.0
d_3H_2O	4.0
	30.0

This digestion was placed at 37˚C for 2-3 hours.

Appendix # 14

Dubose Salt Medium

Components	**Quantity**
10 % K_2HPO_4	1 mL
10 % KCl	1 mL
5 % $NaNO_3$	1 mL
5 % $MgSO_4$	1 mL
Yeast extract	0.4 %
Distilled water	96 mL
Adjust pH = 7.0	

Appendix # 15

SDS-PAGE solutions

Solution I 30% stock

Acrylamide 30%

Bis acrylamide 0.8%

Amberlite 1%

Solution II 4X 250 ml stock

Tris base	45.5 g
SDS	1 g
PH	8.8

Solution III 4X 250 ml stock

Tris base	15.125 g
SDS	1 g
PH	6.8

Stainer for Protein Gel

Coomassie Brilliant Blue	0.5g
Glacial acetic acid	50ml
Methanol	225ml
Water	225ml

The gel was stained for 3-4 hours while shaking

Destainer for Protein Gel

Glacial acetic acid	50ml
Methanol	225ml
Water	225ml

The gel was destained for 2-3 hours while shaking

Transfer buffer 1000 ml

Tris base	50 mM
Glycine	380 mM
SDS	0.1%
Methanol	20%

1X Tang Buffer

Tris base	3 g/L
Glycine	14 g/L
SDS	1 g/L

Appendix # 16

15 % SDS PAGE

Running gel (10 mL)

Solution I	6.67 mL(Appendix # 15)
Solution II	5 mL (Appendix # 15)
d_3H_2O	8.33 mL
APS 10 %	200 µL
TEMED	20 µL

Stacking gel (5 mL)

Solution I	1.5 mL (Appendix # 15)
Solution III	2.5 mL (Appendix # 15)
d_3H_2O	6 mL
APS10 %	100 µL
TEMED	10 µL

25 % SDS PAGE

Running gel (25 mL)

Solution I	10 mL(Appendix # 15)
Solution II	6.25 mL (Appendix #29)
d_3H_2O	8.75 mL
APS 10 %	300 µL
TEMED	30 µL

Stacking gel (10 mL)

Solution I	1.5 mL (Appendix # 15)
Solution III	2.5 mL (Appendix # 15)
d_3H_2O	6 mL
APS10%	200 µL
TEMED	30 µL

Appendix # 17

Western blotting buffers

Tris buffer saline

Tris HCl	10 mM
NaCl	150 mM
PH	8.0

Alkaline phosphatase buffer

NaCl	10 mM
$MgCl_2$	5mM
Tris HCl	100mM
PH	8.0

Appendix # 18

Digestion of pSSZ810(c)

Digestion reaction of insert pSSZ810(b)

Insert(pSSZ810(b))	8.0 µL
EcoRI	1.0 µL
Not I	1.0 µL
RNase	2.0 µL

B (O+)	2.0 µL
d_3H_2O	6.0 µL
	20.0µL

Digestion reaction of vector pPIC3.5K

vector	8.0 µL
EcoRI	1.0 µL
Not I	1.0 µL
RNase	2.0 µL
B (O+)	2.0 µL
d_3H_2O	6.0 µL
	20.0 µL

This digestion was placed at 37°C incubator for 1 hour.

Appendix # 19

Ligation of xylanase fragment approx. 810 bp and pPIC3.5K

	L1 (µl)	L2 (µl)	L3 (µl)
Vector	2.0	3.0	4.0
Insert	5.0	6.0	6.0
Buffer	2.0	2.0	2.0
PEG	2.0	2.0	2.0
T_4 DNA Ligase	1.0	1.0	1.0
d_3H_2O	2.0	6.0	5.0
	20.0	20.0	20.0

This ligation was placed at 16°C for overnight in circulating water bath.

Appendix # 20

Digestion of pSSZ810(c)

DNA	3.0
Kpn I	1.0
B *Kpn I*	2.0
RNAase	2.0
ddd H_2O	12.0
	20.0

This digestion was placed at 37°C in an incubator for 3-4 hrs, run on (0.8%) agarose gel.

Appendix # 21

High Purity Midiprep Protocol for plasmid DNA isolation

1. **Column equilibration:** Add 100 mL of equilibrated buffer (E4) to the column and allow to drain from the column by gravity.

2. **Cell harvest:** Overnight culture media centrifuge at 5000 rpm at 4-20 °C for 5 mins.

3. **Cell suspension:** Add 4 mL of cell suspension buffer (E1). Mix and suspend the cells. This buffer contain Rnase.

4. **Cell lysis:** Add 4 mL of cell lysis soln (E2). Mix gently by inverting the capped tupe 5 times. Do not vortex. Incubate at room temperature for 5 mins.

5. **Neutralization:** Add 4 mL of neutralization Buffer (E3). Mix immediately by inverting the 5 times. Do not vortex. Centrifuge the mixture at 15,000 x g at room temperature for 10 mins.

6. **Column loading:** Take supernatant and load on to the equiliberated column to drain by gravity flow. Discard flow- through.

7. **Column wash:** Wash the column two times with 10 mL of wash buffer (E5). Allow the soln in the column to drain by gravity flow after each wash. Discard flow-through.

8. **Plasmid DNA elution:** Elute the DNA by adding 5 mL of elution buffer (E6). Allow the soln in the column to drain by gravity flow.

9. **Plasmid DNA precipitation:** Add 3.5 mL isopropanol to the elute. Mix and centrifuge the mixture at 15,000 x g at 4 °C for 30 mins. Carefully discard supernatant.

10. **Pellet washing:** Wash the DNA pellet with 3 mL of 70% ethanol and centrifuge at 4 °C for 5 mins. Remove ethanol and dry pellet for 10 mins. Dissolved pelleted DNA in 200 µL of TE Buffer (TE).

Appendix # 22

Digestion reaction of vector pPIC3.5K

vector	8.0µl
EcoRI	1.0 µl
Not I	1.0 µl
RNase	2.0 µl
B (O+)	2.0 µl
ddd H_2O	6.0 µl
	20.0 µl

This digestion was placed at 37°C incubator for 1 hour

Appendix # 23

Yeast extract peptone dextrose medium (YPD) 500 mL

Components	**Quantity**
1%Yeast extract	5.0 g
2%Peptone	10 g
2%Dextrose	25 mL
2%Agar (solid medium)	10 g

100 mg/mL geneticin (available from Invitrogen, Catalog no. K200-01.

For 500 mL (YPD preparation recipe)

1. Combine 5 g yeast extract, 5 g peptone and 5 g agar (in case of solid medium) in 450 mL deionized water.
2. Autoclave for 20 minutes on liquid cycle.
3. Add 50 mL of 10X dextrose (appendix 26) and mix well.
4. Cool YPD to approx. 55-60 °C and appropriate volume of geneticin stock as shown in (appendix 37)
5. Mix well by swirling, but is careful to minimize bubble formation.
6. Pour agar solution into Petri plates. Let plates harden, invert and store at 4 °C. Plates are stable for at least 6 months.

Appendix # 24

Electrocompetent cells formation of *Pichia pastoris*

1. Grow 5 mL of *Pichia pastoris* in YPD in a 50 mL falcon tube at 30 °C for overnight or up to an $OD_{600} = 1.0\text{-}1.5$.
2. Inoculate 500 mL of fresh medium with 2.5 mL of the overnight culture. Grow again to an $OD_{600} = 1.0\text{-}1.5$.
3. Centrifuge the cells at 5000 rpm for 5 minutes at 4 °C.
4. Discard supernatant and resuspend the pellet with 35 mL of ice-cold, sterile water.
5. Centrifuge the cells at 5000 rpm for 5 minutes at 4 °C.

6. Discard supernatant and resuspend the pellet with 25 mL of ice-cold, sterile water.
7. Centrifuge the cells at 5000 rpm for 5 minutes at 4 °C.
8. Discard supernatant and resuspend the pellet with 15 mL of ice-cold, 1 M sorbitol.
9. Centrifuge the cells at 5000 rpm for 5 minutes at 4 °C.
10. Discard supernatant and resuspend the pellet with 1 mL of ice-cold, 1M sorbitol.
11. Freeze the electrocompetent cells in 80 µL aliquots at -70 °C.

Appendix # 25

Minimal Glycerol Medium (MGM) 1000 mL

Components	**Quantity**
1.34% YNB	100 mL of 10X YNB (appendix # 38)
1% glycerol	100 mL of 10X GY (appendix # 39)
4 x 10^{-5}% biotin	2 mL of 500X B (appendix # 40)

For 1 liter (MGM preparation)

1. Autocalve 800 mL of deionzed water for 20 minute on liquid cycle.
2. Combine aseptically 800 mL autoclaved water with 100 mL of 10X YNB, 100 mL of 10X GY, 2 mL of 500X B.
3. Store at 4 °C. The shelf life of solution is approx. two months.

Appendix # 26

10X D (20% Dextrose)

Dissolve 200 g of D-glucose in 1000 mL of water. Autoclave for 15 minutes or filter sterilizes. The shelf life is approximately one year.

Appendix # 27

Breaking buffer (1000 mL)

Components

50 mM sodium phosphate, pH 7.4

1 mM PMSF (phenylmethylsulphonyl fluoride/other protease inhibitors)

1 mM EDTA

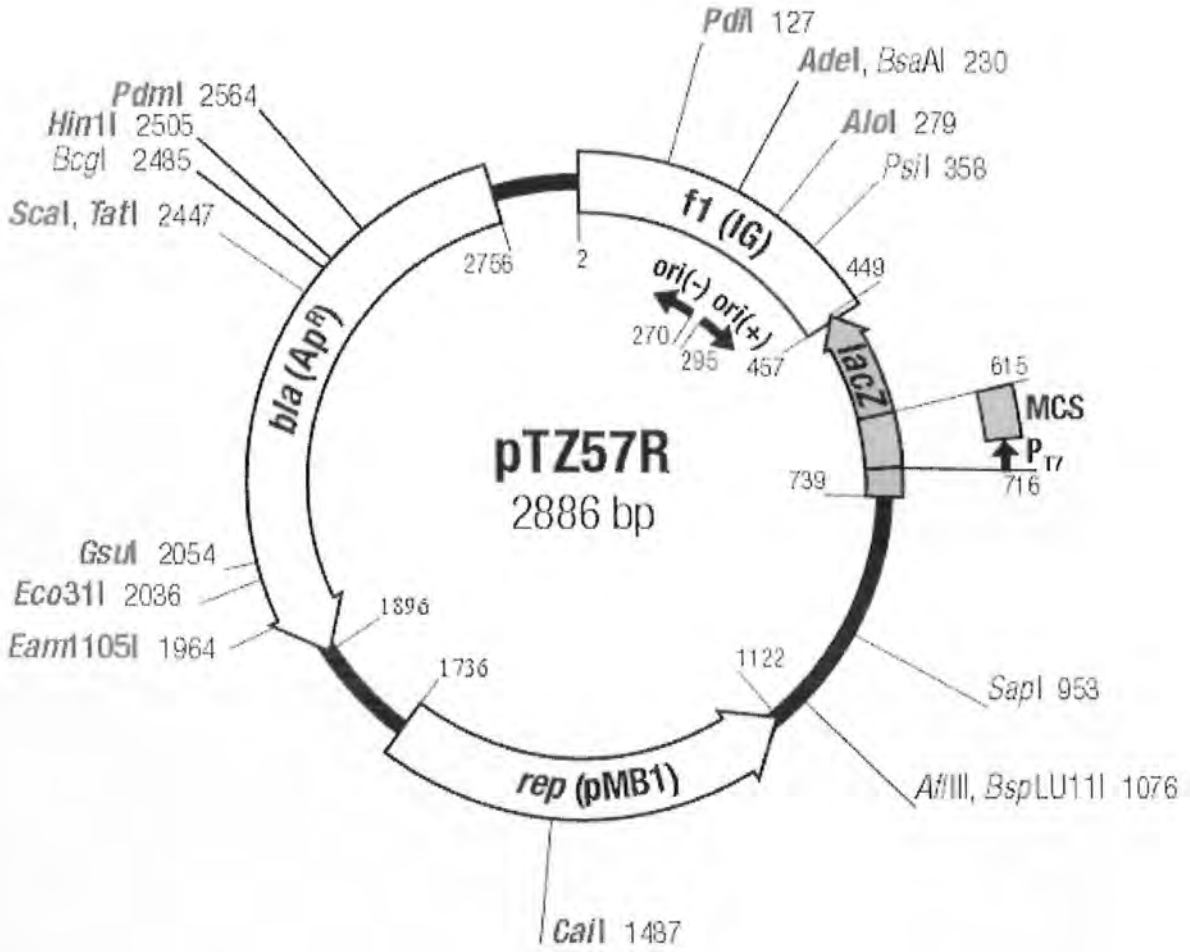
5% glycerol

For 1 liter, dissolve in 900 mL deionized water:

1. 6 g sodium phosphate (monobasic)
2. 372 g EDTA
3. 50 mL glycerol
4. NaOH use to adjust pH and bring volume up to 1 liter.
5. Store at 4 °C.
6. Right before use, add protease inhibitors.

Appendix # 28

PTZ57R

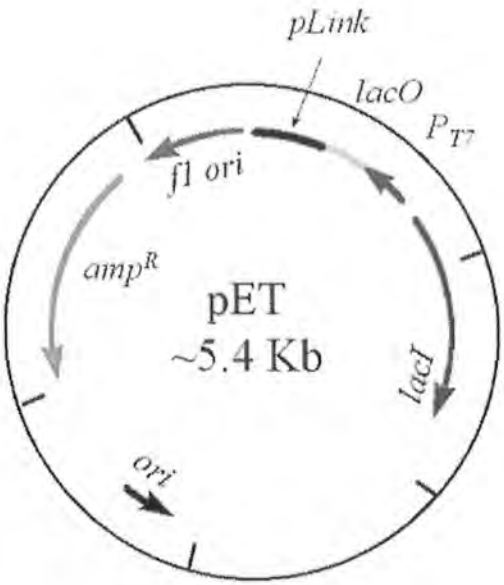


Plasmid pTZ57R, 2886 bp in length, is a derivative of pTZ19R. pTZ57R MCS contains 6 restriction sites with protruding 3'-ends, that are resistant to *E. coli* exonuclease III. This vector is designed for cloning and generation of ExoIII deletions. The exact position of genetic elements is shown on the map (termination codons included). DNA replication initiates at position 1136 (+/- 1) and proceeds in indicated direction. The *bla* gene nucleotides 2688-2756 (complementary strand) code for a signal peptide.

Appendix # 29

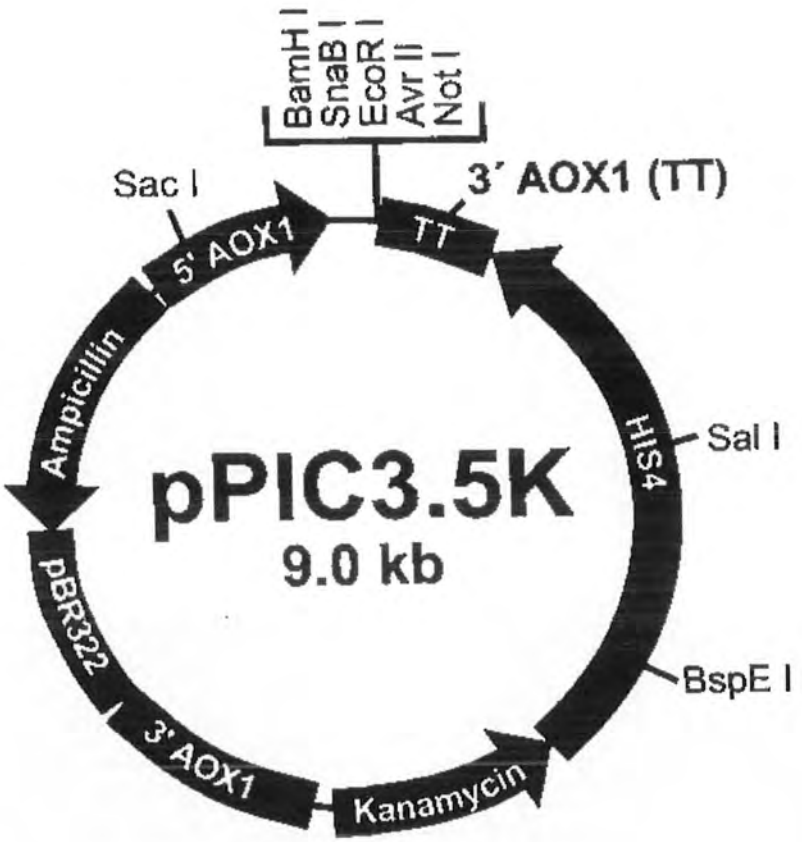
pET 32a(+) vector

The pET 32a(+) is designed for cloning and expression of peptide sequences fused with 109a.a thioredoxin protein. Cloning sites are available for producing fusion proteins. The cloning and expression of the coding strand are transcribed by T7 RNA polymerase. This plasmid contains a drug resistant marker for ampicillin resistance, the lac I gene, the lac operator region 3′ to the T7 promoter and a polylinker region. There are two origins of replication, one is the f1 origin which enables the production of a single stranded vector under appropriate conditions and the other is conventional origin of replication as shown below.



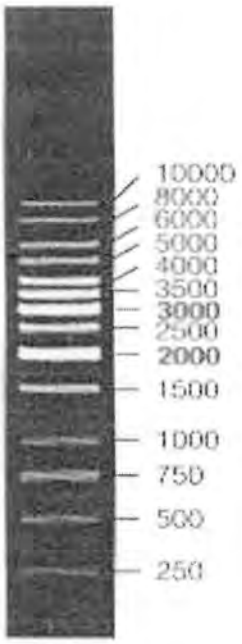
Appendix # 30

pPIC3.5k *Pichia pastoris* vector



Appendix # 31

1kb DNA ladder



Appendix # 32

Ethidium bromide (100 mg/mL)

Add 1 g of ethidium bromide to 100 mL of water. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in the aluminium foil or transfer the solution to a dark bottle and stored at room temperature.

Appendix # 33

Rnase stock solution

The solid (Rnase A) is dissolved in 10 mM Tris. HCl (pH 7.5) and 15 mM NaCl. The solution should be heated at or near boiling (in a water bath) for at least 15 minutes to get rid of Dnase and then cooled slowly to room temperature (Sambrook *et al.* 1989).

Appendix # 34

IPTG (isopropyl-thio-β-D-galactoside) stock solution (0.1 M)

Make a stock solution of 50 mg/mL in distilled water. Use 100 µL/100 mL.

Appendix # 35

Antibiotics

Antibiotics	Stock Conc.	Working Conc.	Solvent
Kanamycine	50mg/ml	50µg/ml	Water
Ampicilline	100mg/ml	100µg/ml	Water
Rafampicine	50mg/ml	50µg/ml	Methanol
Tetracycline	10mg/ml	10µg/ml	90% Ethanol

Appendix # 36

100 mg/mL geneticin

Prepare 30 mL of 100 mg/mL geneticin stock solution in sterile water. Filter sterilize and store frozen at -20 °C. YPD plates containing geneticin at final concentrations of 0.25, 05, 075, 1.0 and 1.25 mg/mL, respectively.

Appendix # 37

10X YNB (13.4% Yeast Nitrogen Base)

Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1000 mL of water. Heat the solution to dissolve YNB completely in water and filter sterilize. Store at 4 °C.

Appendix # 38

10X GY (10% Glycerol)

Mix 100 mL of glycerol with 900 mL of water. Sterilize either by filtering or autoclaving. Store at room temperature. The shelf life of solution is greater than one year.

Appendix # 39

500X B (0.02% Biotin)

Dissolve 20 mg biotin in100 mL of water and filter sterilize. Store at 4 °C. The shelf life of solution is approximately one year.

Appendix # 40

Phenol-chloroform precipitation

The PCR product was diluted with ddH_2O to 100 µl. Equal volume of phenol and chloroform i.e. 50 µl of each was added. The mixture was shaked gently and centrifuged for six minutes. The supernatant was taken in a fresh eppendorf tube. $1/10^{th}$ volume of 3 M sodium acetate (10 µl) with pH 5.4 and 2.5 volume of absolute ethanol (250 µl) is added. The mixture was placed at -20^0C for an hour and then centrifuged for 10 minutes. Supernatant was discarded and the pellet was washed with 70% ethanol. The mixture was centrifuged for two minutes and again supernatant is extracted. The pellet was air dried and dissolved in 20 µl ddH_2O.