BIOPROSPECTING OF THERMOSTABLE XYLANASES FOR LIGNOCELLULOSIC BIOMASS CONVERSION

A project report

submitted in partial fulfillment of the requirements for the award of the degree of

MASTER OF TECHNOLOGY

in

BIOTECHNOLOGY

Under the supervision of

Dr. SUDHIR KUMAR

(Associate Professor)

By

PRAGYA SETHI

(152554)

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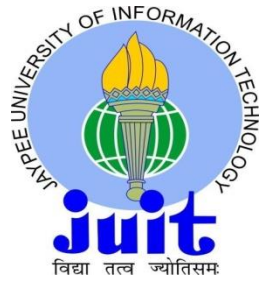

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT SOLAN – 173234

HIMACHAL PRADESH, INDIA

May , 2017

CERTIFICATE

This is to certify that the work entitled **"BIOPROSPECTING OF THERMOSTABLE XYLANASES FOR LIGNOCELLULOSIC BIOMASS CONVERSION"** has been submitted by Pragya Sethi, in partial fulfillment for the award of the degree of Master of Technology in Biotechnology to Jaypee University of Information Technology; Waknaghat Solan has been carried out under my supervision.

This work has not been submitted partially or fully to any other University or Institute for the award of this or any other degree or diploma.

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Date: -

Dr. Sudhir Kumar
Associate Professor
Biotechnology and Bioinformatics
JUIT, Waknaghat, Solan

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Date- PRAGYA SETHI

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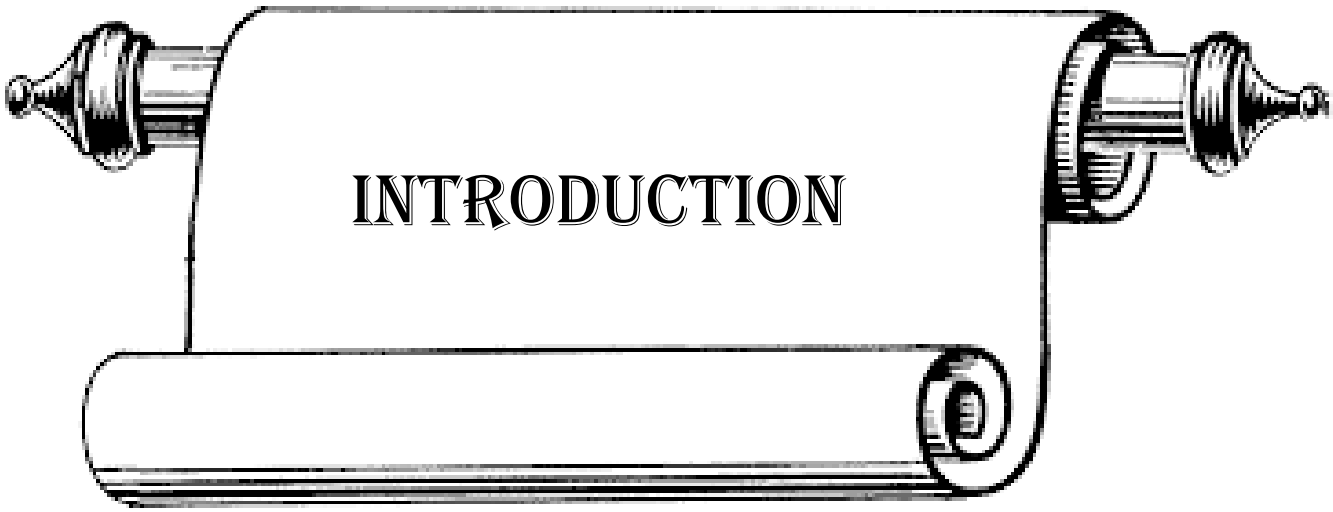
ABSTRACT

BIOPROSPECTIVE OF THERMOSTABLE XYLANASES FOR LIGNOCELLULOSIC BIOMASS CONVERSION

Pragya Sethi, *Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh - 173234*

Xylanases have grabbed great attention in the recent years because of their application in diverse industrial processes such as paper pulp bleaching, baking, textile and biofuel production. This class of enzymes (E.C.3.2.1.8) is known for its significant role in the degradation of hemicellulose fraction of the lignocellulosic materials. Currently, the bacterial sources are the hotspot of research and their bioprospecting from different places is under exploration. The studies have reported that thermophilic bacteria have more potential to produce a thermostable enzyme, which could be of great importance to hands on in industry. In our study, we collected samples from four different sources i.e. pine forest soil, water sample from hotspring (Vashisht in Himachal Pradesh), mushroom compost and biogas slurry. The samples from the respective sources were primarily enriched followed by screening for the xylanase producing bacteria. One of the potent isolate (PS1) was used for the production of xylanase and its unit activity was quantified using DNS assay. The produced xylanase shows activity at thermophilic range of 50 to 70^0C.Thermophilic xylanases are in great demand for lignocellulosic conversion at high temperature. In this context optimisation studies were carried out on the explored substrate (wheat straw) regarding the potent isolate and its characteristics were studied.

Keywords: Xylanase, bacteria, thermostable enzyme, optimization.



INTRODUCTION

1. INTRODUCTION:

The diversity in our environment makes us rich. Many different kinds and types of microorganisms are found in our habitat. The growth of these microorganisms is supported by the environmental conditions around them. This diversity allows great interest in studying the physiological behaviour and their ability to produce enzymes for the benefit of humans. This accounts for great potential in microorganism which is explored and studied in the past years.

The function of enzymes is to speed up the chemical reaction as catalyst without interfering in the reaction. (Reece et al. 2010). Most enzymes are proteins. The enzyme acts on lessening the activation energy without involving in the reaction (Petersen and Anderson 2005). Environmental factors also play a major role in stimulation. Temperature is one such environmental factor that can affect enzyme activity in a positive as well as negative way (Conant 2012). Another factor that affects enzyme is pH (Leake and Read 1990). Enzymes produced by microorganisms are of great industrial potential.

The three chief components that make up the lignocellulosic substrates are cellulose, hemicellulose and lignin, respectively according to their concentration (Verma D, 2012). Hemicellulose is the second most profuse plant polysaccharide available in nature. Amongst them, xylan is the second most abundant. The primary monomers present in most of the hemicelluloses are D-xylose, D-mannose, D-galactose, and L-arabinose. Xylans are hetero polymers comprising primarily of D-xylose as its monomeric unit and traces of L-arabinose (Bastawde 1992). The entire breakdown of xylan requires the action of pool of hydrolytic enzymes which act concurrently to break xylan into its subsequent sugars. The utilisation of those sugar components in the fermentation process could lead to production of bio-fuel or bio-ethanol that studies the second generation of biofuels. This generation eliminates the problems associated with the first generation that majorly related to food vs fuel. Hence the second generation utilises the non edible parts of the plants known as lignocellulosic plant materials. These act as substrates for the microorganisms to synthesise enzymes, which hydrolyse the complex structure of the heteropolymer units.

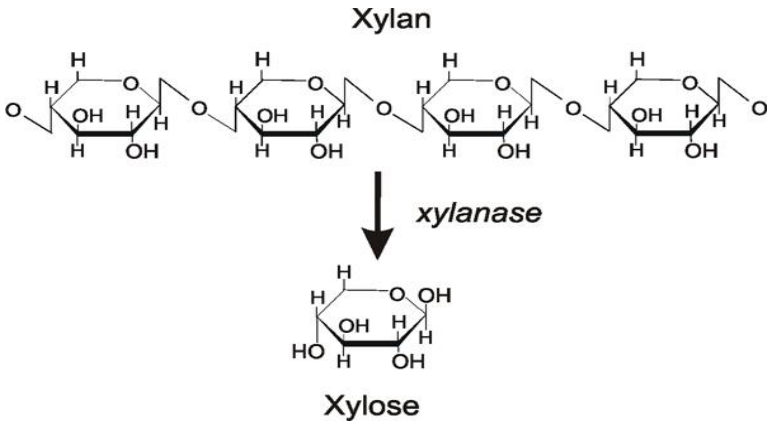


Fig1: Structure of Xylan and its hydrolysis to Xylose (Held, Paul, 2012).

The most suitable feedstock for the conversion of bio-ethanol and bio-fuels would be little cost agricultural residues present in large quantity. This allows greater potential conversion of lignocellulosic biomass to ethanol. The composition of substrates varies from specie to specie. Wheat straw composition includes a complex of cellulose, hemicellulose and lignin. The composition of wheat straw varies from specie to specie also from soil and climatic conditions. The approximate composition of feedstock residue is displayed in the fig below:

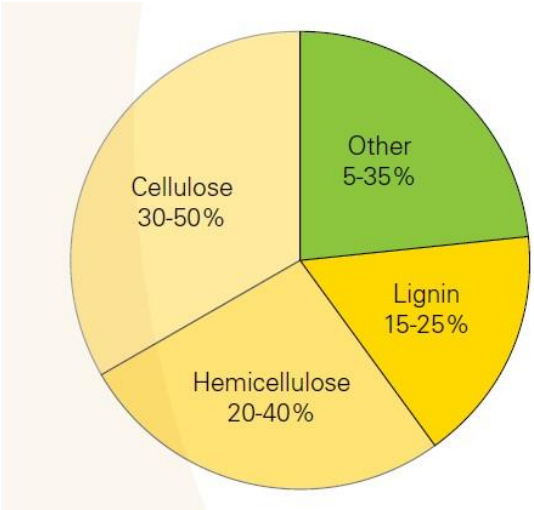


Fig 2: General composition of lignocellulosic biomass feedstock (Lee, DoKyoung, 2007).

The composition varies from specie to specie and the climatic conditions the crop has withstood. This difference is negligible if the crop helps to act as a good substrate and gets easily hydrolysed by the bacterial enzyme. The source of bacteria producing the

enzyme is variant. The preference is mostly given to a thermophilic range of enzymes which are optimised to produce thermostable xylanases.

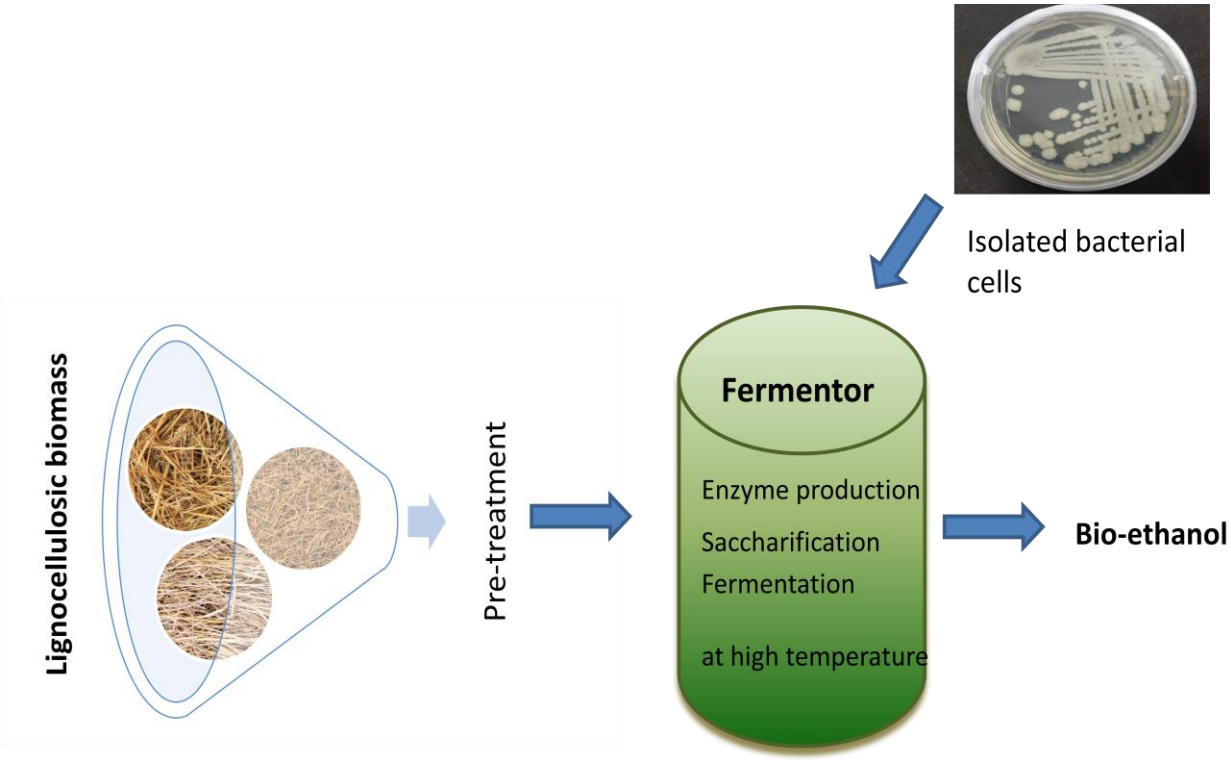
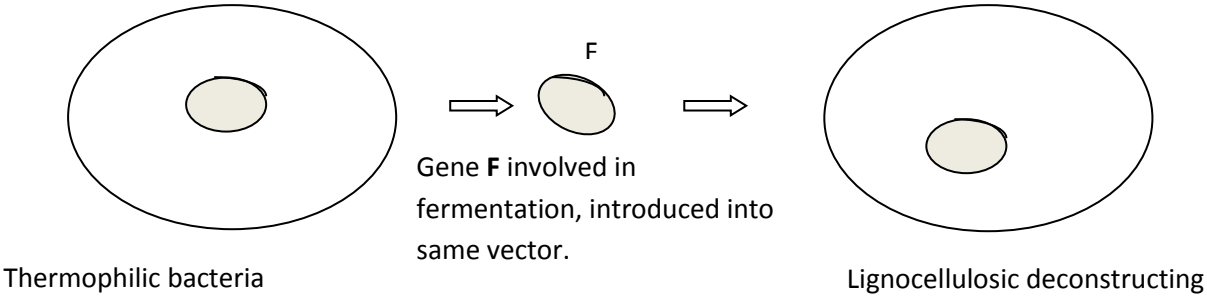
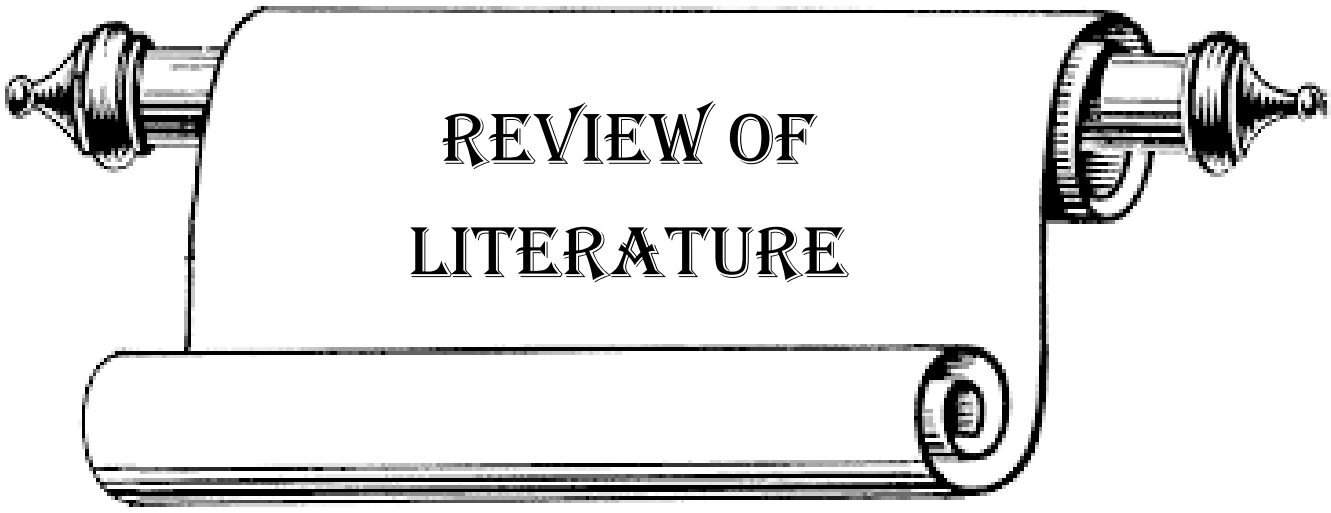


Fig3: The process description of sugars fermented to bio-ethanol in industrial prospects.

The problem associated with this system is the enzyme production even at optimum conditions is less. To overcome this problem, the gene transcribing xylanase enzyme is inserted into host organism (E.coli). This will result in significant production of enzyme at optimum conditions. Also, one step saccharification and fermentation involves the consolidated bio-processing, features enzyme production, hydrolysis and fermentation of biomass in single reactor at the same temperature.



Gene S **Fig 4**: Saccharolytic fermentative thermophile construction process


REVIEW OF
LITERATURE

2. XYLANASES

The reduced environmental toxins is the major advantage of xylanase exploitation in industrial prospects like: paper and pulp kraft bleaching, management of plant waste, conversion of lignocellulosic material to renewable fuels and chemicals and also using enzyme in agro-waste treatment (Collins T, et. al. 2002). The xylanolytic enzyme system that carries out the xylan hydrolysis is normally composed of a collection of hydrolytic enzymes, including endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan-1,4-β-xylosidase, E.C.3.2.1.37), α-glucuronidase (α-glucosiduronase, E.C.3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C.3.2.1.55) and acetyl xylan esterase (E.C.3.1.1.72) (Juturu V,2011). For the complete breakdown on xylan complex, the above enzyme pool act in coordination (Belancic A, 1995).

Xylanases and the microorganisms that make them are presently used in the management of agricultural residual waste, to hydrolyse xylan to renewable fuels and bio-chemicals, and they are used in food management, agro-fiber, and the paper and pulp industries, where the use of enzymes help to reduce their environmental impact (Collins T, 2002). The xylanolytic enzyme system that carries out the xylan hydrolysis is normally composed of a collection of hydrolytic enzymes, including endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan-1,4-β-xylosidase, E.C.3.2.1.37), α-glucuronidase (α-glucosiduronase, E.C.3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C.3.2.1.55) and acetyl xylan esterase (E.C.3.1.1.72) (Juturu V,2011). All the above enzymes act jointly to convert xylan into its constituent sugars monomers (Belancic A, 1995).

The classification of xylan has been proposed in three different ways: molecular weight and isoelectric point (pI) (Wong K K, 1988), the crystal structure (Jeffries T W, 1996) and kinetic properties, or the substrate specificity and product profile. Classification of xylanases havs been primarily performed as GH 10 and 11 based on the hydrophobic group analysis of the catalytic domains and similarities in the amino acid sequences (Verma D, 2012). Xylanase in the GH10 family (or family G) have been identified to have a lower molecular mass with a pI in a range of 8–9.5, while those from the GH11 family (or family F) have a high molecular mass and lower pI

11values (Ahmed S, 2009; Buchert J, 1995). Glycoside hydrolase family 10 consist of endo-1, 4-β-xylanases and endo-1, 3-β-xylanases (EC 3.2.1.32) (Coutinho P M, 1999). GH11 family members exhibit several attractive properties, such as high substrate selectivity and high catalytic efficiency, a small size, and a variety of optimum pH and temperature values, making them fit in various conditions and in many industrial applications (Paës G, 2012). Family 11 is composed only of xylanases (EC3.2.1.8), leading to their consideration as "true xylanases," as they are exclusively active on D-xylose-containing substrates.

Xylanases belonging to GH10 exhibit greater catalytic versatility and lower substrate specificity than those belonging to GH11 (Biely P, 1997; Faulds C B, 2006).

2.1 SOURCES:

Microorganisms are rich source of cellulase-free xylanase enzymes which are synthesised by diverse species of bacteria, fungi and yeast. Bacteria, yeasts and filamentous fungi have been identified as proper candidates to produce xylanases (Kamble and Jadhav, 2012) but the most practical approach has been in screening and isolating the naturally occurring isolate competent of producing xylanase under optimised conditions.

The optimised conditions may vary from specie to specie and also the type of habitat they survive in. The survival of microorganisms depends on the temperature around them. Surviving bacteria in a range of 20-45°C are mesophilic ones whereas surviving bacteria in a range of 50-70°C are thermophilic. Thermostable xylanases are more frequently reported to be produced by extreme thermophilic microorganisms which have the potential to utilize the lignocellulosic substrate (Bhalla et al., 2013, 2014a, b). ''Thermostability'' is defined as ability of an enzyme to retain its dynamic structural conformation at a selected high temperature for a prolonged period. (Bhalla et al 2013). Higher growth temperatures are industrially desirable because temperatures above 50°C could lead to reduced risks of mesophilic microbial contamination (Yeoman et al, 2010). Xylanases from *Geobacillus thermodenitrificans* TSAA1 (Verma et al., 2013), *Bacillus* sp. JB 99 (Shrinivas et al., 2010), *Bacillus licheniformis* 77-2 (Damiano et al., 2006), and *B. flavothermus* strain LB3A (Sunna et al., 1997) also showed their temperature optimum at 70°C. Advantages of using thermostable enzyme in industrial processes:

(i) The solubility of the reactants and products are enhanced, ensuring a higher reaction velocities thus decreasing the amount of enzyme required for the process (Zhang et al., 2011a; Viikari et al., 2007);

(ii) Shorter hydrolysis times;

(iii) Lower risk of contamination, and thus, increased productivity;

(vi) Facilitated recovery of volatile products e.g. ethanol (Taylor et al., 2009); and

(v) Decreased cost of energy for cooling after thermal pre treatment.

The production of endoxylanases is reported by fungi, bacteria, termite gut, algae and other rumen gut micro biota (Kulkarni N, 2009; Wong, K.K.Y, 1988).

In a very recent study new xylanase producing Gram positive bacteria has been found in termite gut (Matteotti et al., 2012; Brennan, YaLi, et al, 2004). Also large scale production of exogenous enzymes combines the disciplines of microbiology, genetics, biochemistry and engineering with the basic principle of fermentation (Sadhu and Maiti, 2013).

Simple and easy growing microorganisms like E.coli and yeasts are used for genetic manipulation of genes encoding for xylanase enzyme (Ahmed et al., 2009; Weng and Sun, 2010). The use of recombinant xylanase is performed in chlorine-free bleaching of kraft pulp in paper and pulp industry (Comlekcioglu, Ugur, et al. 2014).

Table1: Recombinant construction of xylanase producing microorganisms.

SOURCES	**HOST**	**VECTOR**	**MW**	**pI**	**REFERENCES**
Penicillium oxalicum GZ-2	*Pichia pastoris*	pPICZαA plasmid	34.2 KDa	8.2	Liao, Hanpeng, et al. 2015
Nonomuraea Flexuosa	*Trichoderma reesei*		25 KDa	-	Zhang, Junhua, et al. 2011
Thermophilus Bacillus sp. NCIM59	E. coli	pATBX 4.5	35 KDa	4	Kulkarni, Neeta, 1995
Thermophilus Bacillus sp. NCIM59	E. coli	pATBX 4.5	14.5 KDa	8	Kulkarni, Neeta, 1995

Bacillus subtilis DB104	E.coli DH5α	pSKE194		-	Helianti, Is, et al. 2016
Aspergillus niger SCTCC 4OO264	E.coli JM109	pET32a	19.837 KDa	4.19	Yi, Xiuli, et al. 2010
Aspergillus Usamii	*Pichia pastoris* X33	pPIC3.5K and pPICZαA		-	Wang, Jianrong, 2016
Trichoderma reesei ATCC 58350	*Kluyveromyces lactis* GG799 (yeast)	pKLAC1	4.3Ph	-	Fuzi, Siti Fatimah Zaharah Mohamad, et al. 2014

2.2 PRODUCTION

Fermentation is the key technique for the production of various enzymes. Fermentation methods are divided into two categories as Solid State Fermentation (SSF) and Submerged Fermentation (SmF) (Murad and Azzaz, 2010).

Before going for fermentation, one has to treat the substrate chosen for hydrolysis. Pre-treatment allows breakdown of easy bonds after thermal and pressure load, hence enhancing the exposure of hemicellulose to the microorganisms and the removal of lignin. Pre-treatment must gather the following needs:

(1) Recover the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis;

(2) Avoid the degradation or loss of carbohydrate;

(3) Avoid the development of by-products in the process which are inhibitory to the subsequent hydrolysis and fermentation processes; and

(4) Be cost-effective.

The use of Solid State Fermentation (SSF) in the growth of microorganisms on moist solid substrates, like bran, baggase, paddy straw and other agricultural and lignocellulosic waste and paper pulp. Submerged Fermentation (SmF) utilizes free flowing liquid substrates, such as molasses and broth (Subramaniyam and Vimala, 2012). For fermentation studies of fungi SSF is most suitable. Solid state fermentation requires less moisture content while submerged fermentation process requires more moisture which is suited for growth of bacteria. SmF allows production of

approximately 90% of commercial enzymes available in the market because of their advantage of controlled conditions during the process. In the SSF method, a firm contact is created for substrate (insoluble) resulting in an enhanced substrate exposure to the microorganism. Another advantage of SSF over SmF is that in the process of fermentation of substrate the SSF involves less liquid hence lesser the chances of contamination. SSF is theoretically simpler and less expensive. Various fungi reported produce good xylanase amount with SSF (Silva, Roberto da, et al, 2005; Bandikari, Ramesh, 2014; Lakshmi, Garapati Suvarna, et al., 2009).

Bacterial isolates produce preferably a better amount and stable enzyme in SmF conditions rather SSF. (Battan, Bindu, 2006; Nagar, Sushil, et al, 2010; Bocchini, D. A., et al, 2002).

Table2: Review of xylanase-producing microorganisms. (Motta, F. L.,2013)

Microorganisms	**Xylanase**	**Cultivation condition**	**Media**	**Reference**
Streptomyces sp. P12–137	27.8 IU/ml	pH 7.2; 28 °C	Wheat bran and KNO_3	Coman G,2011
Thermomyces lanuginosus SD-21	8,237 IU/g	pH 6.0; 40 °C	Com cob and wheat bran	Ge Y, 2011
Acremonium furcatum	33.1 IU/ml	30 °C	Oat spelt xylan, urea, peptone and yeast extract	Palaniswamy M, 2008
Bacillus circulansD1	8.4 IU/ml	pH 9.0; 45 °C	Bagasse hydrolysates	Bocchini D.A, 2005
Streptomycessp. strain Ib 24D	1,447.0 IU/ml	pH 7.5; 28 °C	Tomato pomace	Rawashdeh R, 2005
Paecilomyces themophila J18	18,580.0 IU/g	pH 6.9; 50 °C	Wheat straw and yeast extract	Yang S.Q, 2006
Aspergillus niger PPI	16.0 IU/ml	pH 5.0; 28 °C	Oat and urea	Pandey P,2002
Penicillium fellutanum	39.7 IU/ml	30 °C	Oat spelt xylan, urea, peptone and yeast extract	Palaniswamy M, 2008
Cochlio bolussativus Cs6	1,469.4 IU/g	pH 4.5; 30 °C	Wheat straw and $NaNO_3$	Arabi M.I.E, 2011
Penicillium Canescens	18,895 IU/g	pH 7.0; 30 °C	Soya oil cake and casein peptone	Assamoi A A,2010
Penicillium clerotiorum	7.5 IU/ml	pH 6.5; 30 °C	Wheat bran	Knob A, 2008

2.3 APPLICATIONS:

Xylanolytic enzymes from microorganism have attracted a great deal of attention in the last decade, particularly because of their biotechnological potential in various industrial processes (Bajpai 1999; Kuhad and Singh 1993; Niehaus et al. 1999; Wong and Saddler 1992), such as food, feed, pulp and paper industries. Potential applications of xylanases in biotechnology industry include, bio-bleaching of wood pulp, treating animal feed to increase digestibility, processing food to increase clarification and converting lignocellulosic substances to feedstock and fuel.

2.3.1 Xylanase in paper and pulp industry

Currently the most promising application of xylanase is in the paper making and wheat straw pulping (Wen-ying, 2008) Enzyme application improves pulp fibrillation and water retention, reduction of beating times in pulps, restoration of bonds and increased freeness in recycled fibers, and selective removal of xylans from dissolving pulps. Xylanase also softens fibers allowing them to undergo easy chemical bleaching (Gangwar, Avdhesh Kumar, 2014). Current efforts are aimed at process optimization, simplification, and cost reduction of enzyme application in pulp industry (Nissen, A. M.,1992).

2.3.2 Xylanase in bread and baking industry:

Xylanases in bread making results in increased volume, reduced stickiness and staling, and increased shelf life. The enzyme can substitute the addition of different emulsifiers and other chemical additives used in bread production. Xylanases improve dough characteristics and bread quality leading to improved dough flexibility, machinability, stability, loaf volume and crumb structure. (Collins, Tony, et al.2006; Butt, Masood Sadiq, et al. 2008). Xylanase enzymes are used to accelerate the baking of cookies, cakes, crackers, and other foods by helping to break down polysaccharides in the dough (Godfrey T et al., 1996).

In biscuit-making, xylanase is suggested for preparing cream crackers lighter and improving the texture, palatability and uniformity of the wafers (Polizeli M.L et al., 2005). Xylanase, when grouped with endo glucanase, takes part in the hydrolysis of arabinoxylan and starch, sorting out and isolating the gluten from the starch in the wheat flour.

2.3.3 Xylanase in animal feed:

Feeding animals with agricultural silage which are pre-treated with xylanase enzyme allows improved nutritional value and allows easy digestion in ruminants. The pre-treatment to enzyme allows release of trapped nutrition on which their specific enzymes act for better digestion.

2.3.4 Xylanase in Agro-waste treatment:

Using ethanol-blended fuel for automobiles can considerably reduce petroleum use and exhaust greenhouse gas emission (Wang et al., 1999). The non-edible parts of the plants are converted to simple sugars by allowing xylanase producing microorganisms to act on agricultural wastes. This process allows synthesis addition of enzyme (xylanase) and substrate (wheat straw, corn cob, corn straw, sugarcane baggase and pine needle) to react and produce simple sugars(xylose), which can be converted to ethanol by fermentation. (Techapun, Charin, et al.2003; Sun, Ye, 2002; Nagar, Sushil, 2012).

2.3.5 Xylanase in value added products formation:

Another industrial use of xylanases is the bioconversion of xylan into higher value added products. As the enzymatic hydrolysis of xylan leads to xylose, different fermentations may occur and a variety of compounds can result from these reactions. One of the most important such product is xylitol, which is used to sweeten food products such as chewing gum, candy, soft drinks and ice cream.(Li, Zhe, et al. 2013). Xylitol can also be used as a natural sweetener in toothpaste and various pharmaceutical products. (Guo, Xiaoxiao, et al. 2013).

Xylanases of microbial origin have great potential and high benefits in industrial application. Xylanase enzyme should be promoted in the food processing industry to replace the chemical emulsifiers and additives. Xylanase enzyme in combination with other enzyme can provide better results.

Table 3: Commercially available xylanase enzyme in market.

COMPANY NAME	PRODUCT	STRAIN AND MODE OF FERMENTATION	APPLICATION
Altech.Inc (USA)	Allzym PT	*Aspergillus niger* (SmF)	Upgrading animal feed
Biotec	Ecosane	*Trichoderma reesei* (SmF)	Upgrading animal feed
Ciba-Geiby Ltd (Switzerland)	Igrazyme 40	*Trichoderma longibrachiatum* (SmF)	Paper and Pulp Industry and animal feed
Iogen Corp (Canada)	Xylanase GS35	*Trichoderma reesei* (SmF)	Pulp bleaching, Pulp cleaning and animal feed processing
Novozymes (Denmark)	Bio-feed plus Novozyme 431 Pulpzyme	*Humicola insolens* (SmF) *Trichoderma longibrachiatum* (SmF) Bacillus sp.	Animal feed and processing Cellulose and paper industry

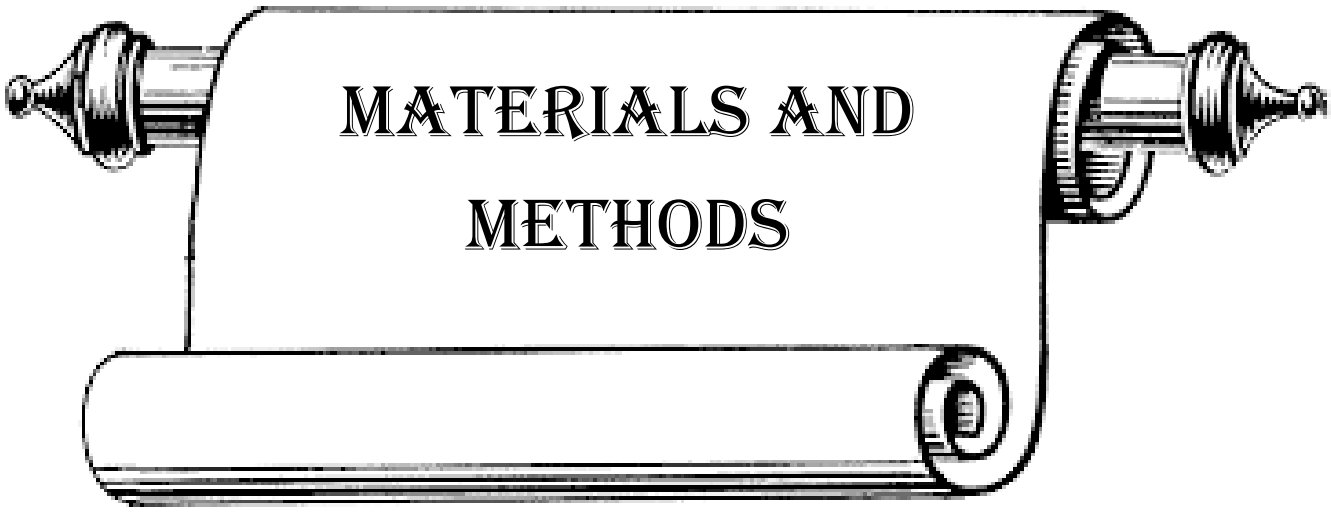
2.4 NEED FOR THERMOPHILIC BACTERIAL XYALANSES:

The thermophilic bacterial isolates are involved majorly in production of thermostable xylanases. These xylanases are of great importance to allow sustainable use of resources and their conversion to value added products. The second generation bio-fuels are of much importance to people as it utilises the non-edible part of plants which are lignocellulosic wastes. Their management in converting them to ethanol and bio-fuels is the topic for current research. Many researchers (Sizova, M. V., et al.2011; Daas, Martinus JA, et al.2016; Bibi, Zainab, et al. "20140; Nagar, Sushil, 2012) have potentially been able to isolate bacteria for the hydrolysis of plant polysaccharide into simple sugars and later their fermentation to ethanol. The bacterial isolates are preferred over fungal isolates, plant and animal cells because of the following reasons:

- The isolation of enzyme from bacteria is cheaper as compared to other sophisticated cells.
- The enzymes produced by bacteria are more predictable and controllable.

- The resources to be supplied to the bacteria to grow under optimized conditions are minimum.
- The fungal origin enzyme has difficulty in down streaming because of mycelial growth.
- Fungus causes more froth formation than bacterial cells.
- Bacterial thermostable xylanases are preferred over fungal xylanases also because of higher temperature optima and thermostability.

Thermophilic xylanases are the need for industrial applications. With wide use in paper and pulp industry, bread and baking industry and biofuels generation, thermostable xylanases have occupied a major portion in toxic free residue and use of lignocellulosic waste conversion to bio-fuel saving the environment from pollutants and chemicals. Higher temperature promotes greater enzyme penetration and cell wall disorganisation of the raw materials. Also higher temperature promotes the growth of only selective bacterial for the enzyme production hence lessens the microbial contamination risk. A thermostable enzyme will also increase the solubility of enzyme and product within less hydrolysis period. These advantages will ultimately result in lower cost of cooling after thermal pre-treatment and a better enzyme activity.


MATERIALS AND
METHODS

3. ISOLATION AND SCREENING OF MICROORGANISMS

Isolation of thermophilic bacteria producing xylanase enzyme activity was performed in two steps. The major emphasis was on highly potent microorganism producing xylanase. The selection of a potent source was a task. Finally, we selected a biogas slurry sample for the isolation procedures. This biogas was fed with pine needles for the past one year and it is maintained by the Environment Research Group, JUIT, Waknaghat, Solan, H.P(Tripathi, et al. 2015).

3.1 SOURCES:

The growth of microorganisms is promoted by their surroundings. The natural habitat has more potential to grow wide range of microorganisms as compared to restricted environment with selective media. For our study, four different sources were selected and screened. The sources were:

- Biogas slurry from the biogas plant fed with pine needles from the past one year, maintained by Environment Research Group, JUIT, Solan, H.P.
- The second source for the study was a natural habitat of pine needles degrading soil compost in the JUIT campus.
- Hot water spring in Vashishth, Manali, H.P., was the third source for screening and isolation of potential isolate.
- The last source was the DMR (Directorate of Mushroom Research), Solan, H.P. The soil compost was taken.

  The selection of sources was greatly thought over. After the screening procedure for different isolates we finally got a potent isolate named PS1. All the other isolates were screened for their ability to produce xylan but the best result was observed in PS1 (data not mentioned). Also, this potent isolate, PS1, is thermophilic and grows easily at 50°C after 24 h.

  Further morphological and molecular characterization was performed for the isolate which would later result in knowing the specie of it. Sequencing of 16s rRNA will tell us the specie name and its Phylogenetic analysis.

Fig 5: Sources screened for the study. A: biogas plant; B: soil sample; C: hot water spring source; D: DMR

3.1.1 PRIMARY SCREENING

Initial screening was performed on CMC and Xylan rich plates. The composition of the same are:

CMC plates: CMC 1%, Peptone 1%, NaCl 0.5%, Yeast extract 1% and Agar 1.5%.

Xylan plates: Xylan 1%, Peptone 1%, NaCl 0.5%, Yeast extract 1% and Agar 1.5%.

Unless and otherwise stated, all the sterilization steps were performed at 121°C , 15lbs pressure for 20mins.

Another screening was performed using the modified Horikoshi media (Horikoshi, Koki.1999). The composition used was: Peptone 5g/ltr, NaCl 5g/ltr, KH_2PO_4 5g/ltr, $MgSO_4$ 5g/ltr and 1% substrate. It was observed that the activity of enzyme was not so good in the presence of modified Horikoshi media. So we switched to another complex minimal media after extensive literature work.

3.1.2 SECONDARY SCREENING

The sample was inoculated in nutrient broth. The substrates used were of three different types: CMC, Xylan and wheat straw. This procedure was continued for 15days at 37°C and 50°C for enrichment of most potent isolate and its ability to grow and utilise the hemicellulose xylan.

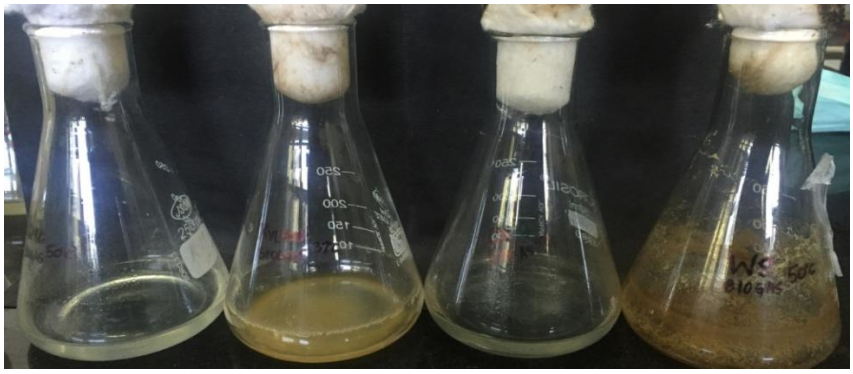


Fig 6: Thermophilic culture flasks after 15days of enrichment on different substrates.

3.1.3 INOCULUM USED FOR FERMENTATION STUDIES

The inoculum was raised in the same medium under similar conditions in 250ml Erlenmeyer flask and 24h old inoculum was used to initiate growth. The properly inoculated media were incubated for 120 days on a rotary shaker (150rpm) at mesophilic and thermophilic conditions. The OD considered for the inoculum was 1.806 at 600nm.

3.1.4 PRODUCTION OF XYLANASE

The production of xylanase was performed for all the isolates observed in the plate. The media selected was complex minimal media with composition as follows. This media provides essential necessary nutrients required for the growth and metabolism of thermophilic bacterial isolates.

Table 4: Composition of minimal media.

K_2HPO_4	1.5g/ltr
KH_2PO_4	3.0g/ltr
$MgSO_4$	0.3g/ltr
$CaCO_3$	0.05g/ltr
NaCl	0.5g/ltr
$NaHCO_3$	0.5g/ltr
NH_4Cl	1.0g/ltr
$FeSO_4$	100µl/ltr
NH_4Fe(III) citrate	100µl/ltr
$MnSO_4$	500µl/ltr
$CoCl_2$	100µl/ltr
$ZnSO_4$	100µl/ltr

$CuSO_4$	100µl/ltr
$H3BO_4$	100µl/ltr
Na_2MoO_4	100µl/ltr
Na_2SeO_3	100µl/ltr
Nicotinic acid	250µl/ltr
Cyanocobalamin	250µl/ltr
p-aminobenzoic acid	250µl/ltr
Calcium pantathenate	250µl/ltr
Thiamine hydrochloride	250µl/ltr
Riboflavin	250µl/ltr
Lipoic acid	250µl/ltr
Folic acid	100µl/ltr
Biotin	100µl/ltr
Pyridoxine hydrochloride	100µl/ltr
Yeast extract	5mg/ltr
L-cystein	0.1g/ltr

3.2 ACTIVITY ASSAY

After performing the production part for 120 days, each day a supernatant was collected and centrifuged at 13,000 rpm at 4°C for 10mins. The soup was taken for activity assay.

3.2.1 Dinitrosalicylic acid (DNS)

It is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at 540 nm. The DNS test will helps in quantifying the amount of xylan converted into xylose which is the reducing sugar by the xylanase produced by the bacteria. The following steps are followed:

1. Sodium hydroxide (6.955gm) and DNS (8.725gm) was dissolved in water.
2. Sodium potassium tartrate (107.475gm) was added.
3. Phenol was melted at 50°C and 2.675ml of it was added to the prepared solution.
4. Sodium meta-bisulphite (2.915gm) was added and mixed well.
5. The solution was stored in a dark bottle.

3.2.2 Sodium Phosphate Buffer preparation (200mM)

Sodium phosphate buffer is used in enzyme assay to maintain a pH of 7.

1. 0.996gm of NaH_2PO_4 (dibasic salt) was dissolved in 35ml of distilled water.
2. 0.69gm of Na_2HPO_4 (monobasic salt) was dissolved in 25ml of distilled water.
3. The dibasic solution prepared was then added into the monobasic solution prepared till the desired pH was obtained.
4. The buffer was then stored in a clean bottle.

Table 5: Activity assay analysis.

	TEST	**ENZYME BLANK**	**SUBSTRATE BLANK**
ENZYME	25 µl	25 µl	-
SUSBTRATE	500 µl	-	500 µl
BUFFER	475 µl	975 µl	500 µl
TOTAL	1 ml	1 ml	1 ml

*Test: the supernatant obtained

*Substrate: beech wood xylan (0.5%).

*Symbol '–': indicates that the particular component is not added to the test tube

The activity was set up using the DNS method. (Miller, Gail Lorenz, 1959).

3.3 PROCEDURE:

- The collected supernatant was centrifuged at 13,000 rpm for 10 minutes at 4°C.
- The supernatant was transferred into a fresh eppendorf.
- Fresh test tubes were labeled for Test; Enzyme blank; Substrate blank and Blank.
- All the reagents were added to labeled test tubes corresponding to the table above.
- Incubation at 70°C was set up in water bath for 5 minutes.
- After incubation the DNS was added (1.5ml) to each test tube to stop the reaction.
- 10minutes of boiling was given to each test tube.
- After boiling, the tubes were cooled down and readings were taken in the spectrophotometer.

- OD was taken at 540nm and graphs were plotted accordingly.

The substrate was prepared in sodium phosphate buffer of 7 pH and was allowed to dissolve 0.5% beechwood xylan, from Himedia.

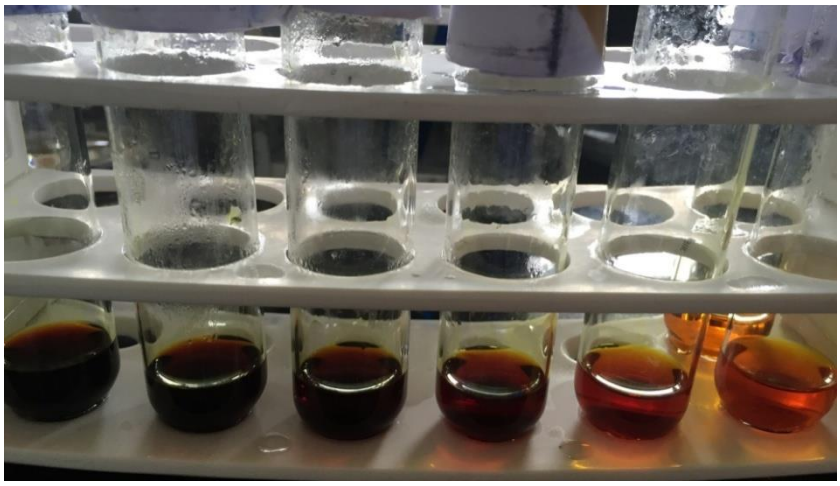
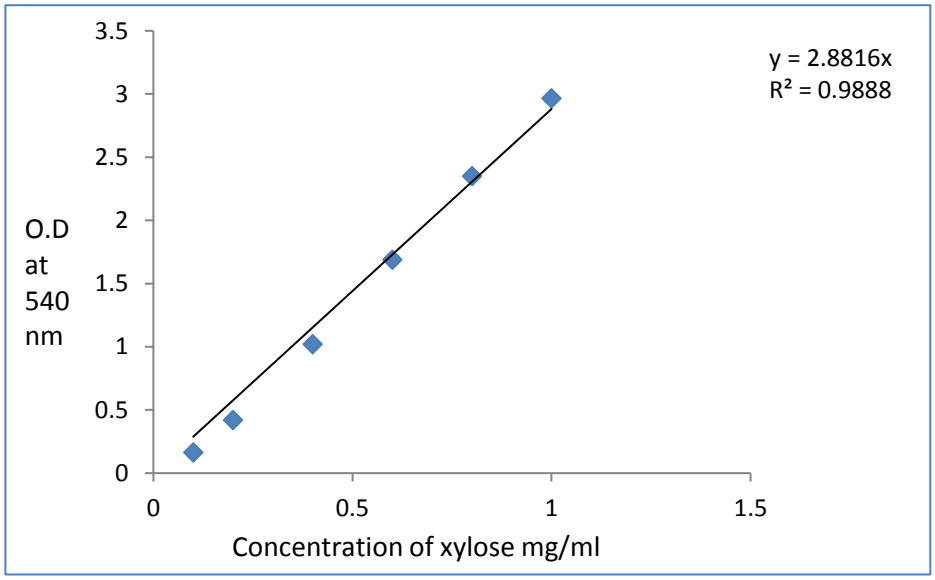


Fig 7: Test tubes with standards.



Graph1: Standard plot for Xylose sugar estimation using DNSA Assay. Stock-1mg/ml.

A setup for Cellulose assay was also performed similarly like xylose assay, using the substrate CMC (Sodium salt of Carboxy methyl cellulose). No activity was observed after performing DNS assay. Hence, the enzyme produced by the bacteria is cellulase-free endo-xylanase.

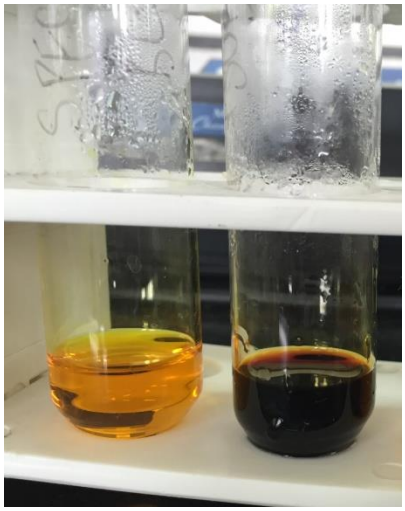


Fig 8: Test tubes with Blank and Test samples for PS1 isolate crude enzyme after performing DNS.

3.4 PRESERVATION OF CULTURES

3.4.1 SLANT PREPARATION (SHORT TERM PRESERVATION)

Agar slants contain nutrient agar as the media, in a test tube where the solidified agar is in slanted position inside the test tube. The slant provides the advantage of a cotton plug that prevents the agar from drying out and also more surface area for the bacteria to grow. The following steps are followed for preserving bacterial cultures on agar slants:

- Nutrient agar medium was prepared and distributed in test tubes, each containing 5 ml of nutrient agar medium.
- The test tubes were covered using cotton plug and were autoclaved.
- While the media was hot, the test tubes were slanted against a solid surface and allowed to solidify.
- The culture was then streaked on the surface of the slants and stored in the refrigerator.

3.4.2 GLYCEROL STOCK PREPARATION (LONG TERM PRESERVATION)

Bacterial glycerol stocks are important for long-term storage. The addition of glycerol stabilizes the frozen bacteria, preventing damage to the cell membranes and keeping

the cells alive. A glycerol stock of bacteria can be stored stably at -80°C for many years. To do so the following steps are followed:

- A solution of 30% glycerol (v/v) was prepared. The solution was then autoclaved at 121°C for 15 min.
- 500µl of the autoclaved glycerol stock was added into an aliquot.
- 500µl of overnight grown culture of the bacteria was added to the aliquot containing glycerol and mixed using a vortex.
- The aliquot was then labelled and stored at -80°C.

3.5 CHARACTERIZATION OF BACTERIA

The basic and observatory characteristics were performed in laboratory for testing the bacterial morphology.

3.5.1 IDENTIFICATION OF BACTERIA

GRAMS STAINING:

The identified bacteria PS1 was countered with Grams stain to identify the type of cell wall. It was observed under microscope that the isolate is rod shaped, gram positive and the arrangement of cells are single and in chain form.

REAGENTS: Crystal violet, Grams iodine, ethyl alcohol and safranin.

PROCEDURE FOR GRAMS STAINING:

- The glass slide was cleaned and microbial smear was prepared.
- The smear was heat fixed.
- The slides were flooded with crystal violet stain and washed after 1 minute with running water.
- Grams iodine (mordant) was added for 1 minute and washed with running water.
- To decolorize the stain, ethyl alcohol (95%) was added for 15-30 seconds and washed with running water.
- Counter stain was added, safranin and kept for 45 seconds to let bacteria with gram negative cell wall attain a pink stain.

- The slide was washed finally and air dried.
- The slides were observed under microscope.

Further plate characterisation studies were also performed which included studying the type of colony formed and its morphology.

3.5.2 CONGO RED ASSAY:

Another study to screen for xylanolytic bacterial isolate was performed which is Congo Red Clear Zone Assay.

Congo red is a diazo dye that binds to carbohydrate polymers (cellulose and xylan). It is red in colour and is used for detecting the presence of xylanolytic bacterial isolate on a plate with isolate. A clear zone is observed that will indicate the presence of potent isolate able to degrade xylan.

REAGENTS: Congo red dye, destaining solution (1M NaCl)

PROCEDURE FOR CONGO RED ASSAY:

- Agar plates were prepared with 0.5% xylan.
- Wells were punctured into the plate to allow pouring of broth isolate onto the well.
- This plate was incubated for 48 h.
- 10ml of 1% congo red was added on top of the plate.
- After 15 minutes the stain was poured off and destaining solution was added.
- The clear zones were observed around the punctured wells.

3.6 TESTING FOR THE FACULTATIVE ANAEROBE:

Another study was performed for the isolate, which was testing whether the isolate is a facultative anaerobe or not. Facultative anaerobes are capable of utilising oxygen and producing ATP, but also they can grow and metabolise in oxygen depletion conditions and still produce ATP. Obligate anaerobes are strictly growing under no oxygen conditions. The condition for anaerobic culture was maintained in serum bottles with nutrient media. The bottles were sealed with septa and nitrogen gas was purged in.

Fig 9: Nitrogen purging in process.

The bottles were kept at optimised conditions i.e. 50°C, 150 rpm for 72 h. Growth was observed in the bottles after 72 h of incubation. Later, the inoculum from purged bottle was incubated into another fresh nitrogen purged bottle and kept on incubation for next 72 h. Meanwhile, the first inoculated bottle sample was spread onto agar plate to study the morphology of the isolate PS1. The plate showed the similar morphology as studied under characteristics of the isolate. It was confirmed that the isolate PS1 is a facultative anaerobe.

3.7 CHEMICAL ANALYSIS OF SUBSTRATE, WHEAT STRAW:

The compositional analysis of lignocellulosic substrate is performed by in three parts determination:

Cellulose concentration; Hemicellulose concentration and Lignin concentration.

3.7.1 DETERMINATION OF CELLULOSE CONTENT:

Cellulose content of the substrate was determined by the method of Crampton and Maynard (1938). One gram of oven dried sample was taken in a 250ml beaker. Then 25ml of acetic nitrate reagent (acetic acid=73.86ml, nitric acid=9.09ml, distilled water=17.04ml) was added and the contents were boiled until the evolution of brown fumes. The residue was then filtered using whatman filter paper. After the filtration, three washings each of water, alcohol and acetone were given. The residue was then transferred into a preweighed crucible and placed in an oven (150°C) overnight. After cooling in a dessicator, it was weighed (W1). Finally, the crucible was kept in a

muffle furnace at 450°C for 1 hour, cooled and weighed (W2). Loss in weight (W2-W1) was observed as the amount of cellulose present in the sample.

Fig 10: Wheat straw after drying in muffle furnace.

3.7.2 DETERMINATION OF HEMICELLULOSE CONTENT:

Hemicellulose content of the substrate was determined by estimating the percentage of NDF and ADF by the method of Georing and Vansoest (1970).

NDF DETERMINATION: Reagent: NDS- Neutral Detergent Solution (SDS=30g/ltr, EDTA=18.61g/ltr, Sodium borate decahydrate= 6.81g/ltr, Disodium hydrogen phosphate=4.56g/ltr, 2-ethoxy ethanol=10ml).

PREPARATION OF NDS: EDTA and sodium borate decahydrate were taken in a beaker containing 200ml of distilled water and dissolving by heating. SDS and 2-ethoxy ethanol were dissolved separately in boiling distilled water and then mixed with above solution. Disodium hydrogen phosphate was separately dissolved in boiling water and then added to above solution. The pH was adjusted to 7 to completely dissolve all the solvents and volume was made upto 400ml with distilled water.

One gram (W) of dried sample was taken in a beaker. Then 100ml of NDS, 2ml decaline and 0.5g sodium sulphite were added in sequence. The contents were boiled for 5-10 minutes and refluxed slowly for 1 hour. The refluxed sample was filtered and transferred into the weighed crucible (A1). It was rinsed with hot water, absolute ethanol and acetone sequentially. The crucible was dried at 105°C for 12 h and weighed (A2). The NDF (%) was calculated as:

NDF (%) = (A1-A2/W) x 100

Fig 11: ADF and NDS in process.

DETERMINATION OF ADF (Acid Detergent Fiber):Reagent : ADS-Acid Detergent Solution (Cetyl trimethyl ammonium bromide (CTAB) 6g in 300ml of 1N sulphuric acid). One gram (W) of the sample was taken in a 500ml beaker, 100ml ADS and 2ml decaline were then added to it. The contents in the beaker were boiled for 10minutes and thereafter refluxed slowly for 1 hour. The digested sample was then filtered and transferred into weighed crucible (A3). The residue was then washed with hot water, ethanol and acetone respectively. The crucible was then dried at 105°C for 12 h and cooled in a dessicator and weighed (A4). The dried weight of the residue was recorded as ADF given by:

ADF (%) = (A3 – A4 / W) x 100

Hemicellulose content (%) in the substrate was calculated as per the formula

HEMICELLULOSE (%) = NDF (%) – ADF (%)

Fig 12: Left: Wheat straw after performing ADF; Right: Wheat straw after performing NDF.

3.7.3 DETERMINATION OF ADL (Acid Detergent Lignin) Georing and Vansoest 1970:

The crucible containing ADF was kept on 500ml conical flask containing water. The contents of the crucible was covered with 20ml of 72% sulphuric acid and stirred with a glass rod to a smooth paste. The crucible was then filled with acid and kept on ice bucket. After 3 h, excess of acid was filtered. The residue was then washed with hot water and acetone till it was acid free. The crucible was dried at 100°C till it was completely dried, cooled in a dessicator and weighed (A5). The crucible was then placed in a muffle furnace at 500°C for 3 h and weighed (A6). The ADL was computed as **ADL (%) = (A5-A6/W) x 100**

The ADL content (%) worked out was equivalent to lignin (%).

Fig 13: Incubation on ice for ADL.

3.8 MOLECULAR CHARACTERISATION OF PS1:

DNA caries the genetic information of the entire living organism. Extraction of DNA from the nucleus is a task to be performed for molecular characterisation of the isolate. The extracted DNA can be quantified and amplified by PCR method with the use of universal primers (27F and 1492R) of IDT (Integrated DNA Technologies). This pair of primer is described as the best pair to amplify the full length 16s r RNA sequences. It gives an amplified product called amplicon which was further used in sequencing studies.

The genomic DNA extraction was performed by the following procedure:

Reagents: TE buffer (pH 8), 10% SDS, Proteinase K, Phenol, Chloroform, 5M Sodium acetate(pH 5.2), Isopropanol and 70% ethanol.

3.8.1 PROCEDURE FOR DNA EXTRACTION

- Colony was inoculated in 20ml of nutrient broth for 24 h.
- After 24 h, the cells were pellet down in 15ml centrifuge tubes at 5000 rpm for 5 minutes.
- 400µl of SDS lysis buffer was added to the pellet and mixed well.
- The above solution was transferred to 1.5ml centrifuge tubes
- 400µl of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and gently mixed by inverting the tubes.
- Centrifugation was performed at 13,000 rpm for 10minutes at room temperature.
- The supernatant was transferred to a fresh eppendorf without disturbing the interface.
- Equal volume of Chloroform: Isoamyl alcohol was added to the supernatant.
- The mixture was centrifuged at 13,000 rpm for 10 minutes at room temperature.
- To precipitate the DNA, $2/3^{rd}$ volume of chilled isopropanol was added along with $1/10^{th}$ volume of sodium acetate (0.5M) and mixed well.
- The solution was incubated on ice for 30 minutes.
- Centrifuged at maximum speed for 15 minutes at 4°C.

- The supernatant was discarded and the pellet was rinsed with 70% ethanol (500µl).
- The centrifugation was performed for 2 minutes at 13,000 rpm.
- The soup was discarded and air dried until all the ethanol is evaporated.
- The pellet was dissolved in DNA rehydration buffer and stored at 4°C.

3.8.2 PROCEDURE FOR PCR AMPLIFICATION

REAGENTS: Master mix (2x), Primers (forward and reverse), DNA template and a thermocycler.

Polymerase chain reaction is a process to amplify DNA by using the specific primers which are complementary to the template. The amplification is performed by using the Taq DNA polymerase which is thermostable.

Conditions for amplification upto 35 cycles:

Table 6: Optimised PCR conditions.

TEMPERATURE	TIME
95°C	5 minutes
95°C	1 minute
51.8°C	1 minute
72°C	1 minute
72°C	7 minute
4°C	Infinity

3.9 OPTIMISATION OF PHYSICO-CHEMICAL PARAMETERS

Optimisation helps in understanding better the bacterial isolate and its functionality to produce the particular enzyme at particular conditions. The best use of the enzyme in the hydrolysis process will be done when all the parameters are known, well studied and quantified related to conditions. Optimisation results in

3.9.1 CULTURE MEDIA USED FOR STUDIES

The culture media used for every optimisation parameter production was minimal complex media. The inoculum used for every production parameters optimisation was 1.806 at 600nm. The culture was revived for 24h at optimum conditions.

3.9.2 EFFECT OF CARBON SOURCE ON THE PRODUCTION OF XYLANASE ENZYME

Using varying carbon source for the production of enzyme allows us to explore the potential of the bacterial isolate to be able to produce enzyme effectively. The varying carbon conditions will help is determining the best suitable source of enzyme production by the isolate. The various sources exploited for the study were: wheat straw, corn strover, sugarcane baggase, rice straw. The major activity noted was utilising the substrate wheat straw.

3.9.3 EFFECT OF INOCULUM SIZE ON THE PRODUCTION OF XYLANASE

The inoculum size also affects the production process. The determination of number of inoculum used helps in optimising the parameters used for pilot scale or for the industrial process.

3.9.4 EFFECT OF TEMPERATURE ON THE GROWTH AND XYLANASE PRODUCTION

Temperature plays a major role in determining the optimum activity of the enzyme and substrate reaction. The isolate was inoculated 500µl in 50ml media with 1% substrate concentration. The production was setup for 120 days at 50°C. The data for variation in temperature conditions were recorded after every 24 h. Incubation of the enzyme and substrate was kept at five different temperatures. We found the best activity at 70°C , which was performed with DNS method.

3.9.5 EFFECT OF pH ON THE XYLANASE PRODUCTION

To study the pH activity of the initial culture medium on the formation of extracellular endo-xylanases, the isolate was grown in nutrient broth for 24h before

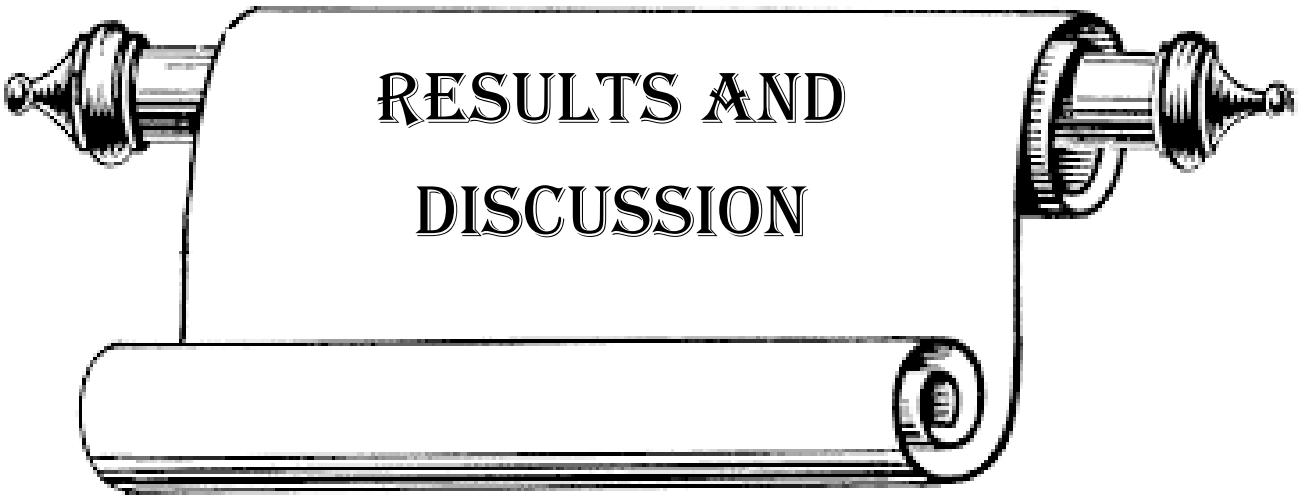
inoculating for production. The initial pH values were recorded and media was prepared. We took five different pH values for our experiment. The data for variation of pH value was recorded after every 24 h.

3.9.6 EFFECT OF VARYING SUBSTRATE CONCENTRATIONS ON XYLANASE PRODUCTION

The difference in substrate percentages gives a broader view for the industrial purpose. The more the substrate the more is the production of endo-β 1.4 xylanase. After reaching the saturation stage, the effect of substrate is stabilised by the presence of constant amount of enzyme. At this point there is no increase in the reaction velocity for the reaction.

3.9.7 EFFECT OF INCUBATION TIME ON THE PRODUCTION PROCESS

The growth of bacteria can be studied in four different phases: Lag phase, Log phase, Stationary phase and Death phase. The incubation time is also linked to the growth metabolism of bacteria. The utilisation of substrate is performed until the bacteria reach the exponential phase, after that; there is a decrease in the enzyme production as well. This may be because of the utilisation of nutrients in the batch media for further growth of the bacteria.


RESULTS AND
DISCUSSION

1. RESULTS AND DISCUSSION

The importance of xylanase in paper pulp, food industry and biofuels generation has initiated the search for microorganisms producing higher level of xylanases. In search for the novel thermostable xylanases suitable for industrial applications, studies were initiated for isolation of microorganisms that could produce xylanases. The screening for the xylanase producing bacteria is little difficult because of the heterogeneity of the plant polysaccharide, xylan (Juturu V,2011). It requires more than two or more enzymes to act efficiently and degrade the xylan complex polymer releasing simple sugars.

The isolation of bacteria with higher competency to produce enzyme is the major focus of the study. Samples were collected from degrading soil waste of lignocellulosic substances like pine needles.

The varying diversity of microorganisms and their nutritional requirements allows the selection of media specific to our use. The settlement was done with minimal media having all the necessary components for the growth and utilisation of substrate.

4.1 IDENTIFICATION OF BACTERIAL CULTURE:

After continuous screening of the bacteria from different sources, one was selected for our future study. The potent isolate named PS1 was able to produce enzyme activity of 27 U/ml after 72 h of production.

Further morphological and molecular studies were performed for the PS1 isolate.

4.1.1 MORPHOLOGICAL AND MOLECULAR STUDIES:

The bacterium was grown on agar plate and the studies were performed accordingly.

Fig 14: PS1 on agar plate.

Colony morphology:

Size: circular

Shape: rod shaped (microscopic view)

Margin: entire

Colour: creamish

Surface texture: smooth, flat

Consistency: highly viscous

Optical features: opaque

Grams' staining was also performed to test the nature of bacterial petidoglycan layer. It was observed that the bacterium is Gram positive and rod shaped under microscopic view. Further characterisation studies were performed.

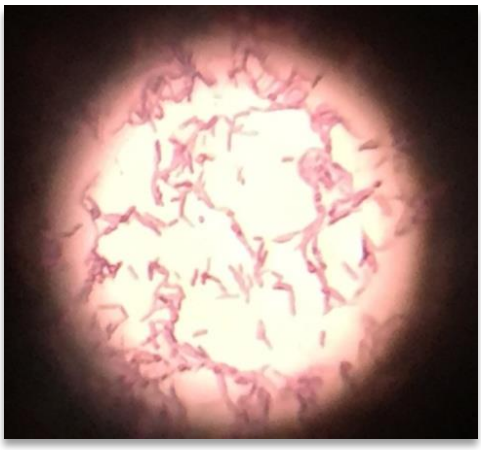


Fig 15: PS1 stained and observed under microscope.

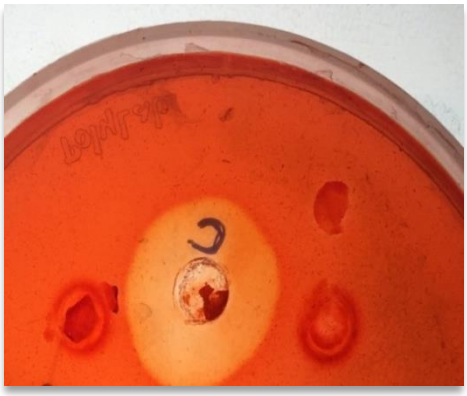


Fig 16: Clear zone formation for PS1 isolate on xylan agar plate.

4.1.2 TESTING FOR FACULTATIVE ANAEROBE

Another study performed was to test for facultative anaerobe. The nature of bacteria when grown in the presence of oxygen and also grown in the absence of oxygen is facultative anaerobe. PS1 was grown under no oxygen condition in the serum bottles and tested for its facultative nature. This exploitation of PS1, degrading xylan anaerobic bacteria could help to extend the potential of xylan and the knowledge of diversity of microbial specie (Niimura, et al. 1987). For this purpose, we have isolated a xylan degrading thermophilic anaerobe from biogas slurry. The purged and inoculated bottles were kept at optimised conditions, 150rpm and 50°C for 48 h. After 48 h the growth was observed at results were studied.

From Fig 17, it was well explained that PS1 could grow in aerobic conditions more rapidly. It could also grow in oxygen depleted environment with slow growth as less turbidity is seen in serum bottle B (Fig 17).

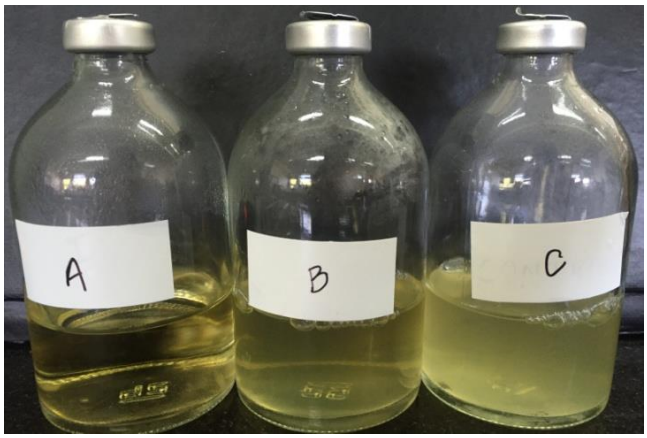


Fig 17: A: Anaerobic condition without inoculum; B- Anaerobic condition with inoculum; C- Aerobic condition with inoculum

Sample was collected from serum bottles and spread onto agar plates to observe the same morphology as of PS1. We observed similar morphology and growth characteristics like PS1 (Fig 18).

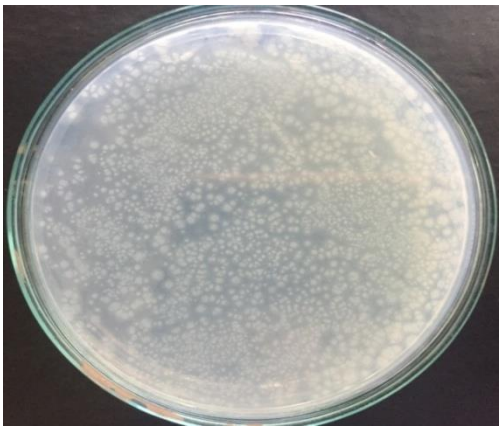


Fig 18: Nitrogen purged inoculum from serum bottles spread onto agar plates.

4.1.3 COMPOSITIONAL ANALYSIS OF WHEAT STRAW

Selection of wheat straw for the purpose of enzyme production was its availability throughout the year. Asia is the largest producer of wheat and rice in the world (Kim S, et al. 2004).

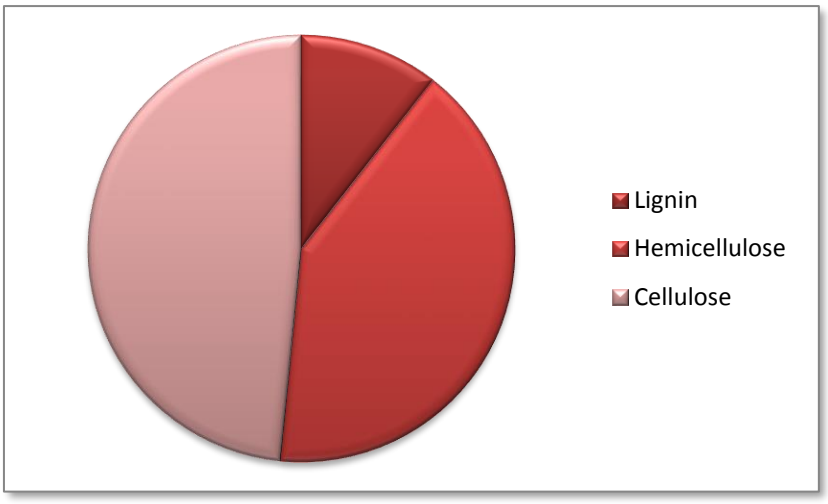
The composition of the wheat straw selected for our study was:

Cellulose 46%

Hemicellulose 39%

Lignin 10%.

The composition of wheat straw selected as substrate signifies the exposure to hemicellulose content of the plant. The isolate PS1 gets more platform for hydrolysis (Graph 2) and produce enzyme. Lignocellulosics degradation is performed by three ways: delignification, for exposure to cellulose and hemicellulose, hydrolysis of xylan and cellulose to release reducing sugar monomers like xylose, arabinose, glucose and mannose by fermentation (Balat, et al. 2008).



Graph 2: Composition of wheat straw used for study.

4.2 MOLECULAR CHARACTERISATION OF THE ISOLATE PS1:

After performing the morphological characterisation, the isolate PS1 was subjected to molecular study. The molecular analysis steps were performed for further identification of PS1. Genomic DNA extraction and 16s rRNA gene amplification were prior steps to examination. A prominent band was observed under the Gel documentation system illuminating UV light.

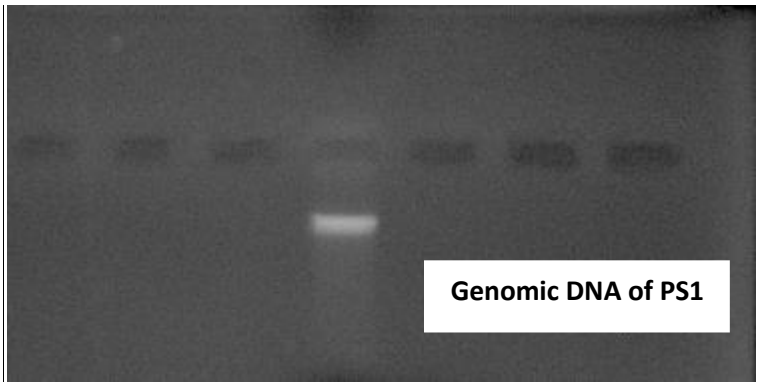


Fig 19: DNA band observed under Gel doc system.

4.2.1 16s rRNA GENE AMPLIFICATION

After extracting genomic DNA from the bacterium, PCR amplification of the template was performed with the universal primers for bacteria. The primers used were 1492R and 27F which are conserved for the 16s rRNA region of the specie. This

amplification would help in sequencing of the 16s rRNA to know the specie of the isolate so that we can identify the conserved sequences of bacteria.

The conditions were optimised for amplification and PCR was run.

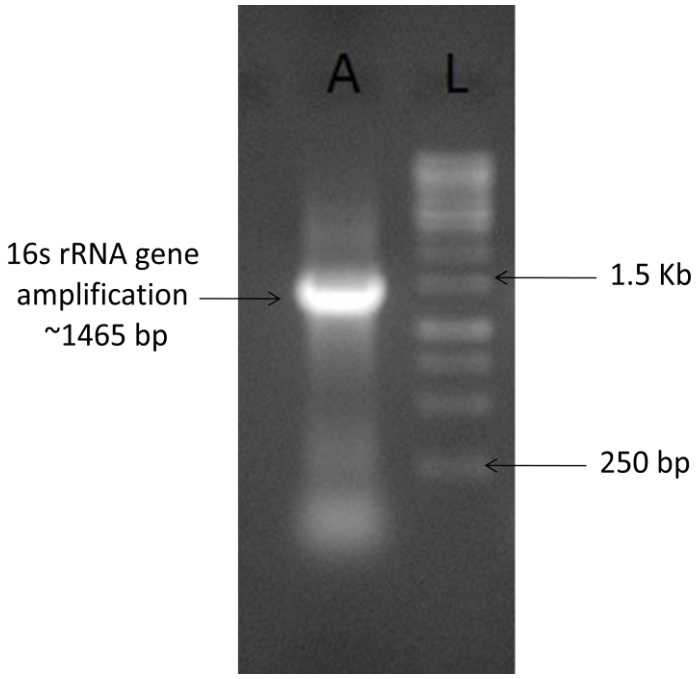


Fig 20: Depicting the amplification of DNA in duplicate. A is are the amplicon of PS1. L is the gene ruler of 1Kb (Thermo fisher).

4.3 CULTURAL PARAMETERS OPTIMISATION:

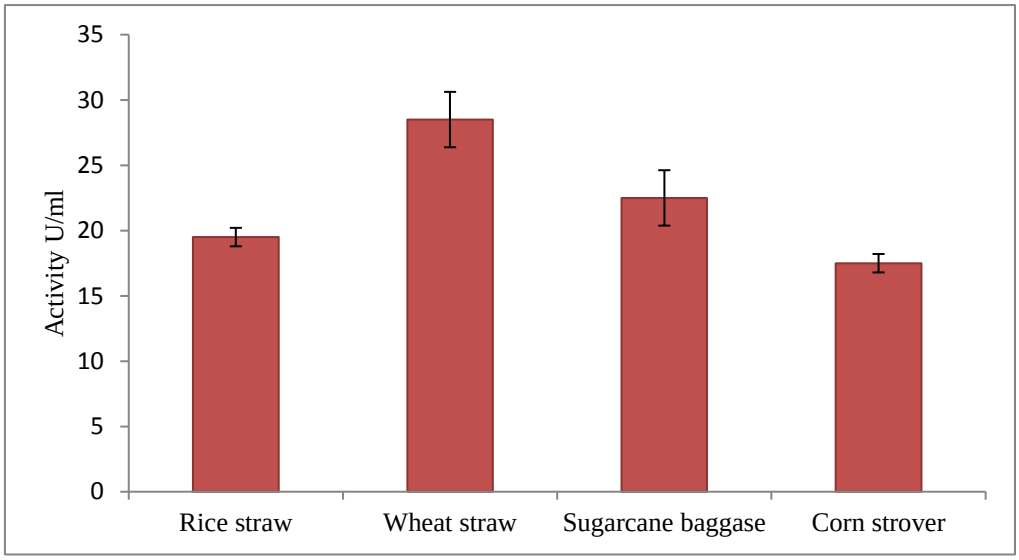
The optimisation studies were performed at different conditions and parameters to get the maximum yield of enzyme. Optimisation was performed as OFAT (One Factor At a Time), (Irfan, Muhammad, et al. 2014). The parameters were selected which could give the enhanced enzyme production a good yield for industrial applications.

4.3.1 OPTIMISATION OF CARBON SOURCE USED:

Bacterial isolate identified PS1 was tested for its ability to utilise different carbon sources. It was observed that PS1 was able to utilise maximum of carbon from wheat straw. From the Graph 3, it was observed that isolate PS1 utilised wheat straw efficiently among all the substrate tested. The maximum enzyme activity reported was 28.5 U/ml after 72 h, similar results with high xylanase production were observed by

using wheat and rice straw, 24 U/ml. (Goswami and Rawat, 2015 and Gupta et al. 2009).

The plant polysaccharides were taken after drying, grinding and sieving them with standard test sieves as per IS 460: 1962 of 60 BSS and 250µm mesh size. The substrates were pre-treated by autoclaving at 150°C for 15 minutes at 15lbs pressure. The study was performed until 120 h and maximum production was noted at 72 h.

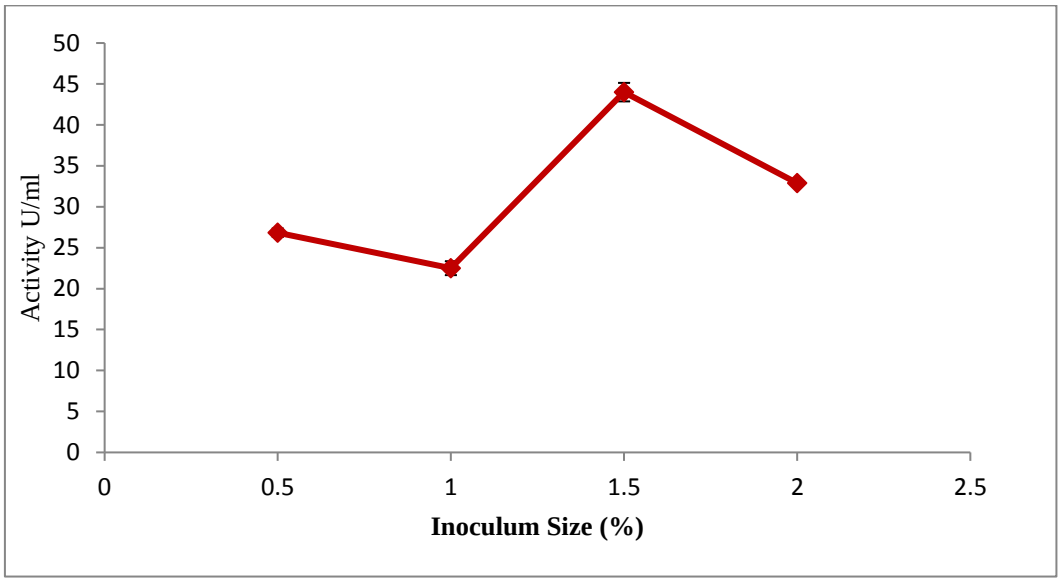


Graph 3: Enzyme activity observed for different substrates.

4.3.2 OPTIMISATION OF INOCULUM SIZE:

The varying inoculum size also has a major impact in production of enzymes (Irfan et al. 2016). Therefore, effect of different inoculum size i.e. 0.5, 1, 1.5 and 2% of bacterial isolate PS1was investigated. The OD, 1.806 of the isolate PS1 was kept constant for every parameter studied. The isolate showed maximum activity (44U/ml) with 1.5% inoculum size (Graph 4). Our results are in good agreement with Irfan et al. (2016), who reported 1.5-2% bacterial inoculum as optimum for the maximum and stable production of xylanase enzyme. Sepahy et al. (2011) reported that the inoculum size of 2% was best for xylanase production by *B*acillus *mojavensis* AG137 in submerged fermentation process. From the Graph 4, it was observed that, with increase in inoculum size from 0.5-1% enzyme production increased. However, further increase in the inoculum size from >1.5% resulted in a decreased enzyme production. Battan et al. (2007), reported a reduced xylanase production in the

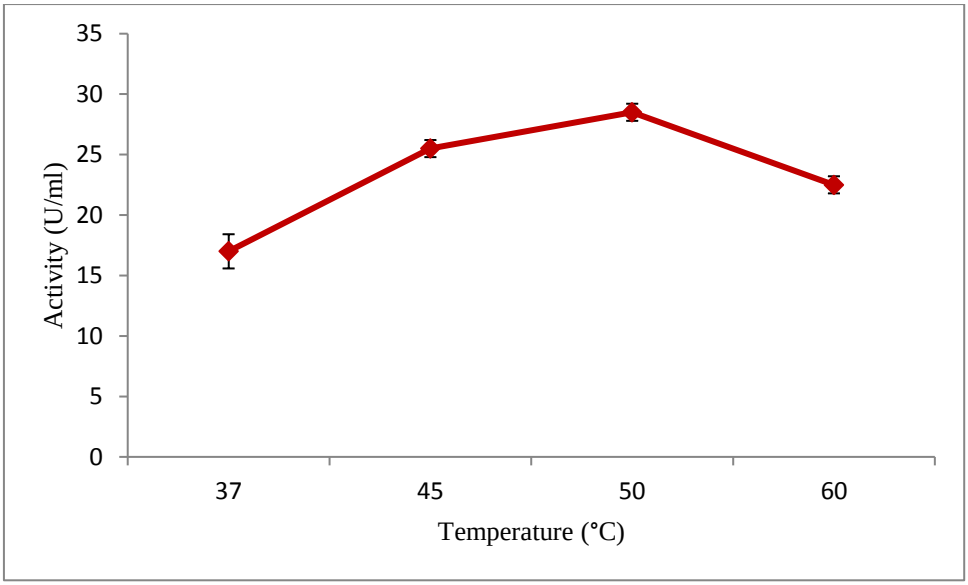
industrial process at higher inoculum size. This has correlation with the depletion of nutrients in the culture media which might not be sufficient for all the bacteria to consume and grow on substrate and produce enzyme.



Graph 4: Optimisation of inoculum size during production.

4.3.3 OPTIMISATION OF TEMPERATURE:

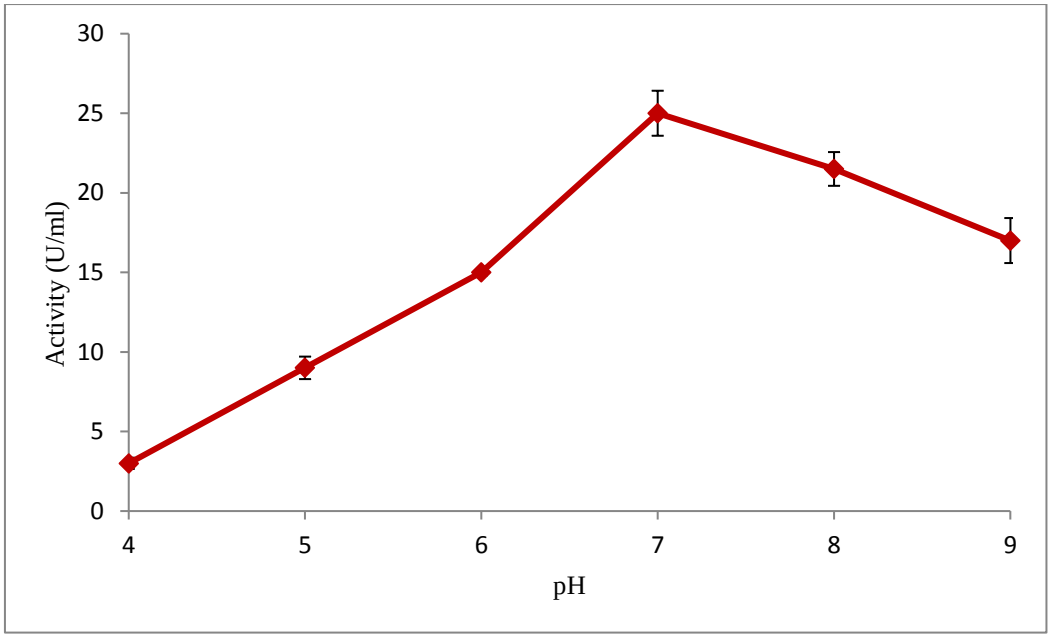
Effect of different temperature range i.e. 37, 45, 50 and 60°C on enzyme production by bacterial isolate PS1 was tested. Graph 5 illustrates an increase in enzyme production from 37-50°C. Further increase >50°C resulted in decreased enzyme production. The maximum enzyme production was 28.5 U/ml at 50°C (Graph 5). The enzyme produced at 50°C with all the optimised conditions was found to be thermostable and gave best activity at 70°C. Irfan et al. (2016) reported maximum activity at 50°C using mesophilic bacteria *B. Subtilis* and *B. megaterium***.** The results may be of good use to the industry after purification of enzyme. Simphiwe et al., (2011), reported maximum yield of xylanase production at incubation temperature of 45 °C to 55 °C using different strains of *Bacillus* sp.



Graph 5: Optimisation of temperature conditions.

4.3.4 OPTIMISATION OF INITIAL pH :

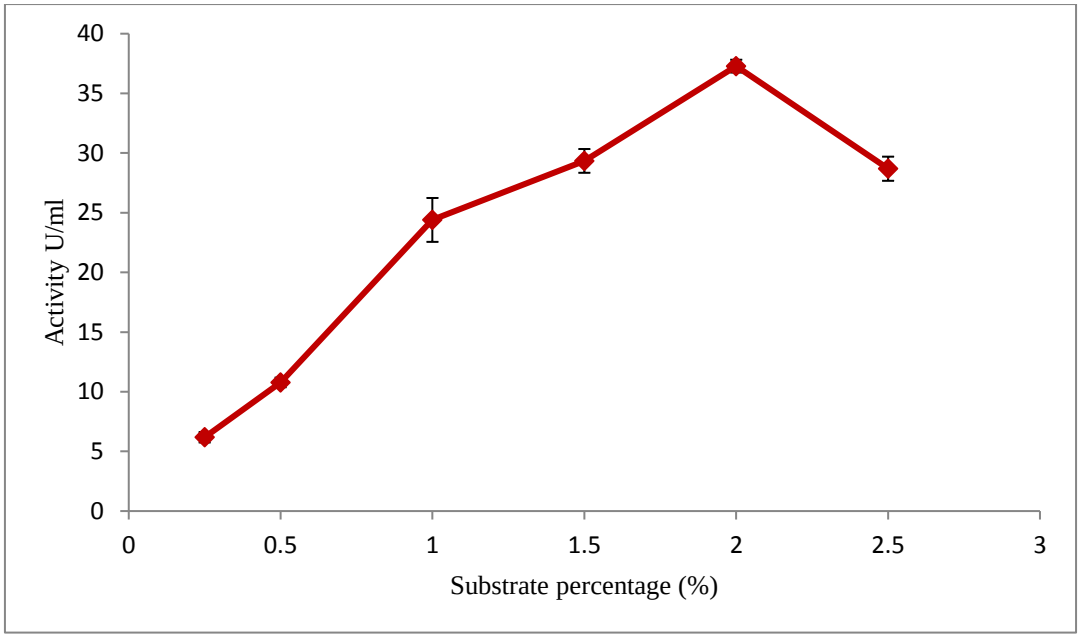
Influence of different pH conditions i.e. 4, 5, 6, 7, 8 and 9 on enzyme production of bacterial isolate PS1 was tested. The production was setup for 120 h at 50°C, pH 7 and 150 rpm in an incubator shaker. The maximum activity was 25U/ml under these conditions in 50ml media (Graph 6). However, at pH >7 enzyme production significantly decreased. Similar findings were reported by Sepahy et al. (2011) who showed optimum pH of 7-8.0 for xylanase production by *B. mojavensis* AG137 in submerged fermentation. Another study of pH optimisation showed that the enzyme activity of *Bacillus pumilus* under controlled conditions produced cellulase free thermostable xylanase at pH 7-8, utilised in paper and pulp kraft process. (Battan, et al. 2007).



Graph 6: pH optimisation conditions for PS1.

4.3.5 SUBSTRATE CONCENTRATION OPTIMISATION:

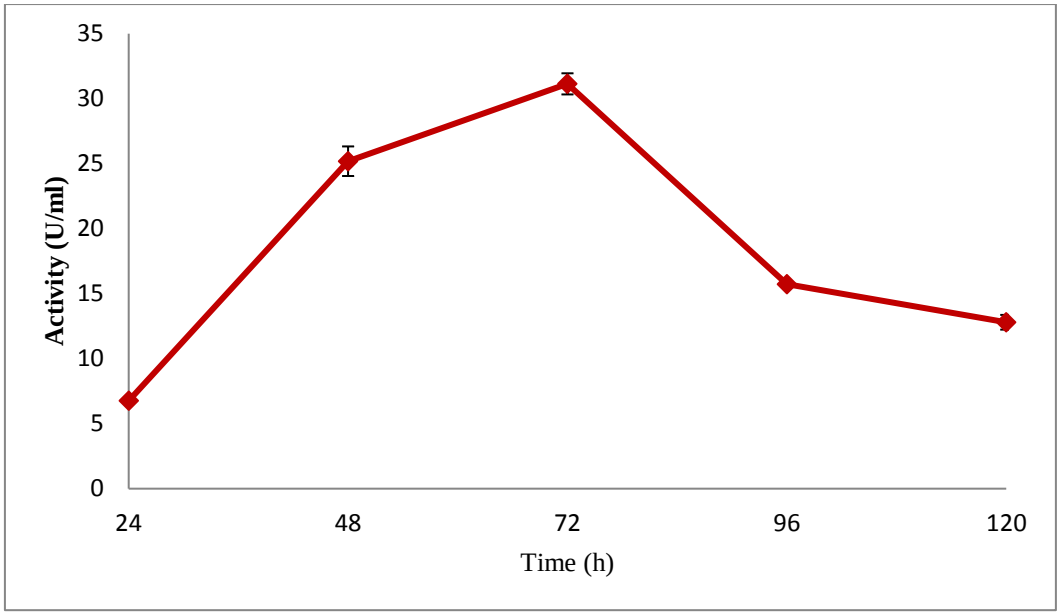
The influence of varying concentrations (0.25, 0.5, 1, 1.5, 2 and 2.5%) of wheat straw of size 250µm mesh work, as per IS 460:1962 standard test sieves, was investigated for enzyme production by isolate PS1. The best activity (37.28 U/ml) was observed at 2% substrate concentration (Graph 7). All the production setups were kept at 150 rpm, 50°C, 50 ml media and pH 7. After 2% , when the bacterial concentration is very high, the increase in substrate concentration does not affect the thermophilic enzyme production process. Saleem, et al, isolated a new strain of *B. subtilis* which produced best xylanase using 0.5% bagasse as a substrate in submerged fermentation. A report compiled that the maximum recovery of hemicellulose was observed when the substrate wheat straw was pre treated at 185°C for 6 min. This will release the xylan from the plant polysaccharide, allowing the bacteria to attack on it and consequently producing the enzyme xylanase. In our study, the substrate used was wheat straw which was pre-treated at 150°C for 15 min at 15 lbs/inch^2 pressure.



Graph 7: Maximum activity observed at 2% substrate concentration.

4.3.6 INCUBATION TIME OPTIMISATION:

Different experiments were conducted to optimise the production of thermostable xylanase. The results (Graph 8) described that the enzyme production incubation was maximum after 72 h. The production was setup at 50°C for 120 h at 150 rpm and 50ml media, keeping the initial inoculum constant every time. The maximum hydrolysing process results in production of sugar after 72 h. Any further acceleration in the hydrolysis process beyond this time resulted in decrease of enzyme production, which might be because of the toxic metabolites produced when the nutrients are absorbed and growth of microbes are inhibited. Similar study was performed with *Bacillus sp.* and the maximum xylanase production was noted in between 48h-72h on the substrate wheat bran and corn corb. (Guptaet.al, 2009).



Graph 8: Conditions for incubation time optimisation.

4.4 DISCUSSION

The xylanolytic bacteria play a major role in conversion of plant xylan complex into simple sugar monomers, which can be utilised by microorganisms as source of nutrition. The purpose of the selection of the source was to identify potent bacteria having the ability to degrade plant xylan complex into its constituent sugar monomers. The *Bacillus* sp. is reported to degrade xylan of plants. The xylanolytic enzyme was the main product after the utilisation of wheat straw by bacteria.

The selection of biogas slurry, fed with lignocellulosic pine needles for the past one year (Tripathi, et al. 2015) was approved as a good source to identify bacteria having ability to hydrolyse xylan. . The microorganisms present in biogas digesters are able to degrade plant lignocellulosic materials (Jiang, et.al, 2011). In our study, the screening method was setup to identify thermostable bacteria with ability to degrade cellulose and xylan. But we found that the potent isolate PS1 could be able to degrade xylan but not cellulose. The biochemical ability of isolate PS1 to convert xylan and its derivatives into acceptable sugars was analysed using screening assays and optimisation studies.

The screening was performed by the Congo red assay in context to the study performed by (Meddeb, et al. 2014). The results indicated confirmation of

xylanolytic bacteria. Another screening method was performed with the crude enzyme isolated after the digestion of substrate by bacteria PS1. The confirmation of enzyme activity was measured by DNS method as performed by many researchers (Alves, et al. 2010, Palaniswamy, at al. 2008).

The activity of the PS1 was found to be 26 U/ml after 72 h at growth optimised conditions of 50°C, submerged production with minimal media and 150 rpm. Many researchers have also reported that the enzyme production at thermophilic growth conditions allows production of thermostable enzyme. (Zambare, et al. 2011; Rastogi, et al. 2010).

The significant increase in the production of xylanase was the major focus after identification of potent bacteria. (Sharma. N, et al. 2013). The parameters, temperature, inoculum size, incubation time, substrate concentration and carbon source, were tested at optimised conditions one after other, keeping in mind the sterility of the flaks and shakers. The isolate was also tested for cellulase production. The results (not mentioned), showed no cellulase activity with the PS1, hence the enzyme produced by isolate is cellulase free endo-xylanase. Enzyme having potency to degrade xylan but not cellulose are widely used in paper and kraft process for the chemical free bleaching of kraft (Comlekcioglu, et al. 2014). Reported earlier, many xylanase producing bacterial species were identified like *Bacillus circulan* (Ratto et al. 1992). Based on morphological, physiological and molecular analysis, isolate PS1 was identified as *Bacillus sp*. These species have been reported as xylanolytic enzymes producers, such as, *B. circulans* AB16 (Dhillon et al. 2000b), *B. circulans* D1 (Bocchini et al. 2002), *B. licheniformis* (Archana, et al. 1997), *B. subtilis* B230 (Oakley et al. 2003). Earlier reported *Bacillus sp*. are able to produce enzyme in the pH range of 6-8 and temperature ranging from mesophilic to thermophilic conditions (37-70°C) (Coughlan, et al. 1993 and Srinivasan, et al. 1999), pp. 137–142). The studied *Bacillus* spp. (PS1) could produce the enzyme at temperature conditions 45-55°C, and pH range of 7-8. The data showed that even though the fastest cell growth was at 50°C, pH 7. The enzyme and protein content were better at pH 7, temperature ranging from 50°C and 70°C. The Bacillus spp. PS1 was able to degrade xylan efficiently of wheat straw. The focus of the study was to increase the thermophilic enzyme activity by optimisation process. After performing the optimisation process,

the activity was found to be increased. This increased activity of endo-xylanase will be helpful in industrial applications, after the purification of enzyme.


CONCLUSION AND
FUTURE TRENDS

5. CONCLUSION

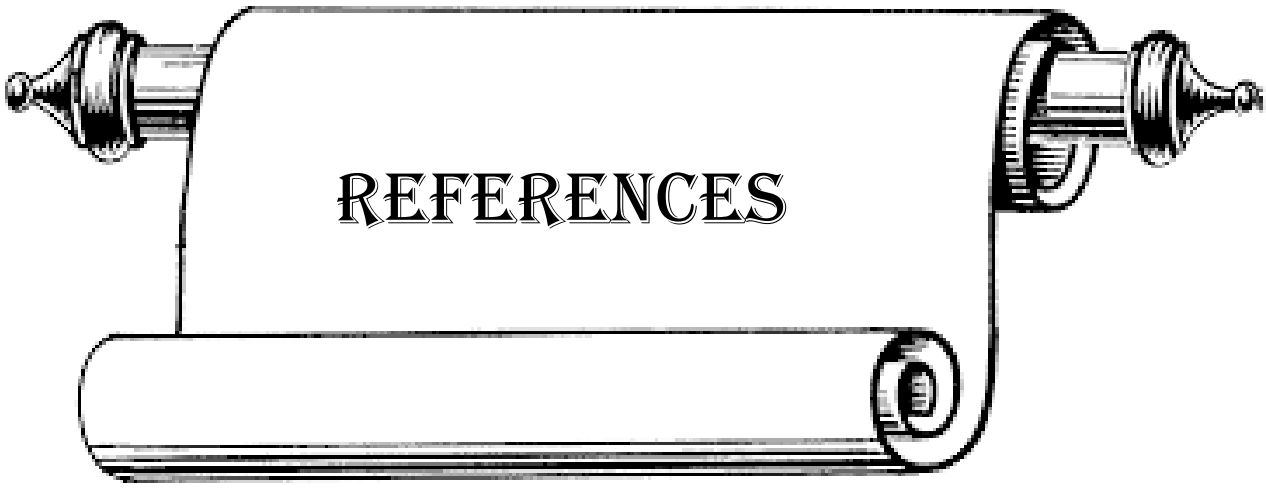
The present study shows that the isolated strain PS1 (whose identification is still under process) has a capability of playing a significant role in the cost effective production of xylanase using carbon sources. The isolate proved to be versatile to the type of carbon source as it was able to utilize all the agricultural residues for the xylanase production.

However it showed highest xylanase yield with wheat straw. Though the xylanase produced is active in thermophilic range, it provides a platform for future research on this strain that is how genetic engineering could be used to make the yield of enzyme increase greater and much stable at high temperature as well. The isolate is capable to produce enzyme at 50°C and the activity of enzyme is noted at 70°C. The purified enzyme can be used in paper and pulp industry as the enzyme is cellulase free xylanase, which could help in improving the quality of paper fibers. Also it will allow chlorine free chemical bleaching helping the environment to be toxic free. The enzyme can be utilised in the conversion of plant polysaccharide complex to simple monomer sugars, by the process of hydrolysis. This will enable production of sugars and their fermentation to renewable fuels and chemicals. However there are many identified isolates having the potency to degrade xylan are found. The isolate PS1 could also contribute to the same.

6. FUTURE TRENDS

The main emphasis is on conservation of resources for our next generation. The bioconversion of lignocellulosic materials into ethanol could be significantly more productive and cost-effective if not only its cellulose fraction was completely hydrolyzed and then fermented, but also all the carbohydrate polymers including xylan and other hemicelluloses. From industrial point of view, it would be desirable if

such enzymes were able to tolerate the high temperatures and mostly low pH levels that are the usual conditions applied during
pre-treatment (Miller and Blum, 2010). At present research groups are focusing their efforts on finding better and more economical ways of producing, refining and chemically altering
xylans and xylo-oligosaccharides to fit their properties to the requirements of the possible markets. Also focus is on the production of recombinant hosts for the high yield of enzymes. Hence purification and enhancement of recombinant enzymes is easier and of more potential in the industrial applications resulting in significant improvement in xylanase production.



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