SCREENING OF XYLANOLYTIC BACTERIA FROM MANURE SAMPLE

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by

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CERTIFICATE

This is to certify that the work entitled "**Screening of Xylanolytic Bacteria from manure sample"** pursued by **Kriti Gupta** (123803) and **Ashima Midha** (123817) in partial fulfillment for the award of degree Bachelor of Technology in Biotechnology from Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This part of work has not been submitted partially or wholly to any other University or Institute for the award of any degree or appreciation.

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CHAPTER 1

Introduction

Xylan is the most abundant non cellulosic polysaccharide made up of linear homopolymers containing D-xylose monomers linked through β-1, 4-glycosyl bonds as shown in Fig. No.1. Isolation of xylan from natural raw material is very difficult without altering or losing the original structure and its association with other components. Xylan is found mainly in the secondary cell wall and is considered to be forming an interphase between lignin and other polysaccharides.

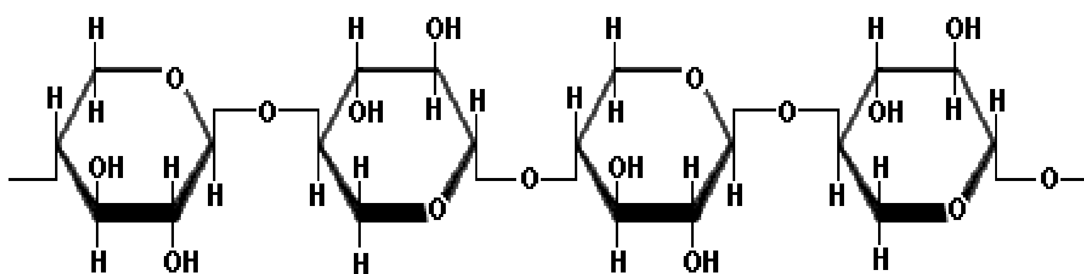


Fig No.1 Structure of xylan showing β-1, 4-glycosyl bonds.

Xylanase (endo-1, 4-β-D-xylanohydrolase) is a hydrolytic enzyme that plays an important role in breaking up of xylan. It is produced by many microorganisms like bacteria, fungi, actinomycetes, and yeast; though enzyme from fungal and bacterial sources has been widely applied in industrial sectors. Bacterial xylanases are preferred as they grow rapidly, need less space, can be easily maintained, and are accessible for genetic manipulations.

Xylanase has evidently increased due its broad variety of biotechnological purposes such as prebleaching of pulp, improving the digestibility of animal feed stocks, alteration of cereal-based stuffs, etc. Cellulase-free xylanases active at high temperature and pH are gaining importance in pulp and paper industry as they reduce the need for toxic chlorinated compounds which makes the bleaching process environment friendly.

The bacteria for production of xylanase were looked for locations where the degradation of lignocellulosic material was taking place. Therefore, the two sites from where the samples were collected for the sources of bacteria were:

1. Herbal waste from Ayurvetpvt. Ltd. Baddi, Solan that contained bark and roots of herbal plants.
2. Mushroom manure from Directorate of Mushroom Research, Solan was taken having hay and straw, which contains lignocellulosic content.

Therefore, the whole idea behind this project is to isolate bacteria that are able to produce alkalophilic xylanase using lignocellulosic biomass as sole carbon source, so as to make the production process cost effective.

CHAPTER 2

Review of literature

Different types of bacteria, actinomycetes and fungi are a rich source of the enzyme xylanase. Along with other bacterial species the Bacillus species of bacteria and filamentous fungi secretes high level of extracellular xylanase, but in case of filamentous fungi such as *Trichoderma*, *Penicillium*, and *Aspergillus* along with xylanase, cellulolytic enzymes are accompanied. For the treatment of pulp, xylanolytic enzymes without any celluloytic activity are required, as cellulase adversely affects the quality of the paper pulp. There have been approaches for overcoming cellulose activity in xylanase preparation, which included treatment with mercurial compounds or cloning selective expression of xylanase genes in heterologous host system. But the most practical approach came out to be screening for naturally occurring microbial strain which are capable of secreting cellulose free xylanases under controlled fermentation conditions.

The early reports of successful isolation of cellulose-free xylanases were from desert sands of Rajasthan, *Chainia*s p. NCL 8251. This sclerotial actinomycete strain secreted 8–10 IU/ml of xylanases on media containing commercial ingredients which are rich in xylan such as cereal bran and 26 IU/ml xylanase was obtained on pure xylan media in submerged culture. The xylanase obtained was active at pH 5-7 and at temperature 55-60° C and no cellulose activity was detected when acted upon caboxymethyl cellulose or filter paper. Cellulase free xylanase activity has also been reported in *Streptomyces roseiscleroticus*, *Saccharomonospora viridis*, and thermolotolerant *Streptomyces* sp. T7, and all these enzymes were active at or around neutral pH.

Furthermore work has been done on xylanase. Horikoshi and Atsuka (1973) were the first to report a xylanase production from *Bacillus* sp. that was active under high pH Srinivasan and Rele (2015) published a review on microbial cellulose free xylanases and their applications in pulp and paper biotechnology. An obligately alkaline *Bacillus* sp. strain isolated by Srinivasan *et al* (1988) from rotting coconut fibers, which secreted high level of xylanase in commercial media containing wheat bran and organic nitrogen, and the enzyme was found to be optimally active at 60° Cat pH 8.0, and the enzyme retained over 75% of its activity at pH 9.0. Several promising *Bacillus* strains, including alkalothermotolerant ones, have been isolated and their xylanase production as well as enzyme characterization has been published. Rajaram and Varma (1990) isolated *B. thermoalkalophilus* from termite infested soil mounds that resulted in high yields of xylanase using cheap agricultural wastes, such as rice husk and bagasse, as substrate. Dey *et al* (1995) characterized an alkalothermophilic (AT) *Bacillus* strain, a xylanase

comprising two components which was active over a pH range of 6–10 and temperature range of at 50–60° C, where as an alkalotolerant *B. Circulans* was described by Nakamura *et al.*, producing high activity xylanase at pH 8–8.5 on a medium containing beechwood xylan. Other reports of thermostable xylanases from various species of *Bacillus* include those of indicated by Gupta *et al (*1992. and Khasin *et al* (1993*).*

The first report of cellulose free xylanase secretion was from an alkalophilic fungus, *Cephalosporium*sp. NCL 87119 was published by Bansod*et al* (1993)., Chandra Raj and Chandra described a cellulose-free xylanase from *Aspergillus fischeri* that was alkali tolerant and stable in the pH range of 5–9.5, which retained its activity at 60°C., Vyas *et al* (1990) reported an alkalophilic *Streptomyces* sp. secreting cellulase free xylanase that was active above pH 9.0.

2.1XYLANSE AND ITS TYPES

Xylanases belong to the family of glycosides (*O*-glycoside hydrolases, EC 3.2.1.x) which catalyse the hydrolysis of 1,4-β-D-xylosidic linkages in xylan leading to the formation of a sugar hemiacetal and the corresponding free aglycone (non-sugar compound remaining after replacement of the glycoside by a hydrogen atom [28]). Xylanases are widespread group of enzymes which are involved in production of xylose which is a primary carbon source for cell metabolism.

Xylanases are been classified in three ways, based on:

- Molecular weight and isoelectric point (pI) [29]
- The crystal structure [30] and kinetic properties
- Substrate specificity and product profile.

The first classification does not sufficiently describe all the xylanases. Exceptions have been identified as all the xylanases do not have a higher molecular mass (above 30 kDa) and low isoelectric point or low molecular mass (less than 30 kDa) and higher isoelectric point. Hence a more satisfying system which classifies xylanases based on primary structure and compares the catalytic domains was introduced [27, 68]. This system analyses both the structural and mechanistic features [27].

According to CAZy database, xylanases (EC3.2.1.8) are related to the following glycoside hydrolase families 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51 and 62. The sequences which are classified in families 16, 51 and 62 appeared to be bi-functional enzymes, which are containing two catalytic domains, unlike the families 5, 7, 8, 10, 11, and 43. The families 5, 7, 8, 10, 11 and 43 have a distinct catalytic domain with endo-1, 4-β-xylanases activity [27]. Based on the same analysis the families 9, 12, 26, 30 and 44 might have residual or secondary activity.

The primary classification of xylanases is done as GH10 and 11 based on the hydrophobic cluster analysis of their catalytic domain and amino acid sequence similarities. The members belonging to GH10 have been studied but the catalytic properties of the remaining families 5, 7, 8 and 43 are still limited.

Xylanases classified in different GH families:

2.1.1 GH families 10 and 11

Xylanases belonging to GH 10 family has a low molecular mass with a pI in a range of 8-9.5, while those from the GH11 family have a higher molecular mass and a lower pI.

GH10 is composed of endo-1, 4-β-xylanases and endo-1, 3-β-xylanases (EC 3.2.1.32) [33]. Members of this family are also capable of hydrolysing the aryl β-glycosides of xylobiose and xylotriose at the aglyconic bond. The enzymes belonging to these families are highly active on short xylo-oligosaccharides, hence indicating the small substrate binding sites. GH10 family xylanases have four to five substrate binding site, a high molecular mass and a low pI and the structure of the xylanase displays an (α/β)8-barrel fold [27,33,37].

On the other side as compared to GH10 xylanases, those belonging to GH11 have high substrate selectivity and catalytic efficiency, a small size and a number of optimum pH as well as temperature values. These properties make them suitable in various conditions with many applications. Family 11 is considered as true xylanases, as this family is only composed of xylanases which are especially active on D-xylose containing substrate. The characteristics of xylanases in GH11 family have low molecular weight and a high pI. Furthermore, the enzymes are more active on long chain xylo-oligosaccharides products and the product by theses xylanases can be hydrolysed by the xylanases of GH10.

Xylanases of GH10 have greater catalytic versatility and lower substrate specificity than those belonging to GH11. According to Davies et al. [40], the binding sites for xylose residues in xylanases are termed sub-sites, the bond cleavage occurs between the sugars residues at the -1 (non-reducing) and the +1 (reducing) ends of the polysaccharide substrate. Hence, xylanases from family 11 cleave the unsubstituted regions of the arabinoxylan backbone, whereas GH10 enzymes cleave the decorated regions, being less hampered by the presence of substituents along the xylan backbone [36].

2.1.2 GH families 5, 7, 8 and 43

GH family 5 also known as family A is the largest glycoside hydrolase family. It consists of 467 sequences with varying activities, including: endoglycosylceramidase (EC 3.2.1.123), cellulase (EC 3.2.1.4), licheninase (EC 3.2.1.73), b-mannosidase (EC 3.2.1.25), glucan 1,3-bglucosidase (EC 3.2.1.58), glucan endo-1,6-b-glucosidase (EC 3.2.1.75), mannan endo-1,4-b-mannosidase (EC 3.2.1.58), cellulose 1,4-b-cellobiosidase (EC 3.2.1.91), endo-1,6-b-galactanase (EC 3.2.1.-), 1,3-bmannanase (EC 3.2.1.-) and endo-1,4-b-xylanase (EC 3.2.1.8) [27]. The product produced by the action of GH family 5 is shorter as compared to the product produced by GH family 7.

GH family 8 also known as family D constitutes cellulases (EC 3.2.1.4) and chitosanases (EC 3.2.1.132), lichenases (EC 3.2.1.73) and endo-1, 4-β-xylanases (EC 3.2.1.8). This family of cold-adapted xylanases was found to hydrolyse xylan to xylotriose and xylotetraose and was most active on long-chain xylooligsaccharides. Enzymes of family 8 catalyse hydrolysis with the inversion of the anomeric configuration.

GH families 7 and 43 contain only few enzymes which show xylanase activity. The enzymes of family 7 have common characters with family 10 and 11. GH family 7 has high molecular weight and low pI and has a small substrate binding site containing approximately four subsites, with the catalytic site in the middle [27]. The enzyme of family 43 have not been studied in detail and the structure of only one enzyme has been determined which indicated that the members of this family may have a five-blade β-propeller fold.

2.2MICROBES PRODUCING XYLANASE

Microorganisms are considered a good source of pragmatic enzymes as they can proliferate at extremely high rates and incorporate biologically active products that can be composed by humans. The advantages of these enzymes over the use of conventional chemistry catalysts are:

i. exhibit high catalytic activity and a high degree of substrate specificity,
ii. produced in large amounts,
iii. highly biodegradable,
iv. pose no threat to the environment and are economically viable.

Microbial xylanases are the favoured catalysts for xylan hydrolysis because of the following reasons:

i. their high specificity
ii. mild reaction conditions
iii. negligible substrate loss and side product generation.

Xylanases derived from microorganisms have many promising applications in the food, feed, and paper pulp industries (Table No. 2). Fungi, actinomycetes and bacteria have been discovered with complete xylanolytic enzyme systems. Some of the most important xylanase producers include *Aspergillus, Trichoderma, Streptomyces, Phanerochaetes, Chytridiomycetes, Ruminococcus, Fibrobacteres, Clostridia* and *Bacillus.*

Table no.1 presents a list of some of the xylanase-producing microorganisms and their activities.

Table no.1: Review of xylanase-producing microorganisms

Microorganisms	**Xylanase**	**Cultivation condition**	**Media**	**Reference**
Penicillium Canescens	18,895 IU/g	pH 7.0; 30 °C	Soya oil cake and casein peptone	45
Streptomyces sp. P12–137	27.8 IU/Ml	pH 7.2; 28 °C	Wheat bran and KNO_3	46

Thermomyces lanuginosus SD-21	8,237 IU/g	pH 6.0; 40 °C	Corn cob and wheat bran and $(NH_4)_2SO_4$	47
Penicillium fellutanum	39.7 IU/mL	30 °C	Oat spelt xylan, urea, peptone and yeast extract	48
Penicillium clerotiorum	7.5 IU/mL	pH 6.5; 30 °C	Wheat bran	49
Acremonium furcatum	33.1 IU/mL	30 °C	Oat spelt xylan, urea, peptone and yeast extract	48
Aspergillus niger PPI	16.0 IU/mL	pH 5.0; 28 °C	Oat and urea	50
Neocallimastix sp. Strain L2	1.13 IU/mL	50 °C	Avicel (pH 10.5) from Serva (Heidelberg, Germany)	51
Cochliobolus sativus Cs6	1,469.4 IU/g	pH 4.5; 30 °C	Wheat straw and $NaNO_3$	52
Bacillus circulansD1	8.4 IU/mL	pH 9.0; 45 °C	Bagasse hydrolysates	53
Streptomycessp. strain Ib 24D	1,447.0 IU/Ml	pH 7.5; 28 °C	Tomato pomace	54
Paecilomyces themophila J18	18,580.0 IU/g	pH 6.9; 50 °C	Wheat straw and yeast extract	55

2.2.1 Bacteria

Bacteria and actinomycetes like *Bacillus* and *Streptomyces* respectively, have been reported to produce xylanase [44]. The extreme thermophile *Rhodothermus marinus* has been reported to

produce α-L-arabinofuranosidase [60], and two different polypeptides with α-arabinofuranosidase activity from *Bacillus polymyxa* were characterized at the gene level for the production of α-arabinofuranosidases [61].

Bacteria have captivated researchers due to their alkaline-thermostable xylanase-producing trait [32]. The optimum pH of bacterial xylanases are, in general, marginally higher than the optimal pH of fungal xylanases [62], which is a suitable characteristic in most industrial applications, especially the paper and pulp industries. *Bacillus spp*. is a significant producer of high levels of xylanase at an alkaline pH and high temperature [32]. If only temperature is considered, a handful of xylanases from different microorganisms have been reported to show optimum activity at higher temperatures. These include *Geobacillus thermoleovorans*, *Bacillus firmus, Streptomyces sp.S27, Actinomadura sp. strain Cpt20* and *Saccharopolyspora pathunthaniensis S582,* which show xylanase activity between 65 and 90 °C [26]. One xylanase, reported from *Thermotoga sp*. [63], has been shown to exhibit temperature optima between 100 and 105 °C.

Table No.2: Cellulase-free xylanase producing microbes reported till date

Bacteria	**Enzyme activity**	**Cultivation Conditions**	**Media**	**Ref.**
Streptomyces sp. Ab106	15 IU	50 °C ; pH 7	agricultural waste cane bagasse	71
Streptomyces olivaceoviridis E-86	1653U/ml	pH 6	Corn cob xylan , beef peptone , 1.5%(v/v) Tween-80	72
Streptomyces sp. QG-11-3	96IU/ml	pH 8.6 ; 60ºC	1% w/v wheat bran, Ca^{2+}	73
Bacillus subtilis- BS05	439.5±2.84 IU	37ºC	Sucrose, K_2HPO_4, NaCl, $MgSO_4.7H_2O$, yeast extract.	74
Bacillus sp. JB-99	4826 IU/ml	70ºC ; 10 pH	Rice bran supplemented with birchwood xylan	75

Clostridium absonum CFR-702	7025 nkatal/ml	75°C ; pH 8.5	1% birchwood xylan	76
Pseudomonas sp. WLUN024	1245 U ml^{-1}	37 °C ; pH 7.2–8.0	Wheat bran	77
Bacillus pumilus	5407 IU/ml	pH 8.0 and at 37 °C	basal medium supplemented with wheat bran (2%, w/v)	78
Cellulosimicrobium sp. *HY-13*	4067 IU/mg	pH 6.0 and 55 °C	oat spelt xylan	79
Streptomycessp.P12-137	9.27 UA/ml	pH 5.5 ; 70°C	Wheat bran	80
Sterptomyces rochei	10 mg/ml	pH 6.0 and 40°C	Xylan hydrolysates extracted from sugar cane bagasse	81
Paenibacillus*sp.N1*	52.30 IU.ml-1	pH 9.0 ; 50^{0}C	12.5%, phenylalanine , xylose,$(NH_4)_2HPO_4$ and Tween 20	82
Streptomyces sp. CS428	4197.1 U/mL	80^{0}C	Wheat bran	83
Terrestrial Streptomyces *sp. CA24*	255 U/mL	pH (6-10) ; 60^{0}C	Oat spelt xylan (0.5%) and yeast extract (0.5%)	84
Thermoanaero bacterium thermosaccharolyticum DSM 571	222.1 U/mg	65 °C ; pH 6.5	beechwoodxylan , Co^{2+}, Mn^{2+}, and Tween 60	85.
Anoxybacillus sp. Ip-C	13.5µmolmin^{-1} mg^{-1} protein	pH 9.0 ; 70^{0}C		86
Bacillus megaterium BRL-0101	2,876 U/mL	50°C ; pH 8.0	mango peel (100 g/L) , yeast extract , peptone, $NaNO_3$ (10 g/L)	87
Bacillus vallismortis RSPP-15	3768U/mL	55^{0}C ; pH 7.0	Birchwood xylan	88

2.3FERMENTATON CONDITIONS FOR PRODUCTION OF XYLANASES

Xylanases are produced by either solid-state or submerged fermentation [24]. Most of the xylanase manufacturers produce these enzymes using submerged fermentation (SmF) techniques (nearly for 90% of the total xylanase sales worldwide) [23]].

The growing interest in using solid-state fermentation (SSF) techniques to produce a wide variety of enzymes, including xylanases from fungal lineage, is mainly due to the economic and engineering advantages of this process [70].

Paradoxically, submerged fermentation allows better control of the conditions during fermentation [66]. The submerged fermentation of aerobic microorganisms is eminent and widely used method for the production of cellulase and xylanase [67]. In general, SmF is the preferred method of production when the preparations require more purified enzymes, whereas synergistic effects from an array of xylan-degrading enzymes can easily be found in preparations obtained by SSF using complex substrates, though the latter is commonly desired in applications aimed at improving animal feed [65].

The choice of the substrate plays an important role in the selection of the fermentation process and the successful production of xylanases. Purified xylans can be exquisite substrates because the low molecular weight compounds derived from them are the best xylanase inducers. The adoption of such substrates has led to elevated yields of xylanase production and a selective induction of xylanases, with accordingly low cellulase activity in a number of microorganisms. But the cost of such substrates are quite high, thus certain alternatives are needed for large scale production.

Some lignocellulolytic substrates such as barley husk, corn cobs, hay, wheat bran or straw have been correlated to pure substrates, and many have accomplished significantly better than isolated xylans (or celluloses) with respect to the yields of xylanase in large-scale production processes. Solid-state fermentation processes are practical for complex substrates, including agricultural, forestry and food processing residues and wastes, which are used as inducing carbon sources for the production of xylanases [65].

The use of richly available and cost-effective agricultural residues, such as wheat bran, corn cobs, rice bran, rice husks, and other similar substrates, to achieve higher xylanase yields via SSF allows the depreciation of the overall production cost of bio-bleached paper. This has promoted the use of this environmentally friendly technology in the paper industry [22].

2.4COMMERCIAL XYLANASES

The commercially available xylanases are from the Sigma Life sciences and they are as follows:

1. **X2753** –Xylanase enzyme from *Thermomyces lanuginosus*, comes in powder form and has an activity ≥2500 units/g. The production is done using recombinant technology and the enzyme is expressed in *Aspergillus oryzae.*
2. **X3876**- Xylanase enzyme from *Trichoderma viride* is available as lyophilized powder and has an enzyme activity of 100-300 units/mg protein.

The available commercial xylanases are produced from fungi. There is no such commercial xylanase available that is prepared from the bacteria.

2.5APPLICATIONS OF XYLANASE

Table No. 3 Potential applications for xylanase

Market	Industry	Application	Function	Ref.
Food	Fruit and vegetable processing, brewing, wine production.	Fruit and vegetable juices, nectars and purees, oils (e.g., olive oil, corn oil) and wines	Improves maceration and juice clarification, reduces viscosity. Improves extraction yield and filtration, process performance and product quality.	[6, 7,8]
	Baking	Dough and bakery products	Improves elasticity and strength of the dough, There by allowing easier	[10, 6,9]

			handling, larger loaf volumes and Improved bread texture.	
Feed	Animal feeds.	Monogastric (swine and poultry) and ruminant feeds	Decreases the content of non-starch polysaccharides , thereby reducing the intestinal viscosity and improving the utilization of proteins and starch. Improves animal performance, increases digestibility and nutritive value of poorly degradable Feeds, e.g., barley and wheat.	[6,12-14]
Technical	Paper and pulp	Bio-bleaching of Kraft pulps	Reduces chlorine consumption and toxic discharges.	[16, 15]
		Bio-mechanical pulping	Facilitates the pulping process and reduces the use of mechanical pulping methods, hence reduces energy consumption.	[6]
		Bio-modification of fibers	Improves fibrillation and drainage properties of pulp, hence improving the process efficiency and the paper strength.	[6]
		Bio-de-inking	Facilitates the de-inking process, reduces the use of alkali.	[6, 17]

	Starch	Starch-gluten separation	Reduces batter viscosity, improves gluten agglomeration and process efficiency.	[18]
	Textiles	Retting of flax, jute, ramie, hemp, etc.	Enzymatic retting reduces/replaces chemical retting methods.	[11, 16, 19]
	Bioremediation/ Bioconversion	Treatment of agricultural, municipal and food industry wastes	Treatment/recycling of wastes. Production of fermentable products, renewable fuel (bioethanol) and fine chemicals.	[11, 20, 21]

CHAPTER 3

Material and methods

3.1Isolation of xylanase producing bacteria

3.1.1. Site characterization and sampling

Samples from two different places were taken (Fig No.1)

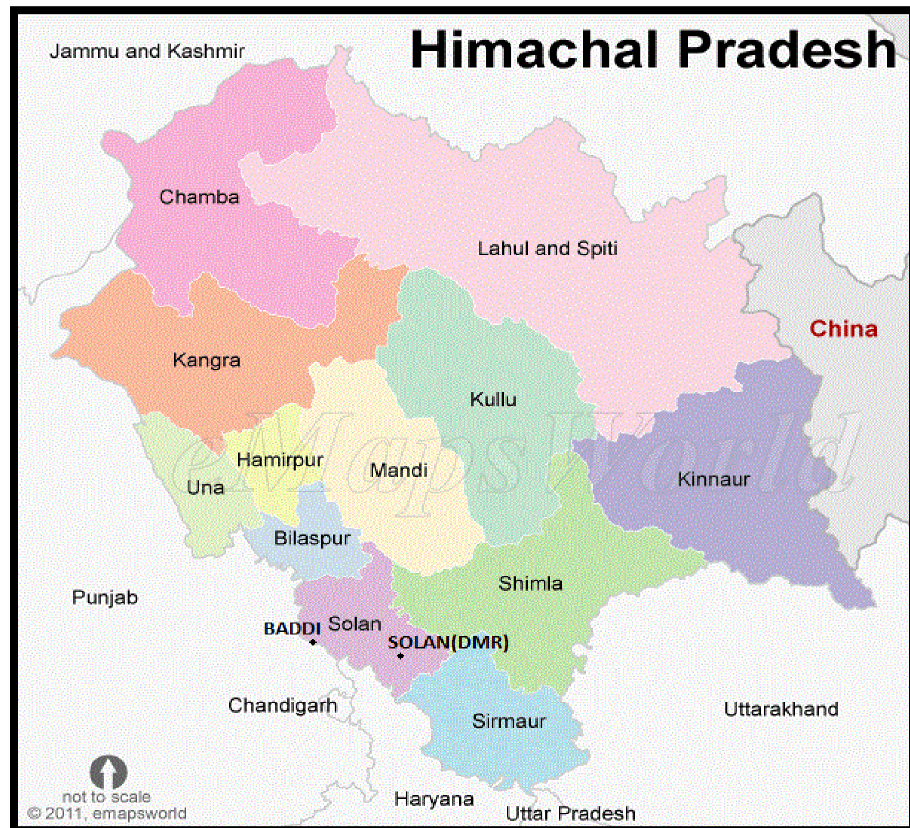
1. Industrial herbal waste Diarok provided by Ayurvet Industry Ltd., Baddi, Solan. The waste consists of remains of medicinally important herbs, collected after industrial operation. The composition of diarok is :

- *Punica granatum*(fruit),
- *Symplocus racemosa*(bark),
- *Andrographis paniculata* (stem),
- *Woodfordia fruticosa*(flower),
- *Salmalia malabarica*(bark),
- *Berberia aristata*(root)
- *Anglemarmelos*(fruit).

This waste was selected as it comprises of bark and root which serve as a good source of xylan.

2. The manure sample from Directorate of Mushroom Research Centre DMRC, Solan was taken. The samples were taken out from bottom and top of a heap of manure, where the soil was in direct contact to the manure. The manure is used for cultivation of mushrooms. The composition of the manure was not disclosed by the DMRC. This manure was selected as it basically contains hay and straw which have high lignocellulosic content.

Fig No.2 Map showing the two sites of sampling i.e. Baddi (diarok) and Solan (mushroom compost)



3.1.2 Methods for isolation of bacteria:

3.1.2.1Isolation from industrial waste

The sample, Diarok, was collected aseptically and the following procedure was followed:

1. Xylan plate containing minimal media with 1% beech wood xylan was prepared.
2. 1 g of waste sample was dissolved in 100 ml of autoclaved distilled water.
3. 1 ml of the dissolved mixture was added to 9 ml of autoclaved distilled water in a test tube which made the dilution 10^{-1}
4. Similarly the dilutions were made till 10^{-6}
5. The two dilutions 10^{-4} and 10^{-6} were spread on the xylan containing agar plate and incubated at 37°C overnight.
6. The pure cultures of the colonies obtained, were made on nutrient agar plates.

3.1.2.2 Isolation from manure sample

A. Enrichment

The enrichment process was carried out in Erlenmeyer flasks containing 50ml media supplemented with 0.5% beech wood xylan (Zambare et al. 2015). The process was carried out in the following manner:

1. 4 flasks of minimal media with 0.5% xylan (beech wood) were prepared.
2. 1gm of soil sample was added to the first flask and kept at shaker incubator at 37°C for 4 days.
3. 1 ml from the first flask was transferred to the second flask and again incubated.
4. Same procedure was followed for the other two flasks.

B. Isolation

1. 0.5% Xylan containing agar plate was prepared.
2. 1 ml from the 4th flask was spread on the plate and then incubated at 37°C.
3. The pure cultures of the colonies obtained were made on nutrient agar plates.

3.2 Qualitative assay for presence of xylanase

3.2.1Preparation for Congo Red assay

Suspension culture of the obtained colonies was prepared, in which the bacteria produced xylanase to break down xylan, which was the only carbon source.

1. 10 ml of nutrient broth was prepared in test tubes.
2. A single colony from the pure culture plate was picked using a loop and added to the nutrient broth under sterile conditions.
3. The test tubes were kept at shaker incubator at 37°C for overnight.

3.2.2 Congo red assay

Congo red is a dye that binds to carbohydrate polymers (e.g. cellulose and xylan). The plates are basically examined for the appearance of yellow zone of hydrolysis around the colonies.

1. 0.5% Xylan plates were prepared and wells were punctured on the plate.
2. The suspension culture was added into the wells and the plate was incubated for 48 hours.
3. Congo red dye was prepared and poured on the plate. The dye was kept for 15 minutes at room temperature.
4. The dye was poured out and plate was destained using 1M NaCl. It was again kept for 15 minutes at room temperature.
5. The clear zones were observed.

3.3Enzyme Assay for quantification of enzyme

Enzyme assays are performed to serve two different purposes:

i. To identify a special enzyme, to prove its presence or absence in a distinct specimen, like an organism or a tissue which is a qualitative approach

ii. To determine the amount of the enzyme in the sample, a quantitative approach.

3.3.1 Suspension culture for enzyme production.

The supernatant will act as a crude enzyme. The procedure to prepare the suspension culture was carried out in a following manner:

1. Minimal media along with 0.5% beech wood xylan was prepared.
2. The media was inoculated with the obtained cultures and incubated at 37°C.
3. Media was taken from the flask after 24 and 48 hours for the enzyme assay.

3.3.2 DNS preparation (for 500ml)

3,5-Dinitrosalicylic acid (DNS) 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.

3.3.3 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.

3.3.4 Enzyme assay

The enzyme assay helps to quantify the amount of enzyme produced by the bacteria, by determining the units of enzyme produced which is calculated by the amount of xylan broken down into xylose sugar. The procedure was carried out in the following manner:

1. 2ml sample was taken in an eppendorf and centrifuged at 5000 rpm for 15 minutes at 4°C.
2. Supernatant was transferred into a fresh eppendorf.
3. 0.5% was prepared as a substrate.
4. In clean test tubes the following components were added.

TableNo.4- Ingredients of enzyme assay for xylanse

	Enzyme	Substrate	Buffer
Test	25µl	500 µl	475 µl
Enzyme control	25 µl	-	975 µl
Substrate control	-	500 µl	500 µl
Blank	-	-	1000 µl

*Test: the supernatant obtained

*Substrate: beech wood xylan (0.5%).

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

5. The test tubes were incubated at 60°C for 30 minutes.
6. 1.5 ml of DNS was added to the test tubes and incubated for 10minutes at 100°C.
7. O.D. was taken at 540 nm.
8. Using the obtained O.D., the concentration of the test samples were calculated using the standard graph.

3.4Preservation 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:

1. Nutrient agar medium was prepared and distributed in test tubes, each containing 5 ml of nutrient agar medium.
2. The test tubes were covered using cotton plug and were autoclaved.

3. While the media was hot, the test tubes were slanted against a solid surface and allowed to solidify.
4. 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:

1. A solution of 30% glycerol (v/v) was prepared. The solution was then autoclaved at 121°C for 15 min.
2. 500µl of the autoclaved glycerol stock was added into an aliquot.
3. 500µl of overnight grown culture of the bacteria was added to the aliquot containing glycerol and mixed using a vortex.
4. The aliquot was then labelled and stored at -80°C.

3.5 Genomic DNA isolation of B3 bacteria

Genomic DNA isolation of the selected isolate was done using Wizard® Genomic DNA Purification Kit of Promega as per manufacturer instruction. The bacterium isolated was a gram positive bacterium and the following steps were followed for the isolation of bacteria:

1. 1ml of the overnight culture was added to a 1.5ml micro-centrifuge tube and centrifuged at 13,000 g for 2 minutes to pellet the cells.
2. The cells were resuspended thoroughly in 480µl of 50mM EDTA and the lytic enzyme was added to the resuspended cell pellet in a total volume of 120µl, and gently pipeted to mix.
3. The sample was incubated at 37°C for 30–60 minutes, centrifuge for 2 minutes at 13,000 g and the supernatant was removed.
4. 600µl of Nuclei Lysis Solution was added and gently pipeted until the cells were resuspended.

5. Incubation was provided at 80°C for 5 minutes to lyse the cells; then cooled to room temperature.
6. 3µl of RNase Solution was added to the cell lysate. The tube was inverted 2–5 times to mix and incubated at 37°C for 15–60 minutes. The sample was cooled to room temperature.
7. 200µl of Protein Precipitation Solution was added to the RNase-treated cell lysate and vortexed vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate.
8. The sample was incubated on ice for 5 minutes and then centrifuged at 13,000g for 3 minutes.
9. The supernatant containing the DNA was transferred to a clean 1.5ml micro-centrifuge tube containing 600µl of room temperature isopropanol and mixed gently till the thread like structure formed a visible mass.
10. The solution was centrifuged at 13,000g for 2 minutes.
11. The supernatant was removed and 600µl of 70% ethanol was added and mixed gently by inverting the tube to wash the pellet.
12. The solution was again centrifuged at 13000g for 2 min, ethanol was gently removed and the pellet was allowed to dry.
13. 100µl of DNA Rehydration Solution was added to the tube and the DNA was rehydrated by incubating at 65°C for 1 hour. Periodically the solution was mixed by gently tapping the tube. Alternatively, the DNA was rehydrated by incubating the solution overnight at room temperature or at 4°C.
14. The DNA was stored at 4°C.
15. The isolated DNA was then used to amplify the 16s rDNA of the bacteria.
16. The primers 27F and 1492R were used having the annealing temperature 51.8°C.
17. The reaction was set for 35 cycles.

3.6Optimization

3.6.1 Substrate optimization

To make the enzyme production cost effective, the agricultural wastes containing xylose sugar were used. Three substrates were selected:

1. Sugarcane bagasse
2. Rice straw
3. Wheat straw.

The selection criteria were based on the following reasons:

1. Earlier reported data
2. Availability around the area
3. Requirement of pre-treatment

The following steps were followed for the enzyme production using the selected agricultural wastes:

1. Minimal media was prepared and the agricultural wastes were washed, grinded and autoclaved.
2. The media was then mixed to the agricultural wastes (1%) and the inoculum was added to it.
3. The flasks were then incubated at 37°C with continuous shaking.
4. The sampling was done after every 24 hours.
5. The supernatant was taken as the crude enzyme and enzyme assay was done.

After selection of the agricultural waste, that were both rice straw and wheat straw, further optimization was done. The substrate and the incubation time were optimized simultaneously. The next parameters used for optimization were pH, temperature and substrate concentration.

3.6.2 pH optimization

For pH optimization, a range of pH was taken from 5 to 10.

1. 6 flasks of each rice straw (1%) and wheat straw (1%) were prepared.
2. 700ml of minimal media was prepared.

3. 100ml of minimal media was taken and different pH (5-10) was set.
4. 50ml of media set at different pH was poured in the each flask of rice straw and wheat straw.
5. All the flasks were autoclaved.
6. 1.5% of inoculum (overnight grown culture) was added to each flask
7. The flasks were kept at 37ºC with continuous shaking for 7days.
8. After the incubation period was over, 1ml of media was taken from each flask and centrifuged at 10,000rpm for 10mins at 4ºC.
9. After centrifugation, the supernatant was taken and stored in a clean eppendorf.
10. Supernatant were taken and enzyme assay was performed.

3.6.3 Substrate concentration optimization

The different concentrations of substrate (rice straw and wheat straw) taken were 0.5% to 3%.

1. Rice straw and wheat straw were grinded and sieved.
2. Flasks containing 0.5% to 3% of substrate concentration were prepared.
3. 50ml of Minimal media (pH 9.0) was added to each flask and the flasks were autoclaved.
4. 1.5% of inoculum was added to each flask.
5. The flasks were kept at 37ºC for 7days.
6. After 7days, 1ml of media from each flask was taken and centrifuged at 10,000rpm for 10mins at 4ºC.
7. Supernatant was taken and enzyme assay was performed.

3.6.4 Temperature optimization

The different temperatures used for optimization were 25ºC, 30ºC and 50ºC. The procedure followed was:

1. 1% of rice straw and wheat straw was grinded and weighed in different flasks. A total of 6 flasks were prepared, three of each substrate used.
2. Minimal media (pH 9.0) was prepared.
3. Both flasks and the media were autoclaved.
4. 50ml of media was poured in each flask. 1.5% of overnight culture was also added to each flask and after that, the flasks were kept at the temperatures selected for the optimization, for 7days.
5. After 7days, from each flask 1ml of media was taken and centrifuged at 10,000rpm for 10mins at 4ºC.
6. Supernatant was taken and enzyme assay was performed.

CHAPTER 4

Results and Discussion

4.1 Source – Diarok

4.1.1 Isolation of bacteria

The screening of the herbal industry waste was done by serial dilution and then plating on beech wood xylan agar plates (0.5%).Two bacterial isolates named B1 and B2, translucent and cream respectively in appearance, consuming xylan were obtained. The pure cultures of the isolated colonies were maintained on nutrient agar plates. The morphological characteristics of the bacteria obtained are discussed in Table No. 5

Table No.5: Morphological characteristics of bacteria isolated from Diarok

CHARACTERISTICS	B1	B2
Shape	Coccus	Coccus
Colour	Off-white	Cream
Texture	Buttery	Viscid
Boundaries	Defines	Lobed
Shape of Colony	Circular	Circular
Elevation	Elevated	Slightly elevated

4.1.2 Qualitative assay

Congo red assay method was performed using xylan(0.5%) agar medium containing xylan as the sole carbon source. The zone of clearance by isolates reflect their extent to xylanolytic activity but the isolated colonies showed no zones (Fig No. 3) around them indicating that no xylanase was produced. It might be possible that the bacteria is not able to show xylanolytic activity on the plate but shows good results in the suspension culture.

Fig No.3 Congo red assay of B1 and B2 colonies, showing no zone formation.

4.1.3 Quantitative assay

Even though no zones were formed around the wells containing bacterial culture, secondary screening was carried out to check the presence of the xylanase activity. For secondary screening the enzyme assay was carried using DNS method. The results obtained after DNS confirmed no xylanase activity by the bacterial cultures. Therefore, new manure sample from Directorate of Mushroom Research, Solan was screened for isolation of xylanase producing bacteria.

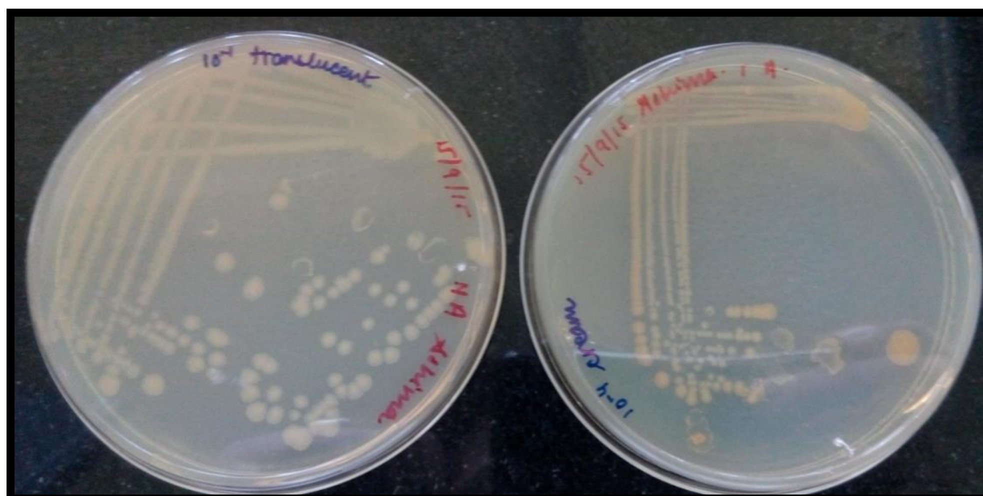
4.2 Source- Manure sample from DMR, Solan

4.2.1 Isolation of bacteria

The primary screening was done by enrichment method which was carried out for 30 days. The culture from the fourth flask (last flask of enrichment) containing minimal media and 0.5% xylan was plated on xylan agar plates (secondary screening). Two bacterial colonies named B3 and B4, yellow and cream coloured respectively were obtained. And pure cultures of these colonies were prepared on nutrient agar plates for further use. The morphological characteristics were studied and shown in table no. 6

Table no.6: Morphological characteristics of bacteria isolated from Manure sample

CHARACTERISTICS	B3	B4
Shape	Coccus	Coccus
Colour	Yellow	White
Texture	Smooth	Sticky
Boundaries	Defined	Defined
Shape of Colony	Circular	Circular
Elevation	Elevated	Flat



B4 **B3**

Fig no. 4 Isolates from DMR compost

4.2.2 Qualitative assay

Congo red assay was done using xylan agar medium. The yellow coloured colony B3 showed a clear zone having a diameter of 2.2cm (Fig No. 4) while the cream coloured colony B4 did not create any zone. Thus the zone created by the yellow coloured colony represented hydrolysis of xylan by xylanase.

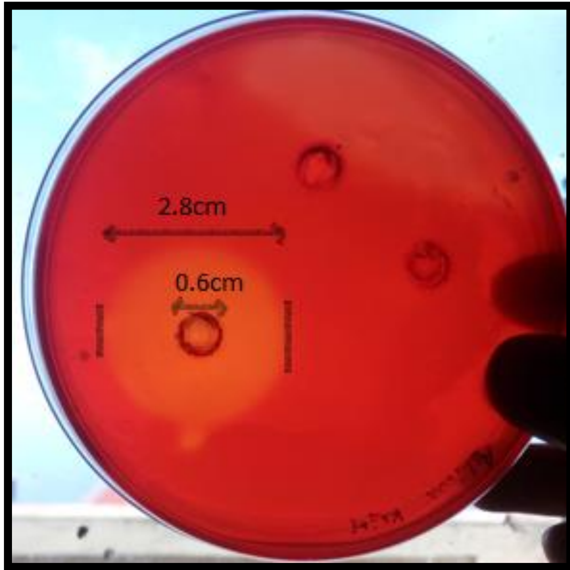


Fig No.5 Congo red assay of B3 and B4 isolates. The zone formation is shown by the B3 isolate. The diameter of the zone (zone + well) is 2.8cm

4.2.3 Quantitative assay

Enzyme assay of bacteria B3 was conducted at pH 7 at 37°C for 48 hours. The supernatant was used as a crude xylanase. This supernatant is utilized to convert xylan into xylose monomers. To quantify the units of enzyme, dinitrosalicylic acid is used which quantifies the reducing sugar. The xylanase production by the bacteria was 4.7 unit/ml, which was calculated using a standard graph of xylose sugar. The test proved that the enzyme produced was extracellular.

4.2.4 DNA Isolation and PCR

DNA (B3 isolate) was successfully isolated and the band clearly seen in the gel doc as shown in figure 5.

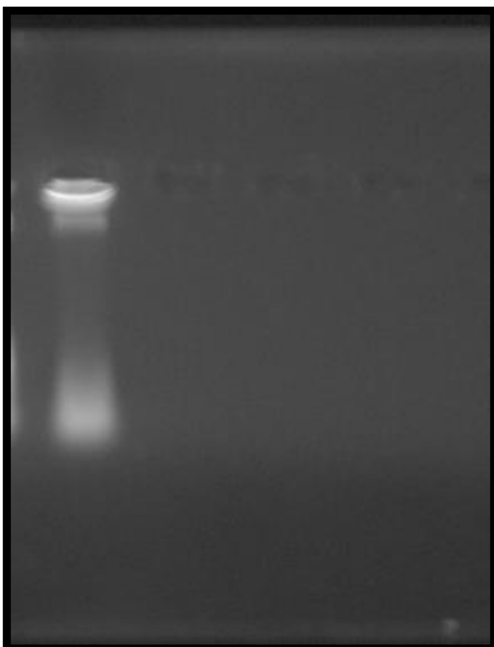


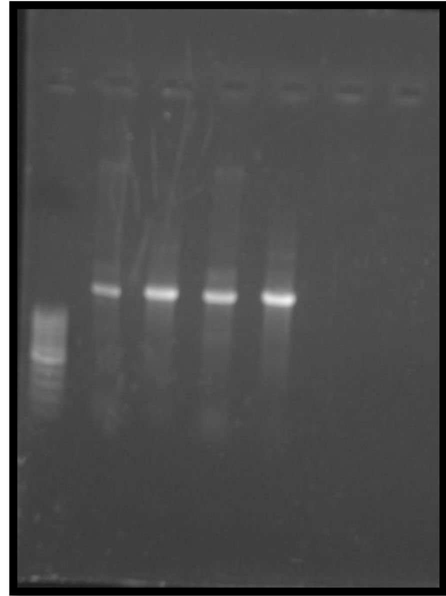
Fig No.6 Isolated DNA was used for the amplification of 16s rDNA for identification of the bacteria.

Lane 2: Isolated DNA

Fig No.7 The bands of DNA after amplification, using universal primers.

Lane 1: 1kb Ladder

Lane 2,3,4&5: PCR products



4.3 <u>OPTIMIZATION</u>

4.3.1Substrate and time course optimization

Different agricultural wastes such as sugarcane bagasse, rice straw and wheat straw were evaluated for xylanase production by using B3 culture isolated from the manure sample(DMR, Solan) in submerged state fermentation. Results (Table No. 7) indicated that maximum xylanase yield of 14.42 U/ml was obtained by rice straw which was followed by wheat straw 12.32 U/ml and sugarcane bagasse 3.733 U/ml, respectively. Similar results with high xylanase production were observed by using wheat and rice straw in reports by Goswami G. and Rawat S.2015 and Gupta et al. 2009.

Xylanase production was checked by incubating the inoculated flasks for various time periods and its was noted that enzyme production was gradually increased with increase in fermentation period and maximum production was achieved after seven days of fermentation period as shown in Table No. 7. As the fermentation period was increased decrease in enzyme production was observed. Increased fermentation time and decreased enzyme synthesis might be due to the depletion of macro- and micronutrients in the fermentation medium with the passage of time, which altered the bacterial physiology resulting in the inactivation of secretary machinery of the enzymes.

Table No. 7: The table represents the enzyme units produced by the bacteria at different hours.

Hours	Sugarcane bagasse(U/ml)	Rice straw(U/ml)	Wheat straw(U/ml)
24	0.453	0.3133	1.27
48	0.74	2.22	4.88
72	1.573	5.366	7.653
96	2.266	7.126	8.0467
120	2.586	8.893	8.6
144	2.513	9.906	8.9
168	3.733	14.42	12.32

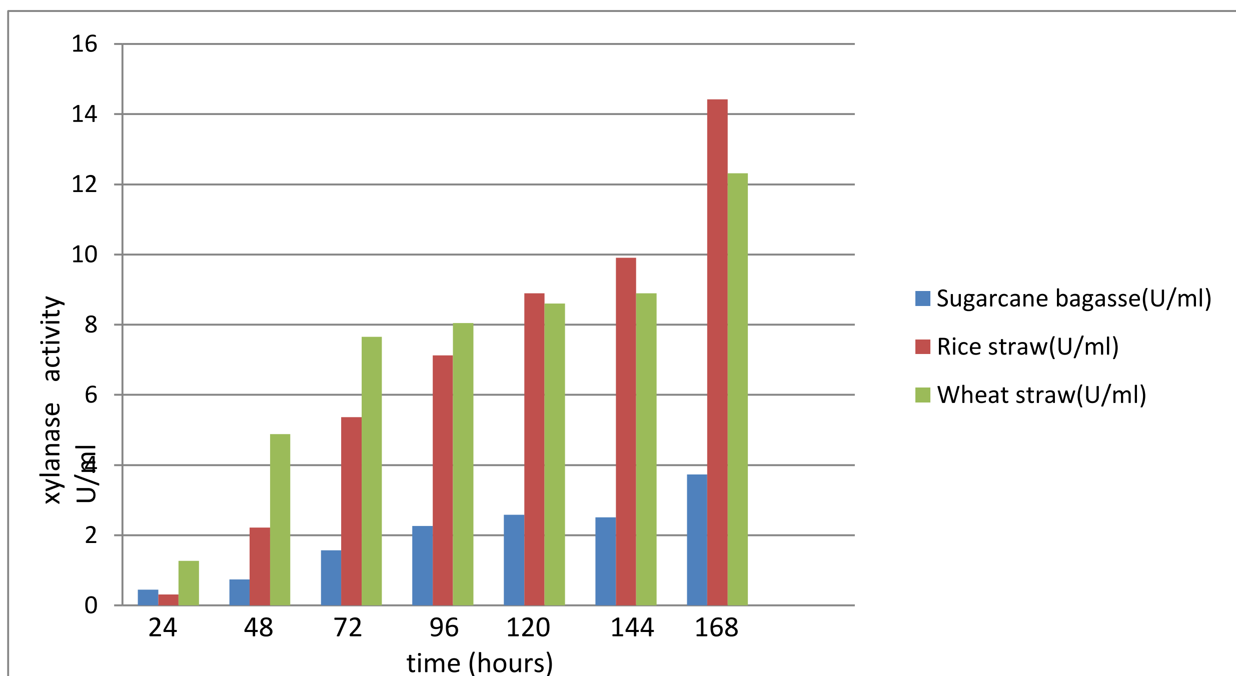


Figure No.8 Selection of substrate and time course for xylanase production by B3 culture in submerged state fermentation

4.3.2Initial pH optimization

To check the optimum initial medium pH for xylanase production, experiments were carried out at different pH of the medium ranging from 5 to10. pH of the medium was adjusted with

1N NaOH and 1N HCl before sterilization. From the experiments it was observed that maximum enzyme production (8.246 U/ml in rice straw and 8.146 U/ml in wheat straw) as observed at pH 9 (Figure No 8). The pH of the medium strongly affects many enzymatic processes and transport of various components across the cell membrane. Similar to our findings, report by Sepahy et al., 2011 showed xylanase at alkaline region.

Table no.8 – Enzyme activity at different initial pH

pH	Rice straw	Wheat straw
5	Negligible	Negligible
6	1.42	0.4466
7	4.34	7.713
8	7.61	8.009
9	8.246	8.146
10	6.37	5.29

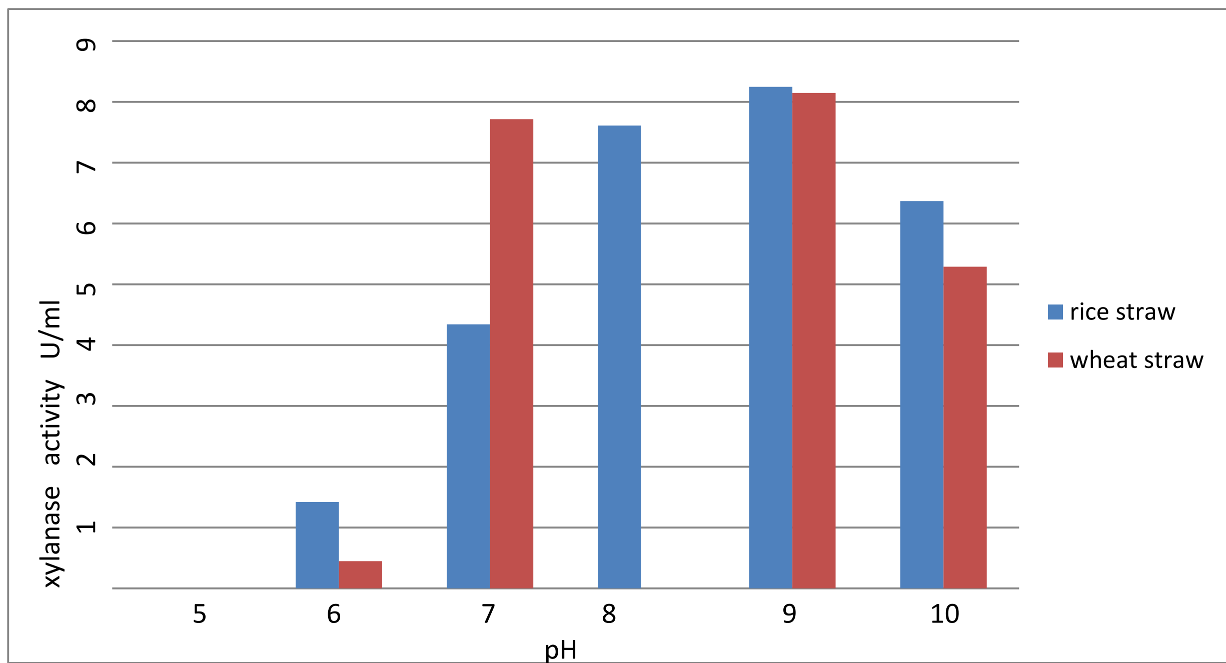


Figure no. 9 Effect of initial pH on xylanase production by B3 culture in SmF.

4.3.3 Substrate concentration optimization

Suitable substrate level for xylanase production was also checked by changing the amount of selected substrate (rice straw and wheat straw) in 500 ml Erlyenmer flask from 0.25 to 1.5 g. Of all these tested concentrations of substrate 1.5 % in 5 showed optimum enzyme production (9.855U/ml for rice straw and 9.413U/ml for wheat straw). As the concentration of substrate was increased above this concentration, decreased in enzyme production and protein secretion were observed as shown in Figure No.9. The similar results were reported in Sepahy et al., 2011 (72) where *B. megaterium* BM07 showed its peak production at 1.5% substrate concentration. High concentration of substrate led to the increase in medium viscosity, which influenced the mixture medium components and oxygen transfer (Gupta et al. 2009).

Table No.9 – Enzyme activity of different substrate at different substrate concentration

Substrate concentration	Rice straw	Wheat straw
0.5%	7.266	4.724
1.0%	9.040	6.151
1.5%	9.835	9.413
2.0%	6.777	6.822
2.5%	5.875	4.191
3.0%	4.960	3.488

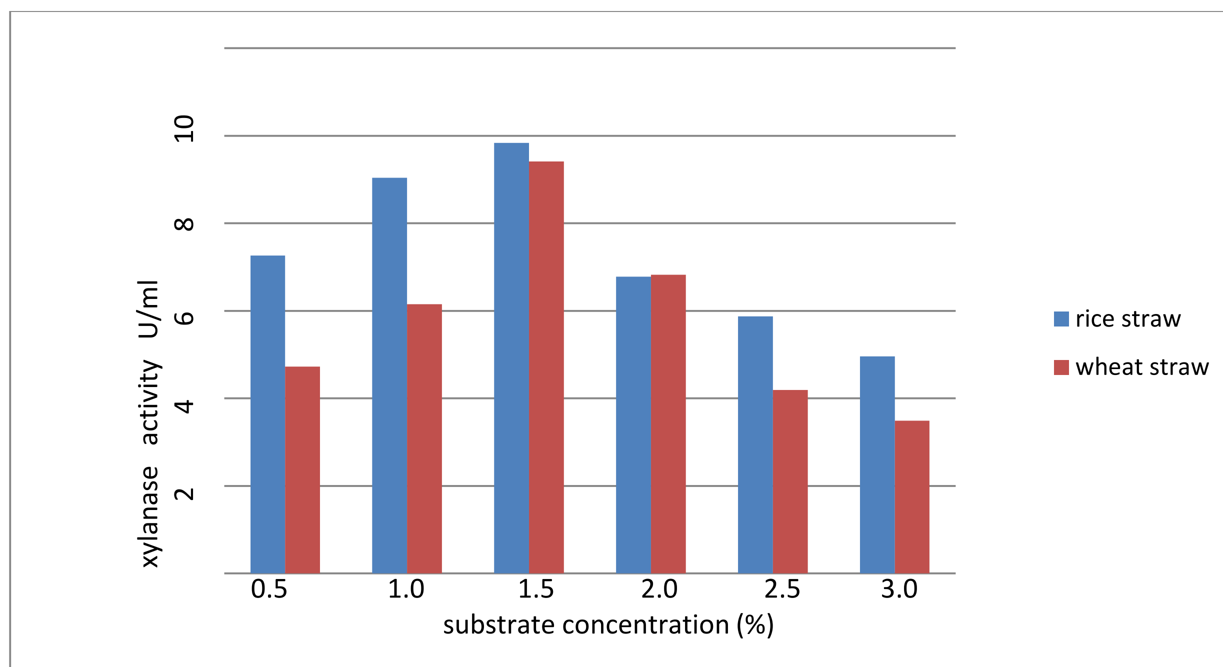


Figure No. 10 Effect of different substrate concentrations on xylanase production by B3 culture in SmF

4.3.4 Temperature optimization

Incubation temperature is also a critical factor, it determines the growth rate of the microorganism and has major effect on levels of enzyme production. Different incubation temperatures were tested ranging from 20 to 50°C. Results of the study indicated that maximum enzyme production was noted at 37°C yielding enzyme activity of 14.42U/ml and 12.32 U/ml using rice straw and wheat straw respectively. Increase in incubation temperature up to 50 °C significantly reduced in enzyme production.

This result shows that the bacterium is mesophilic which works well within a temperature range of 30°C to 37°C but the maximum enzyme production by the bacteria was at 37°C. Similar to findings were reported in Irfan M. et al 2016, the optimum temperature of 37 °C for xylanase production by *B. mojavensis* AG137 in submerge fermentation.

Table No.10 - Enzyme activity of different substrate at different temperature

Temperature	Rice straw	Wheat straw
20°C	0.1067	0.28
30°C	9.288	4.906
40°C	12.661	10.984
50°C	0	0

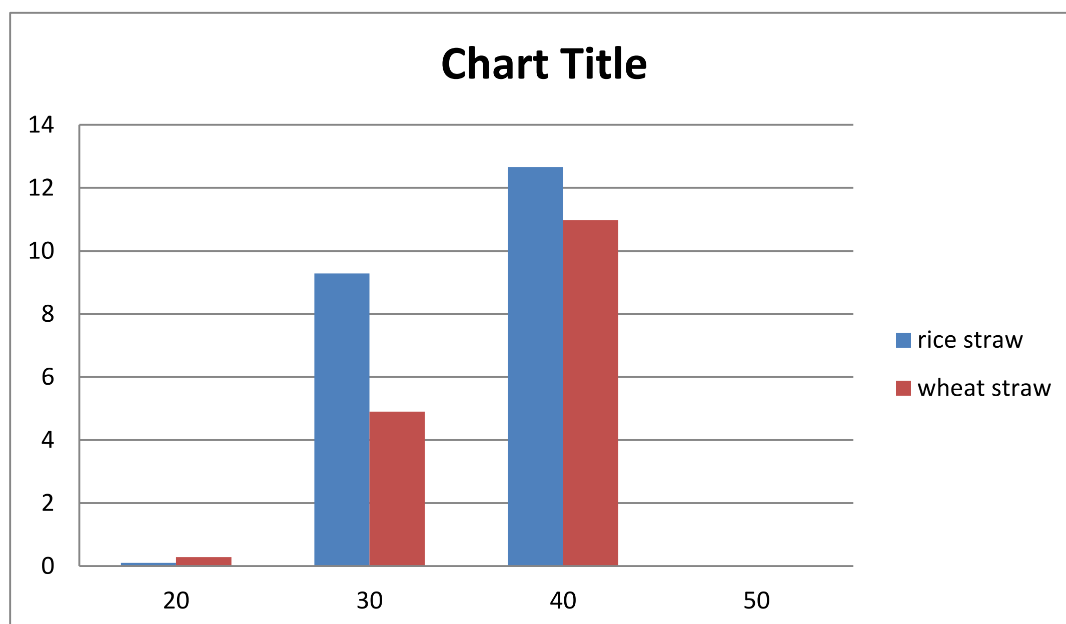


Fig no. 11 Effect of different temperature on enzyme production

CHAPTER 5

Conclusion

CONCLUSION

The present study shows that the isolated strain B3(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 usage of carbon source as it was able to utilize the three agricultural residues (rice straw, wheat straw and sugarcane baggase) for the xylanase production.

However it showed highest xylanase yield with rice straw. Though the xylanase produced is active in mesophilic range, it provides a platform for future research on this strain. Some genetic engineering or site directed mutagenesis techniques could be applied to make the enzyme stable at high temperature as well.

CHAPTER 6

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