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Utilization of Agro-waste Residues for Xylanase Production Using Thermoalkalistable *Bacillus* Isolates

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Abstract:

Thermoalkalistable microorganisms are sources of many enzymes which are applicable to the industrial processes involving extreme conditions of temperatures and hydrogen ion concentrations. There is increasing demand for thermoalkalitolerant xylanases in pulp and paper industry. Lonar lake is an impact crater created by a meteor hitting in basalt rock, situated in Buldhana district, Maharashtra state of India. It is a salt water lake containing carbonates of soda. It has about 1.8 km crater rim diameter and is about 137 meters below the crater rim. It is a nature's bounty for microorganisms having wonderful characteristics and potential for producing industrially useful enzymes.15 Gram positive bacterial cultures, showing polyextremophilic characteristics, were isolated from the soil sample of Lonar lake bank, collected in January, 2008. The pH of the sample was recorded as 10.5 .The organisms were studied for their xylanase production retention capacity at high temperature and high pH. They were further used for xylanase production by growing on different agro-waste residues such as wheat bran, corn cobs, rice husks, banana peels, rice bran and oat flour. The highest activity of this xylan hydrolyzing enzyme was obtained as 2676 Units/ml/min on oat flour by an isolate which was identified biochemically as well as genetically as *Bacillus subtilis*. Wheat bran and corn cobs were also proved good substrates for enzyme production.

Keywords: Lonar lake, *Bacillus subtilis*, Xylanase, Thermoalkalistable, agro waste residue.

1.0 Introduction:

Xylanases are xylan hydrolyzing enzymes. They belong to glycosyl hydrolyses (GH), and they randomly cleave β-1, 4 linkages of the complex polysaccharide xylan (Garbacheva and Radinova, 1977). Many microorganisms are rich sources of xylanases in nature. They include a diverse group of genera and species of bacteria, actinomycetes and fungi (Sunna and Antranikian, 1997). Several organisms secrete a high level of xylanases, however, many of them, particularly, fungi secrete cellulolytic enzymes also (Balkrishnan *et al* 1999). Although all these strains produce detectable amounts of xylanases in the culture filtrate, The productivity of most of these strains is low and they do not meet the requirements of large scale industrial production (Gottschalk, 1994). The majority of microorganisms produce xylanases active within the mesophilic temperature range of approximately 40 – 50^{o} C. and at neutral or slightly acidic pH. (Subramanian and Prema, 2000). During these two decades extensive work is carried out to get alkaliphlic xylanase, as it has increasing demand mainly in the pulp and paper industry, for biobleaching of kraft pulp. Use of xylanase at kraft bleaching stage is effective in reducing requirement of chlorine and in producing a bright, and higher quality pulp (Dhiman *et al.* 2000, Jeffries, 1996). Other important applications of xylanase in technical industries include bio-deinking of recycled papers and fibers gluten separation process, xylose

production, production of pharmacologically active polysaccharides to use as antimicrobials or antioxidants, detergent preparation, production of alkyl glycosidases for use as surfactants, recovery of cellulosic textile fibers and also the bioconversion of biomass into fuel and chemicals. Xylanases used in the pretreatment of forage crops and low quality feed-stuffs to improve the digestibility of ruminant feeds. Use of xylanase in the poultry feed is also recommended in these days. Xylanase has numerous applications in the food industry also (Kapoor *et al*. 1999).They are specially used for preparing white bread and rolls and to enhance the baking volume and quality of the dough in the preparation of bread and other baked products. Xylanases are also used in brewing to increase wort filterability and reduce haze in the final product, in coffee extraction and in the preparation of soluble coffee, and in the fruit and vegetable processing.

Lonar lake is an unique meteorite impact crater situated at Buldhana district of Maharashtra, India (latitude $19^{o}58'$, longitude $76^{o}36'$). It was originated around 50 thousand years ago, due to a meteorite impact on basalt rock. Due to the high alkalinity and salt content in its water it provides a wonderful natural ecosystem for alkaliphilic and halophilic microorganisms those grow at high pH and in high salt contents. Soda lakes of the East African rift valley and those in Jorden are studied for the phylogenetic diversity of alkaliphiles. The literature survey indicates that a good amount of limnological studies of the Lonar lake is made while only few reports deal with its microbial diversity. Wani *et al* (2006) have reported microbial diversity studied using culture independent approach. Recently, cultivable bacterial diversity present in the Lonar lake was studied by phenotypic characterization and 16S rDNA based phylogenetic analysis by Joshi *et al* (2007). Extremophilic microorganisms offer a multitude of actual potential value based applications in the various fields of industrial microbiology and biotechnology. They have ability of secreting great varieties of extracellular enzymes; those are increasingly recommended in different industries having extreme process conditions. These enzymes are also needed in the treatment of harmful waste generated by industries. Another area where these organisms prove very potential is the metabolite production. Production of biodegradable polymers, poly hydroxyalkanoates, from bacterial isolates of Lonar lake sediment was reported (Kanekar *et al*. 2008). Roy *et al* (2004) isolated an aerobic, alkaliphilic xylanolytic bacterium from soda lake water and studied it with respect to the production and characterization of xylanase.

In this paper, study of a 15 thermoalkalistable *Bacillus sp.* isolated from Lonar lake sediment wrt xylanase production is reported. Biotechnological potential of the isolate with respect to the production of extracellular enzymes was studied. The paper presents production of xylan hydrolyzing enzyme production by growing the isolate on different agro-waste residues.

2.0 Materials and Methods:

2.1 Sample collection and isolation of thermophilic bacteria

Sediment soil around the bank of Lonar Lake was collected in plastic bags in the winter season, at the end of January. Thermoresistant and thermophilic bacteria were isolated by applying heat resistant spore selection technique. 0.1 gm sediment was suspended in 10 ml sterile distilled water and subjected to the heat shock at 80^{o}C. for 10 minutes. This was then diluted as 10^{-2} and 10^{-3}. 0.1 ml supernatant aliquot of each dilution was then pour plated on nutrient agar medium. The plates were incubated at 30^{o} C. for 24 hours to select fast growing bacterial isolates. The *Bacillus* isolates were confirmed by Gram staining. Their thermophilic character was further tested by growing the cultures on nutrient agar medium at 60^{o} C.

2.2 Screening for Bacillus isolates having xylan hydrolysis capacity

The selected cultures were streak inoculated on xylan agar medium plates and incubated at 30^{0} C. for 24 hours. The hydrolysis capacity was determined as the ratio of the diameter of zone of xylan clearance to the diameter of the colony in mm by observing well isolated colonies.

2.3 Xylanase production in broth culture

24 hours old single colony of the isolate was inoculated in 2.5 ml oat xylan broth and incubated at 30^{o} C. for 24 hours so as to get

the pellicle. This inoculum was used to inoculate 50 ml production medium, Oat Xylan Medium (OXM). Similarly, another production medium, Xylan Yeast Extract Medium (XYEM), was also inoculated. Fermentation was carried out at 30^0 C. for 72 hours by applying stationary fermentation technique. Same procedure was followed for all selected isolates.
The fermented broth was then filtered to remove the pellicle and centrifuged twice at 8000 rpm, for 20 minutes. The supernatant was collected as the crude enzyme extract and subjected to xylanase assay. EA was calculated for every fermented broth.

2.4 Study of retention of xylanase production capacity at high temperature

Ability ofselected 15 isolates to produce xylanase at 60 ^{o}C. was determined,
so as to select potent enzyme producer with broad temperature range for growth as well as for xylanase production. The inoculum was prepared as described in the previous step, and used to inoculate the production medium OXM, at 5% concentration. The fermentation was carried out at 60^o C. for 72 hours by stationary culture method. The potency of xylanase production was then determined by collecting crude enzyme extract and subjecting it for xylanase assay.

2.5 Study of retention of xylanase production capacity at high PH

In order to select the organism with potential to produce enzyme active at high pH, the fermentation was carried out at pH 10 for 72 hours. Inoculation was done in OXM broth, as described above. The fermentation temperature was maintained at 30^0C. The crude enzyme was prepared. Xylanase assay was performed as described above and EA was calculated. All selected 15 isolates were processed in the same way.

2.6 Cultural and biochemical characterization of three selected isolates

Cultural characters of the selected isolates were studied on nutrient agar plate. Biochemical characterization was performed by referring to the Bergey's manual of Systematic Bacteriology, Vol.2, Ed.1(Sneath, 1986). Ability of the isolate to ferment glucose, mannose, xylose, arabinose, lactose, maltose and mannitol and to utilize glycerol, citrate, propionate and aspartate was studied by growing the organism in the respective media. Also their salt tolerance and thermotolerance was observed.

2.7 Determination of enzyme profile

Ability of these three isolates to produce gelatinase, caseinase, amylase, xylanase, cellulase, and lecithinase was studied by spot inoculating the organism on gelatin agar, milk agar, starch agar, oat xylan agar, carboxy methyl cellulose agar and Dorset egg yolk agar respectively. Zone of clearance of the substrate was noted around the colony, except in the lecithinase test where zone of opalescence was observed. The isolates werealso studied with respect to its ability to produce other enzymes namely, nitrate reductase, phenyl deaminase, desulfurase and tyrosine hydrolase by performing tests using media as per Bergey's manual of systematic bacteriology.

2.8 Xylanase production by utilizing different agro-waste residues

Hemicellulosic agro-waste materials namely, wheat bran, banana peels, rice bran, rice husks, oat flour and corn cobs were used as substrates for xylanase production by the isolate. The substrates were dried and ground to make fine powder. The basal medium was prepared using 0.2%NH_4NO_3, 0.1% KH_2PO_4, 0.1%$MgSO_4.7H_2O$, 0.1%$MnSO_4$, and 0.01%$CaCl_2$. To this agro-waste residues were added at 1% concentration separately, as sole carbon source. 24 hour old inoculums prepared in nutrient broth and showing 0.6 O.D. at 540 nm was used to inoculate the above prepared production medium at 3% concentration. Xylanase production was carried out at 30^o C.

2.9 Enzyme assay

Enzyme activity (EA), for xylanase was determined by treating 0.5% native oat xylan prepared in phosphate buffer of pH 7, with the crude enzyme extracts obtained by fermenting agro-waste residues as described above and estimating the amount of xylose produced due to the hydrolysis of xylan, using DNSA reagent (Sadashivam and Manikam, 1992). EA was calculated as the amount of the enzyme required to form 1µmole of xylose per minute per ml in the reaction mixture and expressed as Units/ml/min.

3.0 Results and Discussion

32 bacterial cultures were isolated on nutrient agar plate, from the heat treated soil sample of the Lonar lake bank. All were Gram positive sporulating rods. 15 cultures grew well at 60^{o} C. Only two isolates showed ability to grow at 80^{o} C. Isolate number 13 and 5 were found with least ability to produce xylanase in XYE medium with EA 380 Units/ml/min and 427 Units/ml/min respectively. On the other hand isolate number 01, 10, 14 and 15 were observed as excellent xylanase producers in OXM with EA 1956 Units/ml/min, 2200 Units/ml/min, 1633 Units/ml/min and 1466 Units/ml/min respectively. Isolate number 14 showed EA 1733 Units/ml/min in XYE broth also. Total **11** isolates were observed as good xylanase producers with EA more than 1000 Units/ml/min (Figure 1). Among them 7 isolates retained more than 50% EA at 60^{o} C. Isolate number 01 retained 99% EA while isolate number 15 retained 98% EA at high temperature (Table 1). 8 isolates retained more than 50% EA, while growing at pH 10 (Table 1). Isolate number10 showed 6.91% increased EA at pH 10. *Bacillus* isolate number 1, 10 and 14 were selected for xylanase production studies. These cultures were selected on the basis of enzyme production capacity at high temperature as well as at pH 10. They exhibited more than 1500 Units/ml/min enzyme activity on XYE as well as OXM. Their morphological and biochemical characters as well as their enzyme profile is tabulated in table no 2, 3 and 4.The screening method used during the project is cost effective as it utilized oat. Other agro-waste materials are also reported (Nagar *et al.* 2012).

In these days of biotechnology, enzyme technology provides the best supportive technology for many industrial processes. Certain industries like pulp and paper industries need enzyme active at high temperature under alkaline pH. Xylanase is one of the extracellular enzymes having increasing demand due to its application in pulp prebleaching step in the paper and pulp industries (Kumar and Satyanarayan, 2011). This reduces the amount of chlorine required for the bleaching step. The effluent of such industries receive lowered amount of chlorine and chlorine compounds, which are toxic and hazardous. Thus use of xylanase not only give better quality rayon grade paper, but also reduce environmental pollution. Lonar lake situated in Buldhana district of Maharashtra, India is a natural bounty of such microorganisms. Alkaliphilic and halophilic bacteria are isolated from water samples and sediments of Lonar lake by many workers. The enzymes of these bacteria are polyextremophilic, due to their improved structures. The present work was focused on isolating thermoalkalistable bacterial species that can be looked upon as a source of xylanase. The most efficient xylanase producer polyextremophilic heat resistant bacterium species isolated was identified as *Bacillus subtilis*. It was observed as a metabolically active isolate, able to produce commercially important extracellular enzymes as xylanase, amylase, casinase and lecithinase.

Xylanase production studies revealed the fact of utilization of agro-waste for the fermentation. Disposal problem of waste results in the generation of heaps of agricultural substances in or near the farm and agro-product industries. Also in the cities heaps of banana waste and corncobs are observed. Many times animals refuse to eat these wastes. This fact was considered for utilization of agro-waste residues as carbon source during the formulation of the production medium. During the experiment oat flour was concluded as the best carbon substrate on which the *Bacillus subtilis* isolate number 10, produced 2676 Units/ml/min xylanase activity. Wheat bran proved second choice medium (Figure 2). One of the major hurdles in commercialization of enzyme production is the cost involved in its production. The xylanase production can be made economical by using agricultural waste under suitable pH and temperature(Sanghi *et al*. 1992, Sugumaran *et al.* 2013). Attempts were made to get xylanase by growing *Bacillus subtilis* isolated from Lonar lake sample on different agro-waste residues. The ecofriendly approach was one aim of the present project.

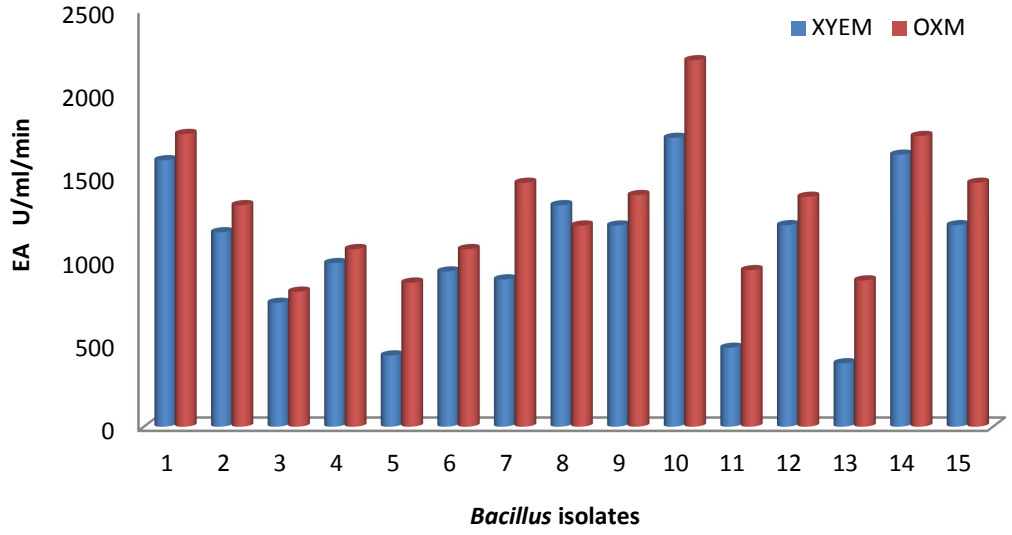

Fig. 1: Xylanase production by *Bacillus* isolates

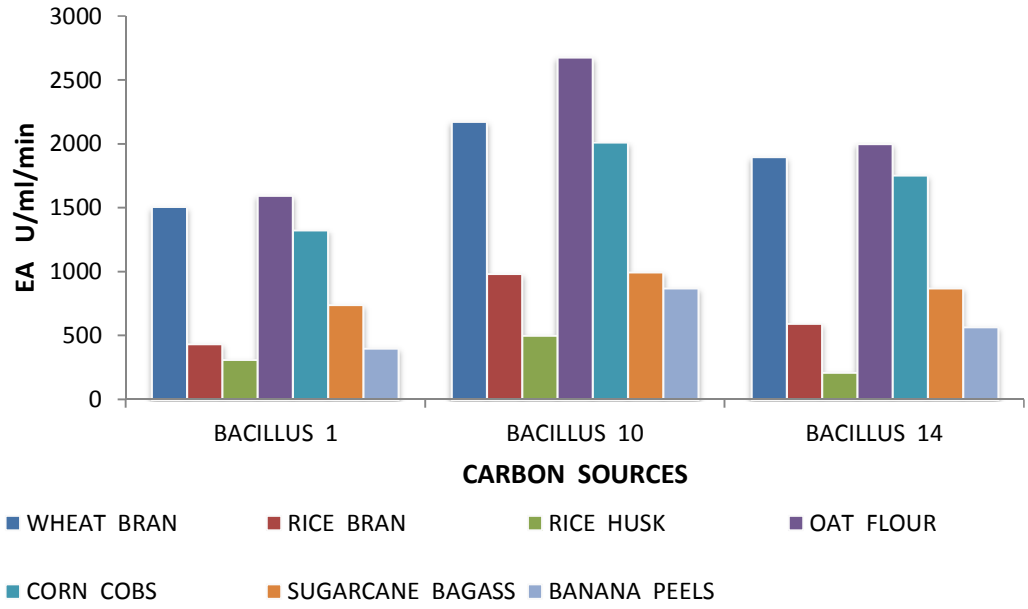

Fig. 2: Xylanase production on different agro-waste residues

Table 1.Percentage retention of xylanase activity

Sr. No.	Isolate Number	% Retention of EA at 60°C.	% Retention of EA at pH 10
01	01	99.00	73.82
02	02	58.31	53.25
03	03	76.58	00.00
04	04	**106.00**	83.61
05	05	21.26	09.67
06	06	81.46	50.59
07	07	50.79	78.66
08	08	44.06	30.60
09	09	**110.78**	50.80
10	10	**112.00**	**106.91**
11	11	03.59	00.00
12	12	63.67	62.89
13	13	21.06	00.00
14	14	98.00	87.69
15	15	01.43	29.53

Table 2. Morphological and cultural characteristics of selected 3 isolates, on oat xylan medium, incubated at 30° C. for 24 hours.

Bacillus Isolate no.	Size mm	Shape	Margin	Color	Opacity	Elevation	Consis - tancy	Gram character	Motility
1	2.5	Circular	Irregular	White	Opaque	Raised	Mucoid	+ ve	Non-Motile
10	3	Circular	Entire	Red tinted	Opaque	Raised Wrinkled	Mucoid	+ ve	Motile
14	1.5	Circular	Irregular	Off white	Opaque	Effuse	Mucoid	+ ve	Motile

Table 3. Biochemical characterization

Sr. No.	Test	Inference		
		Bacillus 01	*Bacillus* 10	*Bacillus* 14
1	Glucose	A	A	A
2	Lactose	-ve	-ve	-ve
3	Mannose	A	-ve	A
4	Arabinose	-ve	-ve	-ve
5	Xylose	-ve	-ve	-ve
6	Maltose	WA	A	-ve
7	Glycerol	-ve	-ve	-ve
8	Citrate Utilization	+ ve	+ ve	+ ve
9	PropionateUtilization	-ve	-ve	+ ve
10	Acetoin production	-ve	-ve	-ve
11	Indole production	-ve	-ve	-ve
12	Growth at 10% NaCl	-ve	+ ve	-ve
13	Growth at 80° C.	+ ve	+ ve	+ ve

A - Acid production. WA-Weak reaction for acid production

Table 4. Enzyme Profile of isolates

Sr. No.	Enzyme	Medium/Reagent used	Inference		
			Isolate 1	Isolate10	Isolate 14
1	Gelatinase	Frezier Gelatin agar	-ve	+ ve	-ve
2	Casinase	Milk agar	-ve	+ ve	+ve
3	Oxidase	Oxidase reagent	+ ve	+ve	+ve
4	Catalase	Hydrogen peroxide	+ve	+ ve	+ve
5	Amylase	Starch agar	+ve	+ ve	+ve
6	Cellulase	CMC agar	-ve	-ve	+ve
7	Xylanase	Xylan agar	-ve	+ ve	+ve
8	Nitrate reductase	Peptone nitrate broth	-ve	+ ve	-ve
9	Phenylalninedeaminase	Phenylalnine agar	-ve	-ve	-ve
10	Lecithinase	Dorset Egg Yolk agar	-ve	+ ve	+ve

Khandeparkar and Bhosale (2006) had reported extracellular xylanase production by thermophilic *Arthrobacter* sp. on wheat bran, rice husks and bagass used as carbon source in solid state fermentation. Batten *et al* (Battan *et al.* 2006) reported wheat bran as the best carbon source under submerged fermentation for the production of high level xylanase using *Bacillus pumilus.* Literature survey showed production of a novel enzyme xylanase V, on oat spelt and birch xylan, using *Aeromonas caviae* ME-1(Kuhad *et al*. 1994). For the production of xylanase wheat bran, sugarcane bagass, orange bagass, corncobs, green grass, saw dust and few other substances were reported as carbon sources in solid state fermentation (Kamble and Jadhav, 2011, Ratankhanokchai and Kyu, 1999, Virupakshi et al. 2005). Gupta and Rita (2009) reported significant repression of enzyme with xylose in medium containing corn cob under SSF .The *Bacillus subtilis*HX-6 strain under study exhibited 3740 Units/ml/min xylanase activity in the crude extract obtained by fermenting oat flour, at alkaline PH 9, and at temperature 60^0 C. This is more by 1164 Units/ml/min than that produced at PH 7 and at temperature 30^0C. This proves the polyextremophilic nature of the enzyme, apart from focusing the oat xylan as the best carbon source for xylanase production. However, good amount of enzyme was obtained on wheat bran, corn cobs, and sugarcane bagass, followed by rice bran and rice husk, and banana peels.

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