Research Article

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CONDITION OPTIMIZATION FOR XYLANASE PRODUCTION USING POLYEXTREMOPHILIC *BACILLUS SUBTILIS* HX-6 STRAIN

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Abstract: Xylanases, (E.C.3.2.1.8), constitute a group of diverse xylanolytic enzymes with varying specificities and action. Polyextremophilic xylanases are required so as to get maximum activity under industrial process conditions, like high temperature and high pH. *Bacillus subtilis* HX-6, was isolated from highly alkaline natural environment of Lonar lake, a meteorite crater in India. The organism had optimum growth temperature 60°C. and optimum pH 9. It produced 3760 U/ml/min xylanase when fermented under optimum conditions of high temperature and high pH, while it produced 2676 U/ml/min at 30° C. with pH 7. The xylanase production was optimized w.r.t. Carbon and nitrogen sources in the production medium, inoculum size, inoculum age, duration of fermentation, production temperature and hydrogen ion concentration.

Keywords: Xylanase, polyextremophilic, *Bacillus subtilis*, Lonar lake.

INTRODUCTION

During these days of biotechnology, biocatalytical reactions are best applied for getting better quality products with low production cost. The use of enzymes is increasingly recommended in many industries to cope mainly with the pollution problems due to disposal of toxic effluents. Microorganisms are commonly employed as the source of enzymes because of the wide varieties of enzymes they produce and also because of the variable characteristics of their enzymes, which make them industrially useful. Endo 1,4 β-Xylanase, is a xylan hydrolyzing enzyme, that belong to glycosyl hydrolase (GH) group and that catalyze random cleavage of the complex plant polysaccharide, xylan[1]. Most xylanases are classified in the families GH 10 and GH 11, having approximate molecular weight 30-45 KDa and 20-22 kDa respectively[2]. Presently, xylanases form the major commercial proportion of hemicellulases as they are used in the wide range of processes, mainly where plant sources are the major raw materials. The major sectors in which xylanases are employed include technical industries, food industries and feed industries. One of the interesting applications of the xylanases is in pulp and paper industry for prebleaching of the kraft pulp. This increases the brightness of the paper and aids in producing rayon grade paper. This also reduces the harsh treatment of chlorine in the next step, resulting great reduction in the amount of the chlorinated byproducts released in the effluent. Thus may cause reduction of the pollution[3, 4]. The process conditions used in industries are generally extreme like high temperature of 80° C.-100° C. and high pH exceeding pH 9. This has focused the need of cellulase free xylanase active with high temperature and high pH optima[5]. Polyextremophilic xylanase have greater stability at extreme conditions of temperatures and pH. They have improved structures due to increased hydrogen bonds, improved internal packing and mainly due to the presence of a series of aromatic residues forming clusters or sticky patches between the molecules. All ecological niches where plant materials are deposited are good natural habitats of xylanase producers. Xylanase producers include a diverse group of genera and species of bacteria, fungi and actinomycetes[6]. Some of the extensively studied bacterial xylanase producers are species of *Aeromonas*[7], *Arthrobacter*[8] and *Bacillus*[9]. Some thermophilic bacteria are exploited for extremophilic xylanases. Xylanase of *Thermotoga* strain FjSS3-B, 1 is one of the most thermostable xylanase reported to date with an optimum temperature 105°C. Xylanases from most of the reported alkaliphilic species have their optimum pH for activity is around neutrality[10]. The majority of alkaliphiles known so far were isolated from soil samples[11]. An alkaliphilic *Bacillus* sp. VI-4 was reported as hardwood kraft pulp isolate[12]. An aerobic, alkaliphilic xylanolytic bacterium was isolated from an alkaline soda lake, in Bangladesh[13].

Naturally occurring alkaline habitats are nature's bounty of novel organisms. In the search of polyextremophilic enzyme producer bacterium, the sediment sample of a natural extreme ecological niche, Lonar lake, a meteorite crater formed nearly fifty thousand years ago near Lonar village, in Buldhana district, Maharashtra, India, was analyzed for alkalinity and used as the bacterial source. Gram positive sporulating rod shaped bacterial species was isolated

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by selective technique and studied for its xylanase production ability. Parameters for xylanase production were standardized.

MATERIALS AND METHODS

Isolation of efficient xylanase producer

Bacillus cultures were isolated by applying heat shock procedure and by using xylan rich agar medium to grow. All colonies with good growth and a zone of xylan clearance around them were sub cultured and tested for their potential for xylanase production in the xylan broth. The isolate growing luxuriously at 80°C. and at pH 10 and showing high xylan hydrolysis ability was selected for further studies.

16S rDNA identification

After cultural, morphological and biochemical characterization[14], the selected bacterial isolate was subjected to genetical identification by performing base pair sequencing of 16S rDNA.

Parameters optimization for xylanase production

Different parameters namely inoculum age, inoculum size, fermentation duration, carbon source, nitrogen source, production temperature, hydrogen ion concentration were standardized. For studying parameters other than pH and temperature, common conditions used were 30°C. and pH 7.

To optimize the inoculum size, 24 hours old slant culture was used. Uniform suspension was prepared in nutrient broth so as to get 0.6 O. D. at 540 nm. This broth culture was used to inoculate xylanase production medium, Oat Xylan Medium(OXM), using various sized inocula as 0.5%, 1%, 2%, 3%, 4%, 5%, and 7% v/v. Xylanase fermentation was carried out at 30° C. for 72 hours. pH maintained was 7. The crude enzyme was extracted by centrifugation twice at 8000 rpm for 20 minutes and used to determine enzyme activity.

Inoculum age was standardized by using 6, 12, 24 and 48 hours old slant cultures to prepare the inocula. 50 ml xylanase production medium, OXM, was inoculated with 3% v/v inoculum and fermentation was carried out by maintaining standardized conditions. The crude enzyme was extracted and assayed for enzyme activity.

For determining the optimum duration of fermentation, xylanase fermentations were carried out by inoculating 24 hour old inoculum in the production medium with 3% concentration as described earlier. Temperature and pH were maintained as 30°C. and 7 respectively. Fermentation was continued for 96 hours with intermittent removal of fermented broths at 24, 48 and 72 hours and assaying the crude enzyme extracts for enzyme activity.

To determine suitable temperature for xylanase production, fermentations were carried out as described above at different temperatures. Different temperatures used were 20°, 30°, 40°, 50°, 60°, 70°, 80° and 90°C.

PH optimum was determined by preparing the fermentation medium in respective buffers having pH as 4, 5, 6, 7, 8, 9 and 10. Buffers used were sodium citrate buffer, phosphate buffer and glycine NaOH buffer. After fermentation the crude xylanase was extracted and assayed to determine enzyme activity.

Hemi cellulosic agro-waste materials viz. Oat flour, wheat bran, banana waste, rice bran, rice husks, and corn cobs, were used as substrates for xylanase production**.** The medium was supplemented with $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$ $MnSO_4$ and $CaCl_2$. Optimum parameters as decided above were applied with fermentation temperature 30°C. and pH 7. EA was determined in the crude enzyme preparations.

Enzyme production capacity of the isolate in the presence of different nitrogen sources was studied by determining EA in the crude enzyme preparations obtained by fermenting fermentation media prepared with oat xylan substrate as mentioned above and supplemented with crude nitrogen sources namely steeped gram seeds, peptone and also, ammonium sulphate at 2% concentration. Optimum parameters as decided above were applied with fermentation temperature 30° C. and pH 7.

Effect of L-phenylalanine, L-asparagine, L-glutamic acid, L-cystine as well as Cu, Co, Mg, Zn, Mn and EDTA was studied by adding these ingredients into the production medium and carrying out the fermentation as described earlier.

Xylanase assay

Xylanase assay was carried out by treating 0.5% native xylan substrate prepared in phosphate buffer, with the enzyme extracts obtained by fermenting the medium and estimating the amount of xylose produced due to the hydrolysis of the substrate, by DNSA reagent according to the Sumner method. EA was calculated as the amount of the xylanase that catalyses the formation of 1 µmole of xylose per minute per ml in the reaction mixture and expressed as Units/ml/min.

RESULTS

Out of 42 *Bacillus* isolates, 17 isolates showed clear zone of xylanase production on oat xylan medium. During further screening program one of these isolates exhibited 12% and 6.91% increased activity when grown at 80° C. and at pH 10 respectively. This strain was selected for further studies. While characterizing this selected strain, it was observed as mucoid, red tinted colony producer, early sporulating *Bacillus*. Its enzyme profile revealed this strain as very efficient, having ability to produce amylase, casinase, gelatinase and xylanase. The strain was unable to produce cellulase on CMC agar medium. It fermented glucose and maltose with the production of acid without gas. It utilized citrate but not propionate. The special characteristics observed were its ability to grow luxuriously at 80°C. and at pH 10 and to tolerate 10% NaCl concentration. The isolate showed 99.99% 16S rDNA similarity with *Bacillus subtilis*. The isolate under study was named as *Bacillus subtilis* HX-6 (Figure 1).

CCCCCCGTCCAAAAAATGCAGTCGAGCGGAAGATGGGAGCTTGCTC
CCTG
ATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTA
AGACTGGGATAACTCCGGGAAACCGGGGCTA
ATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGG
CTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGG
TGAGGTAATGGCTCACCAAGGCAACGATGCGTAG
CCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA
AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTT
CGA
ATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGT
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TTCCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTC
ATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAG
AGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGA
ACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGG
AGCGAAAGCGTGGGGAGCGAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTT
TCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG
AGTACGGTCGCAAGACTGAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA
ACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAG
ATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGG
CACTCTAAGGTGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGC
TACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCG
AGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCG
GATCGCAGTCTGCAACTCGACTGCGTGAAGCTGAATCGCTAGATTCG
CATTTA

Figure 1: 1326 bp 16S rDNA gene sequence of the *Bacillus subtilis* isolate.

Use of 3% inoculum of 24 hour old culture of *Bacillus subtilis* HX-6 gave maximum xylanase production with enzyme activity 2215 U/ml/min. within 24 hours (Table 1).

Xylanase was not produced in the detectable amount until 18 hours. Enzyme activity was detected in 24 hour fermentation medium, that increased gradually up to 72 hours. Maximum enzyme production, 2676 U/ml/min was found in 72 hours fermented broth of *Bacillus subtilis* HX-6. Further incubation did not resulted in correlative increase in the xylanase production. Maximum enzyme production (128 U/mL) was recorded in stationary phase (36 h) of the culture of *Bacillus subtilis* isolated from marine environment by Annamalai *et al.*[15]

Enzyme activity in the range near 510 U/ml/min was detected at 20° C. Xylanase production increased with the increase in temperature. Amazing result was that the *Bacillus subtilis* HX – 6, obtained from Lonar lake sediment sample had highest xylanase production of 3760 U/ml/min at 60° C. and it maintained this ability of xylanase production upto 90°C. Where 65% activity was retained as compared to its activity at 30°C. (Figure 2). *Bacillus subtilis* HX–6 was also found as alkaliphilic strain that had optimum pH in the alkaline range. At pH 9 and temperature 30° C. the maximum enzyme activity recorded was 2633 U/ml/min. Xylanase was not detected at acidic pH below 5 (Figure 3). The isolate, thus revealed as fast growing polyextremophilic bacterium.

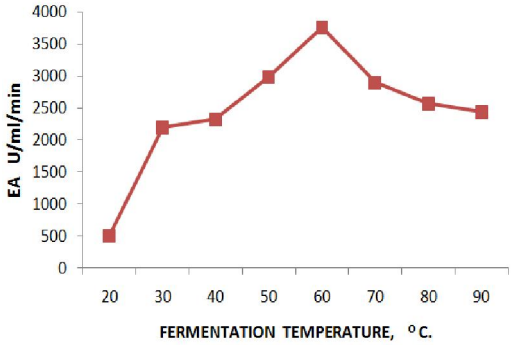

Figure 2: Effect of temperature on xylanase production

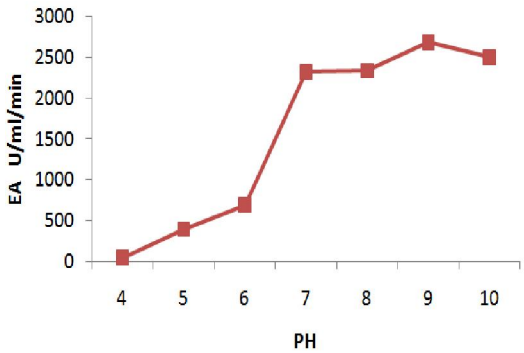

Figure 3: Effect of pH on xylanase production

Table 1: Xylanase activity with different inoculums size, inoculums age and fermentation duration

Inoculum Age –hrs.	EA U/ml/min	Inoculum Size- %	EA U/ml/min	Fermentation Duration–hrs.	EA U/ml/min
1	1393	06	0115	24	2213
2	1666	18	1286	48	2430
3	2213	24	2213	72	2676
4	1733	48	1550	96	1956
5	0920				

Use of crude carbon sources stimulated xylanase production because of their natural xylan content. *Bacillus subtilis* HX – 6 produced maximum xylanase with 2676 U/ml/min EA on oat flour at 30°C. while the least production was obtained on rice husk medium. Wheat bran was second choice substrate (Figure 4). According to Virupakshi *et al.*,[16] Yeast extract, beef extract and xylan enhanced enzyme production, while glucose, lactose and fructose strongly repressed the production process. While studying effect of nitrogen source the results highlighted on ammonium sulphate as the best nitrogen source among those tested for xylanase production while crude sources like peptone and gram seeds had little or no effect in increasing xylanase in the production medium. EA of *Bacillus subtilis* HX-6 was increased by 28% with the addition of 2% ammonium sulphate in the production medium (Figure 5). One more interesting finding of these experiment was that this isolate did not needed additional amino-acids in the medium, though increase in enzyme production was observed when L-asperagine, L- glutamic acid and L-cysteine were added of the medium. 2%-12% increased EA was detected with addition of 5mg% of these amino-acids in the production medium. Also cobalt was detected as enhancer of enzyme production. (Table 2, Figures 6, 7).

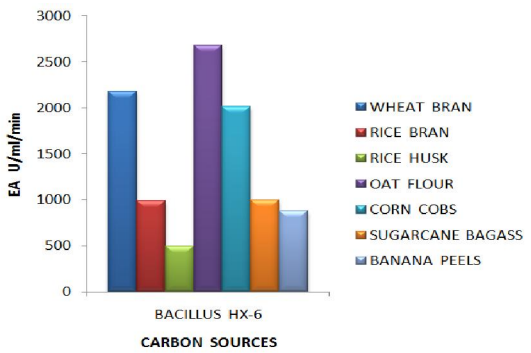

Figure 4: Effect of different carbon sources on xylanase production

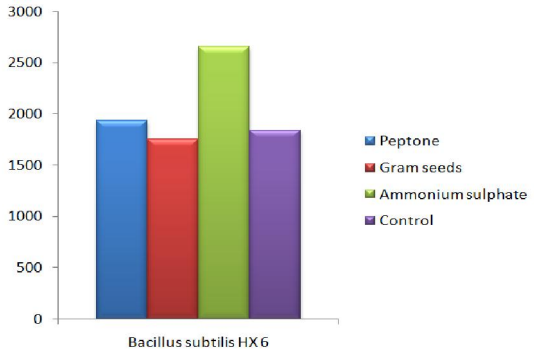

Figure 5: Effect of nitrogen sources on xylanase production

Control-xylanase production medium without additional nitrogen source

Table 2: Xylanase activity with different animo acids and metal ions

Sr. No.	Amino-acids/Metal ions	EA U/ml/min
1	L-phenylalanine	2145
2	L- asperagine	2288
3	L-glutamic acid	2420
4	L-lysine	2134
5	**L-cysteine**	**2464**
6	EDTA	1804
1	$CuSO_4$	1870
2	**$CoCl_2$**	**2860**
3	$Mgcl_2$	2200
4	Zn	1474
5	$MnSO_4$	2200
6	Control	2200

Initial Concentration of amino-acids used - 5mg%
Initial Metal concentration used - 5mM

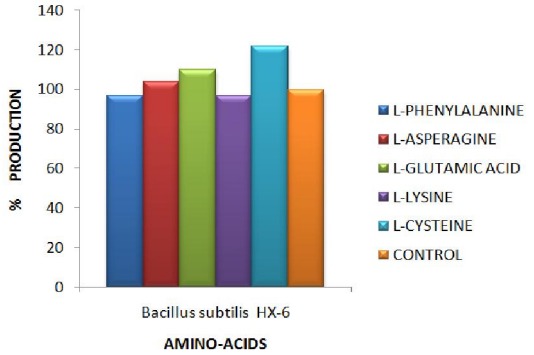

Figure 6: Effect of addition of amino acids on the % yield of xylanase

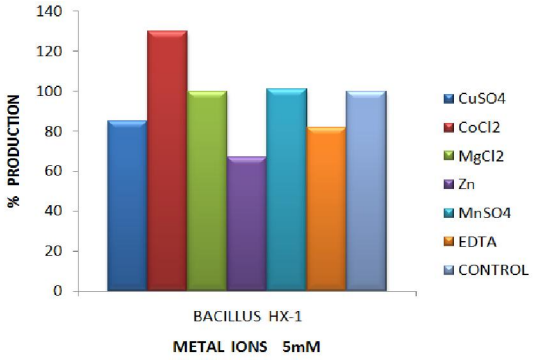

Figure 7: Effect of addition of trace metals on the % yield of xylanase

DISCUSSION AND CONCLUSION

Enzyme technology is a promising sector of fast developing industrial world of biotechnology. Hunting for microbial sources for enzymes is widely open area for research in microbiology. Microorganisms provide basic units where actually many enzymes are produced. Designing an industrial process for microbial enzyme production begins with the isolation of efficient producer strain from the natural sources. The project undertaken concludes with the polyextremophilic *Bacillus subtilis* HX-6 as industrially applicable isolate for the production of xylan hydrolyzing enzyme.

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