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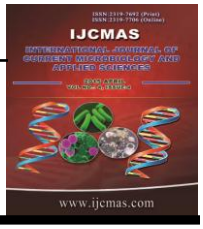

Original Research Article

Production of Bacterial - Xylanolytic Enzyme using Agricultural Waste by Solid State Fermentation

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Keywords

Cellulase free endoxylanase, Bacteria, Optimization, Solid state fermentation, Scale-up

A B S T R A C T

Hydrolysis of xylan, the chief type of hemicellulose is achieved by endo-1, 4-β-xylanase and β-xylosidase among other such enzyme complex. These enzymes are mainly produced by fungi, bacteria, etc. Xylanase depolymerizes xylan molecules into xylose units, a primary carbon source for bacteria and fungi. In this study xylanase producing bacterial isolate from several decaying wood sample exhibiting extracellular xylanase and its phenotypic characteristics were also identified. The optimizations of incubation period, pH, temperature, particle size and moisture concentration were characterized by assay conditions. Maximum xylanase production (16.8U/g in wheat bran) was obtained when substrate was grown at 45°C form 48h at pH 6.5 on 3% substrate concentration. No cellulase activity was found in this purified xylanase fraction. Currently the most important application of xylanases is in prebleaching of kraft pulps to minimize the use of harsh chemicals in the subsequent treatment stages of kraft pulp. Apart from its use in pulp and paper industry, xylanases are also used in baking for improving bread quality by increasing specific bread volume, for must and juice clarification and for liquefying the fruits and vegetables along with pectinases and cellulases.

Introduction

Xylanases (E.C.3.2.1.8) are key enzymes, which play an important role in the breakdown of xylan. Wheat bran and corn cob are a rich source of xylan (28%) and xylose (23%). Therefore these are attractive substrates for production of xylanase & ß-xylosidase enzyme. Xylan, a major component of hemicelluloses, is a heterogeneous polysaccharides consisting of ß-1, 4 linked to D-xylosyl residues on the backbone, but also containing arabinose, glucuronic acid, and arabino glucuronic acid linked to D-xylose backbone (Wong *et al.*, 1988). Enzymatic hydrolysis of xylan is catalyzed by different xylanolytic enzymes such as endo-1,4-ß-xylanase, ß-xylosidase, ά-glucuronidase, ά-arabinofuranosidase and esterase. Among these endo-1,4-β-xylanase (E.C. 3.2.1.8) and ß-xylosidase are the most important enzymes where the first attacks the main internal chain linkages and the second releases xylosyl residues by endwise attack of xylo-oligosaccharides (Bakir *et al.*, 2001). Many of the xylanase producing strains reported in the literature describes the endoxylanase activity only.

Although xylanase production in SSF from fungi and actinomycetes have been reported, only few reports using bacteria showing low enzyme yields are available (Battan *et al.*, 2006; Sindhu *et al.*, 2006). The present study has an objective of optimization and characterizing the enzymes obtained from soil bacterial isolate producing xylanase and β-xylosidase. We also intended to look for a favorable property in terms of activity over a broad range of pH. Xylanase activity in slightly alkaline pH is particularly desirable in operation of biobleaching of pulp. Since chlorine has associated toxicity upon exposure to both humans and environment, partial or full replacement of chlorine as commercial bleaching agent by xylanase, an industrially important enzyme, may be a possibility.

Industries that have demand for xylanase are paper and pulp, baking for improving dough handling and quality of baked products, during extraction of coffee, plant oils, starch, for improvement of nutritional properties of silage and grain, and in combination with pectinase and cellulase for clarification of fruit juices. The enzyme finds applications in textile industry for degumming of plant fiber sources as well as to enhance fiber quality (Bindu *et al.*, 2007; Aysegul *et al.*, 2008).

Materials and Methods

(i) Materials: Oat spelt xylan (Himedia Laboratories Pvt. Ltd., India) was used for enzyme assay. Wheat bran was procured from a local market and sieved (0.5mm. particle size). Corn cob was prepared by stripping corn of all the kernels, drying, grinding and sieving (0.5mm particle size).

(ii) Microbial strain: The bacterial strain used in this study was isolated from decayed woody materials. The optimum growth temperature of this strain was 45^{o}C.

(iii) Inocula preparation: The culture was maintained on wheat bran & corn cob, yeast extract, peptone, agar slants (4:1:1:3%w/v), stored at 4^{0}C and sub cultured routinely after every three four weeks. For inoculum preparation the culture was grown at 45^{0}C for 48h and was used to inoculate 50ml of fermentation medium.

(iv)Xylanase production in SSF

1) Mineral Salt Medium: Erlenmeyer flasks containing 10g of wheat bran/corn cob and 35ml of mineral salt solution (MSS) g/l: $MgCl_2.6H_2O$, 6.6; K_2HPO_4, 0.5; KH_2PO_4, 0.5; $(NH_4)_2SO_4$, 2.0g; pH 6.7) were autoclaved for 20 min at 15 p.s.i., cooled, inoculated with 10% (v/w) of inocula (24h old) and incubated at 45°C. At the desired intervals, the flasks were removed and the contents extracted were with 50ml of 0.02 M phosphate buffer (pH 7.0).

2) Enzyme extraction: Enzyme was extracted with 50ml 0.02M phosphate buffer (pH 7.0) and squeezed through a wet muslin cloth. The enzyme extracted was centrifuged at 1500 rpm for 30min. The clear supernatant was used in the enzyme assay.

3) ***Xylanase assay:*** Xylanase activity was measured by incubating 0.5ml of 0.4% (w/v) oat spelt xylan in 0.02 M phosphate buffer (pH 7.0) and 0.5 ml of suitably diluted enzyme extract at 45°C for 30 min. The release of reducing sugar was measured as xylose by dinitro salicylic acid method (Miller, 1959). One unit (U) of xylanase is defined as the amount of enzyme that releases 1 µmol xylose/ml/min under the assay conditions.

4) ***β-Xylosidase assay:*** The reaction mixture (2.1ml final volume) containing p-nitro phenyl β-D xylopyranoside (1.5mg/ml) dissolved in sodium acetate buffer (2ml, 0.1M, pH 4.0) and suitable diluted culture filtrate (0.1ml) was inoculated for 15 minutes at 50ºC. The reaction was terminated by the addition of glycine buffer (2ml, 0.4 M, pH 10.8) and the absorbance was read at 430 nm.

(v)Optimization of culture conditions for enzyme production: Xylanase production was studied at different incubation period (24 to 96h), pH (5–7), temperature (30–50°C), particle size (30–70mm) and moisture content (50–90%) assayed for production of xylanase & β xylosidase with wheat bran & corncob as substrates.

Results and Discussion

(i) ***Xylanase production and morphological characteristics:*** The culture was isolated locally from a sediment sample collected from various decaying wood. Culture showed good growth at 30°C in MSS medium of pH 6 supplemented with 0.5 % birch wood xylan. Its ability to produce xylanase enzyme was further confirmed when it formed orange digestion halos on birch wood xylan plates, when treated with congo red and washed with 1M NaCl. The isolate is gram positive, pluromorphic, facultative anaerobe, motile, oxidase positive, catalase positive and reduced nitrate to nitrite (Table 1).

16S rDNA analysis has become the reference method for bacterial taxonomy and identification. It provides suitable phenotypic data that can be used to determine both close and very distant relationships between the species Bull T. A. 1992. The Nucleotide sequences of the partial 16SrRNA gene of the isolate identified as *Bacillus licheniformis*

Aligned Sequence: (1307 bp)

AATGCGAGCTTGCTCGGTGATGTTAG
CGAGCGGACGGGTGAGTAACTCGTG
GGTAACTGCCTGTCATACTGGGATAA
CTCCGGGAAACCGGGGCTAATACCG
GATGCTTGATTGAACCGCGGGTTCAA
TTATAAAAGGTGGCTTTGGATTACCA
CTTACGGATGGTCCCTCGGCGCATTA
GCTCGTTGGTGAGGTAACGGCTCACC
AAGGCCCAATGCGTAGCCGACCTGA
AAGGGGGATCGGCCACCTGGGACTG
ATACTACGCCCGGACTCCTACGGGAG
GCTTCCTAGGGAATCTTCCTCTGTGG
ACGAAACTCTGACGGACCAACGCCG
CTTGTCTGATGAAGGTTTTCGGATCTT
AAGACTCTGTTGTTACGGATCAACAA
GTACCGTTCCAATAGGGCGGTACCTT
GACGGTACCTAACCAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGTGGCAAGCGTTTTCCG
GAATTATTGGGCGTAAAGCGCGCGCA
GGCGGTTTCTTAAGTCTGAGTGAAAG
CCCCCGGCTCAACCGGGGATGGTCAT
TGGAAACTGGGGAACTTGAGTGCAG
AAGAGGAGAGTGGAATTCCACGTGT
AGCGGTGAAATGCGTAGAGATGTGG
AGGAACACCAGTGGCGAAGGCGACT
CTCTGGTCTGTAACTGACGCTGAGGC
GCGAAAGCGTGGGGAGCGAACAGGA
TTAGATACCCTGGTAGTCCACGCCGT
AAACGATGAGTGCTAAGTGTTAGAGG
GTTTCCGCCCTTTAGTGCTGCAGCAA
ACGCATTAAGCACTCCGCCTGGGGAG
TACGGTCGCAAGACTGAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGAAGC
AACGCGAAGAACCTTACCAGGTCTTG
ACATCCTCTGACAACCCTAGAGATAG
GGCTTCCCCTTCGGGGGCAGAGTGAC
AGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGATCTTA

GTTGCCAGCATTCAGTTGGGCACTCT
AAGGTGACTGCCGGTGACAAACCGG
AGGAAGGTGGGGATGACGTCAAATC
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ACACGTGCTACAATGGGCAGAACAA
AGGGCAGCGAAGCCGCGAGGGTAAG
CCAATCCCACAAATCCGTTCTCAGTT
CGGATCGCAGTCTGCAACTCGCCTGC
GTGAAGTCGGAATCGCTAGTAATCGC
GGATCAGCATGCCGGAACCGTC

The above sequence is showing maximum similarity with *Bacillus licheniformis*.

(ii)Effect of incubation period, pH, and temperature

Xylanase production by bacterial isolate under SSF showed that a low level of xylanase activity was detected in earlier stages of incubation and enzyme activity steadily reached a maximum level (15.8 U/g) by 48h of incubation (Fig.1) there was a decrease in enzyme activity (12.4 U/g) with further increase in incubation period. Similar findings have been reported with β-xylosidase where enzyme production reached a maximum level by 48h (3.8 U/g) in wheat bran medium. The reduction in xylanase and β-xylosidase yield after optimum period was probably due to the depletion of nutrient available to microorganism or due to proteolysis (Flores *et al.*, 1997).

The effect of media pH on enzyme production is shown in figure 2. The optimum pH for enzymes production was found to be 6.5. Each microorganism holds a pH range for its growth and activity with optimum value between around this range. Initial pH influences many enzymatic systems and the transport of several species of enzymes across the cell membrane (Poorna and Prema, 2006). The results of influence of temperature on xylanase production are shown in Figure 3, showing that the optimum temperature for xylanase production was 45^{o}C at 48h of incubation with pH 6.5.

(iii)Effect of Particle Size Effect of particle size of substrates on enzyme production is shown in Fig.4. Particle size of 500µm was found best for maximum xylanase production (15.9 U/g) and β-xylosidase production (4.2U/g). The results agree with Poorna and Prema (2006), where 500µm particle size gives maximum xylanase production with wheat bran.

(iv)Effect of moisture contents: The moisture content in SSF is an important factor that determines success of the process. Moisture content higher than optimum moisture level causes decreased porosity of substrate, alternation in particle size, gummy texture, and lower oxygen transfer (Raimbault and Alazard, 1980). A lower moisture level leads into a reduction in solubility of nutrients of solid substrate it leads to a lower degree of swelling and to a higher water tension (Ikasri and Mitchell, 1994). As indicated Fig.5, xylanase production was optimum (15.2U/g) and xylosidase (3.9U/g) with wheat bran.

(v)Scale-up of xylanase production under SSF: Xyalnase production was enhanced by scaling up the solid state system by using enamel trays contains 40g wheat bran. When SSF was performed in trays for xylanase production using bulk quantities of wheat bran (40g), xylanase production was higher if compared with Erlenmeyer flasks. Cultivation in large enamel trays yielded 32.8U/ml (xylanase) & 14.5U/g (β-xylosidase) in static state and 31.6U/g (xylanase) & 10.5U/ml (β-xylosidase) at 110 rpm when compared to the value obtained in 250 ml flasks (16.8U/ml) xylanase & (3.8U/g) β-xylosidase. The improvement of

enzyme production in trays, more than flasks, may be due to efficient aeration, better mass, and heat transfer.

Wheat bran, an abundantly available agro-residue in India was successfully used as a solid state support by the bacterial isolate at 45°C for xylanase & β-xylosidase production and it has not been reported so far. The results presented here show that optimization of process parameters resulted in higher production of xylanase and β-xylosidase. Scaling up under SSF also resulted in significant improvement in both enzymes production.

The results obtained in the present study had an optimum pH of 6.5 and temperature of 45^0Cfor both enzymes. The xylanases were stable for more than 30 min at 45^0C. The xylanases produced by bacterial isolate in this study were stable over a range of temperature (30.0–50.0°C). In addition, the cost of enzymes production will be greatly reduced with inexpensive agricultural substrates such as wheat bran and corncob. Further optimization process will have to be carried out for maximum enzymes production by other genetic engineering methods.

Table.1 Morphological characteristics

Character	***Strain***
Shape and Size	Rod and 2.0 µm–3.0 µm
Gram Character	*Positive*
Growth Temperature	30ºC–50ºC
Agar Slant Growth	*Smooth, Small, White colonies*
Oxidase Activity	*Positive*
Catalase Activity	*Positive*

Figure.1 Effect of incubation time on endoxylanase and β-xylosidase production by bacterial isolate in SSF, temperature 35°C

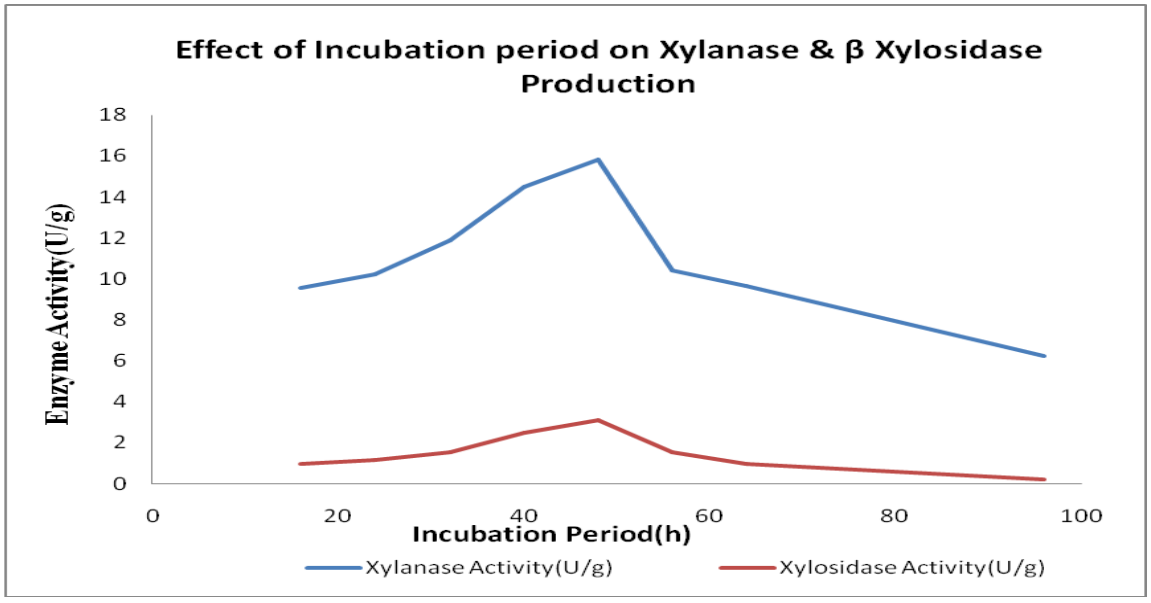


Figure.2 Effect of pH on endoxylanase and β-xylosidase production by bacterial isolate in SSF at 48h

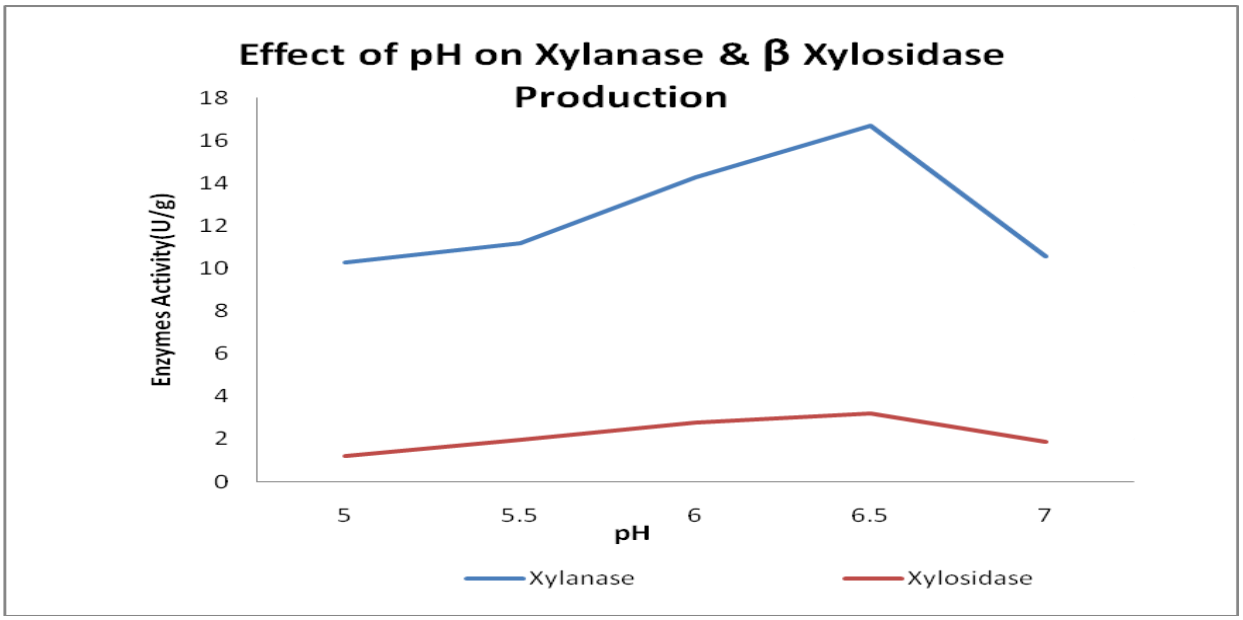


Figure.3 Effect of temperature on endoxylanase and β -xylosidase production by bacterial isolate in SSF at 48h & pH 6.5

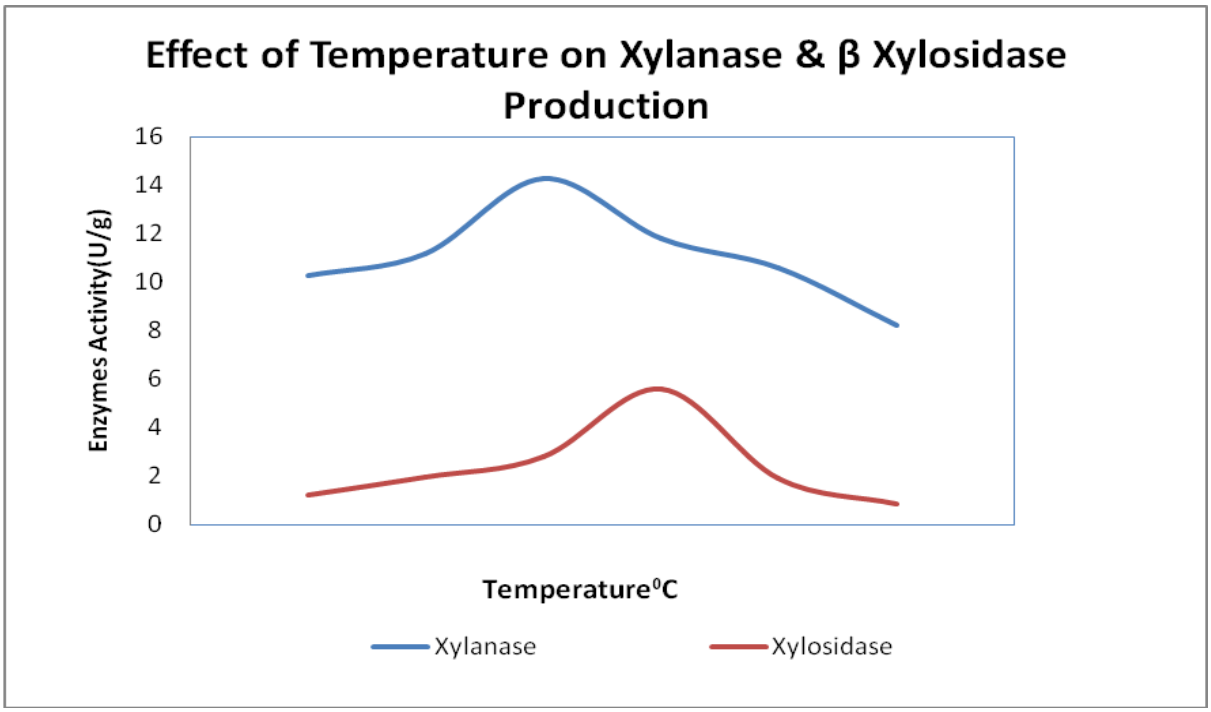


Figure.4 Effect of particle size on endoxylanase and β xylosidase production by bacterial isolate at 48 h, pH 6.5 & temp 45°C

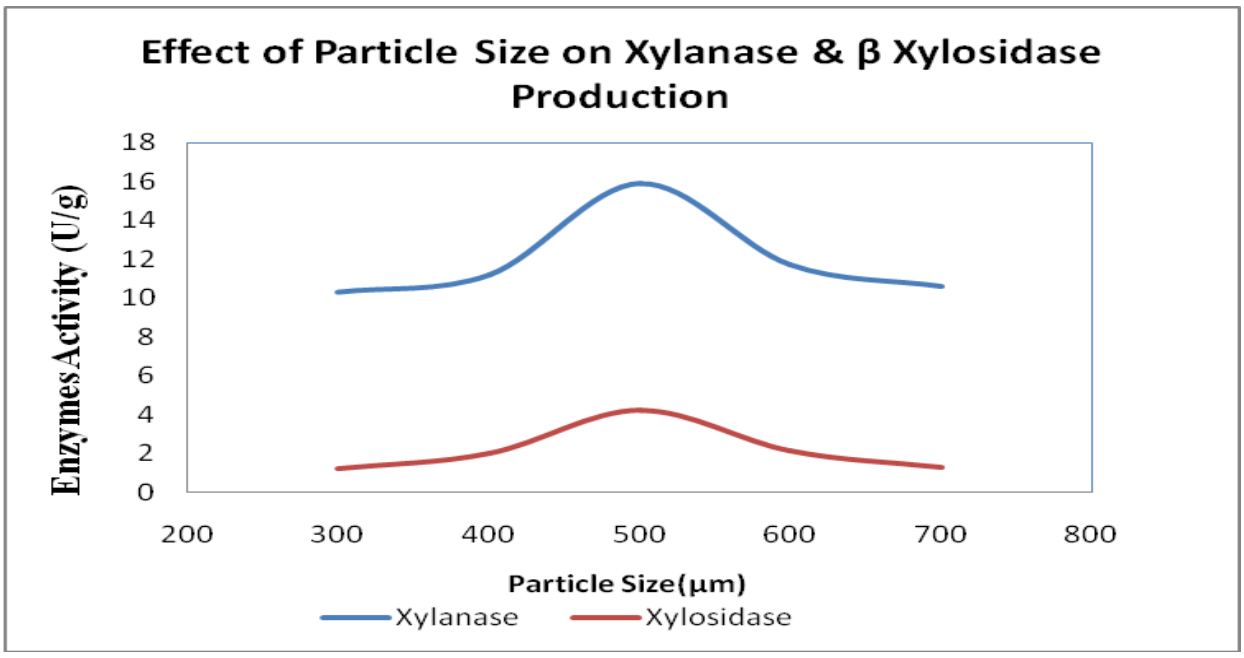
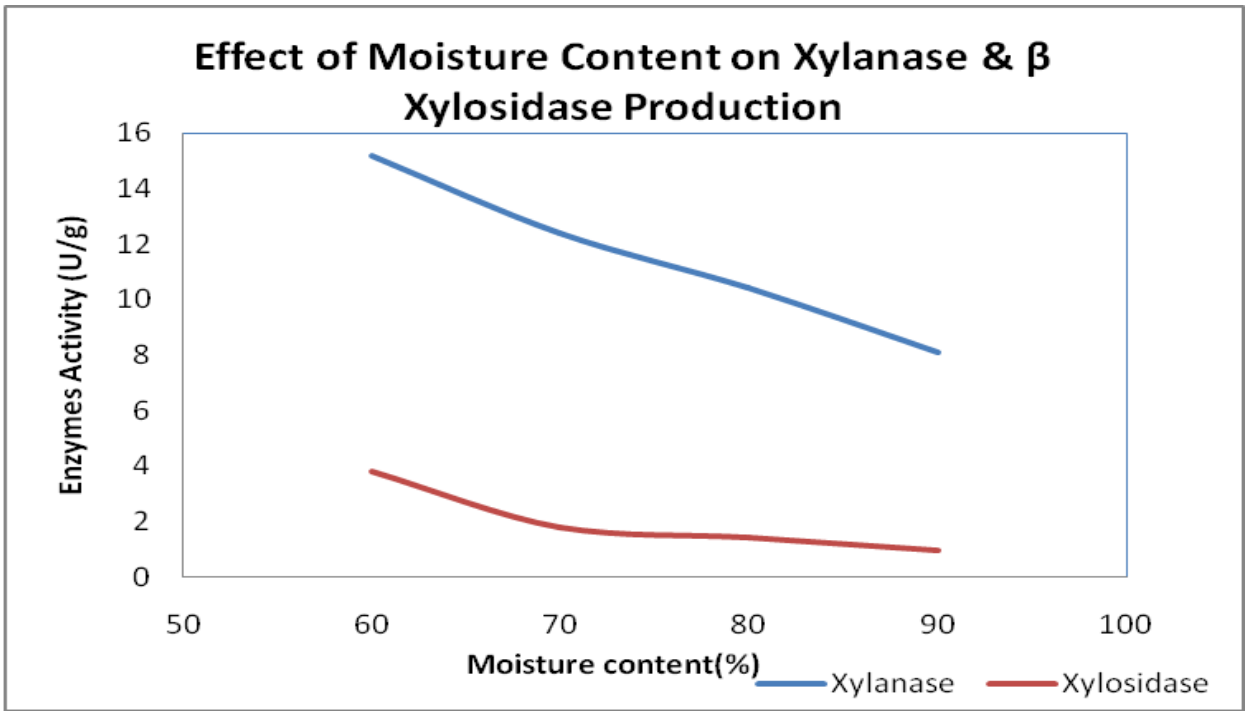


Figure.5 Effect of particle size on endoxylanase and β-xylosidase production by bacterial isolate at 48h, pH 6.5, 45°C & particle size 500 µm



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