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Research article

ISOLATION AND IDENTIFICATION OF XYLANOLYTIC ENZYME FROM AN EFFECTIVE STRAIN *BACILLUS LICHENIFORMIS* ISOLATED FROM THE DECAYING WOOD

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ABSTRACT: A new species of Bacillus licheniformis produced extracellular xylanase under submerged fermentation when wheat bran is used as carbon source. The xylan is the most common hemicellulosic polysaccharide in food industry and agricultural wastes, comprising a backbone of xylose residues linked by β-1,4 glycosidic bonds. Bacillus licheniformis has been shown to be a promising organism for enhanced production of xylanases & β-xylosidase under submerged fermentation (SmF). The optimization of cultural conditions and carbon, nitrogen sources for enzymes production. The bacterial strain Bacillus licheniformis was cultivated using as substrate xylan, wheat bran, corn straw, corncob, and sugarcane bagasse. Wheat bran has been a good xylanase (16.8U/ml) & β xylosidase (5.6U/ml) activity after 48h of fermentation. Maximum enzyme activity was observed in xylan as carbon source and peptone as nitrogen source. Both crude enzymes were characterized and a bacterial xylanase shows optimum pH for xylanase activity at 6.5 & β xylosidase were found to be 6.0. The optimum temperatures were 450C for both and they were thermally stable up to 500C. The parameters of Vmax and Km obtained using Line weaver-Burk plot method were 277.7µmol / min/mg and 5.26 mg /L correspondingly.

Key words: Xylanase, β xylosidase, Bacillus licheniformis, V_{max} and $K_{m.}$

INTRODUCTION

Xylanases (E.C.3.2.1.8) are key enzymes, which play an important role in the degradation of xylan into oligomers & xylose. Xylan is hemicellulosic polysaccharide & the second most abundant biopolymer after cellulose found in the plant cell wall. Xylan is associated with cellulose and lignin phenolic residues & also interacts with polysaccharides such as pectin & glucan. Xylan, a major heterogeneous polysaccharides consisting of β-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 K.K.Y.1988). Biodegradation of xylan is catalyzed by different xylanolytic enzymes such as endo-1,4β -xylanase, β-xylosidase, ά-glucuronidase, ά-arabino furanosidase and esterase. Xylanases (1, 4 β -D-xylan xylanohydrolase, EC 3.2.1.8.) are endoenzymes which release xylo oligosaccharides and xylose residues from xylan, while the xylosidases [l, 4-(3-D- xylan xylohydrolase), EC. 3.2.1.37)] hydrolyze xylo oligosaccharides resulting in the xylose residues.

Industrial production of enzymes on large scale is associated with substrate &30-40% of the production cost is affected by the cost of growth complex. The use of solid agro residues represent one of the most energy-rich resources & cost effective. For this, agricultural wastes sugarcane baggasse, wheat bran, rice bran and corn cob is agricultural wastes which are abundant in several countries and can be used as raw materials for developing biotechnological process of industrial interest. Commercial wheat bran consists of 30% cellulose, 27% hemicellulose, 21% lignin & 8 % ash (Gawande P.V.1999). Xylan is the major hemicellulosic polysaccharide of food industry and agricultural wastes, where it comprises up to 20-35% dry weight (Senthilkumar S.R. 2005). Wheat bran and corn cob are a rich source of xylan (28%) and xylose (23%). Therefore these are attractive substrates for production of xylanase & β-xylosidase enzyme. Although xylanase production in SSF from fungi and actinomycetes have been reported, only few reports using bacteria showing low enzyme yields are available (Battan B. 2006; Sindhu I. 2006).

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Industries that have demand for endoβ-1, 4-xylanase are paper and pulp, in the pre bleaching process to reduce the use of toxic chlorine chemicals (Wong K.K.Y. 1992). In bread and bakery industry, for improving dough viscosity, bread volume, shelf life and quality of baked products (Roman owska I. 2003). Xylanase also used in extraction of coffee, plant oils, starch, for improvement of nutritional properties of silage and grain, and in combination with pectinase & 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 B.2007; Aysegul E.Y.2008) and the production of fuel ethanol and chemical feedstock (Damaso M.L.T.2003; Sunna A.1997).

Among various xylanase producing bacteria *Bacillus licheniformis* has been proposed for xylanase production. To increase the xylanase activity cultural condition was monitored as cultivation time, temperature, pH, inoculums size. Therefore in this paper a *Bacillus licheniformis* can produce xylanase from agricultural waste materials and the optimization of biosynthesis conditions (carbon and nitrogen sources, carbon: nitrogen ratio), kinetic behavior (pH, temperature, enzyme: substrate ratio) from submerged fermentation.

MATERIALS AND METHODS

Isolation & screening of the bacterium For isolation of xylanolytic bacteria 1g of sample (decaying wood, alkaline soil, dry humus, paper industry waste) was enriched in yeast extract, peptone, xylan & agar stants(2:1:0.5:3%w/v) under shaking(240 rpm) for 24-48h at 45^0C. Colonies developed were assayed by xylaolytic activity (on the xylan agar plate). Colonies showing haloes around them were picked up & purified. Samples were stored at 4^0C and sub cultured routinely after every three four weeks. A total of 18 xylanolytic bacterial strain were isolated & screened further for their xylanolytic producing ability under submerged fermentation at 45^0C in 50 ml production media (basal salt solution BSS) containing 1g of wheat bran/corn cob and of BSS g/l: $MgCl_2.6H_2O$, 6.6; K_2HPO_4, 0.5; KH_2PO_4, 0.5; $(NH_4)_2S0_4$, 2.0g; pH 6.7 were autoclaved, inoculated with 10% (v/w) of inocula (24h old). At the desired intervals, the flasks were removed and the contents extracted were with 50ml of 0.02 M phosphate buffer (pH 7.0).

Characterization of the bacterium

Culture was studied for morphological, physiological and biochemical characteristics following the standard procedures Bernier R.(1983). The morphological properties and taxonomic characteristics of the bacteria were studied by the methods in Bergey's manual of systematic bacteriology Sneath P.H.A (1986).

Preparation of substrates

Oat spelt xylan (Himedia Laboratories Pvt. Ltd., India) was used for enzyme assay. The substrates wheat bran, rice husk, rice straw, sugarcane bagassae were washed 2-3 times in distilled water and then boiled with distilled water for 10-15 min. The water was then decanted and substrates were dried in an oven and powdered using mortar and pestle. The powder was then sieved (0.5mm particle size).

Xylanase purification

The above strain cells were centrifuged at 36,000g for 55 sec & used as crude enzyme. The purification of the crude enzyme was done at 4^0C. The xylanase was precipitated with 80% ammonium sulphate saturation. Than the precipitated was collected at 28,000 X g for 10 min dissolved in 50 mM glycine NaOH buffer (pH 9.0) & dialyzed against the same buffer. The elution was done from 0-0.5M NaCl. The xylanase active fraction was eluted at 0.25M NaCl gradient.

Enzyme extraction

Enzyme was extracted with 10ml of 50 mM glycine-NaOH buffer (pH 9), and vortexes thoroughly to extract the xylanase enzyme and squeezed through a wet muslin cloth. The enzyme extracted was centrifuged at 15000rpm for 30min at 4^oC. The clear supernatant was used in the enzyme assay.

(I)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 G.L.1959). One unit (U) of xylanase is defined as the amount of enzyme that releases 1 µmol xylose/ml/min under the assay conditions.

(II)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, 0.4M, pH 10.8) and the absorbance was read at 430 nm Lachke A. H (1988).

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Optimization of culture conditions for xylanase & ß-xylosidase production The optimization of the growth conditions was carried out based on stepwise modification of the governing parameters for xylanase production by using Miller method (Miller G.L, 1959). The Bacillus licheniformis was grown at different incubation period (16 to 96h) evaluated by 16h interval in different media, pH (5.0-10.0), temperature (30- 60°C), and various substrates (wheat bran, corn cob, rice husk, rice straw, sugarcane bagassae) proportion of 1.0% (w/v) assayed for production of xylanase & β xylosidase.

Effect of carbon & nitrogen sources on xylanase production The effect of various carbon sources: lactose, arabinose, raffinose, cellulose, mannitol and xylan, at the concentrations (1% w/v) were examined using salt medium, at ambient temperature 45^0C for 48h. Similarly nitrogen sources: yeast extract, peptone, ammonium nitrate, sodium nitrate, ammonium sulphate, and meat extract at the concentrations (1% w/v) were used.

Kinetic determinations Initial reaction rates of using oat spelt xylan as substrate were determined at different substrate concentrations ranging from 0.5 mg to 8 mg /ml of 50 mM 0.02 M phosphate buffer (pH 7.0) at 55^oC. The kinetic constants K_m and V_{max} were estimated using linear regression method of Lineweaver H. (1934).

Optimization of reaction conditions: The various buffer system at concentration of 50 mM {Sodium phosphate (pH 6, 7), Tris HCl (pH 8, 9), carbonate bicarbonate (pH10), glycine NaOH (pH10-11)}, reaction temperature (30^0C to 80^0C) and the pH optima from 6.0 to10.0 for up to 2h at optimum temperature. The relative enzyme activity was determined at 15 min interval during the 2h period of incubation.

RESULT AND DISCUSSION

Isolation & screening of the bacterium: To study the experiment, bacterial strains are isolated from the different soil of different areas. Bacterial strains are incubated at 45^0C for 48h & isolated from the basis of xylanolytic clear & transparent zone (on the xylan agar plate). About 18 bacterial strains, which formed clear zones around their hole on xylan agar plates were picked up for further studies. A total of 18 xylanolytic bacterial strain were screened further for their xylanolytic producing ability under submerged fermentation at 45^0C in 50 ml production media (basal salt solution BSS). These strains were grown on wheat bran for 96 hr of incubation at pH76.7 & 45^0C, maximum xylanase production was observed, that is 12.4 U/ml. The production of xylanase by wheat bran is reported in many papers under SSF (Heck J.X 2006, Malavirchi K. 2003). So finally selected that strain on staining method, microscopic examination.

Characterization of Bacteria: The bacteria were maintained by the pure culture technique, at last that strain was maintained for xylanase production. Some morphological & biochemical as well as partial 16S rRNA sequencing had been done. And it was found that 16S rRNA gene of the strain revealed 95% identity with *Bacillus licheniformis*. (Table-1).

Table 1: **Microscopic, biochemical & physiological characteristic of *Bacillus licheniformis***

Tests	Result
Shape	Circular
Color	Pinkish
Opacity	Opaque
Margin	Regular
Gram Nature	Gram positive
Shape of the cells	Rods
Motility	Motile
Oxidase Test	Positive
Catalase Test	Positive
Casein Hydrolysis	Positive
Starch Hydrolysis	Positive

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*

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Aligned Sequence: (1307 bp)

AATGCGAGCTTGCTCGGTGATGTTAGCGAGCGGACGGGTGAGTAACTCGTGGGTAACTGCCTGTCATACT
GGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCGGGTTCAATTATAAAAGGTG
GCTTTGGATTACCACTTACGGATGGTCCCTCGGCGCATTAGCTCGTTGGTGAGGTAACGGCTCACCAAGGC
CCAATGCGTAGCCGACCTGAAAGGGGGATCGGCCACCTGGGACTGATACTACGCCCGGACTCCTACGGGA
GGCTTCCTAGGGAATCTTCCTCTGTGGACGAAACTCTGACGGACCAACGCCGCTTGTCTGATGAAGGTTTT
CGGATCTTAAGACTCTGTTGTTACGGATCAACAAGTACCGTTCCAATAGGGCGGTACCTTGACGGTACCTA
ACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTTTCCGGAAT
TATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGAGTGAAAGCCCCCGGCTCAACCGGGGATGG
TCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGC
GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGG
GTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA
ACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGGGGGCAGAGTGACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTG
ATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGA
TGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAAGGGCAG
CGAAGCCGCGAGGGTAAGCCAATCCCACAAATCCGTTCTCAGTTCGGATCGCAGTCTGCAACTCGCCTGC
GTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGGAACCGTC

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

Optimization of culture conditions for xylanase & β-xylosidase *production*

Xylanase & β xylosidase activity at earlier stages of incubation was found a very low level as 16h and reached maximum at 48h of incubation(15.8U/ml & 3.12 U/ml) after this activity reduction occurred , firstly slowly upto 72h(as shown in Fig 1) & later more drastically at 96h. The maximum enzyme production stage of any organism is largely dependent upon the type of microbial strain and its genetic make-up as well as on cultural and environmental conditions during the growth of the organism. The xylanase production was decreased due to due to the depletion of nutrient available to microorganism or due to proteolysis (Flores M.E.1997).

The effect of media pH on both enzymes production is shown in Fig2. pH was the most important factor to characterize the enzyme. The productivity of xylanase was highest at 6.5 for xylanase & 7.0 for β xylosidase and effective at a range from pH 5-8. Alkaline xylanases are more familiar due to their application in the pulp and paper industry, and xylanases in acid group could be a key enzyme to improve the bioconversion of lignocellulosic materials (Parachin N.S., 2009). Which was same pH(6.0) as reported previously for xylanases from P. oxalicumZH-30(Li. Y. et al 2007), B. subtilis ASH(Naga S. et al 2010),B. subtilis(Sa-Pereura P et al, 2002)and Bacillus sp.AQ-1(Wahyuntari B. et al,2009). Xylanase showed 100% activity for 1 h at pH 6.5. pH 6.5 was the most favorable for stability. The optimum value of pH depends mainly on microorganisms because each microorganism holds a pH range for its growth and activity. Initial pH influences many enzymatic systems and the transport of several species of enzymes across the cell membrane (Poorna C.A. 2006). Kuhad (2006) observed maximum production of xylanase by Bacillus sp. RPP-1 at pH 7.0. The activity of xylanase & xylosidase from Bacillus licheniformis was gradually increased with increasing temperature & optimum temperature for xylanase production was 45^{o}C at 48h which declined after 50^{0}C(Fig3). The maximum activity was found at 45^{0}C for xylanase & xylosidase. As stability of the enzyme is important. Xylanase was more stable at temperature 30^{0}C to 40^{0}C for 2h. At temperature increases the stability decreased. Rajshre D.K. 2012 reported Bacillus arseniciselenatis DSM 15340, 50°C was found to be the most favorable for enzyme activity & more stable at temperatures 30°C and 40°C for 4h of incubation and retained almost 93% activity.

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The enzyme production by bacillus species was also affected by substrates. To optimize the substrates condition in shake flask cultivation, the experiments were carried out under the variation of different substrates as wheat bran, corn cob, rice husk, rice straw, sugarcane bagassae at 1%w/v. The result revealed that better enzyme activity of 15.8 U/ml and 3.72 U/ml as wheat bran substrate. There are also reports on use of xylan (Lopez., 1998), soyameal waste (HeckJ.X., 2002) and rice bran (Virupakshi S., 2005) as a substrate for the xylanase production by Bacillus spp

Kinetic Parameters

The kinetic parameters K_m and V_{max} of the enzyme were determined from Lineweaver-Burk double-reciprocal plots of xylanase activity at 45°C using various concentrations of oat spelt xylan as substrate. The K_m and V_{max} values of xylanase were 5.26 mg/mL and 277.7 µmol/min/mg, respectively. Wang et al. reported that K_m and values of xylanase isolated from Bacillus sp. NTU-06 were 3.45 mg/mL and 387.3 µmol/min/mg, respectively [C. Y. Wang, et al 2010]. Xylanases isolated from Aeromonas cavie 171 ME-1 and Bacillus sp. strain 41m-1 showed similar values of 260 to 350 µmol/min/mg protein [Kubata B. K. et al,1994].

Effect of carbon & nitrogen sources on xylanase production

The Bacillus species utilized all carbon sources tested (Fig4) and induced highest level of xylanase production with oat spelt xylan followed by mannose and lactose. The use of oat spelt xylan as an inducer increases the cost of enzyme production. When different carbon sources were used in the production medium, maintaining other physical parameters and nitrogen source(peptone) constant, the highest enzyme activity was obtained in oat spelt xylan(18.98 Unit/ml xylanase & 3.99 β-xylosidase), and least in cellulose(2.87Unit/ml xylanase & 0.98 β-xylosidase) while raffinose also show decrease production of enzymes. A series of different carbohydrates have been studied for *Aspergillus niger* growth L. Olsson et al 2003. Typical nitrogen sources for Bacillus species cultivation was found to be peptone (16.8 U/ml xylanase and 3.89 U/ml β-xylosidase)) as it shows highest yield. Very low level of enzyme was produced in the presence of inorganic nitrogen (ammonium sulphate in case of xylanase & sodium nitrate in xylosidase) Where as yeast extract, meat extract and ammonium nitrates can also stimulate enzyme production but not as high as peptone (Fig 5). There are also reports on use of soya meal waste (Heck et al., 2002) as a substrate for the xylanase production by Bacillus spp.

Purification of xylanase

The purification of xylanase had been done at different steps: ammonium-sulphate precipitation, DEAE-sepharose as described above. The xylanase was purified 15.4 fold, with a final yield of 11.2 %.

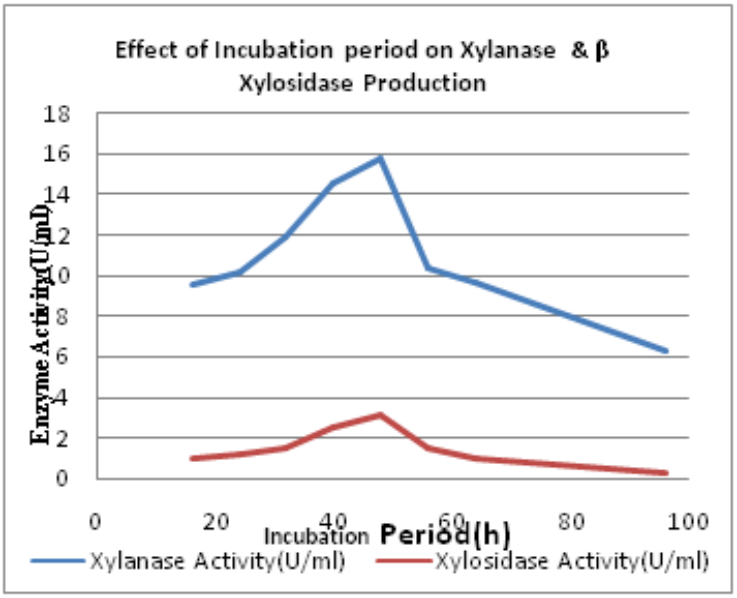

Fig-1: Effect of incubation period on activity of xylanase & β-xylosidase from *Bacillus liheniformis*

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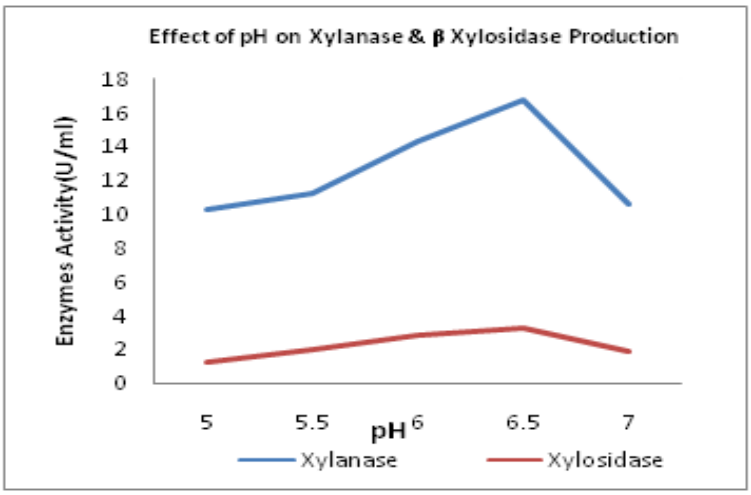

Fig-2: Effect of pH on activity of xylanase & β-xylosidase from *Bacillus liheniformis*

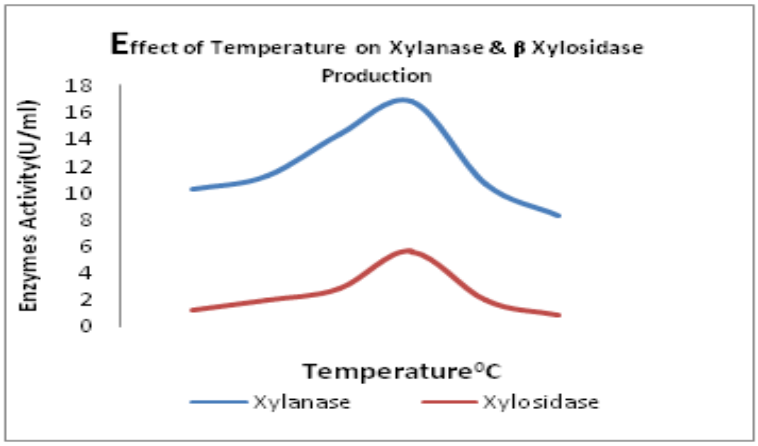

Fig-3: Effect of temperature on activity of xylanase & β-xylosidase from *Bacillus liheniformis*

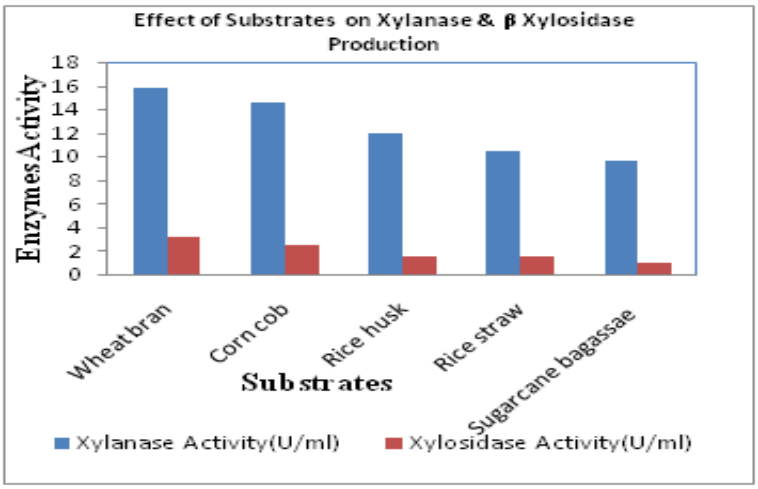

Fig-4: Effect of substrate on activity of xylanase & β-xylosidase from *Bacillus liheniformis*

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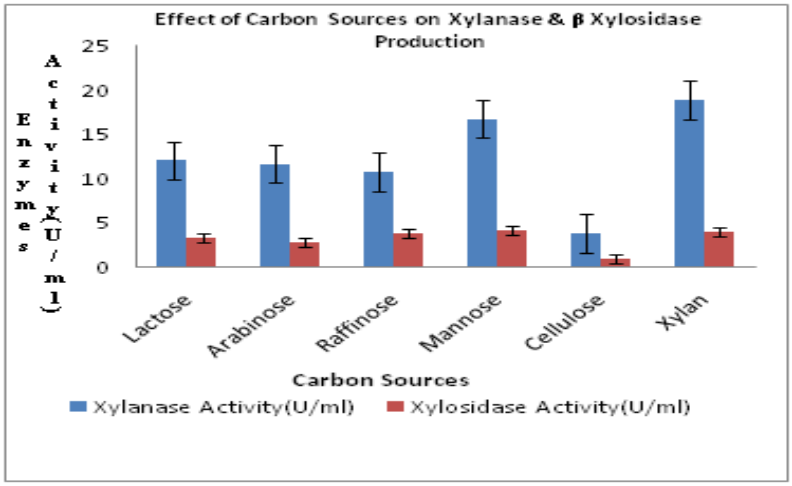

Fig-5: Effect of Carbon Sources on activity of xylanase & β-xylosidase from *Bacillus liheniformis*

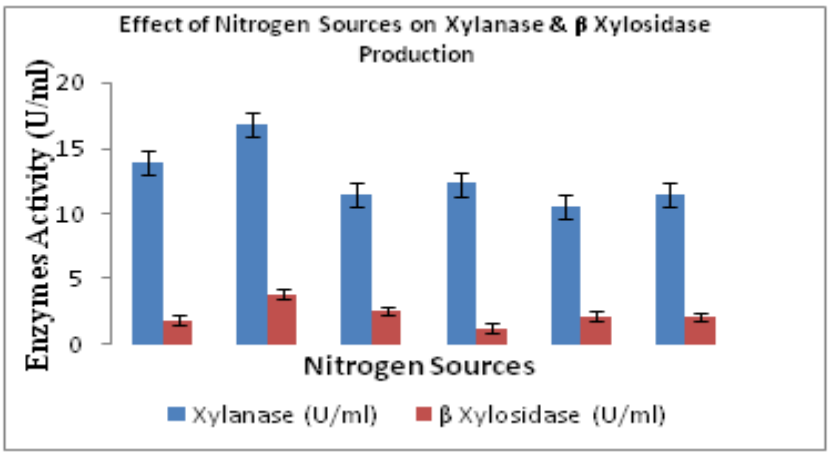

Fig-6: Effect of Nitrogen Sources on activity of xylanase & β-xylosidase from *Bacillus liheniformis*

CONCLUSION

Xylanase could be produced by Bacillus licheniformis species. To enhance its activity, physical parameters were optimized. The highest xylanase activity was obtained from cultivation temperature at45°C. The pH of culture medium also effected on xylanase and xylosidase production and the optimal pH for xylanase production was around normal at pH6-7. The substrates wheat bran shows maximum activity. Then, xylanase production under the optimized and non-optimization condition was compared; higher activity of 1.32 folds was achieved under the improved condition.

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