OPTIMIZATION OF PROCESS PARAMETERS AND SCALE-UP OF XYLANASE PRODUCTION FROM *BACILLUS AMYLOLIQUIFACIENS* SH8 IN A STIRRED TANK BIOREACTOR

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Bacillus amyloliquifaciens SH8 has been explored as a potential xylanolytic in-house producer for xylanase production in a bioreactor by optimizing process conditions. The scale-up of the fermentation process up to 3.5 L along with the optimization of physical parameters yielded a significantly increased xylanase yield – of 67.80 IU/mL, in 8 h of fermentation at an inoculum size of 10% with an aeration rate of 1.0 vvm and agitation speed of 300 rpm. The extracellular purified enzyme was found stable at pH6.0 and an optimal temperature of 90ºC. Metal ions, such as Zn^{2+}, Ca^{2+} and Mn^{2+}, increased the residual enzyme activity, whereas Cu^{2+}, Mg^{2+} and Na^{2+} strongly inhibited the enzyme activity.

Keywords: xylanase, wheat bran, purification, characterization, gene encoding, scale-up

INTRODUCTION

Xylan is the major constituent of hemicelluloses and is the second most abundant biopolymer after cellulose. It is the major renewable source with high potential for degradation into useful end products.[1] Xylan, a heterogeneous polysaccharide, is an integral part of the lignocellulosic structure consisting of β-1-4 linked D-xylosyl residue. Xylanases are responsible for the hydrolysis of xylan and the subsequent release of xylo-oligosaccharides in the form of xylose and xylobiose.[2] They are used in textile processing, for recovery of hexoses and pentoses, in animal feed processing, food industry *etc*. Their most important application, however, is for pulp bleaching in the pulp and paper industry.

Microorganisms are the most preferred source of industrially important enzymes for commercial applications. There are many microbes that have been reported to produce xylanases, such as fungi, actinomycetes and bacteria, but among them, bacteria are most often preferred due to their high pH and temperature optima, which facilitate their use for industrial purposes. The processes of pulping and bleaching during paper manufacturing are carried out at high temperatures and alkaline pH, therefore alkaline thermostable xylanase enzymes are necessary. Moreover, the enzyme should be free of cellulases, which may destroy the cellulose microfibres and thus deteriorate paper quality. Moreover, the production of xylanases from bacteria can be advantageous as the enzyme production rate is normally higher due to the high growth rate of bacteria.[3] Commercially, the use of xylan as a carbon source is uneconomical for the large scale production of xylanase because of its high cost. On the other hand, agricultural waste products containing hemicelluloses are generated worldwide and their use as by-products in biotechnological processes provides an alternative substrate, while also offering a solution for the environmental problems caused by their accumulation.[4] In addition, the increasing industrial demand for xylanolytic enzymes requires the development of new processes to ensure the economic feasibility of xylan-containing material hydrolysis at a commercial scale.[5] Stirred tank bioreactors are widely used in

biotechnological processes, providing high heat and mass transfer rates (due to efficient mixing).

Therefore, the present study was undertaken with the aim to optimize the fermentation conditions of the in-house hyperxylanolytic bacterial strain *B. amyloliquifaciens* SH8for large-scale production of xylanolytic enzymes in a stirred tank bioreactor, followed by purification and characterization of the enzyme.

EXPERIMENTAL

Microorganism and maintenance

Bacillus amyloliquifaciens SH8 JX129360.1, an in-house hyperxylanolytic bacterium, was explored for xylanase production and purification.[5] The cellulase-free xylanase strain was isolated from compostand exhibited maximum xylanase activity, *i.e*. 46.50 IU/mL in Basal salt medium after 72 h, at pH 5.5, a temperature of 45ºC, inoculum size of 10%, and 1.25% wheat bran as carbon source. The culture was preserved on a nutrient agar slant at 4ºC.

Estimation of enzyme activity

Selected isolates were quantitatively assayed by growing them in Reese medium (HIMEDIA, Mumbai) at 50ºC and 120 rpm. Xylanase activity in the culture broth was assayed in triplicates. 0.2 mL of crude enzyme was mixed with 0.3 mL of citrate buffer (pH 5.0) and 0.5 mL of xylan solution (kept overnight at 37ºC in citrate buffer of pH 4.0, then centrifuged;the obtained clear suspension was used), and incubated at 45ºC for 10 min.The reaction was terminated by adding 3 mL of dinitrosalicylic acid.[6] The absorbance was measured (on a Thermo electron spectrometer) against the control at 540 nm, using xylose as a standard.

Protein estimation

Total protein content in the fermented mash was estimated following the method of Lowry.[7]

Fermentation studies in a bioreactor

The bioreactor studies were carried out in a 7.5 L lab scale bioreactor (New Brunswick Scientific, New Jersey, USA) with 3.0 L working volume.The optimized medium from the shake flask studies was used to run the bioreactor for the production of xylanase.It contained 1% wheat bran, tryptone 9.0g/L, NH_4Cl 2.0g/L, $ZnSO_4$ 0.20g/L and $CaSO_4$ 0.20g/L. The fermentor was sterilized at 121ºC for 15min. The samples were collected periodically after 1 h of fermentation to monitor xylanase production and total protein.

Effect of different inoculum age and size on xylanase production

Different inoculum age (*i.e.* 2, 4 and 6 h) and size (5, 10 and 15%) were evaluated for the production of xylanase in the7.5 L stirred tank bioreactor with 10% (v/v) inoculum atagitation speed of 150 rpm and aeration rate of 1.0 vvm. Samples were withdrawn regularly at different intervals ranging from 2, 4 up to 10h for estimating enzyme production.

Effect of aeration and agitation rate on xylanase production

The fermentor was operated with 7.0 L of growth medium to optimize the aeration and agitationrates for the maximum yield of xylanase. Different aeration rates (0.5, 1.0 and 1.5 vvm) and agitation rates (100 rpm, 200 rpm and 300 rpm) were evaluated for 10% (v/v) of inoculum and 4 h inoculum age under aseptic conditions.

Determination of growth kinetics in the bioreactor

Microbial fermentation, particularly that relating to enzyme production, does not essentially follow the conventional kinetic model of growth-limiting biomass substrate and product formation proposed by Monod (Eq. 1). Therefore, the logistic equation is used as an alternative empirical function[8] for microbial cell growth:

Monod equation: $\mu = \mu_m S/(Ks+S)$ (1)

$1/\mu = K_s/K_m.1/S+1/\mu_{max}$

where μ = specific growth rate, μ_{max} = maximum specific growth rate, S = substrate concentration, Ks = substrate saturation constant (*i.e.*substrate concentration at half/max).

In Monod's model, the growth rate is related to the concentration of a single growth-limiting substrate though the parameters $\mu_{max}$and Ks.

$Y_{x/s} = dx/ds$ (2)

$Y_{x/s} = X_m-X_o/S_o-S_m$

$\mu = dx/dt.1/x$ (3)

In addition to this, Monod also related the yield coefficient (Yx/s) (Eq. 2) to the specific rate of biomass growth (m) and the specific rate of substrate utilization (q) (Eq. 3).

$q_p = \alpha\mu+\beta$

$q_p = dp/dt = \alpha dx/dt + \beta x$ (4)

where α = growth associated constant,

β = non-growth associated constant.

Luedeking-Piret equation (Eq. 4) was applied for comparison between experimental (actual) results and the model prediction for xylanase production. During this modeling, the rate of xylanase production is linearly proportional to the biomass growth rate.

Purification of crude enzyme

For purification of the xylanase from *B. amyloliquifaciens* SH8, two purification strategies were followed. The xylanase was initially concentrated by lyophilization and then subjected to ion exchange chromatography. All the subsequent steps were carried out at 4ºC. The lyophilized culture was dissolved in a phosphate buffer. The highest xylanase activity was pooled and applied to DEAES-300 a Sephadex column

(10×1.0 cm) equilibrated with0.05M of phosphate buffer at pH 7. The lyophilized protein material was eluted with a stepwise gradient at a flow rate of 3 mL in 6 min in a glass column with the dimensions of 31×2.5cm. DEAE sepharose fractions having the highest xylanase activities were pooled and subjected for enzyme characterization.

Molecular weight analysis

The molecular weight of *B. amyloliquifaciens* SH8 was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as described by LaemmLi,[9] using medium range molecular markers.

Characterization of purified enzyme

Effect of pH

Each enzyme has its specific pH for efficient working. To determine the optimum pH of the xylanase produced by *B. amyloliquifaciens* SH8, the enzyme was assayed at different pH values. Purified xylanase was evaluated by incubating the enzyme for 10 min in the presence of an appropriate buffer, *i.e.* 0.05M HCl-glycine (pH 3.0), 0.05M citrate buffer (pH 4.0-6.0), 0.05M sodium phosphate buffer (pH 7.0-8.0) and 0.05M NaOH-glycine buffer (pH 9.0-10.0, 11.0, 12.0).

Effect of temperature

To check the optimum temperature of the xylanase produced by *B.amyloliquifaciens* SH8 under submerged fermentation, experiments were performed by incubating the reaction mixtures at different incubating temperatures, ranging from 30 to 100ºC with a regular interval of 10ºC.

Effect of metal ions

The enzyme was further characterized to check the metal profile for activation with various metal ions: (5mM) K^+, Hg^{2+}, Na^+, Ca^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+} and Mg^{2+}were incubated at 60ºC for 10 min.

Effect of substrate concentration

The relative activity of xylanase towards different substrates,*i.e.* 1% birch wood xylan, oat spelt xylan, Avicel, cellulose and carboxymethylcellulose in 0.05M of citrate buffer (pH 6.0) was analyzed by incubating the sample at 90ºC for 10 min.

Effect of different concentration of birch wood xylan

Different concentrations of substrate in the range of 0.2%, 0.4% and up to 2.0% in 0.05M citrate buffer (pH 6.0) were incubated at 90ºC for 10 min.

Kinetic parameters determination

The kinetic parameters K_m and V_{max} were estimated by linear regression from double-reciprocal plots according to Lineweaver and Burk.[10]

Encoding of xylanase gene from *B.amyloliquifaciens* SH8

The xylan specific DNA was amplified from *B. amyloliquifaciens* SH8 chromosomal DNA, as prepared by the method given by Sambrook and Russel.[11] The reaction mixtures containing the sequence of the primers were (5'-CATGTGTATGTGCTTCCTCGTC-3') (5-'AATGTCCGGTATGGCTTCAG-3') and(5'-GCAGTCATGTGTAT GTGCTTCC-3') (5-'CTTCAGCCGTCCATTTTCG-3') of expected amplicon size of ~700 bpusing a gradient PCR reaction. The annealing temperature of 55.0ºC was found the most suitable for the amplification of cellulase encoded gene *Bacillus amyloliquefaciens* SH8as a clear expected amplification product of ~700 bp was obtained. The PCR product was purified and submitted for sequencing to Xcelaris, Ahmadabad, India.

RESULTS AND DISCUSSION

Scale-up for enzyme production

Xylanase production was enhanced by scaling up the submerged state system to a large scale stirred tank bioreactor. When the bioreactor was used for xylanase production using a bulk quantity of wheat bran, xylanase production was higher compared to that in Erlenmayer flasks. The improvement of xylanase production in the bioreactor, compared to that in flasks, may be due to efficient aeration, as well as better mass and heat transfer.

Optimization of physical parameters for xylanase production

Effect of inoculum age

The inoculum age had a pronounced effect on the xylanase production. The highest xylanase titers were observed at an inoculum age of 8 h, *i.e.* 41.75 IU/mL (Fig. 1), which was followed by those of 10h (37.11 IU/mL) and of 6 h (30.64 IU/mL). The minimum xylanase production was observed at 2 h of fermentation time (15.11 IU/mL). There was a lag phase of approximately of 2 h before xylanase synthesis commenced, and biomass production began approximately after 2 h, while Figure 1 exhibits lesser xylanase production at inoculum ages of 2 h and 6 h from *Bacillus amyloliquefaciens* SH8 than at an inoculum age of 4 h. The inoculum age was thus demonstrated to be an important parameter to ensure dynamic growth in a stirred tank bioreactor.[12] The younger culture of *B. amyloliquefaciens* SH8, being non-sporulating, entered the growth phase very soon, while the cultures older than of 4 h produced spores, which took a longer time to germinate, and therefore, the enzyme production was low. The optimization of inoculum age and size, initial pH, temperature and agitation enhanced the xylanase activity to about 520 IU/mL after the

scale-up of the fermentation process up to the 14 L bioreactor in 36 h of fermentation, which is 1.22 fold higher than that obtained at the shake flask level.[13]

Effect of inoculum size

Different inoculum sizes, *i.e.* 5%, 10% and 15%, were used for assessing the activity of xylanase in the stirred tank bioreactor by *B. amyloliquefaciens* SH8. The inoculum size of 10% showed maximum xylanase titers of 48.91 IU/mL after 8 h of fermentation time, which was followed by 10h (36.86 IU/mL) and 6h (36.29 IU/mL). The minimum xylanase titers were observed at 2 h of fermentation time (16.76 IU/mL). A graphical representation of the data obtained for each of three bioreactor experiments is presented in Figure 2. The highest xylanase titers were observed at an inoculum size of 10%. The larger volume of the stirred tank bioreactor also specifies the need of a higher inoculum concentration for optimum xylanase production. An optimum inoculum size of 10% (v/v; 9 $\times 10^6$ CFU/mL) produced the maximum xylanase activity. Cell growth also was the highest at the inoculum size of 10%. Inoculum size influences the substrate utilization in the medium, resulting in the higher cell concentration at a time, and consequently higher production of the enzyme. However, at higher inoculum size, enzyme activity was ceased due to depletion of oxygen on account of the high cell concentration and the competition among bacterial cells for nutrients.

Effect of aeration rate

Maximum xylanase titers of 56.80 IU/mL were attained after 8 h of fermentation time at an aeration rateof 1.0 vvm, which was followed by 10h (37.86 IU/mL) and 6h (36.29 IU/mL). The minimum xylanase titers were observed at 2 h of fermentation time (16.76 IU/mL). There was a lag phase of approximately of 2 h before xylanase synthesis commenced and biomass production began approximately after 2 h, while the aeration rates of 0.5 vvm and 1.5 vvm showed lesser xylanase production, as depicted in Figure 3.

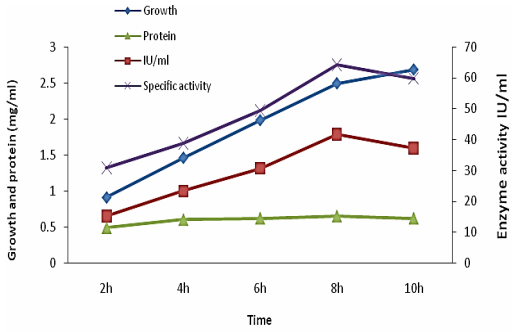


Figure 1: Xylanase production in a bioreactor at 4 h inoculum age

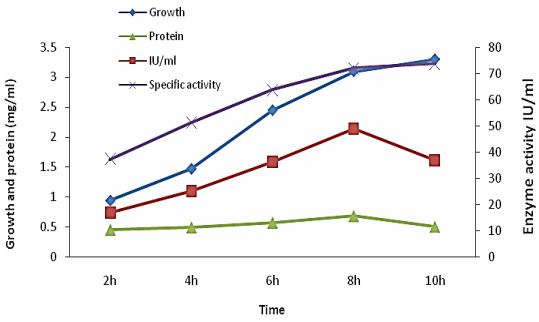


Figure 2: Xylanase production in a bioreactor at 10% inoculum size

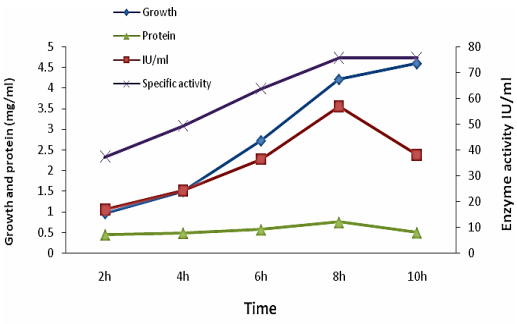


Figure 3: Xylanase production in a bioreactor at 1.0 vvm aeration rate

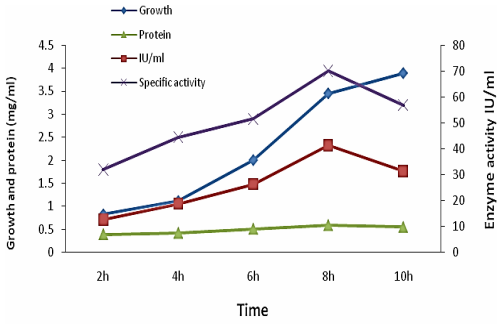


Figure 4: Xylanase production in a bioreactor at 100 rpm agitation rate

An aeration of less than 1.0 vvm might not provide enough oxygen for cell growth. Whereas, an aeration rate greater than 1.0 vvm might have exceeded the oxygen need, proving toxic/lethal for cell growth, and thus resulted in lower enzyme activity. The maximum xylanase activity in the

mono-agitator system was at achieved at the agitation speed of 200 rpm and aeration rate of 1.0 vvm.[14]

Effect of agitation rate

An agitation rate of 200 rpm led to maximum xylanase titers of 67.80 IU/mL after 8 h of fermentation time (Fig. 4), which was followed by the xylanase titers at 10 h (37.86 IU/mL). The minimum xylanase titers were observed at 2 h of fermentation time (16.76 IU/mL) at agitation rates of 100 and 300 rpm. There was a lag phase of approximately 2 h before xylanase synthesis commenced, and biomass production began approximately after 2 h. In contrast, an agitation rate lower or higher than 200 rpm resulted in a drastic decrease in xylanase units, *i.e.* 42.34 IU/mL and 52.10 IU/mL at 100 and 300 rpm, respectively. Agitation brings about proper mixing of the production broth, as well as increases the oxygen transfer and thus has a tremendous effect on the productivity of the system. The optimization of agitation for maximum production of any culture needs to be carefully monitored, as it has both beneficial and deleterious effects (rupture of cells and thus less growth, change in cell morphology, foam production at high agitation *etc*.). The optimization of process parameters performed using response surface methodology and the central composite design for maximum xylanase production revealed increased xylanase production for 2.0% wheat bran, 0.2% KNO_3 and 0.1% xylose.[15]

Physiological behavior of *Bacillus amyloliquefaciens* SH8 in the stirred tank bioreactor

Most of the growth processes are explained in terms of Monod or logistic equations (unstructured models). A logistic equation permits simple calculation of the fermentation parameters related to biological and geometrical significance, using sigmoid profiles, independent of substrate concentration.[16,17] The sigmoidal growth pattern of *Bacillus amyloliquefaciens* was analyzed using a logistic equation for its variation against time during the log phase. The idea of microbial growth kinetics has been dominated by an empirical model originally proposed by Monod.[8] The Monod model introduced the concept of a growth-limiting substrate (Fig. 5).

$\mu = \mu_{max} S/Ks+S$ (Batch growth) (5)

$1/\mu = Ks/ \mu_{max}.1/s +1/ \mu_{max}$

where μ = specific growth rate, μ_{max} = maximum specific growth rate, S = substrate concentration, Ks = substrate saturation constant (*i.e.* substrate concentration at half μ_{max}).

Bacillus amyloliquefaciens SH8 showed a conventional growth pattern during batch fermentation, in which the exponential phase lasted for 8 h and was followed by a stationary phase upto 10 h. The production of xylanase was started in 2 h and reached its maximum in 8 h.

In Monod's model, the growth rate is related to the concentration of a single growth-limiting substrate though the parameters μ_{max} and K_s. In addition to this, Monod also related the yield coefficient ($Y_{x/s}$) to the specific rate of biomass growth (m) and the specific rate of substrate utilization (q):

$Y_{x/s} = dx/ds$ (6)

$Y_{x/s} = X_m - X_0/S_0-S_m$

Model prediction for cell growth was performed with the experimental results with the coefficient of determination R^2= 1.0, which revealed that this kinetic model is appropriate for predicting the experimental cell growth:

$Y_{x/s} = dx/ds$

$Y_{x/s} = 0.96$

$1/\mu_{max} = 0.06$

μ_{max}= 14.92 min^{-1}

KS/μmax = 0.0006 from slope

Ks = 0.00892 mg/mL

Kinetic analysis for xylanase production

The Luedeking-Piret equation[10] was applied for the kinetic modeling of xylanase production. The Luedeking-Piret model indicates that the rate of xylanase production (q_p) is linearly proportional to the *B. amyloliquifaceins* SH8 cell growth rate and cell biomass (Fig. 6a, b):

$q_p = \alpha\mu + \beta$ (7)

where α = growth associated constant andβ non-growth associated constant, both may vary under different fermentation conditions.

The effect of various process parameters (pH, temperature, aeration, agitation rates, substrate concentration, and dissolved oxygen (DO) concentration) on xylanase production by *Penicillium citrinum* MTCC 9629 in a 5 L stirred bioreactor resulted in 2.5 times higher enzyme activity as compared to the shake flask fermentation after 96 h.

The growth kinetics of *P. citrinum* MTCC 9620 in the bioreactor was studied using the Monod, Moser, Contois and Edward equations. Based on *R*2, SE and pattern of residuals, the

microbial growth kinetics of *P. citrinum* MTCC 9620 was effectively represented by the Moser equation.[18]

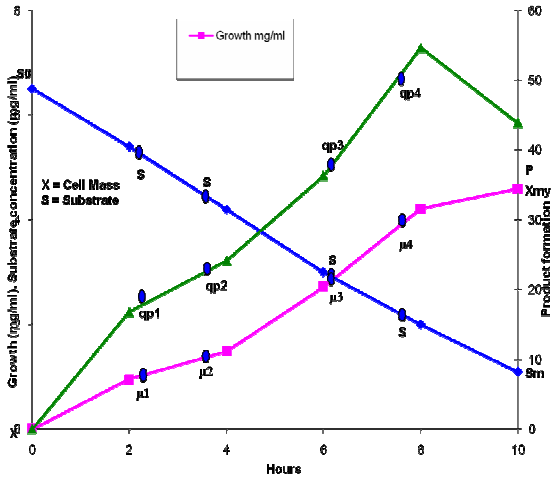
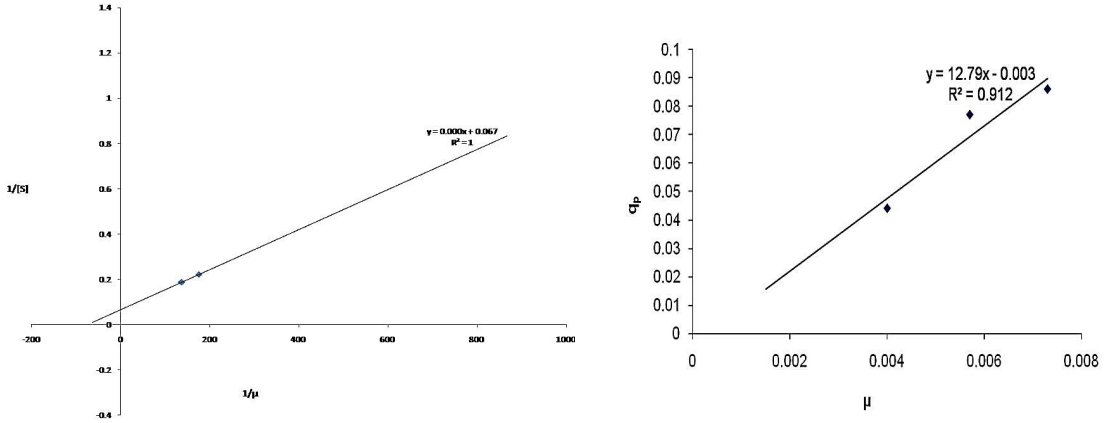


Figure 5: Determination of growth kinetics of *B. amyloliquefaciens* SH8 in a stirred tank bioreactor



(a) (b)

Figure 6: Comparison between experimental results and model prediction for (a) specific growth rate (μ) and substrate utilization [S],(b) for product formation

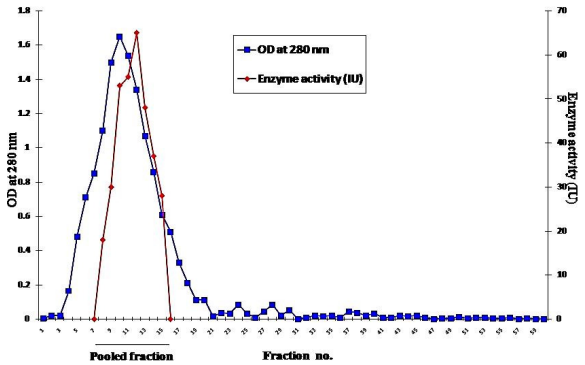


Figure 7: Gel exclusion chromatography of the xylanase active fraction (obtained after ion exchange chromatography) of *B. amyloliquefaciens* SH8 on Sephadex G-100

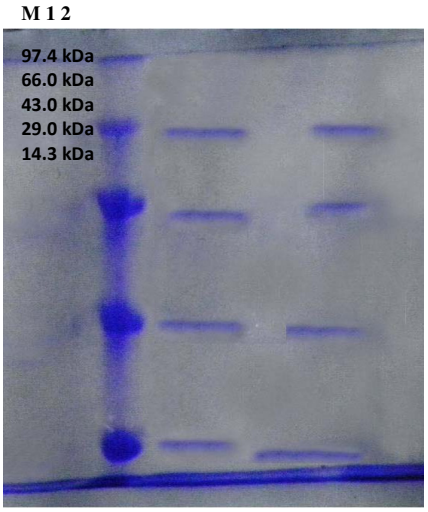


Plate 1: SDS PAGE of partially purified xylanase of *B.amyloliquifaciens*SH8 (Lane 1 = Partially purified xylanase by ion exchange chromatography; Lane 2 = Partially purified xylanase by gel chromatography; M = Marker)

Purification of xylanase

For the purification of *B. amyloliquifaciens* SH8 xylanase,t he sample was first lyophilized, and then subjected to ion exchange and gel exclusion column chromatography to obtain pure xylanase. The precipitate resulting from lyophilization was dialyzed and further used as starting material for chromatographic purification by CM-Sepharose and Sephadex G-100. The used purification protocol led to a 4.88 fold increase in purity with 22.90% xylanase yield (recovery). The enzyme activity of the purified enzyme fraction was 79.6 IU/mL (Fig.7). The lyophilized enzyme preparation was stored at (-20ºC) for further use. By SDS-PAGE, the molecular weight of the xylanase was estimated to be 14.3-97.4kDa (Plate 1).

Microorganisms havebeen shown to possess multiple forms of xylanase with varying characteristics.[19] The two bands observed in the present study were inseparable by any separation technique used, this implied that they were either subunits of the same proteins or products of the same gene with minor variations as a result of differential posttranslational modification, such as proteolysis, glycosylation,[19] indicating the multifunctional characteristics of xylanase. The molecular weights of purified xylanases of *B. pumilus* were observed to be 14, 35 and 60 kDa and were within the range of molecular weight for xylanases, *i.e.* 11-85 kDa.[20] Xylanases derived from *B. firmus* depicted an increase in specific activity from 1.75 U mg^{-1} to 34.08 U mg^{-1} with 45 kDa enzyme and to 3.67 U mg^{-1} with 23 kDaenzyme.[21]

Properties of enzyme

Effect of pH

The optimum activities for xylanase were recorded at pH 6 (81.83 IU/mL) using phosphate buffer (Fig. 8).The pH optimum for bacterial xylanase was found to be in the acidic range. When the pH of the medium was changed on either side of the optimum range from acidic to neutral pH and alkaline pH, a decline in enzyme activity was observed. Similarly, xylanase producing *Trichoderma ressei* F418 showed maximum xylanase activity at pH 5.5, using alkali pretreated rice straw with stability at 50ºC, showing specific activity towards xylans.[22] The pH influences many enzymatic systems and the transport of several species of enzymes across the cell membrane.[23] An unfavourable pH may limit the growth and consequently xylanase production by substrate inaccessibility. The optimum pH 6 found in this study was similar to the xylanase from *A. giganteus*[24] and *Marasmius* sp.[25] Thus, the *B. amyloliquifaciens* xylanase can be used in the paper industry where optimal pH 6 is required.[25]

Effect of temperature

Temperature has a profound influence on enzyme activity. The utilization of enzymes in industrial processes often encounters the problem of thermal inactivation of the enzyme. In the present study, xylanase of *B. amyloliquifaciens*

SH8 showed optimum activity at 90ºC (99.89 IU/mL) (Fig. 9). As the denaturation of the enzyme protein occurs at elevated temperature, the enzyme activity was drastically reduced above 90 ºC. The thermal stability of xylanase is an important property due to its potential application in several industrial processes, the use of such enzymes has been expected to greatly reduce the need for pH and temperature adjustments before the enzyme addition. Also, xylanase from *Steptomyces*sp. AMT-3 was found to be active over a range of temperature 55-60 ºC and at pH 6.[27] Anotherpurified xylanase from *Termitomyces* sp. exhibited maximum activity at 65-70ºC at pH 5.6.[28]

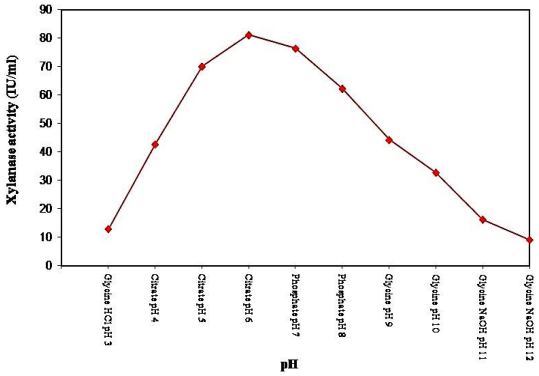


Figure 8: Effect of different pH on enzyme activity of partially purified xylanase produced by *Bacillus amyloliquefaciens* SH8

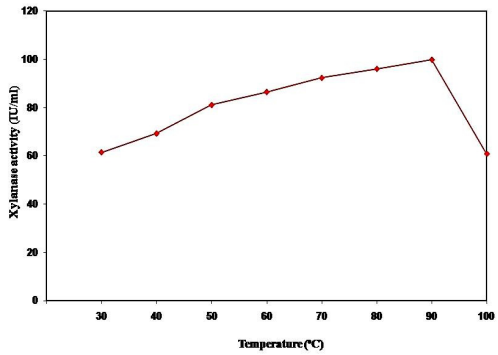


Figure 9: Effect of different temperature on enzyme activity of partially purified xylanase produced by *Bacillus amyloliquefaciens* SH8

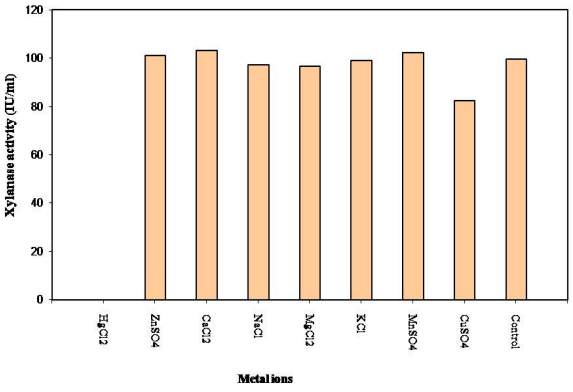


Figure 10: Effect of different metal ions on enzyme activity of partially purified xylanase produced by *Bacillus amyloliquefaciens*SH8

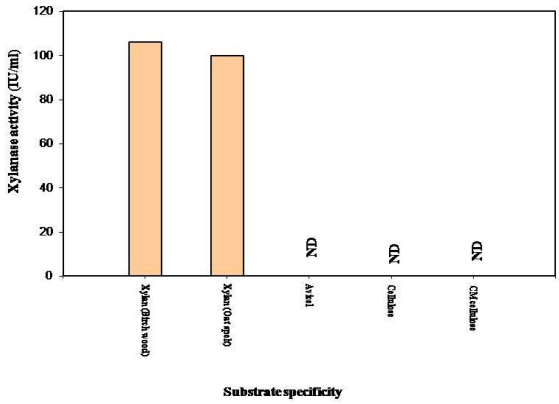


Figure 11: Effect of different substrate on enzyme activity of partially purified xylanase produced by *Bacillus amyloliquefaciens* SH8

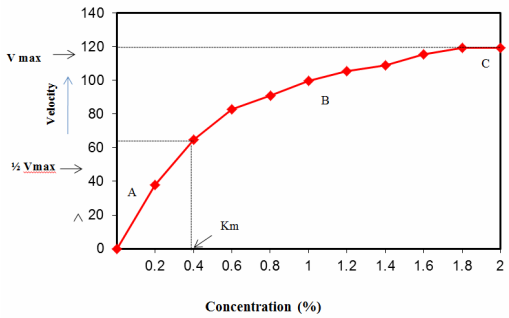


Figure 12: Effect of different substrateconcentration (%) on enzyme activity of partially purified xylanase produced by *Bacillus amyloliquifaciens* SH8

Effect of metal ions

The effect of some metal ions that generally occur in pulp was investigated, due to the potential use of the xylanase in pulp and paper production. Zn^{2+}, Ca^{2+} and Mn^{2+} (101.10, 103.80 and 102.45 IU/mL) had a greater effect than other metal ions on enzyme activity (Fig. 10). Other studies have also shown that Ca^{2+} enhances xylanase activity, while Cu^{2+}, Na^{+} and Mg^{2+} ions have a negative effect on xylanase activity. The activity of analkalophilic bacterium *Bacillus* sp. AP4 producing extracellular xylanase purified by ionexchange chromatography was shown to be influenced by the presence of Zn^{2+} and Ca, whereas Pb, Mn and Hg strongly inhibited the enzyme activity.[29] The purified enzyme had an apparent molecular weight of 42 kDa and showed optimum activity at 70ºC and pH 7.[30] Metal ions of Mn^{2+} and Co^{2+} increased enzyme activity twofold, while Cu^{2+} and Fe^{2+} reduced the activity fivefold, as compared to the control. Xylanase from *Bacillus* sp. SSP-0 was completely inhibited by Hg^{2+}, while in *Bacillus subtilis* Mn^{2+} ions enhanced xylanolytic activity 2.7 fold, whereas Fe^{3+} completely inhibited it.[31] The enhanced production of polygalacturonase from *Bacillus* sp. MG-cp 2 was observed under submerged fermentation from Tween-60, DL-serine and folic acid by 2.7 fold (240.0IU), 4.0 fold (360 U/mL) and 3.8 fold (342.0 U/mL).[32]

Effect of substrate specificity

A variety of substrates were tested for their ability to serve as substrates,*i.e.* 1% birch wood xylan, oat spelt xylan, Avicel, cellulose and carboxymethylcellulose. The xylanase has a high specificity and activity towards various polysaccharides used as a substrate,thus, it catalyses the hydrolysis of birch xylan (106.1 IU/mL) and oat spelt xylan (99.89 IU/mL), but it could not hydrolyze cellulose, Avicel and CMC (Fig. 11). Thus, this enzyme is cellulase-free and proves the true nature of the xylanase enzyme. The results are in agreement with those of Srinivasan.[33] The enzyme purified from *Cellulosimicrobium* sp. MTCC 10645 exhibited greater binding affinity exclusively for xylans, but not for Avicel, CMC, cellobiose, starch, or p-nitrophenylxylopyranoside. Parachloromercuric benzoate and iodoacetamidewere found stimulatory, while potassium permanganate, cysteine, and cystine markedly reduced the activity.[34] *Bacillus subtilis* SJ01 degraded xylan substrates (C1:0.24 U/mg, C2: 0.14 U/mg birchwood xylan) with higher efficiency than amorphous cellulose substrates (C1: 0.002 U/mg, C2: 0.01 U/mg carboxymethylcellulose).[35]

Effect of different concentration of birch wood xylan

Enzyme activity was found to be greatly affected by substrate concentration. The enzyme exhibited a gradual increase in enzyme activity from 0.2% to 1.8% (68.23 to 119.5 IU/mL) reaching a constant value of 119.5 IU/mL at 1.8 and 2.0% of substrate concentration, respectively. The stability in enzyme activity at increasing substrate concentration reflects the attainment of the saturation point of substrate, further leading to nil or negligible increase in enzyme activity. In general, an increase in the substrate concentration gradually increases the velocity of the enzyme reaction within the limited range of substrate levels. A rectangular hyperbola is obtained when velocity is plotted against the substrate concentration. Three distinct phases of the reaction are observed as shown in Figure 12. Maximum xylanase activity (35 U/mL) was observed after 96 h at 35ºC and pH 6 in 1% oat spelt xylan from *A. terreus* UL 4209.[36] The K_m value of xylanase for birchwood xylan and for oat spelt xylan was 6.5 and 2.6 mg/mL, respectively, whereas the K_m values of xylanase for the substrates were 26.61 and 23.45 mg/mL.[37]

Kinetic parameters

For the purpose of comparison, the kinetic parameters were determined in particular for the birch wood xylan as substrate. The enzyme obeyed the Michaelis Menten equation. The specificity is largely dependent on the nature of the substrate tested. The K_m (5.83mg/mL) and V_{max} (166.67 µ/mol/mg/min) values are reported in Figure 13. Although the value of V_{max} is in favor of birchwood,the small K_m value in our study shows that partially purified xylanases have high affinity for the substrate. This is of significance in industrial use of the enzyme, as to product a good conversion rate, the substrate concentration needs to be high for the enzymes with low K_m values. *Chaetomium thermophile* NIBGE xylanase produced with oat spelt xylanwas found to have K_m and V_{max} values of 12.5 mg/mL and 83.31 IU/mg.[38]T he extracellular purified xylanase from *Aspergillus niger* C3486 had a K_m value and V_{max} of 0.104 mg/mL and 24.8 µmol min^{-1} mg^{-1}, respectively.[39]

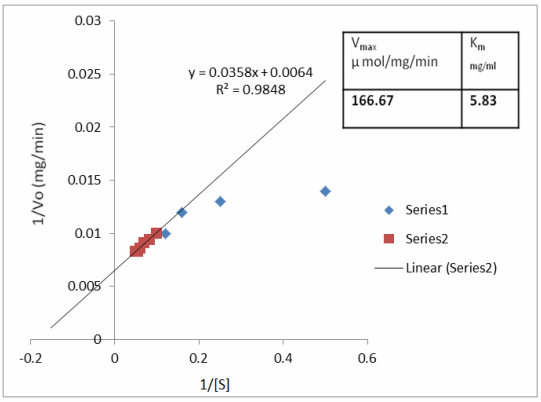


Figure 13: Lineweaver–Burk double reciprocal plot for the determination of K_m and V_{max} of partially purified xylanase from *Bacillus amyloliquefaciens* SH8

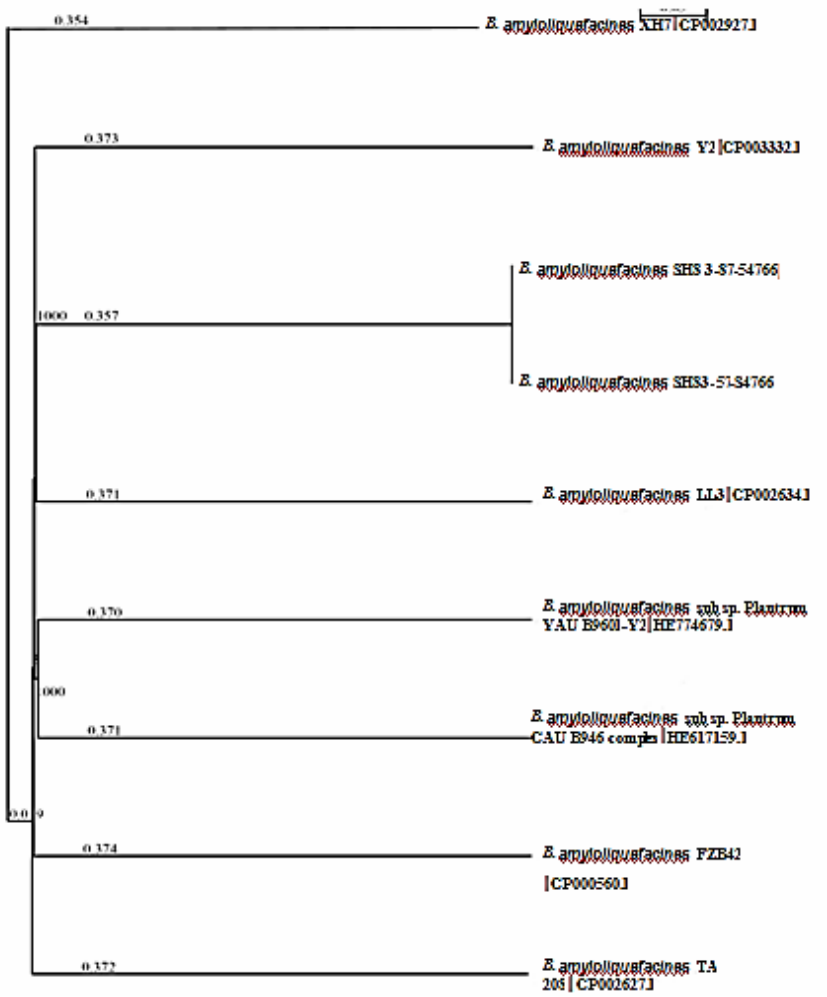


Figure 14: Neighbor tree constructed in Clustral X showing phylogenetic relationship of *B.amyloliquifaciens* SH8 among the genus *Bacillus* based on a distance matrix analysis of xyn gene sequence. The scale bar represents the number of changes of nucleotides per sequence position. The numbers at the nodes show the bootstraps (percentages) obtained with 1,000 replicates

Table 1
Sequence alignment of xylanase gene from *Bacillus amyloliquefaciens* SH8 using BLAST

Accession No.	Sequences producing significant alignments					
	Description	Max. score	Total score	Query coverage	E value	Max. ident.
CP000560.1	*B. amyloliquefaciens* FZB42, complete genome	1098	1098	97%	0.0	96%

Table 2
Sequence alignment of xylanase protein from translated nucleotide by *Bacillus amyloliquefaciens* SH8 using xBLAST

Accession No.	Sequences producing significant alignments					
	Description	Max. score	Total score	Query coverage	E value	Max. ident.
YP_006328295.1	Endo-1,4-beta-xylanase D [*B. amyloliquefaciens*Y2] >gb|AFJ61570.1| endo-1,4-beta-xylanase D [*B. amyloliquefaciens* Y2]	423	423	94%	3e-147	97%
EKE47070.1	Polysaccharide deacetylase [*B. amyloliquefaciens* subsp. plantarum M27]	435	435	94%	2e-152	97%

Gene encoding for xylanase production

A 850bp DNA fragment harbouring the entire xylanase encoding sequence was amplified from chromosomal DNA from *B. amyloliquifaciens* SH8 and showed 96% homology with *B. amyloliquifaciens* (Acc. No CP000501) (Tables 1 and 2). Specific primers of the xylanase gene of *B. subtilis* strain R5 gene resulted in the amplification of 0.65 kbp DNA.[40] Sequencing of the xylanase gene from different *Bacillus* sp. and further expressing them successfully in clones has been cited in the literature either for improving enzyme production or for constructing a clone of desirable characteristics for various applications in relevant industries. Similarly, the extracellular xylanase (xynA) was amplified as a 770bp DNA fragment from *Bacillus subtilis* 168 chromosomal DNA by PCR. The sequences of xynA and eglS were identical xylanase sequences submitted in the GENEbank.[41]Xylanase activity was optimal at pH 5.0 and 60ºC.The xylanase gene (xynA) encoded a 42 residue prepropeptide and a 191 residue mature protein. The xynAprotein showed the highest sequence identity of 69% to *Aspergillus niger*. XynB(Da 174549) belongs to the glycoside hydrolase family 11.[42]

CONCLUSION

The present study indicates that xylanase from the cellulase free strain of *Bacillus amyloliquifaciens* SH8 is active under acidic conditions and is thermophilic. Moreover, it shows high hydrolytic affinity for natural xylan and only a lower activity for CMC. The application of a lignocellulosic substrate for large-scale production of xylanolytic enzymes in a stirred tank bioreactor has been investigated. The characteristics of the xylanase from *B. amyloliquifaciens* enhance its industrial potential, making it suitable for effective applications in juice clarification and in the baking industry, as well as in the paper and pulp industry and in the feed industry.

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