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PURIFICATION, CHARACTERIZATION AND GENE ENCODING OF XYLANASE PRODUCED FROM *BACILLUS TEQUILENSIS* SH0 ISOLATED FROM COMPOST USING LOW COST WHEAT BRAN AS SUBSTRATE

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Abstract

A potential bacterial isolate exhibiting extracellular cellulase-free xylanase activity by *Bacillus tequilensis* SH0 was isolated from compost. The isolate demonstrating maximal xylanase activity after optimization using COFAT i.e. basal salt medium at 96 h, pH 5.5, temperature 45ºC, inoculums size 10%, carbon source-wheat bran (1.25%). The multistep purification techniques used were ion exchange chromatography and gel exclusion chromatography. The molecular weight was found in range of 14kDa- 97.4 kDa. The purified xylanase showed optimal activity of 41.30 IU/ml at 90ºC, pH 6.0 on xylan and also depicted cellulase free nature. The K_m and V_{max} of partially purified xylanase from *B.tequilensis* SH0 were 1.55 mg/ml and 125.0 µ mol/mg/min. Encoding of genes responsible for xylanase production was done using gradient PCR.

***Keywords*:** xylanase, wheat bran, submerged fermentation, gene encoding.

Introduction

Agricultural waste is one of the major environmental pollutants, their biotechnological conversion is not only a remedy for environmental problems but also the source of suitable microbial byproducts like food, fuel and chemicals (Milala et al., 2005).The use of agro wastes not only helps to overcome the problem of solid waste management but also allows the development of useful biotechnological processes from cheap natural resources (Timande, 202). India, being an agricultural country, has abundant agricultural waste produced from different crop residues. Thus, the use of agricultural waste in xylanase production will decrease the impact of agricultural waste on the environment. The cost of carbon source plays major role in the economics of xylanase production. Hence an approach to reduce to the cost of xylanase production is the use of lignocellulosic materials as substrate rather than opting for the expensive pure xylans. Xylanases are the group of enzymes that are involved in the hydrolysis of xylans and arabinoxylan polymers. These enzymes include endo-1,4-β-xylanase, β-xylosidase, a-arabinofuranosidase and acetylxylan esterase . Xylanases hydrolyze 1,4-β-D-xylosidic linkages in xylan to produce xylo-oligosaccharide (Irfan , 2012).

Xylanases are receiving increasing attention because of their potential applications in digestibility of animal feed, bioconversion of lingo-celluloses into feed-stocks, fuels and improving pretreatment of pulps prior to bleaching in pulp and paper industry (Salles et al., 2005). Biobleaching is a process where xylanolytic enzymes, instead of chlorine, are used to break the xylan- cellulose- lignin and compounds during pulping process. Cellulase-free xylanases active at high temperature are gaining importance in pulp and paper technology as alternatives to the use of toxic chlorinated compounds (Sharma et al., 2009). Xylanases are being used, primarily, for the removal of the lignin–carbohydrate complex (LCC) that is generated in the kraft process and acts as physical barriers to the entry of bleaching chemicals. A prerequisite in the pulp and paper industry is the use of cellulase free xylanases that ensure minimal damage to the pulp fibres and also generate rayon grade or superior quality dissolving pulps (Sharma, 2009). Keeping in view the increasing demand of cellulase free xylanase in industry, the present study was aimed to cost effective production of xylanase, its purification and characterized produced from *Bacillus tequilensis* SH0 under submerged fermentation.

Material and Method

Isolation of microorganism

A bacterial strain was isolated from compost sample by serial dilution technique. The pure culture was maintained on nutrient agar slant and stored at 4 ºC. The pure culture was identified by phenotypic, genotypic characterization as given below:

Characterization

Morphological and Biochemical test:

The conventional morphological and biochemical tests as described by Aneja (2003) were performed. Main physiological tests performed were catalase, urease, H_2S production, MR/VP test, gelatin hydrolysis, fermentation of glucose, citrate and indole test.

Molecular studies:

Genomic DNA was extracted from representative isolate acocoding to Sambrook and Russell, 1989. The 16 S rRNA gene was PCR amplified using the PCR primers i.e. forward BITS-1(5'AGAGTTTGATCCTGG) and reverse primer BITS-4-(5'-TACCTTGTTACGACTT). PCR product so obtained after amplification with specific primers at standardized amplification temperature was clean up using PCR clean up kit (Real Genomics Hi yield™ Make).

Separation of bases was carried out by commercial available services of (Xcelaris, Ahmdabad, India). Sequences similarity searches were performed on the national centre for biotechnology information (NCBI) database using NCBI-BLASTN megablast. Matrices of the evolutionary distance was computed from the sequences alignment by calculating pair wise, the phylogenetic trees was inferred by a neighbor–joining method.

Production of xylanase from *B. tequilensis* SH0

Bacterial isolate was grown in 50 ml of nutrient broth and incubated at 37±2^{0}C for 24 h. As soon as the substantial growth of the isolate was observed in the broth, the optical density was set to 1.0 using sterilized distilled water. 5 ml of inoculum was added to each 45 ml of Riviere's broth **(**Riviere, 1961) containing 1% xylan for xylanase production in 250 ml of Erlenmeyer flasks and the flasks were incubated at 37±2^{o}C for 5 days at 120 rpm. After incubation the culture contents were centrifuged at 10000 rpm for 15 min (4^{o}C). The supernatant was collected.

Estimation of xylanase activity

Xylanase assay

The activity of xylanase in the culture filtrate was determined by Millers method (Miller et al., 1959).
One international unit (IU) of enzyme activity represents µmoles of xylose released ml of enzyme per min**.**

Protein assay:

The protein concentrations were determined by the Lowry method (Lowry et al., 1951). Bovine serum albumin was used as standard.

Optimization of xylanase production under submerged fermentation

The optimization of different cultural conditions was carried using classical one factor at a time (OFAT) approach as given below.

Effect of medium

The nutritional requirement of the *B.tequilensis* SH0 was determined by adding various nutrient supplements into the medium i.e. TGY medium (Garg et al., 2009), Emerson medium (Garg et al., 2009), Mineral medium (Boochini et al., 2008), Basal salt medium (Dhillon and Khannna, 2000) and Mandel's medium (Kheng and Omar, 2005), xylanase assay was done after 5 days of incubation.

Effect of temperature

The effect of xylanase activity on different fermentation temperatures was evaluated i.e. from 25, 35,...., 65°C @120 rpm for 5 days.

Effect of pH

The pH of optimized media was set at 4, 5, 6, 7, 8, 9 & 10 and activity of xylanase was determined after incubation of 5 days at 50ºC under constant shaking at 120 rpm.

Effect of inoculum size

Different inoculum sizes i.e. 1.0%, 2.5%, 5.0 %....15.0 % were used to study their effect on enzyme production. The investigation was carried out at 30°C, 5 days of fermentation and keeping all other conditions at their optimum levels.

Effect of carbon source

Different substrates i.e. xylan, xylose, wheat bran, lactose, fructose, dextrose, sucrose, arabinose and D- ribose were added @ **5**% in each of the flask. After 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C.

Effect of different concentration of carbon source

Different percentage of carbon was investigated by different concentration of xylan such as 0.5%, 1.0%, 1.5%....., 3.0% for investigating its effect on xylanase.

Effect of incubation time

5 ml of inoculum was added to 45 ml of Basal Salt medium in each of 250ml of Erlenmeyer flasks. The flasks were incubated at different incubation time ranging from 12h, 24h, 36h…..120h at 120 rpm. After mentioned incubation time, the culture contents were centrifuged at 10,000 rpm for 15 min at 4°C.

Purification of xylanase

Lyophilization:

Culture supernatant containing extracellular xylanase showing sensitivity to ammonium sulfate was directly subjected to concentration by lypholozation in a round bottom flask (20ml) in a lyophilizer for 5 h.

Ion Exchange Chromatography

DEAE-cellulose (2 g) was suspended in 200 ml of distilled water for 48 h and packed in a glass column (10x1.0 cm). Concentrated supernatant containing enzyme was loaded on ion exchange column. Fractions of 3 ml each were collected and monitored for xylanase activity and the protein content was measured at 280 nm. The active fractions were pooled and used for electrophoresis and gel filtration chromatography (Bollag, 1991).

Gel Filtration Chromatography

Sephadex G-100 (5g) was suspended in 500 ml of distilled water for 48 h. It was packed into the glass column having dimensions of (31× 2.5 cm) avoiding entrapment of any air bubble in the gel bed. It was then equilibrated with three bed volumes of 0.1M Phosphate buffer (pH 6.9). Lyophilized protein sample (2 ml) was loaded on the Sephadex G-100 column. It was then eluted with three bed volumes of 0.1M phosphate buffer (pH 6.9) and 3 ml fractions were collected. A flow rate of 3 ml in 6 min was maintained. The most active fractions were pooled and stored at 4^{o}C. Purity of sample was checked out by SDS polyacrylamide slab gel electrophoresis (Bollag, 1991).

SDS-PAGE

The molecular mass of purified cellulase free xylanase was determined by using 10% SDS-polyacrylamide gel following the procedure of Lammeli (1970) using 12% polyacrylamide gel. The gel was stained with Cossmic brilliant blue. The protein content was estimated by Lowry et al. (1951).

Characterization of cellulase free xylanase

Effect of pH on xylanase activity and stability

The pH profile of partially purified xylanase was evaluated by incubating the enzyme for 10 min in the presence of 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 on enzyme activity

The optimum temperature for partially purified xylanase for the hydrolysis of oat spelt xylan (1%) in 0.05M of citrate buffer at pH 6.0 was determined by incubating the enzyme for 10 min at different temperature ranging from 30^{o}C, 40^{o}C, 50 ^{o}C, 60 ^{o}C, 70 ^{o}C, 80 ^{o}C, 90 ^{o}C, 100^{o}C.

Effect of Metal Ions

Xylanase in 0.05M citrate buffer (pH 6.0) with various metal ions i.e. each of 5 mM each of K^{+}, Hg^{2+}, Na^{+}, Ca^{2+}, Cu^{2+}, Mn^{2+},Zn^{2+}, Mg^{2+} was incubated at 90^{o}C for 10 min for studying the effect of metal ions on its activity.

Substrate Specificity

The hydrolytic activity of enzyme against 1% birch wood xylan, oat spelt xylan, avicel, cellulose and carboxyymethyl cellulose in 0.05M citrate buffer (pH 6.0) was analysed by incubating the samples at 90^{o}C for 10 min to evaluate its substrate specificity.

Effect of Different Concentration of Birch Wood Xylan

The different concentrations of substrate ranging from 0.2%, 0.4%.....2.0% in 0.05M citrate buffer (pH 6.0) were incubated at 90^{o}C for 10 min.

Determination of Enzyme Kinetics

The effect of birch wood xylan concentration ranging from 2.0, 4.0……& 20.0 mg/ml on partially purified xylanase activity in 0.05M citrate buffer (pH 6.0) were incubated at 90^{o}C for 10 min under optimal assay conditions. The kinetic parameters of Michaelis- Menton constant, Km, maximal reaction velocity and Vmax were determined by linear regression according to Lineweaver and Burk double-reciprocal plot.

Encoding of xylanase gene from *B.tequilensis* SH0

Genomic DNA of isolates was isolated by using DNA purification kit (Banglore Genei, make). The DNA was quantified by using standard protocol (Sambrook and Russel, 1989). The selected sets of primers used were (5'-GGGGAAGGCTGGCTTATATC-3') (5-'ATGGTGTGCCTTCACTTTCC-3') forward and reverse (5'-AGGGCAACCTCATGTCAAAG-3') (5-'GTTAGCGGCAGTTCTTCGAG -3') using a gradient PCR reaction consisted of 35 cycles of 92^{o}C for 1 min, 55^{o}C for 1 min, 72 for 1 min (ASTEL, make). Eluted PCR products of SH0 were sequenced by commercial available services of (Xcelaris, Ahmdabad, India).

RESULTS AND DISCUSSION

Isolation of microorganisms

The culture was isolated locally from a compost sample, culture showed good growth at 45^{o}C in Riviere (1961) medium supplemented with 1% of xylan. Its ability to produce xylanase was confirmed through quantitative estimation under submerged fermentation yielding 13.30 IU/ml of xylanase initially. The isolate was gram positive, oxidase positive, hydrolysis casein and gelatin (Table 1). The isolated bacterial strain was identified by 16sRNA as *Bacillus tequilensis* SH0 **(Plate 1)**. The sequence was deposited in Genbank database and had been assigned an accession number NCBI-JX129359 and its phylogenetic tree had been given as in **Fig 1**.

Optimization of xylanase production

Effect of media

B.tequilensis SH0 showed maximum xylanase activity in basal salt medium i.e. 21.20 (**Fig 2)**. Highest xylanase production using defined medium may be due to the presence of nitrogen, carbohydrate and other compounds in adequate quantity that could be utilized easily by the growing isolate thus enhancing the cell ability to produce xylanase enzyme (Basar et al., 2010). Sodium chloride present in the medium probably helped in maintaining the osmotic balance of the medium while magnesium sulfate was a cofactor for a variety of metabolic reaction. Basal salt medium contains

ammonium sulfate and ammonium chloride as its major ingredients and both being rich source of nitrogen might have exerted positive influence for highest xylanase. Dhillon *et al.* (2000) isolated *Bacillus circulans* AB16 from garbage dump, the highest xylanase activity was in basal medium of 55 IU/ml. *B. pumilus* showed a 3.4 fold increase in the xylanase production was achieved using the optimized culture medium consisting of (g/l): K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, NaCl, and peptone, yeast extract and wheat bran. Similarly, a thermophilic *Arthorobacter* sp. MTCC 5214 produced optimal extracellular xylanase in medium supplemented with modified salt medium of pH 9 supplemented with 0.5% birch wood was isolated from locally from a sediment sample collected from Mandovi estuary, west coast of India (Khandeparker and Bhosle, 2006). Kamble and Jadhav (2012) studied the xylanase production in Erlenmeyer flasks containing fresh basal salt medium supplemented with 1% oat spelt xylan.

Effect of temperature

The fermentation temperature is also important in production of xylanase enzyme activity. Referring to **Fig 3**, the highest enzyme activity of xylanase was obtained at 50°C of the fermentation temperature which was 21.20 IU/ml. Optimum temperature range obtained for potential enzyme producing bacteria in the present study has reflected their thermpohilic nature, thus suggesting the strong possibility of their being an asset for commercial use. Roy and Rowshanul (2009) examined the effect of temperature on activity of xylanase against oatspelt xylan from *Bacillus cereus* and observed that xylanase activity showed maximum activity at 40°C. Wahyuntari *et al*. (2009) reported xylanase production from *Bacillus* sp. AQ1 at 40^{o}C and Kumar *et al*. (2010) reported the xylanase production from *Bacillus pumilus* at 40^{o}C.

Effect of pH

Enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution on both substrate and in particular enzyme molecules. pH is known to affects the synthesis and secretion of xylanase. During optimization study pH ranged from 5 to 9. Among which, maximum xylanase production (26.10 IU/min) was achieved at pH 6 significant higher than others and least xylanase titers was observed at pH 10.0 i.e. 0.46 IU/ml and 0.12 IU/ml **(Fig 4)**. The extracellular pH has a strong influence on the pathways of metabolism and product formation by microorganism. Changes in the external pH alter the ionization of nutrient molecules and reduce their availability to the organism thus lowering their overall metabolic activity (Willey *et al.*, 2008). Abou- Dobara *et al.* (2011) evinced that maximum xylanase production was achieved after 48 h, at 50^{o}C and pH 6 for *Bacillus coagulans and* 60h, at 50^{o}C and pH 7.0 by *Bacillus licheniformis.* Similarly Sanghi *et al.* (2010) examined that *Bacillus* sp. produced maximum cellulase-free-xylanase enzyme titre of 283 IU/ml at pH 7.0.

Effect of inoculum size

Effect of inoculum size on xylanase production was evaluated in Fig.5. Different inoculum size used were 1.0%, 2.5%, 5.0 %, 7.5%, 10.0%, 12.5% and 15.0% (V/V). Highest xylanase production from *B. tequilensis* SH0 i.e. 26.1 IU/ml was obtained @ 10% inoculum having statistically significant differences over other inoculum sizes. inoculum size of 10%(V/V), a balance between proliferating biomass and available nutrient was seen. Enzyme activity is maximum at optimal level because at this point because equilibrium is maintained between inoculum size and availability of substrates while the decline in enzyme yield at larger inoculum size might be due to formation of thick suspensions and improper mixing of substrates in shake flasks. With subsequent increase in inoculum size, competition for carbon source increases and results in rapid depletion of macro and micro nutrients and thus inhibits their growth and enzyme production (Omojasola *et al*., 2008).

Effect of carbon source

The varied effect of different substrates for xylanase production i.e. xylose, arabinose, ribose, mannose, xylan, dextrose, fructose, sucrose, lactose and wheat bran has been expressed in Fig 6.. Statistical analysis showed significant variation among all the substrates for enzyme titers. xylanase titer varied from 4.56 to 32.3. *B. tequilensis* SH0. Optimal level of xylanase were recorded when wheat bran was used as a carbon source in all the three selected hyperenzyme producers. Some of the carbon source used in the medium supports the good growth as well as good enzyme synthesis, while other supports the good growth as well as good enzyme synthesis (Satyanarayanan, 2007). Geeta and Gunasekaran (2010) used wheat bran as carbon source for optimization of xylanase production. Gupta and Kar (2009) recorded maximum xylanase production from *Bacillus* sp. in wheat bran residue supplemented with mineral salt medium.

Effect of different carbon source

The significant variation among different substrate concentrations used for xylanase production ranging from 0.25%, 0.5%....to 3.0% from hyperxylanolytic *B. tequilensis* SH0 has been shown in Fig 7. Increase in substrate concentration level other than optimum showed a consistent decrease in cellulase production. Whereas effect of substrate concentration on xylanase production from potential bacteria revealed *B. tequilensis* SH0 exhibited statistically significant higher xylanase activity at 1.25% substrate concentration i.e. 36.8 IU/ml. Minimum xylanase production i.e. 4.2 IU/ml was observed at 3.0 % substrate concentration for hyperxylanolytic bacteria. Substrate concentration is an influencing factor that affects the yield and initial rate of hydrolysis of xylan (Da Silva *et al*., 2005). Very low substrate concentration fails to trigger enzyme production to desirable level because most of the inoculum remains without substrate and hence resulting in minimum secretion of enzymes. Optimum substrate concentration normally results in an increase in the yield and reaction rate of the hydrolysis (Regina *et al.*, 2008). However, high substrate concentration can cause substrate inhibition, which substantially lowers enzyme production (Liu and Yang, 2007 and Singhania *et al*., 2007). Sanghi *et al.* (2007) used 2% wheat bran as a carbon source for xylanase production (8,964 U/g). Kapoor *et al.* (2009) evaluated different agro-residues for xylanase production and among them maximum production was observed in 1.0% wheat bran (1220±56.0 IU/ml).

Effect of incubation time

Time course plays a very critical role in metabolic activity and growth. In present study xylanase production was determined starting from 2nd day up to 5 th day of inoculation at regular interval of 24 h **(Fig 8)**. The obtained results indicated that, the highest yield of xylanase was 41.30 IU/ml at 96h of incubation. Enzyme production when compared with the growth profile peak in yield was noticed at 96 h i.e. stationary phase. A decline in enzyme activity afterwards was probably due to proteolysis or due to depletion of nutrients available to microorganisms, causing a stressed microbial physiology resulting in inactivation of enzyme (Flores *et al*., 1997). Irfan *et al.* (2012) recorded maximum xylanase production at 48 h under shake conditions while under static conditions at 72 h (408 ± 2.9 IU) of fermentation time from *B. subtilis* using various agricultural wastes. Similarly Kumar *et al.* (2009) recorded optimum cellulase production after 6 days under submerged fermentation in different substrates from newly isolated *Bacillus* sp. FME.

Purification of Enzyme

Production of xylanase

Lyophilization:

The xylanase containing cell free supernatant was concentrated for 6h in lyophilizer (Allied Frost, make). In the present study the direct method of concentration by lyophilization was opted because of high sensitivity of xylanase enzyme towards ammonium sulphate. Xylanase lost its complete activity during salt saturation method even at initial concentration of ammonium sulfate. Though, ammonium sulphate is a recommended method to stabilize protein but at times it may prove toxic to some enzymes probably depending upon the nature of the of protein and also due to the possible traces of metals which might be detrimental to the protein of interest (Bollag and Edelstein, 1991). Phongdara et al., 1996 purified xylanase from *Pichia stiptitis* by centrifugation and further lyophilization of the supernatant and loaded on Sephacyl S-300 eluted at a rate of 0.5ml/min and fraction of 2.0ml was collected. A novel high molecular weight endoxylanase (XylnF1) from *Aspergillus fumigatus* MKU1 strain was purified to homogeity by combination gel electrophoresis and electro-elution method. The purity was estimated by SDS-PAGE with the molecular mass of 43kDa. The apparent Km and Vmax were 6.25 mg/mL and 0.05 mmol/mL/min respectively.

Ion Exchange Chromatography

Lyophilized crude enzyme was subjected to ion exchange chromatography column DEAE cellulose. Filterate was eluated from that column as a single protein peak obatined Phosphate buffer pH (6.9) was used as elution buffer. The fractions of 0.1 M showed highest enzyme activity by calorimetric method i.e. 6-12 were pooled and concentrated further by lyophilization as shown in **Fig. 9.** Damiano et al., 2003 purified crude xylanase from an alkalophilic *Bacillus licheniformis* 77-2 by gel filteration (G-75) and ionic exchange chromatography, the molecular mass of purified xylanase was estimated to be 17kDa(X-I) and 40kDa(X-II). Similarly Sanghi et al., 2010 reported one step purification and characterization of an extracellular cellulase free xylanase from a newly isolated alkalophilic *Bacillus subtilis* ASH. Xylanase was purified to homogeneity by 10.5-fold with ~43% recovery using ion-exchange chromatography through CM-Sephadex C-50. The purified enzyme revealed a single band on SDS-PAGE gel with a molecular mass of 23 kDa.

Gel Exclusion Column Chromatography:

The active fractions of the anion exchange chromatography were further purified by gel exclusion column chromatography using sephadex G-100. A total of 60 fractions with flow rate 6min/3ml each were collected in chromatographic vials under cool environment. The optical density of each collected fraction was measured at 280 nm. The maximum protein was observed in fraction no 10 to 17 i.e. 3.1, 34.2, 36.0, 57.89, 51.20, 42.0, 31.0, 13.0 IU **(Fig. 10)**. Table 2 summarizes the procedure for the purification of extracellular xylanase from *B.tequilensis* SH0 with 5.30 purification fold and final specific activity of 211.96 in gel exclusion chromatography, indicating the degree of purification and yield for each step. Heck *et al*. (2005) isolated purified xylanase *Bacillus circulans* BL53 by ammonium sulphate fractioning, cation-exchange and gel filtration chromatography. A purification factor of 428-fold was achieved, with the purified enzyme presenting a specific activity of about 37Umg^{-1} protein. Similarly A highly thermostable alkaline xylanase was purified to homogeneity from culture supernatant of *Bacillus* sp. JB 99 using DEAE-Sepharose and Sephadex G-100 gel filtration with 25.7-fold increase in activity and 43.5% recovery (Shrinivas et al., 2010).

SDS–PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (1970) to determine the molecular weight of xylanase 14 and 97.4 kDa (**Plate 2).** Microorganisms have been commonly shown to posses multiple forms of xylanase with varying characteristics (Wong et al., 1998). 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, gylcosylation (Breccia et al., 1998) indicating the multifunctional characterstics of xylanase**.** Similarly Damiano *et al.* (2006) reported that the alkalophilic bacteria *Bacillus licheniformis* 772 produces significant quantities of thermostable cellulase-free xylanases. The molecular masses of the enzymes were estimated to be 17 kDa (X-I) and 40 kDa (X-II), as determined by SDS-PAGE. Aygan and Arikan (2009) observed that a multifunctional endoxylanase producing *Bacillus* sp. was isolated from soil optimum temperature of 37°C. Analysis of the enzyme by SDS-PAGE revealed 4 active enzyme bands, which were estimated to be 66.5 kDa, 80.6 kDa, 95.5 kDa, and 108.4 kDa.

Characterization of xylanase

Effect of pH

Most enzymes have a characteristic pH at which their activity is maximal, above or below this pH, the activity declines. The effect of various pH was determined in different buffers with the pH values ranging from (3, 4, 5……12)

on the purified xylanase activity was depicted in **Fig 11.** The enzyme exhibited high activity in a range spanning 5.0 to 7.0 was found maximum at pH 6.0 (62.20 IU/ml). When the pH of the enzyme medium was change on either side of optimum range from acidic to neutral pH and alkaline pH, a decline in enzyme activity was observed. Roy and Rowshanul (2009) purified extracellular xylanase from *Bacillus cereus* showed optimum activity at pH 6.0. Similarly Kumar et al., 2010 reported that extracellular xylanase from *Bacillus pumilis* MTCC 8964 showing optimum pH of enzyme activity was pH 6.0. Sa-Pereira *et al.* (2002) reported that enzyme showed an optimum pH of 6.0 by *Bacillus subtilis*.

Effect of temperature

The effect of various temperatures regime on the enzyme activity of purified xylanase was presented in **Fig. 12** ranging from 30°C, 40°C…..100°C. It was found that the enzyme exhibited maximum activity at 90°C (74.90 IU/ml). When the temperature increased above 90°C the activity of the enzyme was affected negatively and gradually reduced. As the denaturation of the enzymatic protein occurs at elevated temperatures therefore, after certain level of temperature increase (above 90°C), the enzyme activity decreased rapidly. Shrinivas *et al.* (2011) reported a highly thermostable alkaline xylanase was purified to homogeneity from culture supernatant of *Bacillus* sp. JB 99, enzyme was optimally active at 70°C. Kiddinamoorthy *et al.* (2008) reported the purified enzyme had an apparent molecular weight of 42 kDa and showed optimum activity at 70°C.

Effect of metal ions

Xylanase activity was assayed in the presence of metal ions. The results shows that Hg^{2+} was strong inhibitor of enzyme activity while Cu^{2+}, Na^{+} and Mg^{2+} had a slight inhibition effect on enzyme activity as depicted in **Fig. 13**. The inhibition of the enzyme was generally obtained with ions that reacted with sulfydryl groups such as Hg^{2+}, suggesting that there was an important cysteine residue in or close to the active site of the enzyme. Inactivation of xylanase by Hg^{2+} has been reported by others authors also. K^{+} ions were found inert and it donot affect the activity of this enzyme. However, stimulatory effects were obtained with Zn^{2+}, Ca^{2+} and Mn^{2+} (77.10, 78.0 and 76.88 IU/ml). Chivero *et al.* (2001) purified endoxylanase from tow isolates of *Bacillus* sp. Endoxylanase activity of the for isolate SB9-a was inhibited by Hg^{2+}, Ag^{+} and Mn^{2+} ions, while Fe^{3+}, K+, Ca^{2+} and Cu^{2+} ions stimulated xylanase activity. Khandeparkar et al.2011, studied the effect of metal ions on extracellular xylanase isolated from thermoalkalophilic *Arthrobacter* sp., the activity of the enzyme increased in the presence of metal ions such as Co^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+} and decreased in the presence of Hg^{2+}.

Effect of substrate specificity

The substrate specificity by the purified xylanase for different substrates was depicted i**n Fig 14**. The xylanase enzymes exhibited different specificity and activity towards various polysaccharide used here as substrates. Xylanase of *Bacillus tequilensis* SH0 strongly hydrolyzed Birch wood xylan (82.10 IU/ml) followed by oat spelt xylan (76.10 IU/ml). However, it could not hydrolyze cellulose, avicel and CMC. Thus, this enzyme is cellulase free proves the true nature of xylanase enzyme. Kamble and Jadhav (2012) purified enzyme from *Cellulosimicrobium* sp. MTCC 10645 exhibited greater binding affinity exclusively for xylan but not for avicel, CMC, cellobiose, starch, or p-nitrophenyl xylopyranoside. Parachloromercuric benzoate and iodoacetamide were found stimulatory, while potassium permanganate, cysteine markedly reduced the activity.

Effect of different substrate concentration

The effect of various substrate concentrations was determined in values ranging from (0.2, 0.4…….2.0%) on the purified xylanase activity is depicted in **Fig. 15**. 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% (39.12 to 96.87 IU/ml) reaching a constant value to (96.87 IU/ml) at 1.8 and 2.0% of substrate concentration, respectively. The stability in enzyme activity at increasing concentration of reflects the attainment of saturation point of substrate furthermore leading to nil increase in enzyme activity. 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. At low substrate concentration at phase A, the velocity of the reaction is directly proportional to the substrate level. In the second phase B, substrate concentration is not directly proportional to the enzyme activity. In the third and final phase C, the reaction is independent of the substrate concentration.

Kinetic pararameters

The kinetic parameters of for hydrolysis of birch wood xylan were determined by plotting the initial velocities towards different concentrations of substrate as shown in **Fig 16**. The enzyme showed Michaelis-Menten behaviour, and the kinetic value for birch wood xylan substrate was calculated. The kinetic parameters (Km and Vmax) were determined using Line weaver-Burk plot at 90^0C (pH 6.0) with different concentrations of Birchwood xylan ranging from 2-20 mg/ml. The K_m and V_{max} of purified xylanase were 10.99 mg/ml and 166.67 µ mol/mg/min. This indicates that the specificity of towards birch wood xylan substrate is high (K_m value is low). Similarly Monisha et al., 2009 isolated bacterium identified was *Bacillus pumilus* producing xylanase and demonstrated maximal activity at 350C and at pH 7.0. Xylanase had Km of 4.0 mg ml-1 and Vmax of 0.068×10^{-4} mM min-1 mg-1. Similarly *Bacillus licheniformis* producing cellulase free xylanase with Km and Vmax values were 1.8mg/ml and 7.05 U/mg protein (X-I), 1.05mg/mL and 9.1 U/mg protein (X-II) (Damiano et al., 2006).

Encoding of xylanase gene from *B.tequilensis* SH0

The primers were selected by taking into account nucleotide sequences of xylanase encoded genes of *Bacillus tequilensis* SH0 available after searching in the Gene Bank databases i.e. EMBL and NCBI. The multiple amplification

was achieved with both set of primers, i.e. (5'-GGGGAAGGCTGGCTTATATC-3') (5-'ATGGTGTGCCTTCACTTTCC-3') and (5'-AGGGCAACCTCATGTCAAAG-3') (5-'GTTAGCGGCAGTTCTTCGAG -3') of expected amplicon size 509 bp and 2020 bp using a gradient PCR reaction **(Plate 3)**. The genes so obtained of this strain are under the process of cloning in *E.coli* for further commercial use. Jalal *et al*. (2009) used specific primers of xylanase gene of *B. subtilis* strain R5 gene resulted in the amplification of 0.65 kbp DNA. The PCR amplified DNA fragment was ligated into cloning vector and then *E. coli* cells were transformed. Sequencing of xylanase gene from different *Bacillus* sp. and further to express them successfully in clones has been cited in literature to improve either enzyme production or to construct a clone of desirable characteristics for various applications in relevant industries. Similarly Khandeparker et al., 2011 isolated a halotolerant xylanase from marine bacteria *B.subtilis* cho40.

Conclusion

A hyperxylanase producing strain was isolated from compost. *B. tequilensis* SH0 on the basis of their highest xylanase activity were selected for the further optimization under SmF. *B. tequilensis* SH0 showed highest xylanase activity i.e. 41.30 IU/ml in Basal salt medium at 96h, pH 5.5, temperature 45^{o}C, inoculum size 10%, carbon source-wheat bran and 1.25% wheat bran concentration. It had shown capability to utilize natural waste i.e. wheat bran as a substrate for growth and produce high xylanase. Purified xylanase having a molecular mass of 14kDa-97.4 kDa was thermophilic in nature, possessing high thermostability. Partially purified enzymes of the bacterial isolate had broad range activity between pH 4.0-10.0 with optimum peak at 6.0. Hg^{2+} was strong inhibitor of enzyme activity while Cu^{2+}, Na^{+} and Mg^{2+} had a slight inhibition effect on enzyme activity, while stimulatory effects were obtained with Zn^{2+}, Ca^{2+} and Mn^{2+}, showed highest substrate specificity towards birch wood xylan . The kinetic parameters (Km and Vmax) were determined using Line weaver-Burk plot at 90^{o}C (pH 6.0) with different concentrations of birchwood xylan ranging from 2-20 mg/ml. Xylanases from this bacterium may potentially applicable in enzymatic hydrolysis of xylan especially in kraft pulp prebleaching process.

Acknowledgment

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Annexure

Figure

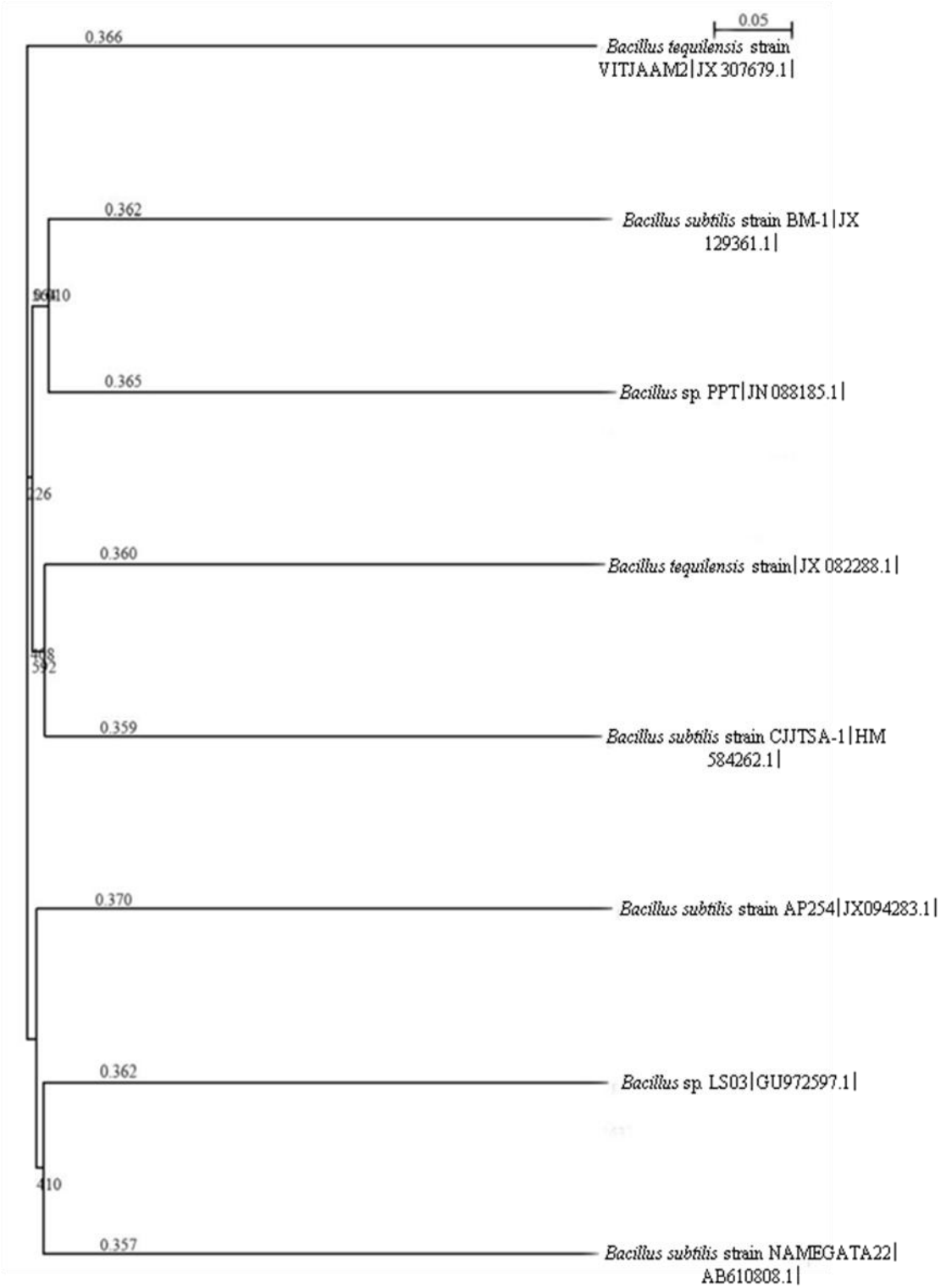


Fig. 1 Neighbor-joining tree constructed in clustal X showing phylogenetic relationship of *B. tequilensis* SH0 among the genus *Bacillus based on a distance matrix analysis of 16S r*RNA sequences. The scale bar represents the number of changes of nucleotides per sequence position. The numbers at the nodes show the bootstrap values (percentages) obtained with 1,000 replicates.

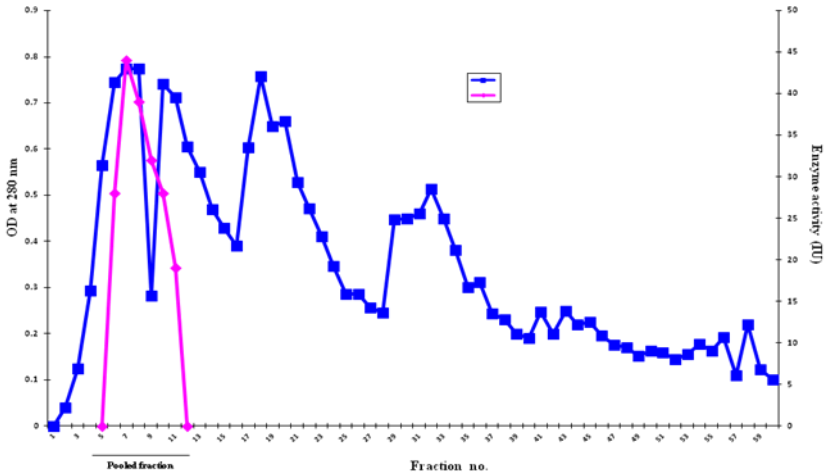


Fig. 2 Ion exchange chromatography of extracellular crude xylanase of *Bacillus tequilensis* SH0 on DEAE Cellulose

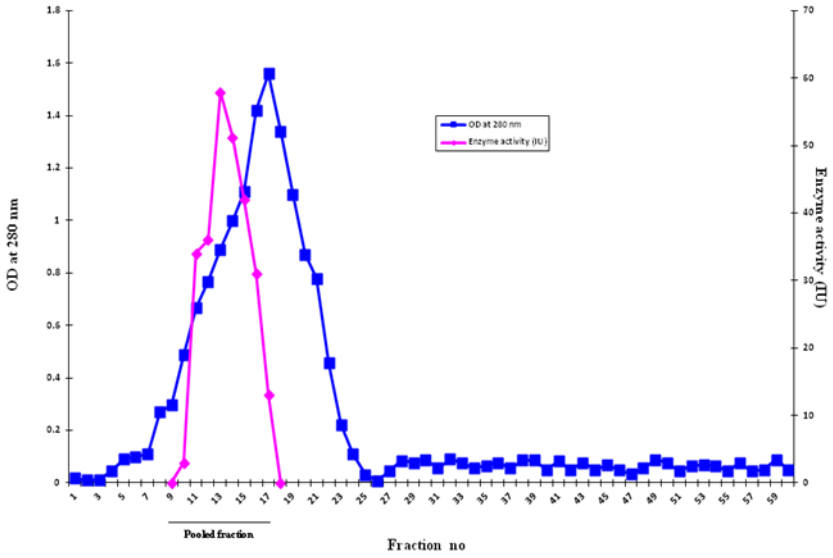


Fig 3. Gel exclusion chromatography of xylanase active fraction (obtained after ion exchange) of *B. tequilensis* SH0 on sephadex G-100

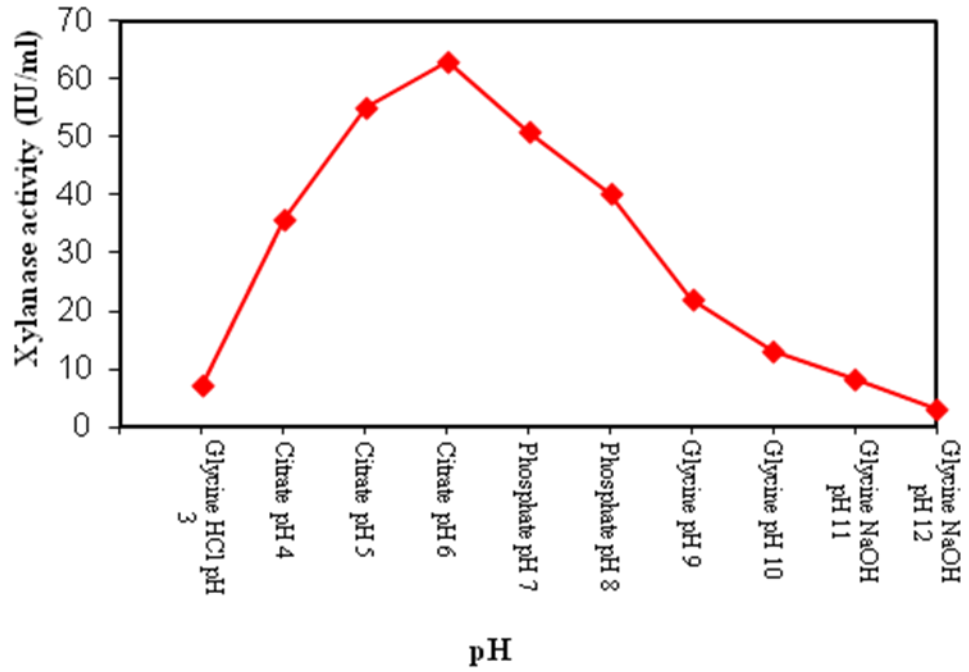


Fig 4. **Effect of different pH on enzyme activity of partially purified xylanase produced by *Bacillus tequilensis* SH0**

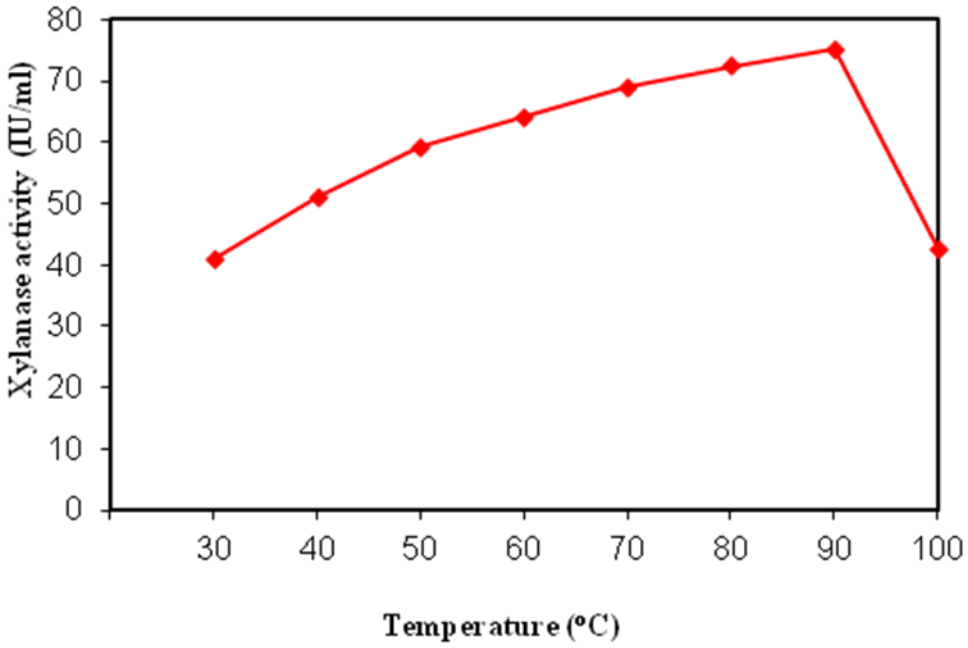


Fig 5. **Effect of different temperature on enzyme activity of partially purified xylanase produced by *Bacillus tequilensis* SH0**

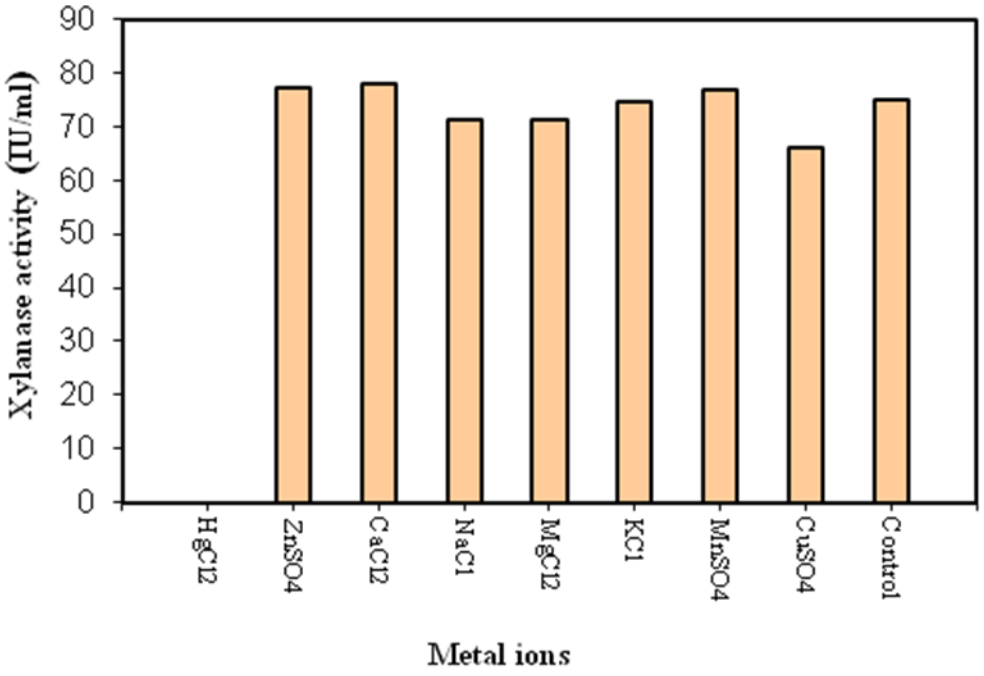


Fig 6. **Effect of different Metal ions on enzyme activity of partially purified xylanase produced by *Bacillus tequilensis* SH0**

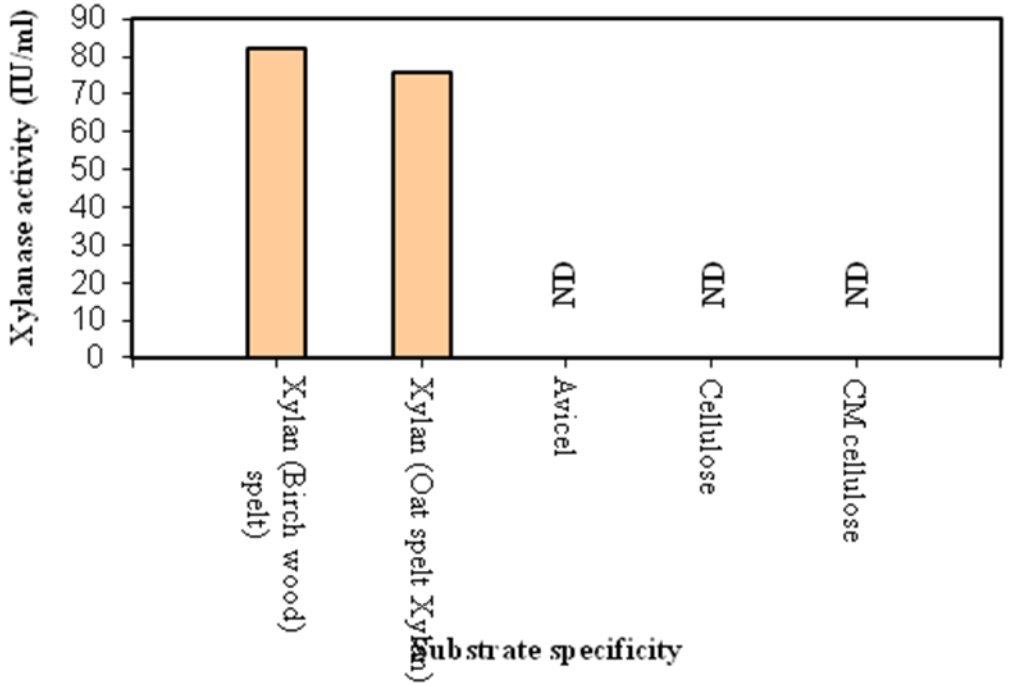


Fig 7. **Effect of different substrate on enzyme activity of partially purified xylanase produced by *Bacillus tequilensis* SH0**

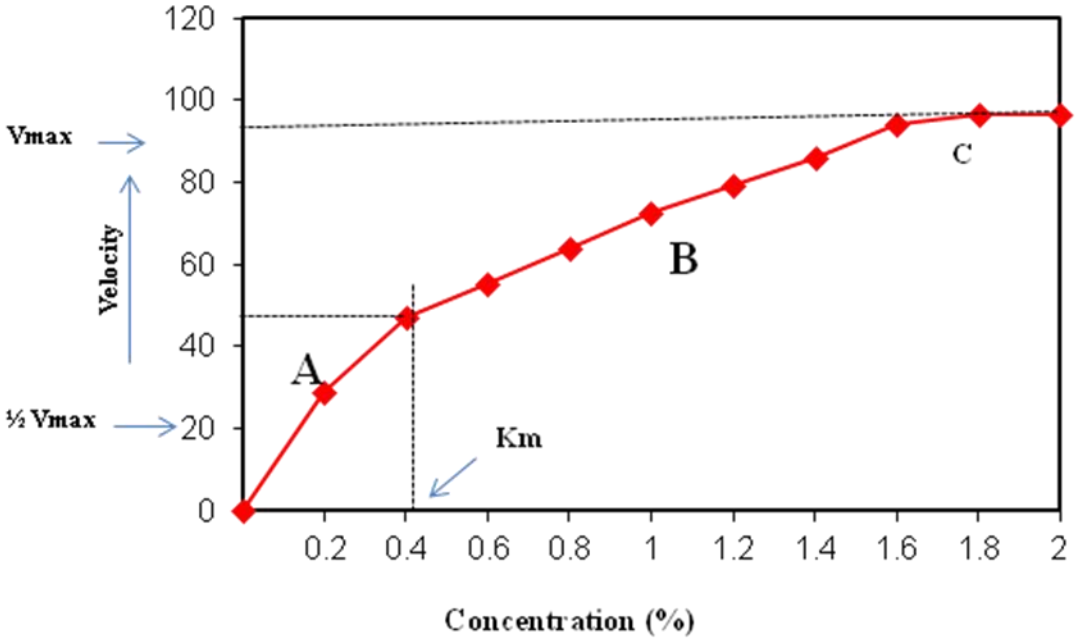


Fig 8. **Effect of different Concentration (%) of substrate on enzyme activity of partially purified xylanase produced by *Bacillus tequilensis* SH0**

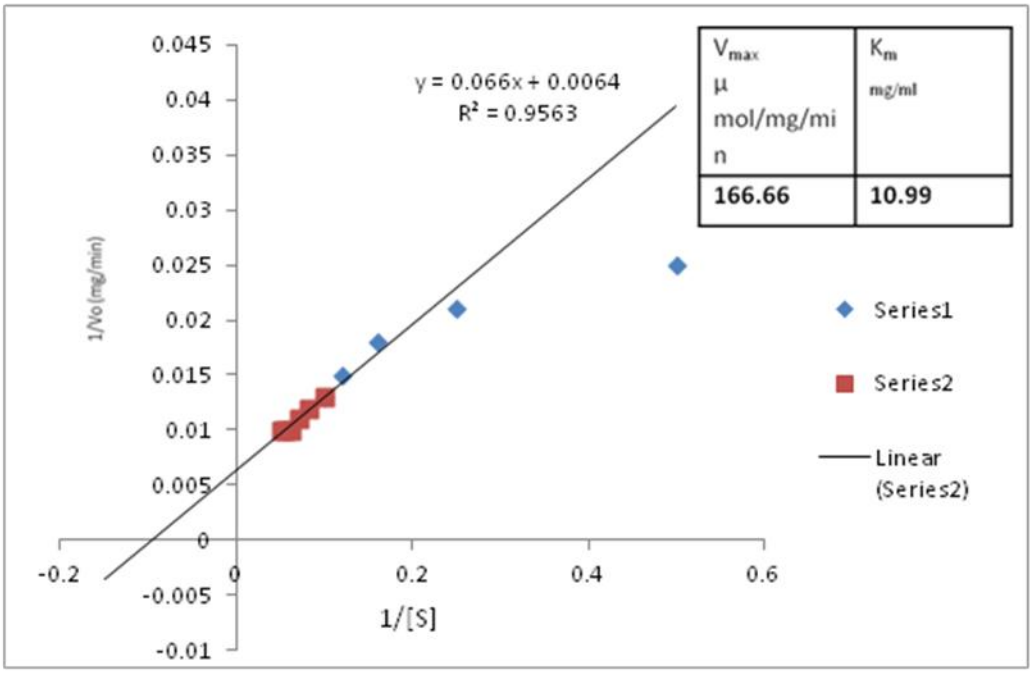


Fig 9 **Lineweaver –Burk double reciprocal plot for the determination of Km and Vmax of partially purified xylanase from *Bacillus tequilensis* SH0**

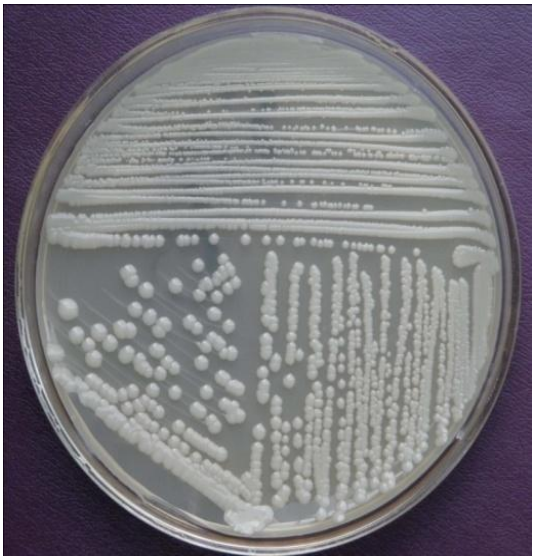
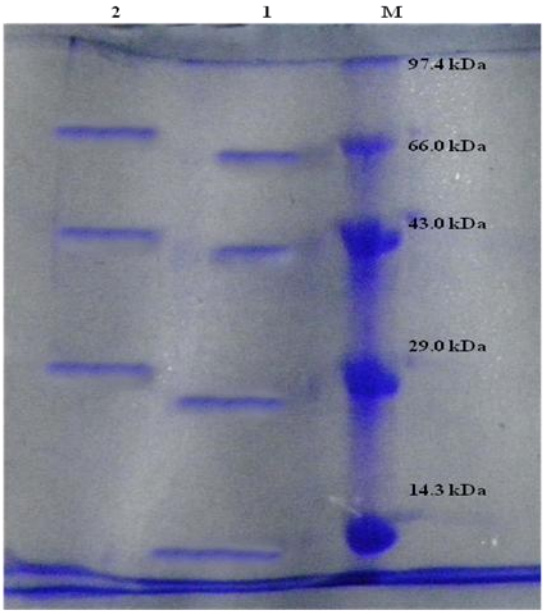


Plate 1. Morphology of *Bacillus tequilensis* SH0 (24h)



Lane 1. = Partially purified xylanase of ion exchange chromatography
Lane 2. = Partially purified xylanase of gel exchange chromatography
M = Marker

Plate 2. SDS PAGE of partially purified xylanase of *Bacillus tequilensis* SH0

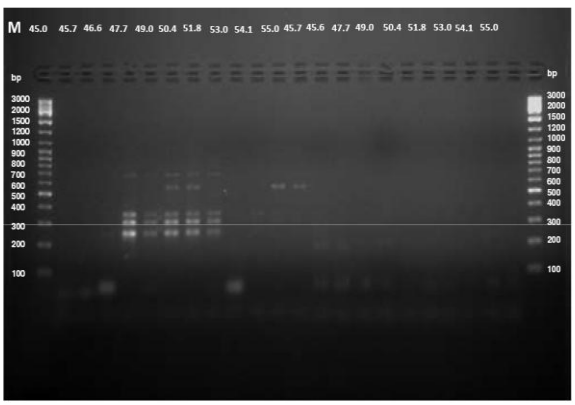


Plate 24. PCR amplification gene encoding of xylanase gene of chromosomal DNA of *Bacillus tequilensis* SH0 at different annealing temperatures

Tables

Table 1. Biochemical characteristics of screened cellulolytic and xylanolytic bacterial isolates

Biochemical tests	**SH0**
Growth on MacConkey	nlf
Indole test	-
Methyl red test	-
Voges Prauskauer test	-
Citrate utilization test	-
H_2S production	-
Gas production from glucose	-
Casein hydrolysis test	+
Gelatin hydrolysis test	+
Starch hydrolysis test	-
Nitrate reduction	+
Catalase test	+
Oxidase test	(+)
Urea hydrolysis	-
Esculin hydrolysis	-
Arginine dihydrolase	+
Tween 20 hydrolysis	+
Tween 40 hydrolysis	+
Tween 60 hydrolysis	+
Tween 80 hydrolysis	+
Acid production	
Raffinose	+
Maltose	-
Galactose	-
Sucrose	-
Dextrose	-
Arabinose	-

Table 2. Purification stages of xylanase produced by *B. tequillensis* SH0

Steps	**Volume (ml)**	**Activity units IU/ml**	**Total* activity**	**Protein** mg/ml**	**Specific*** activity**	**Purification♦ fold**	**Yield♦♦ %**
Crude	100	41.00	4100	1.18	34.75	1.0	100
Lyophilized	20	49.56	4956	1.30	38.12	1.0	100
Ion exchange	2	54.71	5471	0.75	72.95	1.82	57.69
Gel filtration	2	59.35	5935	0.28	211.96	5.30	21.53

* Total activity was determined by the multiplication of volume and activity

** Protein concentration was determined by Lowry's method

*** Specific activity is the activity unit/protein concentration

♦ Purification fold is increase in the specific activity

♦♦ Recovery % is remaining protein concentration as % of the initial protein concentration