



## Understanding of diverse xylanolytic activity of *Bacillus* strains under extremophilic *in-vitro* conditions

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### ABSTRACT

*Bacillus* strains have wide distribution from soil to plants to digestive system of animals including human. Most of its strains produces xylanases enzymes that are being useful in pulp and paper industries as well as biotransformation of hemicellulose to simple carbons molecules. In nature, celluloses and hemicelluloses in plants are most abundant complex carbon source on earth. The xylanases hydrolysis  $\beta$ -1,4-D xylan bonding of the hemicellulose. Among bacteria, xylanases shows diverse activities under environmental extremophile conditions. In this comparative study, xylanase activity was assessed in six *Bacillus* wild-type strains i.e. *B. amylolique faciens*, *B. cereus*, *B. licheniformis*, *B. pumilus*, *B. thuringiensis*, *B. subtilis* 168 under low to high pH and temperature stressed conditions. Cell culture of these strains was raised in BXN (supplemented 0.5% xylan) liquid medium of 20-hrs of incubation with 250 rpm shaking at 37°C. Maximum cell multiplication was observed in *B. pumilus* and *B. cereus* while total proteins in cell culture of *B. cereus* and *B. subtilis* 168. Higher xylanase activity was observed in *B. pumilus* ( $9.669 \pm 0.003 \text{ U.ml}^{-1}$ ) and *B. cereus* ( $8.319 \pm 0.003 \text{ U.ml}^{-1}$ ). The enzyme stability was observed in *B. pumilus* that showed  $1.251 \pm 0.003 \text{ U.ml}^{-1}$  and  $0.420 \pm 0.002 \text{ U.ml}^{-1}$  activities in acidic (pH 4.5) as well as basic (pH 10.0) from low (28°C) to high (50°C) temperature stressed conditions respectively. Comparative analysis of this study suggests, *B. pumilus* and *B. cereus* produces stable xylanase that could be suitable source of industrial point of view. In general, *B. subtilis* 168 is non-pathogenic and best extracellular protein producer. It might be nicer to use *B. subtilis* 168 for the production of stable xylanases and helpful in industrial revolutionization.

**Keywords:** *Bacillus* strains, xylanase, xylan, extremophiles, pulp bleaching, reducing sugars.

### INTRODUCTION

The world is abundant with cellulose as a by-product of photosynthetically fixed free carbon, while its 20% is hemicellulose of autotrophic organisms [1]. Xylan is major component [2,3] and insoluble in nature. Prokaryotes including sessile eukaryotes readily solubilize xylan enzymatically in the useful xylose [4-6], xylitol and ethanol [7,8]. Xylan is a 2<sup>nd</sup> most predominant polysaccharide with conserved backbone of 1,4- $\beta$ -D-xylose residues [9,10]. It contribute one-third renewable organic carbon in nutrition by xylanolytic microbes [10-12]. A large number of bacterial strains with xylanolytic activity have adopted diverse ecological niches either stay in or on host body (phyllosphere), debris, soil, aquatic or even to animal digestive tracts [13]. They are surviving under extreme to moderate conditions with systematic variant xylanolytic activity.

From few decades, importance of cellulases and hemicellulases (especially xylanases) have been increasing due to their application in the production of ethanol from lignocellulosic residues [15,16]. Mainly for the production of hydrolysate from agriculture waste, xylanases have been studied extensively for food processing, animal feed

digestibility, clarification of drinks or beverages, bio-bleaching of paper pulp and plant oil extraction [17,18]. In coming days, xylanolytic enzymes are opening new doors for possible bio-conversion of plant wastes to simple sugars [19].

In pulp and paper industry, *xylanase* remain an important alkalino-thermophilic enzyme [19-21] with exciting cost effective in bleaching processes[22,23]. Increasing demand of *xylanase* is coupled with increasing costs of its production as well as purification from cellulose. Presently, its variable stability under harsh industrial conditions still has questions. Optimization of *xylanase* for high temperature and acido-alkalic resistance has great importance for its industrial application. Target is achievable through the selection of a suitable strain with stable enzyme and moderate strain with high efficiency of secretion of enzymes.

A variety of *xylanases* has been identified among bacterial species. All forms of *xylanase* are not useful at industrial level because of their level of efficiency as well stability under extreme *in-vitro* conditions. To search out an efficient and stable *xylanase* enzyme is the need of time-scenario. Available energy rich natural organic sugar resource are remaining limited with the increasing level of human population in next few decades. Meanwhile, hemicellulose (xylan) is a big and free natural carbon source, which can be useful as functional food additives and *xylanase* as alternative sweeteners production of safe and cheap food with certain beneficial properties[24-27].

In this experiment, *in-vitro xylanase* activity was assessed in six wild type *Bacillus* strains i.e. *B. amylolique faciens*, *B. cereus*, *B. licheniformis*, *B. pumilus*, *B. thuringiensis*, *B. subtilis* 168 to select the bacterial strain able to produce a stable xylanase enzyme. Enzymatic assays were performed to find out the optimal stability of crude *xylanases* of each strain under high to low temperature and pH applied conditions in comparative almost close range to food processing and kraft pulps are bleached in industries.

## EXPERIMENTAL SECTION

### 2.1. Selection of wild-type strains of *Bacillus* and culture medium

For this experiment, six wild-types strains of *Bacillus*(i.e. *B. amylolique faciens*, *B. cereus*, *B. licheni formis*, *B. pumilus*, *B. thuringiensis*, *B. subtilis* 168) were selected. These strains were streaked from glycerol stock freeze at -80°C on LB agar medium. Single colony was cultured for overnight in LB liquid medium. For the production of *xylanase*, each culture was maintained in XBN [xylan in bacterial nutrient basal medium (w v<sup>-1</sup>) 0.5% peptone, 0.1% NaCl, 0.2% K<sub>2</sub>HPO<sub>4</sub>, 0.01% CaCl<sub>2</sub>, 1% MgCl<sub>2</sub>, 0.1% yeast extract, 0.5% oat-spelt xylan and pH 7.0] medium and incubated at 37°C until OD ~1.2 was reached.

### 2.2. Inoculum preparation

The overnight cell cultures were transferred in the 250ml Erlenmeyer flasks that contain 50 ml bacterial nutrient basal medium (as described above). The initial OD<sub>600</sub> was adjusted 0.0025 and incubated at 37°C in a rotary shaker for 20 hrs with 250 rpm shaking speed. Meanwhile, cell concentrations of all cultures of different strains were adjusted with distilled H<sub>2</sub>O to 3% T (transmittance) at 600 nm.

### 2.3. Determination of *xylanase* activity

The *xylanase* (1,4-β-D-xylan *xylano hydrolase*, EC 3.2.1.8. xyl) activity was determined by the quantification of the produced xylose. The crude enzyme in supernatant of cell culture (OD<sub>600</sub> ~1.20) and 0.5 % xylan as a substrate were mixed in 50 mM phosphate buffer (pH 7.0) as the method [28]. Reducing sugars were analyzed with DNS method[29]. *Xylanase* activity was calculated by the determination of xylose produced by enzyme by measuring absorbance at 540 nm from standard curve prepared from 0 to 500 μg xylose.

### 2.4. Preparation of xylan solution

Exact 1.0 g xylan was dissolved in ddH<sub>2</sub>O and mixture was heated to up to boiling point and then stirred upto cool down to room temperature. Its volume was raised to 100 ml with ddH<sub>2</sub>O and stored at 20°C.

### 2.5. Effect of pH and temperature on *xylanase* activity

For the purpose to study the effect of pH on the activity of *xylanase* three buffers i.e. 0.1M sodium acetate buffer(pH 4.5), 0.1M sodium phosphate (pH 8.0), glycine-NaOH (pH 10) were prepared[30]. The reaction mixture of each strain was prepared by mixing 0.9 ml xylan solution with 1 ml of buffer with respective constant pH and in final 0.1 ml of cell culture (crude enzyme) was added. Mixture was incubated at 28-50°C for 40 minutes (Table 1).

## 2.6. Quantification of total proteins in cell culture

The 20 hrs cell culture used as crude *xylanase* enzyme was subjected for determination of total protein contents in according to method reported by Sedmak and Grossberg[31]. Readings were quantified with BSA (Bovin serum albumin) which was used as standard.

## 2.7. Statistical Analysis

The ANOVA (analysis of variance) was performed for various cell growth characters in 6 bacterial strains by F-test. Strain potential mean comparisons was assessed with the DMRT (Duncan's Multiple Range Test) as suggested by[32].

## RESULTS AND DISCUSSION

The *xylanases* are being included among the important industrial enzymes. A number of prokaryotes are able to produce this enzyme including *Bacillus* strains among the bacteria, fungi and yeast. According to Pandey et al.[33], production of *xylanases* in yeast is very low ( $17.1 \text{ IU.ml}^{-1}$ ) while could be hosted in bacterial systems [34-35]. The yield of *xylanase* in bacterial strains is reported around  $41 \text{ IU.ml}^{-1}$  in *Bacillus licheniformis* 77-2 [36] and  $1,120 \text{ IU.ml}^{-1}$  in *Bacillus subtilis* by recombinant *xylanase*[37]. Different fungal strains are also able to produce *xylanase* but reported with very low yield rate. Like as *Aspergillus nidulans* and *Streptomyces cuspidosporus* yields  $40 \text{ IU.ml}^{-1}$  and  $105 \text{ IU.ml}^{-1}$  of *xylanase*[38,39] respectively. Some other strains have really higher rate of production of *xylanase* around  $5,740 \text{ IU.ml}^{-1}$  as in *Schizophyllum commune* but it is obtained in 11 days of incubation[40]. Long incubation of *in-vitro* system is useless or not very economic at commercial level.

*Bacillus* species are best for the production of a lot of number of proteins including *xylanase*. The secretion of enzymes depends on the provision of suitable substrate in culture medium [41,42]. The presence of xylan in the conventional cell culture medium could be relevant and best for the optimal production of enzymes. Even each strain is variable for the secretion of a specific enzyme in the medium because of variation in substrate type, and time of incubation. In this experiment, *Bacillus* wild-types strains (i.e. *B. amylolique faciens*, *B. cereus*, *B. licheniformis*, *B. pumilus*, *B. thuringiensis*, *B. subtilis* 168) were cultured in XBN (xylan in bacterial nutrient basal medium) liquid medium for 20 hrs. Culture incubation is definable for that time with the production of enzyme occurs around in 16hrs of incubation that corresponds to cell growth log phase. The 20 hrs aged cultures were harvested after the measurement of  $\text{OD}_{600}$  and supernatant was subjected for the determination of enzyme activity under various environmental stressed conditions. Maximum cell growth rate was observed in *B. cereus* and *B. pumilus* strains and minimum in *B. thuriengensis*, while total proteins were higher *B. subtilis* 168 and *B. cereus* (Fig 1).

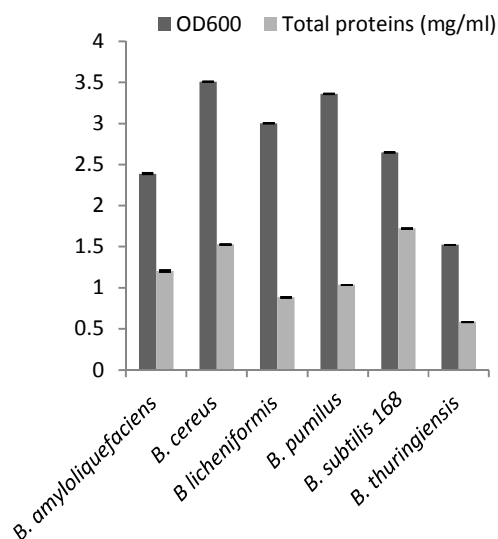


Figure 1. Comparative cell growth rate and level of total proteins secreted in BXN-medium by different *Bacillus* wild-type strains in 20-hrs culture supplemented with xylan as a major bacterial nutritional substrate

Table 1. Comparative effects of pH on xylanolytic activity of *Bacillus* wild-type strains under different incubation temperatures after 40-min

#s	Bacterial strains	Enzyme Activity (U.ml <sup>-1</sup> )			Significance @ 5%
		28°C	37°C	50°C	
<b>a. pH 4.5</b>					
i.	<i>B. amyloliquefaciens</i>	<sup>c</sup> 0.221±0.010	<sup>a</sup> 0.451±0.010	<sup>b</sup> 0.398±0.010	***
ii.	<i>B. cereus</i>	<sup>b</sup> 0.426±0.003	<sup>a</sup> 0.757±0.003	<sup>c</sup> 0.331±0.003	***
iii.	<i>B. licheniformis</i>	<sup>b</sup> 0.121±0.004	<sup>a</sup> 0.207±0.004	<sup>c</sup> 0.092±0.004	***
iv.	<i>B. pumilus</i>	<sup>c</sup> 1.251±0.003	<sup>a</sup> 2.118±0.003	<sup>b</sup> 1.842±0.002	***
v.	<i>B. subtilis 168</i>	<sup>b</sup> 0.020±0.003	<sup>a</sup> 0.121±0.003	<sup>c</sup> 0.014±0.003	***
vi.	<i>B. thuringiensis</i>	<sup>b</sup> 0.012±0.003	<sup>a</sup> 0.022±0.003	<sup>ab</sup> 0.019±0.003	ns
<b>b. pH 8.0</b>					
i.	<i>B. amyloliquefaciens</i>	<sup>b</sup> 0.702±0.010	<sup>a</sup> 1.256±0.010	<sup>c</sup> 1.247±0.009	***
ii.	<i>B. cereus</i>	<sup>b</sup> 6.063±0.003	<sup>a</sup> 8.319±0.003	<sup>c</sup> 5.143±0.003	***
iii.	<i>B. licheniformis</i>	<sup>b</sup> 1.001±0.004	<sup>a</sup> 1.226±0.004	<sup>c</sup> 0.872±0.004	***
iv.	<i>B. pumilus</i>	<sup>b</sup> 7.819±0.003	<sup>a</sup> 9.669±0.003	<sup>b</sup> 8.012±0.002	***
v.	<i>B. subtilis 168</i>	<sup>c</sup> 0.468±0.003	<sup>a</sup> 0.833±0.003	<sup>b</sup> 0.678±0.003	***
vi.	<i>B. thuringiensis</i>	<sup>b</sup> 0.384±0.003	<sup>a</sup> 0.445±0.003	<sup>a</sup> 0.447±0.003	***
<b>c. pH 10.0</b>					
i.	<i>B. amyloliquefaciens</i>	<sup>b</sup> 0.011±0.010	<sup>a</sup> 0.323±0.100	<sup>c</sup> 0.028±0.009	***
ii.	<i>B. cereus</i>	<sup>b</sup> 0.056±0.003	<sup>a</sup> 0.172±0.003	<sup>c</sup> 0.044±0.003	***
iii.	<i>B. licheniformis</i>	<sup>b</sup> 0.033±0.004	<sup>a</sup> 0.987±0.004	<sup>c</sup> 0.022±0.004	***
iv.	<i>B. pumilus</i>	<sup>c</sup> 0.143±0.003	<sup>a</sup> 1.118±0.003	<sup>b</sup> 0.420±0.002	***
v.	<i>B. subtilis 168</i>	<sup>b</sup> 0.037±0.003	<sup>a</sup> 0.119±0.003	<sup>b</sup> 0.035±0.003	***
vi.	<i>B. thuringiensis</i>	<sup>b</sup> 0.010±0.003	<sup>a</sup> 0.025±0.003	<sup>a</sup> 0.019±0.003	**

The variation in cell culture growth was observed in 6 *Bacillus* wild-type strains. This differential cell multiplication may occur due to the sensation of cell to the applied nutritional environment, secretion of substrate degradation enzymes and then uptake of substrate as its nutrition. This difference among the strains can reflect the growth efficiency *Bacillus* strains in the presence of xylan substrate as a source of bacterial nutrition in the medium. Higher rate of cell growth means that high production of *xylanase* that convert the complex form of xylan substrate to simple sugars, which could be easily uptake by the cells as source of nutrition. Best degradation and uptake can be assumed in *B. cereus* and *B. pumilus* because they are growing at higher and optimal rate than other strains.

Meanwhile, xylan is being a complex organic polymer with high molecular mass. It could not penetrate or uptake the xylan into the cell wall of bacterial strains. Both deficiency of simple organic carbon source and xylan complex stress in cell culture are playing a key role in activation of *xylanase* biosynthesis. The secretion of *xylanase* in culture medium is involved in the fragmentation of xylan into xylose or its derivatives (i.e. xylobiose, heterodisaccharides, xylooligosaccharides) and glucose that bacteria easily uptake to fulfill its nutrition need[43,44]. Further, *xylanase* hydrolyze the xylan to xylooligosaccharides and xylose is released by  $\beta$ -*xylosidase* from non-reducing ends of *xylanase* product (xylooligosaccharides). In final, *acetyl esterase* complete the xylan degradation [45,46].

The yield of *xylanase* in the medium and level of its activity both are variable characters among the bacterial species even within *Bacillus* strains also. For the analysis of *xylanase* activity, supernatant of 20 hrs old cell culture of all *Bacillus* strains was used as *xylanase* crude enzyme mixture. However it is not a pure solution with *xylanase*, while it is an indicator overall secretion of cellulolytic activity (Fig 1). This crude enzyme sample was used for the measurement of *xylanase* activity, while *cellulase* may also present in the crude of strains that are able to synthesize this enzyme. According to industrial point of view *xylanase* must be *cellulase*-free because its presence losses the fiber strength [47]. For that culture filtrate could be injected during pulp treatment [48].

Stability or activity of *xylanase* is a specific property of each strain, its high production rate by a specific strain does not mean for it being more active stable than the strains with low level of *xylanase* production. For the characterization of *xylanase* under *in-vitro* temperature and pH stressed conditions, crude *xylanase* was subjected for xylan fragmentation reaction. After 40 min of incubation of reaction mixture, the data obtained is presented in Table 1. Maximum enzyme (*xylanase*) activity was observed in *B. pumilus* at pH 8.0 and 37°C (9.669±0.003 U.ml<sup>-1</sup>), and in *B. cereus* at pH 8.0 and 37°C (8.319±0.003 U.ml<sup>-1</sup>). However, at pH 10.0, a significant instability in enzymatic activities was observed along the changing temperature levels, while stability was retained almost same even at 50°C with pH 8.0.

The past reports about the optimal pH for the activity of *xylanase* enzyme have revealed that xylanolytic activity of enzymes produced by *Bacillus* spp like pH 7.0[48,49] and *Thermoana bacterium* spp [50]. In most cases, if the enzyme is stable at high pH but cannot retain its activity at high temperature and vice versa. In relation to both environmental stresses, maximum activity was observed in *B. pumilus* and *B. cereus* with  $1.251\pm 0.003$  U.ml<sup>-1</sup> to  $0.420\pm 0.002$  U.ml<sup>-1</sup> and  $0.426\pm 0.003$  U.ml<sup>-1</sup> to  $0.044\pm 0.003$  U.ml<sup>-1</sup> at pH 4.5 to pH 10.0, respectively. The produced enzyme by these bacteria was also stable or active at the temperatures ranged from 28°C to 50°C (Table 1, Fig 2). This stability of *xylanase* in these strains is comparable with thermophilic, alkalophilic and acidophilic bacteria. *Xylanase* from latter source could be more suitable for industrial application for paper bleaching processes at different stages without changes of the pH.

The *xylanase* reaction mixtures with different pH conditions were incubated at different temperatures for 40 min, while *xylanase* activity was analyzed for each strain under the controlled and optimal *xylanase* activation conditions. According to the observed results, the enzymes are considered as stable at 40°C reasonably, while at 28°C and 50°C, minimum enzymes activity (except few) was observed. Less activity of enzyme at 28°C could be because of less activate state of enzyme but with defined enzyme structure, while at 50°C less activity might be due to denaturation of enzyme. This inactivation or denaturation of enzyme is not good for industrial purposes, while this problem of enzyme instability can be solved by the addition of 50% glycerol (v/v) solution [51-53]. The results of this experiment confirm that stability of *xylanase* from *Bacillus* strains could be maintained as well as improvable by the supplementation of glycerol. This protective effect of glycerol might be beneficial for other bacterial strains also.

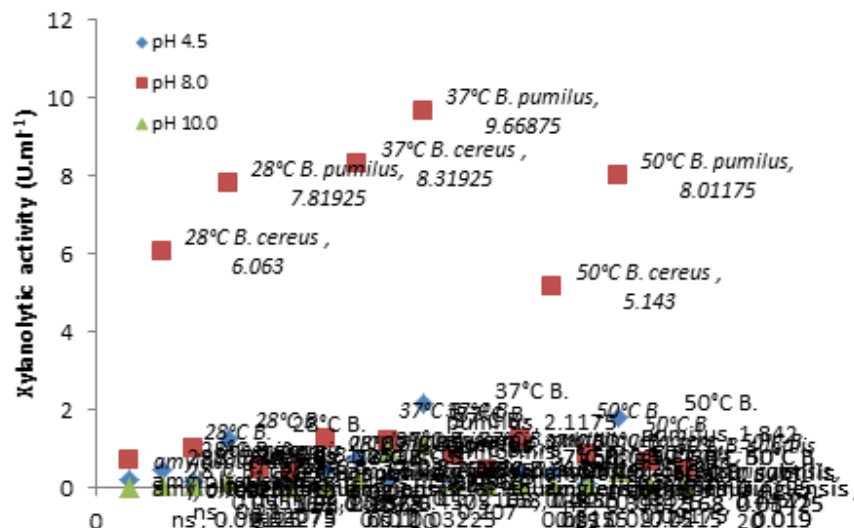


Figure 2. Comparative xylanolytic activity (U.ml<sup>-1</sup>) of *Bacillus* strains under different pH and temperature stressed condition.

## CONCLUSION

Among the selected *Bacillus* strains, some strains showed comparatively low cell multiplication rate. In these strain, not sure for its ability to produce *xylanase*. The *B. cereus* and *B. pumilus* were observed well multiplying *Bacillus* strains in liquid medium supplemented with xylan as a major carbon source. This xylanolytic activity was considered as best under extremophile conditions either at lower to higher temperature or pH conditions ( $\geq 37^\circ\text{C}$ ) or  $\text{pH} \geq 8.0$  respectively (Fig 2). *Xylanase* activity in *B. amylolique faciens* and *B. thuringiensis* under alkalophilic condition as well as at high temperatures less stability in comparisons to other strains. The beneficial *xylanase* activity has shown by *B. cereus* and *B. pumilus* in alkaline medium with higher stability at 50°C. This property of *xylanase* produced by *B. pumilus* and *B. cereus* might be significant for the purpose to use and fulfill the demand of pulp bleaching, food and beverage processing industries under high temperate and alkaline conditions. This study suggests for owing to *xylanase* with novel properties produced by either *B. cereus* or *B. pumilus*, if multiplied in *B. subtilis* would help for the availability of this enzyme at low cost. Even could be more cheaper when *xylanase* is biosynthesized by the utilization of plant residues.

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