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Research Article

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*Xylanase*as exoprotein of *Bacillus* strains: Evolutionary trends in its activities under *in-vitro* extremophillics

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ABSTRACT

Xylanases hydrolysis β-1,4-xylan of hemi-cellulose that exist in diverse forms among bacterial strains. Each strain specific xylanase display variation for xylan hydrolysis under specific environment like as extremophillic in particular to thermophiles, alkaliphiles, and acidiphiles. In this study, activity of xylanases secreted by six Bacillus strains i.e. B. amyloliquefaciens, B. cereus, B. pumilus, B. licheniformis, B. subtilis 168, B. thuringiensis in BXN liquid (supplemented with xylan as major nutrient or carbon source) medium were assayed under thermo-alkali-acidiphilic conditions to search its superior phenotype. In SDS-PAGE, higher total exoproteins in nutritive liquid medium of B. subtilis 168, B. amyloliquefaciens and B. pumilus and cell growth rate remain higher inB. subtilis 168 to B. pumilus and B. cereus. Best xylanolytic activity was shown by B. pumilus, B. licheniformis and B. cereus at neutrophilic conditions while does not remain persistent under alkaline, acidophilic and thermophilic (50°C) conditions except B. pumilus. For industrial point of view, xylanase production from these strains might be useful but required in relatively better quality and quantity. This lack of cellulose free xylanase availability could be permissibly fulfilled by non-pathogenic B. subtilis 168 through recombinant biotechnology. Production of xylanase of B. licheniformis or B. pumilus with B. subtilis 168 as a model system could be usefulfor industrial revolutionization.

Keywords: *Bacillus* strains, *xylanase*, gene sequences, reducing sugars, proteomics, evolutionary tree.

INTRODUCTION

Cellulose and hemicellulose are most prominent natural polysaccharide of photosynthetically fixed carbon by plants, while xylan is a major component of hemicellulose of plant cell wall. Chemically, it is comprised on conserved backbone of 1,4-β-D-xylose residues substituted with glucuronic acid, 4-O-methyl-glucuronic acid and arabinose [1,2]. Xylan contribute one-third renewable carbon source of nutriment and degradable sugars like as xylose, glucose by many xylanolytic microbes [2-4]. This renewable natural organic resources are recycled by a number of biological systems including bacterial strains equipped with xylanolytic activities to release their organic nutrients from plant cells at the time of colonization [4,5]. A number of xylanolytic bacterial strains have adopted diverse ecological niches typically from live plant body (phyllosphere), plant debris, soil, aquatic or even to animal digestive tracts [5]. Although bacterial xylanolytic systems in soil or digestive tracts have been studied in detail, the only limited information is known regarding to pin point variation in xylanolytic activity among the soil-born bacterial strains in relation to their proteomic sequences.

Xylanase(1,4-β-D-xylan *xylano hydrolase*, EC 3.2.1.8. *xyl*) is an important xylanolytic alkalino-thermophilic industrial enzyme[6,7]. Application of these enzymes may be exciting cost effective prospects to replace toxic

chemical based bleaching [8]to bio-bleaching in paper and pulp-industry[9,10]. With the passage of time, demand of bio-bleaching is increasing even purification of *xylanase* from *cellulose* is increasing its production cost. Variable stability as well as its activity under harsh industrial conditions still raising questions. Optimization of *xylanase* for high temperature and acido-alkalic resistance has great importance for industrial application. Target is achievable even phenotype might be variable among bacterial strain to strain. Sum up of beneficial trait in correspond to *xylanase* stability and activity into one heterologous protein against extremophillic conditions can be fruitful.

Improvement of *xylanase* activity has been considerable for its most potential broad variety of applications in bio-bleaching of wood-pulps in paper, textile and food industries. Nowadays, chlorine is a major bleaching agent in food industry especially for production of oligosaccharides from hemicellulose. Xylan may be a cheapest source of functional food additive, when *xylanase* is used alternative sweeteners for the production of safe and cheap food with certain beneficial properties[11-14].Therefore, in this study *in-vitro xylanase* activity of xylanolytic based variable six species of *Bacillus* were assessed at high to low temperature and pH to find out its superior phenotype. A co-relation between enzyme activity and its respective protein sequences is studied. Identification of best phenotype of *xylanase* among these heterologous bacterial species could allow us to introduce a durable *xylanase* as a leading cheapest bio-processer of food, paper, wood-pulp and textile industries[5,15].This way of study for the detection of a useful peptide might be helpful study in future for further technical improvement of *xylanase* or other enzymes.

EXPERIMENTAL SECTION

2.1. Bacterial strains and their culture medium

Six xylanolytic specific *Bacillus* strains namely *B. amyloli quefaciens, B. cereus, B. pumilus, B. licheniformis, B. subtilis* 168 and *B. thuringiensis* were streaked from glycerol stock on LB plate. Overnight (O.N.) grown single colonies of each strain were cultured in LB liquid medium for overnight at 37°C with constant shaking (250 rpm). Main culture for the production of *xylanase*, 0.01ml overnight pre-culture was sub-cultured in bacterial nutrient basal medium [BXN (w v^{-1}) 0.5% peptone, 0.1% NaCl, 0.2% K_2HPO_4, 0.01% $CaCl_2$, 1% $MgCl_2$, 0.1% yeast extract, 0.5% oat-spelt xylan and pH 7.0]. This main culture was incubated at 37°C, 250 rpm for 4hrs[16].

2.2.Analysis of *xylanase* activity

For *xylanase* activity, by-product (reducing sugars) of *xylanases* secreted by each strain was determined in a mixture of crude enzyme supernatant of cell culture (OD_{600} < 1.50) and 1% birchwoodxylan as a substrate in 50 mM phosphate buffer (pH 7.0)[17]. Reducing sugars were measured with DNS method[18]. *Xylanase* activity was calculated from the level of xylose produced by enzyme and it defined in term of units as μM $ml^{-1}min^{-1}$(U. ml^{-1}) in according to xylose standard curve.

2.3. Quantification of *xylanase* in liquid medium

For visual localization of *xylanase* protein and its activity, SDS-PAGE was performed [19,20]. Sample for SDS-PAGE was prepared with supernatant (without culture dilution or protein precipitation) of cell culture by mixing in 2xLDS sample buffer supplemented with reducing agent. Sample mixture was heated for 10 min at 95^0C before gel loading. Protein bands visualized by staining SDS-PAGEin Coomassie brilliant blue R250 (0.025% coomassie blue, 45 ml ddH_2O, 10 ml ethanol, 10ml acetic acid) for 4h and destined in destining solution (67% water, 25% ethanol, 8% acetic acid)for overnight. For photographs, gel was scanned.

2.4. *Xylanase* time-tree homologous modeling

The *xylanase* sequences (available online) from NCBI and UniProtein data-base were selected (Table 1, Fig 2). Protein structural comparison for evolutionary and maximum likelihood relationship among *Bacillus* strains were developed by using UPGMA method[21] and JTT matrix-based model[22,23].Real time method was applied for the calculation of divergence time frame for branching points in topology and branch lengths of inferred tree [24]. Poisson correction method was employed for the computation of evolutionary distance for optimal tree (Fig 4) with sum of 12.9732 branch length. This analysis based on 16 amino acid sequences with omission of gaps as well as missing data. Total 210 positions among the final dataset were used for evolutionary analyses in MEGA6[23].

2.5. Statistical Analysis

The ANOVA (analysis of variance) was performed for data or treatment significance of enzyme activity secreted by the selected *Bacillus* strains by applying F-testas suggested by Gomez & Gomez, [25]. Each treatment was comprised on five replicates.

RESULTS AND DISCUSSION

Bacterial cells adopt their growth with the change in nutrient availability in a systematic way. Yet in doubt that changes in protein expression largely associated with a kind of signaling system for sensing adaptation and simplification of different available environmental nutrient sources. Among the growing cell's environment, proteins are dominant players of cell in terms of its functional and survival with about half of total cell dry mass. Presence or absence even changes in level of most proteins in the medium or even within the cell depend on growth phase and conditions. Many aspects of cell's physiology come to change in according to the available growth medium. These changes seem to be coordinated with growth rate rather than culture conditions or function of proteins [16,26]. In this experiment, growth profiles were determined for six *Bacillus* strains with variant level of growth in basic bacterial BXN nutrient (supplemented with xylan as major carbon source) medium. Maximum growth rate (OD_{600}) was observed in *B. subtilis* 168, *B. pumilus* and *B. cereus*than *B. licheniformis*, *B. amylo liquefaciens* and *B. thuringiensis*. A distinct pattern of total protein contents secreted by these strains in liquid medium. This character was maximum in *B. subtilis* 168 and *B. amylo liquefaciens*(Fig 1).

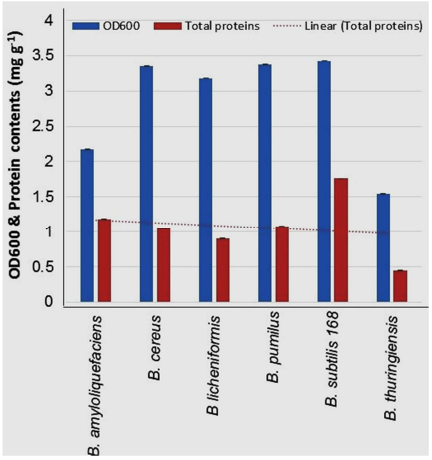

Figure 1. Comparative cell growth rate (OD_{600}) and level of total proteins (mg g^{-1}) secreted by six xylanolytic based *Bacillus* strains in BXN (supplemented with xylan as a major nutritional substrate) liquid medium after 4-hrs of main culture (250rpm, 37°C)

*Bacillus subtilis*168 secretes numerous heterologous enzymes that are involved in degradation of a variety of substrates including agriculture wastes. Being a soil bacterium able to survive under systematic harsh environment. Almost 60% industrial-enzyme available in market are secreted by *Bacillus* species, while secretion of heterologous proteins is often severely hampered especially their origin is gram -ve bacteria or eukaryotes[27-30]. Level of total proteins secreted by *Bacillus subtilis* 168 in liquid medium is relatively high than *B. cereus* and *B. pumilus*(Fig 1,2).

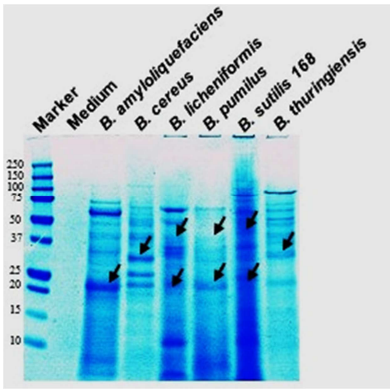

Figure 2. Comparative pattern of total proteins and localization of *xylanase*(*almost comparable totable 1*) secreted in BXN (xylan as a major carbon source) liquid medium after 4-hrs of incubation (250rpm, 37°C) by xynlanolytic *Bacillus* strains

Lane 1 -protein marker, lane 2 liquid medium, lane 3 to 8 -B. amyloliquefaciens[31,32]– baXA (23.3 kDa); B. cereus[33] – (32kDa); B. licheniformis[34,35] – xyl-I (23 kDa) and xyl-II (40 kDa), B. pumilus[36-39] - xyl (23 kDa & 40 kDa); B. subtilis 168 [40,41] –xylA (20.38 kDa) andxylC (43.9kDa) &B. thuringenesis[42] – xyl (28.55kDa).

Table 1. Explanatory structural comparative features of *xylanase* among xylanolytic *Bacillus* strains (information collected from online database).

#s	Strains	Bp	aa	kDa	Characteristics
a.	*B. amyloliquefaciens*	642	213	23.254	*Endo-1,4-Beta-xylanase*
b.	*B. cereus*	765	254	28.553	*Xylanase/chitin/polysaccharide deacetylase*
c.	*B. licheniformis*	642	213	23.315	*Endo-1,4-Beta-xylanase*
d.	*B. pumilus*	525	174	20.431	*Endo-1,4-Beta-xylanase*
e.	*B. subtilis* 168	1076	213	20.38	*Endo-1,4-Beta-xylanase*
f.	*B. thuringiensis*	765	254	28.553	*Xylanase/chitin/polysaccharide deacetylase*

On the basis of cell growth pattern and level of total protein secretion, target *xylanase* enzyme production was characterized with the determination of their *xylanase* activity in a reaction mixture contain 1.0 % xylan as a carbon source. The pH ranged 4.0-10.0 were adjusted to study its effect on the activity of *xylanase* (Fig 3). At lowest and highest pH intensity most the *xylanases* secreted by different *Bacillus* strains showed diverse phenomena. Maximum *xylanase* activity in relation to pH were measured~8.50 and ~12 U. ml^{-1} at pH 8, 37°C in *B. cereus* and *B. pumilus* respectively. At pH 10, 37°C*xylanases* from *B. pumilus* and *B. licheniformis* showed its working state. For the study on effect of temperature on *xylanase* activity, supernatant of all of six *Bacillus* strains were assayed under specific pH conditions at different temperatures ranged from 28°C to 50°C (Fig 3). The crude mixture of x*ylanase* of *B. cereus* and *B. pumilus* strains in liquid culture exhibited maximal activity at 50°C and also well showing activity lower range to high temperature from 40-50°C.

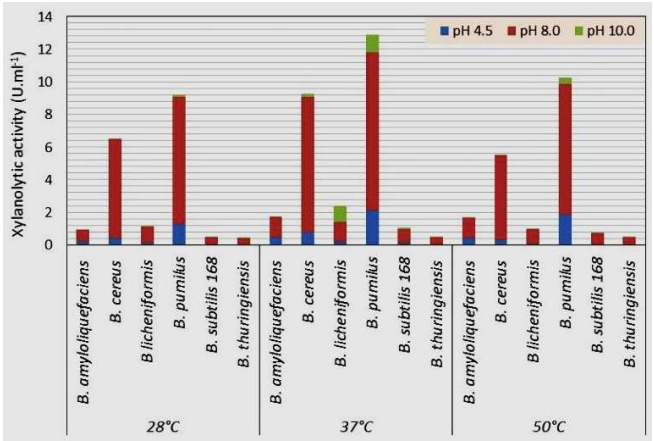

Figure 3. Expressed *xylanase* activity (U.ml^{-1}) by six*Bacillus* strains under different extremophillic (pH and temperature stresses) conditions

Active form of *xylanases* secreted in the liquid medium of *B. licheniformis, B. cereus* and *B. pumilus*was observed from low to high temperature and pH than other strains. It is a best point for their selection as being useful for industrials point of view. Since thermal stability of *xylanase* is a very important fact for its potential applications in several industries. These superior forms of *xylanases* secreted by *B. licheniformis, B. cereus* and *B. pumilus*strain that could be good candidates for industrial applications. Before bring them to industry, it is an important subject to modify them for the purpose to change their host specification for the enhancement of its production rate as an exoprotein with economic down-stream processes.

Despite of level of secretion and activity of the enzymes depends on the applied nutrient substances, while many gaps remain in lack of understanding of connections between activity of specific enzymes and its stability with growth rate. Primarily it is unclear the interaction of level of gene expression or secretion of proteins in medium and growth rate [43,44].Identification of new microbial species with secretion of biomaterial degrading bio-catalyst is remain of great interest since last two decades. The enzymes especially useful for commercial purposes include pulp and paper processing, brewing and production of animal feeds [45-47]. Among the bacterial species a large variety of xylanase are existed. It is resulted because of course of evolution and their genome has changed either via gene acquisition and its loss with the passage of time for the adaptation of a specific applied environment [48]. Probably, with regard to *Bacillus* strains it reflects a specific history for the adaptation of a habitat. They have presented a history of a phylogenetic evaluation for being acidiphiles, alkaliphiles and thermophiles. In contrast to genomic characteristic, the comparative sequence analysis of orthologous proteins based analysis might be less altered but shows principal evolutionary relationships among *Bacillus* strains[49]. Evolutionary phylogenetic and maximum likelihood trees for *xylanases* peptide sequences were computed (Fig 1,A and B respectively).

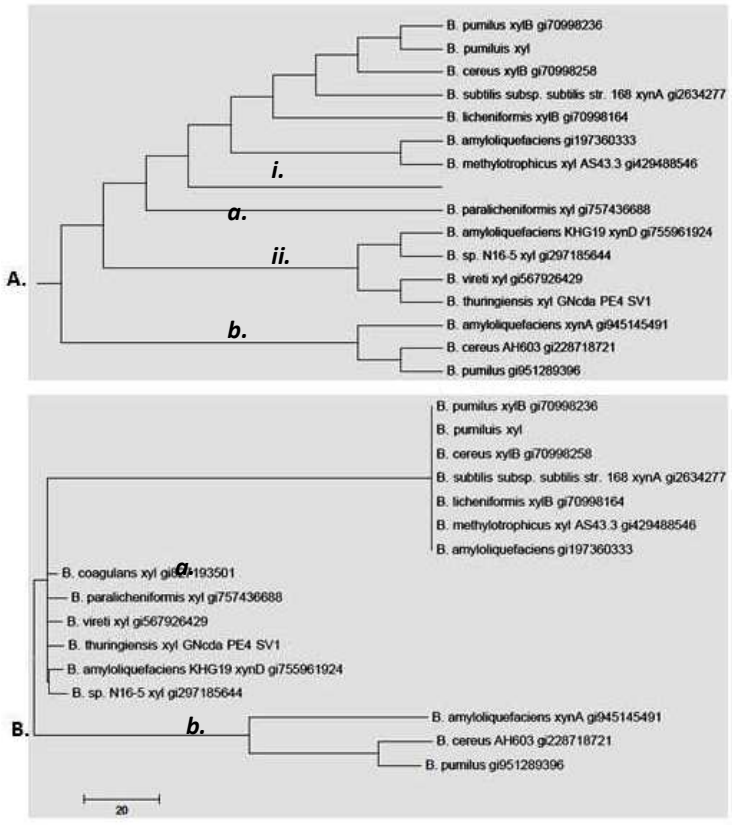

Figure 4. Evolutionary (A) and maximum likelihood (B) phylogenetic trees computed for *xylanases* secreted by six selected xylanolytic based *Bacillus* strains with some other xylanolytic bacterial clusters

According to the computational relationships based on protein sequences of *xylanase* two major groups are observed (Fig 4_A). The *xylanases* of selected six bacterial strains have presented diverse growth rate, total protein secretion (Fig 1), *xylanase* activity (Fig 3) and size of *xylanase* (Fig 2, Table 1). Same diverse phenomena also seen in their

taxonomic grouping on the basis of *xylanase* peptide sequences (Fig 4).Most persistent as well as less persistent *xylanases* were observed in group A (*B. cereus, B. licheniformis, B. pumilus, B. thuringiensis*), while moderate in group B (*B. amyloliquefaciens, B. subtilis* 168 including *xyl*A of *B. cereus* and *B. pumilus*). Group taxa Ais further grouped into two *i*-active (*B. cereus, B. licheniformis, B. pumilus*) and *ii*-non-active (*B. thuringiensis*) forms of *xylanase* low to high temperature and pH. Higher *xylanase* activity may be linked with polypeptide forms in *B. cereus, B. licheniformis, B. pumilus* (Table 1, Fig 2-4). Maximum likelihood tree suggest the multiplication of *xylanase* of group *b*(Fig 4_B) in *B. subtilis* 168 through recombinant biotechnology could increase its production to reach the required amount at industrial level.

CONCLUSION

According to present investigation, active forms of endo-1, 4-β-*xylanase* at high temperature(~50°C)and pH (10.0) to low (28°C and 4.5 *respectively*) observed in *B. licheniformis, B. pumilus* and *B. cereus* strains. These strains are superior with *xylanase* activity, while could be inferior in production of *xylanase* in the medium. It indicates significance for their utility in a number of industrial processes at relatively high temperature and pH and vice versa. To fulfill the huge industrial requirement, pre-requisition is to find out best exoprotein producing system. The *B. subtilis* 168 is one safe and efficient bacterial strain that has ability to produce a large number of heterozygous proteins in the medium. On the basis of the increasing demand of *xylanase*, owing with novel enzyme properties (from *B. licheniformis, B. pumilus* and *B. cereus* strains) and selection of best heterologous protein secretor *Bacillus subtilis*168would be helpful to meet the industrial demand. Cost effects of enzymes could be reduced with usage of agricultural residues as a cheap bacterial nutrition source for the production of *xylanase* enzyme.

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