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Stable activity of extra-cellular *xylanases* and its phylogeny in different *Bacillus* species

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Abstract

The production of cellulose free *xylanase* has increased its demand for waste treatment in pulping and bleaching industrial processes. Four *Bacillus* strains were selected on the basis of their habitation for the production of *xylanase* in xylan based nutrient culture. The secreted *xylanase* was referenced with protein marker for size of 20.3kDa in *B. subtilis* 168, 20.4kDa in *B. pumilus,* 28.6kDa *B. cereus* and 23.3kDa in *B. amyloliquefaciens* on SDS-PAGE. High activity of *xylanase* was observed in *B. pumilus* than *B. amyloliquefaciens* and *B. cereus* under harsh extremophillic culture conditions like as 28°C with pH 4.0 and 50°C with pH 10.0. The phylogenetic tree shows divergence of *xylanase* produce by *B. pumilus* from other 4 *Bacillus* species because of its evolution in proteins sequence for adaptation to severe habitate-conditions. In optimum culture medium, maximum cell biomass produced by *B. pumilus* ($p \leq 0.05$) and significantly higher total extra-cellular protein contents in *B. subtilis* cultures. It might be permissibly nice, if origin of *xylanases* could be excised from *B. pumilus* and produced with *B. subtilis* 168 that could bring a revolution in pulp and paper industry. Preference for *B. subtilis* 168, it is being a safe bacterium for neighborhood.

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Introduction

Extra-cellular *xylanases* (1,4-β-D-xylan *xylano hydrolase*, EC 3.2.1.8. *xyl*) are hydrolytic enzymes, which is being commercially important industrial enzyme. It can withstand at certain level of alkalino-thermophilics conditions but depends on its source organism (Chapla *et al*., 2012; Saleem *et al*., 2012). *Xylanase* is produced as extracellular polypeptide by a variety of microbes including bacteria, fungi and yeast (Sunna and Antranikian, 1997; Tani *et al*., 2014). The substrate of *xylanase* is xylan, while a major component of hemicellulose. On earth, it is the 2nd most abundant polysaccharide in plants after cellulose. Chemically, xylan is a heterogeneous carbohydrate with backbone of 1,4-β-D-xylose residues and substituted with variable branches of glucuronic acid, arabinose and 4-O-methyl-glucuronic acidic complexes (Selvendran, 1985; Burton *et al*., 2010; Scheller and Ulvskov, 2010).

The microbial colonization on the plants has shown xylanolytic activities and proved that xylan is a renewable natural organic source and it is contributing one-third renewable carbon source (Collins, 2005; Dodd and Cann, 2009; Binder *et al*., 2010). On the basis of available carbon sources various bacterial strains have adopted differential xylanolytic activities under diverse ecological niches from phyllo-sphere to digestive tracts of animals (Collins *et al*., 2005; Pinto-Tomas *et al*., 2007; Mirande *et al*., 2010). A variety of microbial cells have shown xylanolytic activities with limited information regarding to their xylanolytic activity and its stability at proteomic level. Production and screening of cellulose free *xylanase* seems to be exciting in favor to achieve cost effective prospects for replacing the toxic chemical based industrial bleaching and pulping processes (Kapoor *et al*., 2008; Saelee *et al*., 2016) to bio-bleaching and bio-pulping (Khristova *et al*., 2006; Saleema *et al*., 2009; Singh *et al*., 2013). With the increasing industrial research, the demand of bio-bleaching is increasing.

The production of *xylanase* in the nutrient culture with xylan as a carbon source is minimizing its down-stream purification of *xylanase* from *cellulose*. It could be helpful in minimizing the xylanase production cost and economic availability to industries.

Multiple forms of *xylanases* are produced by several microorganisms to imply an effective hydrolytic strategy on β-1,4 xylan. Each is specialized with their variable level functional efficiency for the degradation of complex polysaccharides. These specialized *xylanases* may be useful for food processing as well as paper and paper industry (Prade, 1996; Viikari, 1994). Recently, interest in *xylanases* has increased markedly in pulping and bleaching processes due to its *cellulase* free availability (Wong *et al*., 1988). Still chlorine is a major bleaching agent for the production of oligosaccharides from hemicellulose, while xylan is next functional food additive source when *xylanase* is a useful alternate safe and cheap sweeteners with certain health beneficial properties (Suurnäkki *et al*., 1997; Mechaly *et al*., 2000; Ramalingam and Harris, 2010). The findings for alternate to chlorine step is requiring further mega study in the search of stable *in-vitro xylanase* activity among the variable strains of *Bacillus*. In this study, some *Bacillus* strains are selected on the basis of their performance in terms to *xylanase* production. Selection of a suitable active xylanolytic polypeptide under high to low temperature and pH could be helpful or suitable for industrial applications. A mutual co-relation in enzyme activity and its polypeptide sequences is further helpful to pin-out the properties of the selected specific *Bacillus* species for future use.

Materials and methods

Bacterial strains and nutrient cultures

The four strains of *Bacillus* i.e. *B. subtilis* 168, *B. pumilus, B. cereus* and *B. amyloliquefaciens* were cultured from its previous identified glycerol stocks for *xylanase* production. Firstly strains were streaked on LB plates for their activation. Single-cell colony of each strain was cultured in LB liquid medium (Bertani *et al*., 2006) and culture was grown at 37°C with 250rpm shaking for overnight (O.N.).

Cell biomass and production of extra-cellular proteins

For the production of extracellular *xylanase*, main culture was raised by the inoculation of 1% (v/v) overnight seed culture of *Bacillus* species in Erlenmeyer flasks containing 500 ml of liquid

bacterial xylan nutrient (BXN) medium. Briefly, composition of BXN (w v^{-1}) medium comprised on 0.1% yeast extract, 0.5% peptone, 0.5% oat-spelt xylan, 0.01% $CaCl_2$, 0.1% NaCl, 1% $MgCl_2$, 0.2% K_2HPO_4 with pH 7.0 (Haq, 2015). The BXN *Bacillus* cultures grew as submerged fermentation for 24 h at 37°C and 250 rpm. The grown cell culture of each strains was harvested with centrifuge at 5000g for 5min after measurement of cell biomass of each culture with OD_{600}. The supernatant of the cell culture is used as crude source of *xylanase* enzyme.

Quantification of total extra-cellular proteins in liquid medium

Total protein contents in culture supernatant were quantified with Bradford assay (Bradford, 1976) than proteins of supernatant were precipitated for visualization of *xylanase* on SDS-PAGE (Avila-levy *et al.*, 1980; Laemmli, 1970). For precipitation, 10 ml culture supernatant was saturated up to 80% with ammonium sulphate and incubated at 4°C for over-night. The precipitates were separated with centrifuge at 10000 g for 20min than pallet was washed with acetone. After complete evaporation of acetone the pallet was dissolved in 0.5ml deionized distilled water. Finally, samples for SDS-PAGE was prepared by mixing in 2xLDS protein sample buffer (containing reducing agent). The sample mixture was heated at 95^0C for 10min and then cool down in ice before loading the gel. Respective protein bands of *xylanase* were visualized after staining SDS-PAGE in Coomassie Brilliant Blue R250 (0.025% coomassie blue, 10ml ethanol, 45 ml ddH_2O and 10ml acetic acid) for 4h. The gel was destined in destining (67% water, 25% ethanol, 8% acetic acid) solution for gel clearance. Photograph of the stained gel was taken with scanner.

Measurement of xylanase activity

The *xylanase* activity was measured with the estimation of reducing sugars released from hydrolysis of xylan (birch-wood xylan, Fluka, USA). The *xylanase* reaction was conducted by following the method of Bailey's group (Bailey *et al.*, 1992). Briefly, the reaction mixture was raised by adding 0.4ml phosphate buffer (50mM, pH 7.0), 0.2 ml supernatant (a crude protein mixture) containing extracellular *xylanases* and 0.9ml of 1% birch-wood xylan as a substrate. This xylanolytic reaction mixture was incubated for 30 min at 37°C. The reaction was stopped with 1.0ml DNS (3%, w/v). Reducing sugars were measured by following the DNS method (Breuil and Saddler, 1985). After adding DNS, solution was boiled in water-bath for 5 min. Absorbance of reaction was measured at 540nm with UV-Visible spectrophotometer (Hitachi, Tokyo, Japan). *Xylanase* activity was calculated against D-xylose standard curve and it is defined in term of units as μM ml^{-1} min^{-1} (U. ml^{-1}).

Effect of temperature and pH on activity of xylanase enzyme and its stability

For the determination of thermo-stability and acido-alkalic-stability of *xylanase* a series of reactions were conducted. Different levels of temperature (28°C, 37°C and 50°C) and pH (acidic, basic and neutral pH were maintained with sodium acetate buffer - pH 4.0-5.0, phosphate buffer - pH 6.0-8.0 and carbonate-bicarbonate buffer - pH 9.0-10.0) were adjusted. The supernatant contain crude proteins all *Bacillus* species were mixed in buffers of variable pH and incubated at different controlled temperatures in water bath separately as shown in Fig. 2. After 1 h, *xylanase* activity reaction was conducted for the estimation of reducing sugar contents as mentioned above in *xylanase* activity measurement.

The xylanase homologous time frame

The homozygosity in *xylanase* was drawn on the basis of its protein sequences (available online) on NCBI and UniProtein data-base of each selected *Bacillus* strain (Table 1). The structural comparisons among these *xylanase* sequences were developed with UPGMA method to determine the evolutionary trend (Sneath and Sokal, 1962). Further real time method was also applied on same *xylanase* sequences to calculate the level of divergence time frame on the branching points in topology as well as branch lengths on inferred tree (Tamura *et al.*, 2012). The real time analysis is based on the heterologous amino acid sequences. The comparative amino acid sequence gaps and missing or un-available data were omitted.

After data screening, the final dataset of *xylanase* polypeptide sequences was subjected to MEGA (7.0) software for evolutionary trend analysis (Tamura *et al.*, 2013).

Statistical Analysis

In this experiment, total 9 treatments (based on pH and temperature) were employed on 4 strains of *Bacillus* genus. Each treatment was comprised on three replicates. On final, the data of each treatment was subjected for ANOVA (analysis of variance) by applying F-test as suggested by Gomez & Gomez (Gomez and Gomez, 1984). It was performed to sketch out a trend of data significance for the extracellular *xylanase* enzyme activity of four selected *Bacillus* strains under differential environmental stressed conditions.

Results and discussion

Nutritional and physical conditions of habitat never remain suitable for the survival of living organisms. It changes time to time either with weather or due to co-habitants. All organism including bacteria are adopting their growth patterns systematically in response to the availability of nutrition at their localities. The continuous unbalanced environmental conditions causes to change the level and type of cellular as well as extra-cellular proteins expression. Such alterations in protein based biological functions are associated with a kind of signaling system of the cell. These senses leads to adopt the available differential nutrient sources with the biosynthesis of efficient proteins. Always proteins remain dominant players of cell for its survival with normal physiological functions under changing environmental conditions.

The identification of cellular functional proteins that remains stable and shows activity under extremophillic conditions could be useful in industrial point of view. Like as *xylanases* are produced by wide range of microbes due to their high metabolic diversity (Sanghi *et al.*, 2010). Strains of *Bacillus* genus are surviving from normal to severe ranges of nutritional as well as physical conditions. They could be exploited in the interest of *xylanase* production for industrial uses (Bajaj and Singh, 2010). Meanwhile, industrial processing conditions are harsh in terms of extreme temperature, pH and might be due to the presence of inhibitors for enzyme's activation and its stability.

Four diverse habituated *Bacillus* strains namely *B. subtilis* 168, *B. pumilus, B. cereus* and *B. amyloliquefaciens* are being positive for *xylanase* activities (Table 1). Optimization of growth parameters remain major step for enzyme production in industrial enzymology (Adhyaru *et al.*, 2014). Like as screening of enzyme stability under various physical conditions of fermentation which usually depends on temperature and pH of cell culture including cations in order to meet industrial basics industries (Mayende *et al.*, 2006; Motta *et al.*, 2013; Khusro *et al.*, 2016).

A number of studies have been conducted on many *Bacillus* species for their capability and efficiency for hydrolysis of lignocellulosic biomass. However, *Bacillus* species are capable for *xylanase* production when agricultural wastes supplied as sole carbon sources. Most exploited species for the production of *xylanase* is *Bacillus subtilis* while very few reports on other three *Bacillus* species for its biosynthesis as well as extremophillic stability comparatively.

Bacterial population is producing a variety of *xylanase* which depends on the availability of niche type. Evaluation of *xylanase* production as well as its stability can be a challenge to discern *xylanase* activity on birch wood xylan as its substrate. Maximum biomass production was observed in *B. pumilus* and *B. cereus*, while minimum in *B. amyloliquefaciens*.

This significant ($p<0.05$) difference could be best indication for the activity and likeness of *xylanase* for xylan (Fig. 1). A dramatic difference in the production of extra-cellular proteins, especially *B. subtilis* secreted higher ($p<0.05$) than other *Bacillus* species (Table 1, Fig 1).

Table 1. Comparative pattern of *xylanase* polypeptide in *Bacillus* species on SDS-PAGE secreted in BXN liquid culture after 4 hrs.

#s	*Bacillus* strains	kDa	Characteristics		References
a.	*B. subtilis* 168	20.38	*Endo-1,4-Beta-xylanase*		Blanco, Vidal, Colom, & Pastor, 1995
b.	*B. pumilus*	20.431	*Endo-1,4-Beta-xylanase*		Bim & Franco, 2000
c.	*B. cereus*	28.553	*Xylanase/polysaccharide deacetylase*		Habib, 2009
d.	*B. amyloliquefaciens*	23.254	*Endo-1,4-Beta-xylanase*		Xu, Liu, & Dai, 2015

* In SDS-PAGE, M: Marker protein, W: Water, 1-4 are extracellular protein samples of Bacillus strains and arrows indicate expected xylanase band of each strain.

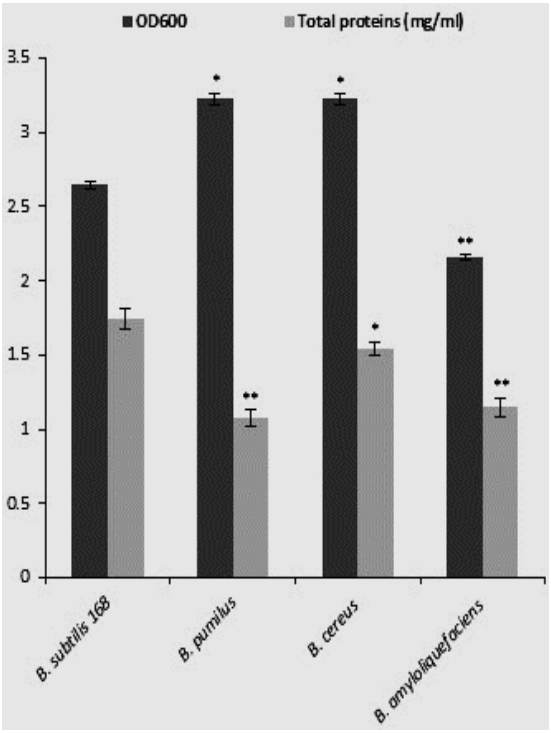

Fig. 1. Cell growth rate (OD_{600}) and total protein contents (mg ml^{-1}) in BXN (xylan as a substrate) liquid culture of *Bacillus* strains after 4-hrs.

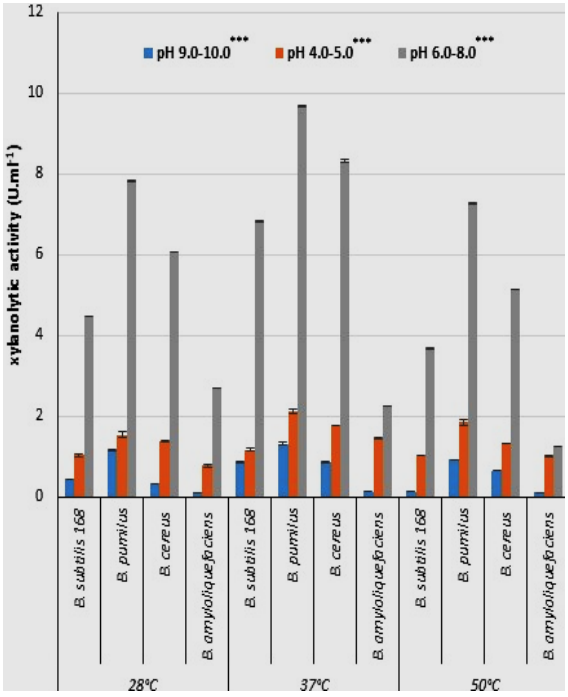

Fig. 2. Comparative xylanolytic activity (U.ml^{-1}) under different pH and temperature stressed culture conditions on *Bacillus* strain.

The cell biomass as well as protein secretion levels by various *Bacillus* species depend on cell's growth phase and its culture conditions. A number of cell's physiological parameters changes in correspondence to available cell growth nutrition.

Culture medium composition seem to coordinate with rate of increase in cell biomass rather than with functional activities of proteins (Haq 2015; Barenholz *et al.*, 2015). For industrial purpose stability of *xylanase* is one among other most important factors. Crude *xylanase* in supernatant secreted by *Bacillus* sp. shows stable activity under various temperature and pH conditions (Fig 2).

At neutral pH (7.0-8.0), maximum xylanase activity was observed all species. It was significantly higher in *B. pumilus*. With the decrease in pH (acidic), higher activity was measured in the same species at all the three levels of temperature (28°C, 37°C and 50°C). However in basic reaction mixtures (with pH 9.0-10.0) more than 90% *xylanase* activity was decreased in comparisons to neutral reaction mixtures. With the alterations in pH as well as temperature, there might be denaturation of proteins including *xylanases*. Even high temperature with basic pH has shown severe impact on *xylanase* activity as well as its stability (Saxena *et al.*, 2007; Raul *et al.*, 2014). The enzyme stability trend especially in *B. pumilus* and *B. cereus* in reaction mixtures regulated at high temperature

and lower to higher pH could be suitable for industrial point of view than other *Bacillus* species as reported by Marques *et al*., (1998). These bacterial strains have shown optimal activity at 37°C and also able to retained enzyme activity under acid to basic as well as lower to high temperature conditions.

On the basis of pH and temperature tolerant *xylanase* activity could be further evaluated on the basis of their proteomic uniqueness. *Bacillus* strains were analyzed for phylogeny (Zhi *et al*., 2015). *B. pumilus* occupy its position in a separate group than others strains of Bacillus genus (Fig 3). In according to multi-locus sequence typing (MLT) as in previous studies have proved that specific sequence types may be over-expressed under non-host conditions (Bergholz, Noar, and Buckley 2011; Goto and Yan, 2011). However, *xylanase* from *B. cereus* also performed stable activity but less stable than *B. pumilus* and phylogenetic tree has identified its position among the less stable *xylanase group* (Rhodes and Kator, 1988; Lin *et al*., 1996). These findings on the basis of *xylanase* activity and phylogenetic group of its host strains of *Bacillus* (Herzer *et al*., 1990; Wirth *et al*., 2006), even the branch length differences among them suggests a pattern of differential degrees of host adaptation (Touchon *et al*., 2009). Greatest probability of host adaptation to its ecological niche remain parallel to its physiological stability.

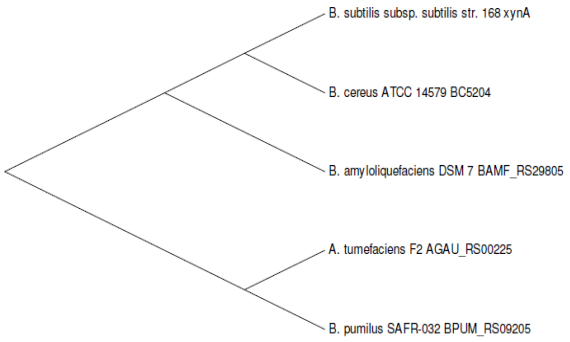

Fig. 3. Phylogenetic tree of *xylanases* polypeptide of *Bacillus subtilis* 168 with some selected strains of *Bacillus* genus cluster. For log value of maximum likelihood was selected at evolutionary invariable 0.235% sites for 5 amino acids in total of 213 positions (Tamura *et al*. 2013).

Conclusions

The potential 4 species have shown their ability to hydrolyze birch wood xylan. The *Bacillus pumilus* has shown significantly higher cell biomass and *Bacillus subtilis* extra-cellular protein secretion. Xylan hydrolysis efficiency and protein secretion potential are two drastic phonemic characteristics existed in two variant bacterial species. *B. pumilus* showed high stability under acidic to basic as well as low to high temperature than other *Bacillus* species. In spite to that *B. cereus* also shown nice activity but less than *B. cereus* and also it got position among the less stable strains through phylogenetic analysis. This study suggests, a forward way how to select a strain from the population of all bacterial strains on the basis of their extra-cellular protein production potential and stability of secreted products. The selection of stable enzyme could resist harsh industrial conditions. If isolation of *xylanase* enzyme from *B. pumilus* and production in *B. subtilis* may be helpful to reach the industrial requirement. It is safe and efficient bacterial strain that has ability to produce maximum number of heterologous proteins in the medium.

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