

## Molecular Characterization of *Thermostable xylanase* Producing *Thermoactinomyces vulgaris* HSB4

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(Received: 11 July 2016; accepted: 29 September 2016)

This study will evaluate *Thermoactinomyces vulgaris* HSB4 (KR698305) thermophilic microbial isolate for production of thermostable xylanase isolated from water spring in Aljouf, Saudi Arabia. Culture parameters for xylanase production by *T. vulgaris* were optimized. The enzyme was concentrated by spinning through a centriplus, centrifugal ultrafiltration Millipore membrane with a total yield of 25%. The relative molecular mass of this xylanase determined by SDS-PAGE was ranging from 67-43 KDa. This extracellular xylanase therefore could be defined as thermostable with distinct properties that make the enzyme applicable for different industrial purposes.

**Keywords:** Thermophilic, *Thermoactinomyces vulgaris* HSB4, Xylanase.

Xylan is the primary constituent of hemicellulose in grasses used for commercial cattle feed much there is increased interest in searching for effective hydrolases for xylan which might serve as feed additives to improve the absorption of nutrients by ruminants and maximize production efficiency of dairy cows and beef cattle. Currently enzyme treatment of feed is a widely accepted and utilized practice in the highly regulated poultry industry, but feed enzyme technology is developing in the cattle industry (Beauchemin *et al.* 2006). But more research could potentially change this.

Xylan is a heterogeneous natural polymer and thus requires diverse xylanases (Collins *et al.* 2005) for complete breakdown to monomers. Xylan

degrading enzymes include those that degrade the main chain which are endo- $\beta$ -1,4-xylanase (EC 3.2.1.8) and  $\beta$ -xylosidase (EC 3.2.1.37); and side chain-cleaving enzymes that include  $\alpha$ -glucuronidase (EC 3.2.1.139),  $\alpha$ -L-arabinofuranosidase (EC 3.2.1.55), acetylxyylan esterase (EC 3.1.1.72) and feruloyl esterase (3.1.1.73) (Biely 1985; Biely 2003; Ryabovaa *et al.* 2009; Beg 2001). Xylanases are produced by bacteria, fungi and actinomycetes (Beg *et al.* 2001; Dutta *et al.* 2007). Among xylan degrading bacteria, the strains of *Aeromonas*, *Bacillus*, *Bacteroides*, *Cellulomonas*, *Microbacterium*, *Paenibacillus*, *Ruminococcus* and *Streptomyces* have been reported (Dutta *et al.* 2007).

This study will evaluate several thermophilic microbial isolates for production of thermostable xylanases. Selected microbial isolates will then be characterized using 16s rRNA gene sequence analysis of the V3 region of genomic DNA. Further studies will focus on purification and characterization of selected unique xylanases.

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## MATERIALS AND METHODS

### Study sites and sampling

Water and sediment samples were collected in sterile containers from the springs in Aljouf region, Saudi Arabia (Figure 1), Kasr Kaff (31°22'37.10"N, 37°35'28.71"E) and Ain Hawas (31°23'28.13"N, 37°29'58.86"E). The temperature ranged between 45-50 °C. All samples were kept at +4 °C until reaching the laboratory for the microbiological investigation and the physicochemical properties according to standard methods (APHA, 1995).

### Isolation of culturable bacteria

For enumeration and isolation from water and sediments, the samples were placed on four different media using a standard spread plate technique and incubated at 45°C, 55°C and 65 °C for 2-7 days. The media used to enrich aerobic thermophilic bacteria were nutrient agar (peptone 0.5 %, beef extract 0.3 %, NaCl 0.5 % and agar 1.8 %), and LB-medium (1 % tryptone, 0.5 % yeast extract, and 1 % NaCl). After incubation, plates were continually observed for the appearance of bacterial and the total viable count was recorded for each sample on the different media used. Single colonies with distinct morphology were selected for phenotypic and genotypic characters.

### Screening for xylanase activity

All isolates were screened for xylanase activity as accomplished by using xylan agar. Xylanase-producing bacteria were identified by a clear halo around the colony. Xylanase activity was determined by incubating 0.1 mL of culture filtrate with 0.9 mL of 1% (w/v) oat-spelt xylan (Sigma Chemical Co., St. Louis, Mo.) in 0.05 M citrate buffer, pH 5.0 at 50°C for 30 min (Bailey *et al.*, 1992). The reaction was terminated by adding 1 µmL of dinitrosalicylic acid (DNSA) reagent. The reaction mixture was then placed in a boiling water bath at 100°C for 5 min and thereafter cooled to room temperature (Miller, 1959). Absorbance was read at 540 nm using a PYE UNICAM SP6-250 visible spectrophotometer. Xylose (Sigma Chemical Co., St. Louis, Mo.) was used as standard. Xylanase activity was expressed as 1 µmol of reducing sugar (xylose equivalent) released per minute per milliliter of enzyme solution.

## Identification of Bacteria

### Phenotypic studies

Bacterial isolates were first Gram stained and examined under the light microscopy. Sporulation was examined under compound microscope. Catalase and oxidase tests were performed. The isolates were then subjected to some physiological tests on nutrient agar plates for 1-3 days: growth at 37, 45, 65 and 70 °C; in the presence of 0.5, 1, 3 and 6% (w/v) NaCl at 55 °C; at pH 8, 9 and 10 at 55 °C. Citrate utilization was determined by the appearance of a change in colour of citrate agar medium. The fermentation of various sugars was examined by changes in the colour of Andrade peptone water containing 0.1% Andrade indicator. The sensitivity to antibiotics was tested at 65 °C for 24 h in TY agar media. For the determination of oxygen requirements the strains were grown in TY broth for 24 h. All morphological, physiological and biochemical characterization tests were performed according to the methods described by Harley and Prescott (2002).

### Molecular methods

#### 16S rDNA gene sequencing

The 16S rRNA gene sequence obtained from the strain HSB4, by MacroGen Company in Korea (<http://www.macrogen.com>), was compared with other bacterial sequences by using NCBI BLASTn (Altschul *et al.*, 1997) for their pair wise identities. Phylogenetic trees were constructed in MEGA 5.0 version ([www.megasoftware.net](http://www.megasoftware.net)) using neighbor joining (NJ) algorithms. The sequence used in the analysis was deposited in GenBank, EMBL in Europe and the DNA Data Bank of Japan with an accession number KR698305.

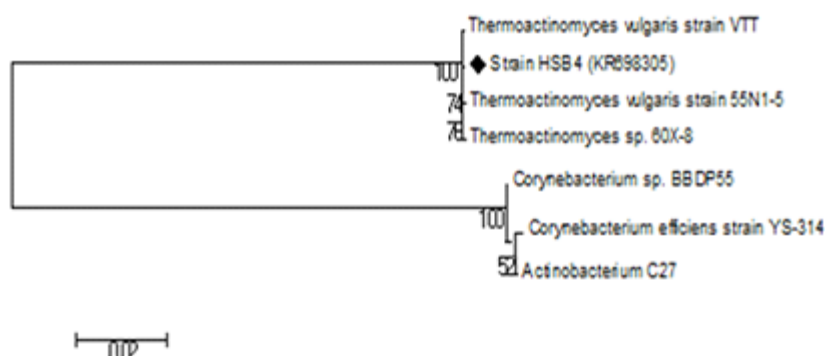
## RESULTS AND DISCUSSION

Taxonomic affiliation of the 16S rRNA sequences of the isolate HSB4 was retrieved from classifier programme of Ribosomal Database Project II version 9.0 (<http://rdp8.cme.msu.edu/html>). The 16S rRNA sequence of the isolate was blasted using megablast tool of GenBank (<http://www.ncbi.nlm.nih.gov>). This revealed that the isolate was a *Thermoactinomyces vulgaris* strain. Representatives of maximum homologous (93~98%) sequences of each isolate were obtained

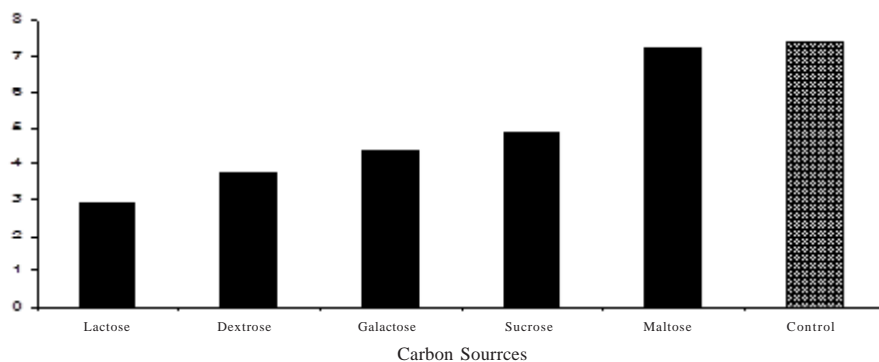
from seqmatch programme of RDPII and were used for the construction of phylogenetic affiliation. The phylogenetic tree was constructed using only the culturable *Thermoactinomyces vulgaris* strains (Fig. 1). Based on the morphological, physiological, and biochemical characteristics and phylogenetic analysis, the isolate HSB4 was identified as

*Thermoactinomyces vulgaris*.

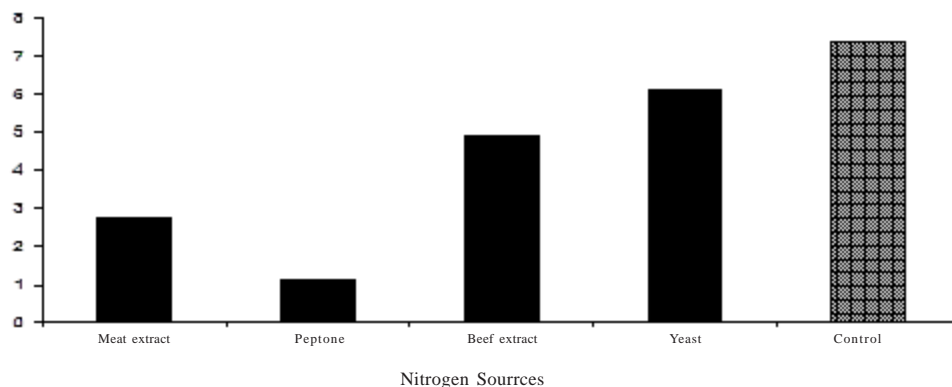
The production of xylanase was decreased in media containing carbon sources other than starch. Particularly, maltose was found to be the best carbon source (Fig. 2). The xylanase activity was approximately 1% lower than that of the control. Xylanase production by *T. vulgaris*



**Fig. 1.** Neighbor-joining tree (partial sequences ~950 bp) showing the phylogenetic relationships of archaeal 16S rRNA gene sequences of isolated strain, *Thermoactinomyces vulgaris* HSB4 (KR698305), to closely related ( $S \geq 98\%$ ) sequences from the GenBank database.



**Fig. 2.** Effect of different carbon sources on xylanase production. The cells were cultivated in the production medium. All the data are given mean  $\pm$  SD,  $n = 6$ .

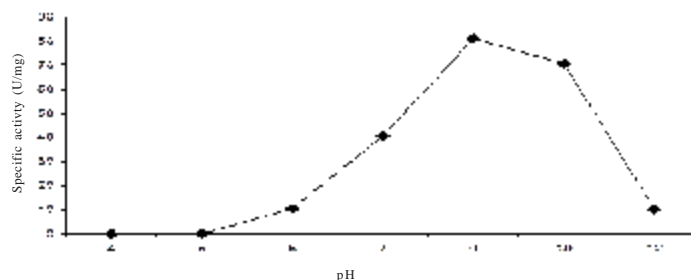


**Fig. 3.** Effect of different nitrogen sources on xylanase production. The cells were cultivated in the production medium. All the data are given mean  $\pm$  SD,  $n = 6$ .

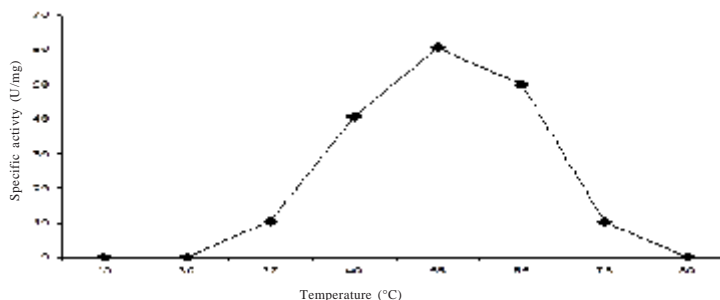
HSB4 was constitutive since the enzyme synthesis took place not only in the presence of xylan but also in other carbon sources. On the optimization process, yeast extract was found to be the most promising nitrogen source for the production of xylanase e followed by beef extract (Fig. 3).

pH is one of the most important factors that determine the growth and morphology of microorganisms as they are sensitive to the

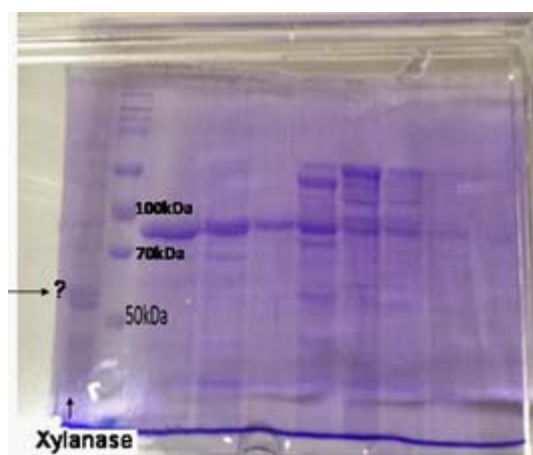
concentration of hydrogen ions present in the medium. pH is known to affect the synthesis and secretion of xylanase just like its stability. The results suggested that there is a stimulation of enzyme synthesis at pH 7.0 and optimum production was obtained at pH 9.0 (Fig. 4). In acidic medium, the results are insignificant and increasing the initial pH of the medium above pH 9.0 resulted in decrease of the xylanase production. This



**Fig. 4.** The effect of pH on xylanase production. The values are mean  $\pm$  SD, n = 6.



**Fig. 5.** The effect of temperature on xylanase production. The values are mean  $\pm$  SD, n = 6.



**Fig. 6.** SDS-PAGE profile of purified xylanase production.

showed that the bacterium required an alkaline pH for the production of xylanase.

The effect of temperature on xylanase production is related to the growth of the organism. Hence the optimum temperature depends upon whether the organism is mesophilic or thermophilic. The effect of temperature on bacterial growth and xylanase production was studied. The production of enzyme and bacterial growth was determined at different temperatures ranging from 30 to 80°C and optimum enzyme production was obtained at 55°C (Fig. 5). After 40°C the enzyme production increased dramatically. This organism did not produce xylanase at 27°C although it grew well at this temperature.

The culture supernatant (459 mg protein) was used as a starting material for the purification of amylase from *T. vulgaris* HSB4. The enzyme was

purified by a three-step strategy including ammonium sulphate precipitation and dialysis, ion-exchange chromatography, and gel filtration. The enzyme was concentrated by spinning through a centriplus, centrifugal ultrafiltration Millipore membrane with a total yield of 25%. The relative molecular mass of this xylanase determined by SDS-PAGE was ranging from 43-67 KDa (Fig. 6).

#### ACKNOWLEDGEMENTS

This work was funded by the Deanship of Scientific Research (DSR), Aljouf University, Aljouf, KSA, under grant no. (35/278, 2015). The authors, therefore, acknowledge with thanks DSR technical and financial support.

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