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Production of novel halo-alkali-thermo-stable xylanase by a newly isolated moderately halophilic and alkali-tolerant *Gracilibacillus* sp. TSCPVG

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ABSTRACT

An aerobic xylanolytic *Gracilibacillus* sp. TSCPVG growing at moderate to extreme salinity (1–30%) and neutral to alkaline pH (6.5–10.5) was isolated from the salt fields near Sambhar district of Rajasthan, India. β-xylanase (18.44 U/ml) and β-xylosidase (1.01 U/ml) were produced in 60 h in the GSL-2 mineral base medium with additions of (in g/l) Birchwood xylan (7.5), yeast extract (10.0), tryptone (8.0), proline (2.0), thiamine (2.0), Tween-40 (2.0) and NaCl (35) at pH 7.5, 30 °C and 180 rpm. The β-xylanase was active within a broad salinity range (0–30% NaCl), pH (5.0–10.5) and temperature (50–70 °C). It exhibited maximal activity with 3.5% NaCl, pH 7.5 at 60 °C. It was extremely halotolerant retaining more than 80% of activity at 0 and 30% NaCl and alkali-tolerant retaining 76% of activity at pH 10.5. The acetone precipitated xylanase was highly stable (100%) at variable salinities of 0–30% NaCl, pH of 5.0–10.5 and temperatures of 0–60 °C for 48 h. HPLC analysis showed xylose, arabinose and xylooligosaccharides as hydrolysis products of xylan. This is the first report on hemi-cellulose degrading halo-alkali-thermotolerant enzyme from a moderately halophilic Gram-positive *Gracilibacillus* species.

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1. Introduction

Xylan, being the major structural and second most abundant polysaccharide in plant cells, accounts for approximately 1/3rd of all renewable carbon on earth. It is a heteropolysaccharide with a homopolymeric chain of 1,4,β-D-xylosidic linkages with the backbone comprising of *O*-acetyl, α-L-arabinofuranosyl, α,1,2-linked glucuronic or 4-*O*-methylglucuronic acid [1]. The complete hydrolysis of xylan requires endo-1,4-β-D-xylanases (EC 3.2.1.8), β-D-xylosidases (EC 3.2.1.37), α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139) and acetylxylanesterases (3.1.1.72) [1]. Xylanases are produced by plethora of organisms including bacteria, algae, fungi, protozoa, gastropods and arthropods [1].

Extremophiles are organisms evolved to live in a variety of extreme environments like deep sea hydrothermal vents, hot springs and hyper saline environments. They can be classified as thermophiles, psychrophiles, acidophiles, alkalophiles, halophiles and others [2]. Halophiles have gained attention due to their extensive mechanism of adaptation to extreme hypersaline environments and differentiated based on salinity into non-halophile (<1.2% NaCl), slight halophile (1.2–3%), moderate halophile (3–15%) and extreme halophile (>15% NaCl) [3]. Their exoenzymes exhibit unique structural and biochemical characteristics. The intriguing stabilities of these enzymes under extreme high saline conditions are still unknown [4]. It is speculated that this could be due to the presence of relatively large number of negatively charged amino acid residues on their surface to prevent precipitation [5]. However, hydrolytic enzymes from halophiles are not only interesting from the basic scientific viewpoint but, they may also be of potential interest in many industrial and biotechnological applications, owing to their stability and activity at low water levels [6,7].

Thermo-alkalophilic xylanases have many applications in paper and pulp industries, as they can be used as an alternative to toxic chlorinated compounds [1]. Xylanases of highly thermo-stable nature and tolerance to low pH are beneficial to animal feeds [1]. In addition, thermo-stable xylanases can be effectively used with cellulases to hydrolyze the lignocellulosic biomass generated in bioethanol production [8]. There is much interest in the production of xylanase under halophilic conditions, as there are very few studies on this enzyme from halophilic bacteria. Xylanase production has been shown in only four halophilic species viz., halophilic archaeon *Halorhabdus utahensis* [9], a novel Gram-negative gamma Proteobacterium strain SX15 [10], strain CL8 [11] and *Chromohalobacter* sp. TPSV 101 [4].

In the present study, the production of an unusual halo-thermo-alkali-stable xylanase by a moderately halophilic and alkali-tolerant bacterium, *Gracilibacillus* sp. TSCPVG, isolated from

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the soil samples collected from the salt fields in the Sambhar district of Rajasthan, India is reported.

2. Materials and methods

2.1. Bacterial strain isolation and culture conditions

Gracilibacillus sp. TSCPVG was isolated in our laboratory from soil samples collected from the salt fields in the Sambhar district of Rajasthan. The isolate was maintained on Great Salt Lake-2 (GSL-2) medium [10] of the following composition (g/l): NaCl, 35 g; Birchwood xylan, 7.5; citric acid, 0.5; tryptone, 2; yeast extract, 2; $MgSO_4{\cdot}7H_2O$, 10; KCl, 5; NH_4Cl, 2; $NaHCO_3$, 1; KH_2PO_4, 0.5; trace metal solution, 2 ml/l; metal chloride solution, 5 ml/l. The trace metal solution consisted of (/l): HCl (32%), 10 ml; $FeCl_2{\cdot}4H_2O$, 2 g; $CoCl_2{\cdot}6H_2O$, 250 mg; $MnCl_2{\cdot}4H_2O$, 100 mg; $ZnCl_2$, 70 mg; H_3BO_3, 6 mg; $Na_2MoO_4{\cdot}2H_2O$, 40 mg; $NiCl_2{\cdot}6H_2O$, 70 mg; $CuCl_2{\cdot}2H_2O$, 2 mg; $AlCl_3{\cdot}6H_2O$, 60 mg; $Na_2Wo_4{\cdot}2H_2O$, 6 mg. The metal chloride solution (g/l): $CaCl_2{\cdot}2H_2O$, 100; $FeCl_2{\cdot}4H_2O$, 8; $MnCl_2{\cdot}4H_2O$, 8; 10 mM HCl. The pH was adjusted to 7.5 with 0.1 N NaOH prior to autoclaving. The trace metals and metal chloride solutions were autoclaved separately and added after cooling. This medium was used for all further studies on the production of xylanase. Cultures were grown at 30 °C at 180 rpm in 250 ml Erlenmeyer flask containing 50 ml of medium. The strain was identified based on the typical cultural, morphological and biochemical characteristics and 16S ribosomal nucleic acid (rRNA) gene sequencing. The 16S rRNA gene sequence was submitted to GenBank with the accession number FJ526393.

2.2. Effect of culture conditions on TSCPVG for maximum growth and xylanase production

2.2.1. Effect of NaCl, pH and temperature

TSCPVG was grown at different NaCl concentrations (0–30%) with pH 7.5 at 30 °C; different pH (6–10.5) with 3.5% NaCl at 30 °C and at different temperatures (20–40 °C) with pH 7.5 and 3.5% NaCl. As xylan caused some turbidity in the medium, cell growth was measured by total cell protein [12]. Increase in the total cellular protein and extracellular protein was estimated every 6 h by Lowry's method using bovine serum albumin (BSA) as standard [13]. Extracellular xylanase activity was measured in the culture supernatant. Residual xylan in the fermentation broth over time was estimated by phenol sulphuric acid method using xylose as standard [14].

2.2.2. Effect of carbon, nitrogen sources, vitamins, amino acids, surfactants, salts and metal ions

For investigating the effect of alternative carbon sources on xylanase production, birchwood xylan was replaced with 0.75% (w/v) monosaccharides, disaccharides and polysaccharides.

To investigate the effect of alternative nitrogen sources on xylanase production, yeast extract (0.2%), tryptone (0.2%) and ammonium chloride (0.2%) were replaced by 0.2% of different organic and inorganic nitrogen sources. A combination of yeast extract with other nitrogen sources like tryptone was also tested.

The effect of amino acids, vitamins and surfactants were studied by adding them separately (after filter sterilization) at a final concentration of 0.2% (w/v).

Effect of salts was tested by replacing 3.5% NaCl with 3.5 or 7% of KCl, Na_2SO_4, CH_3COONa, $NaNO_3$, and $C_6H_5O_7Na_3$. Metal salts like $CaCl_2$, $MgSO_4$, $MnSO_4$, $ZnSO_4$, $CoCl_2$, $FeSO_4$ and $NiCl_2$ were tested at 0.1% (w/v).

The medium for enhanced cell growth and β-xylanase production was obtained using different combinations of the components selected from the above screening. In all the cases cultures were grown in duplicates and mean was taken. SD values were less than 0.05%.

2.2.3. Xylanase assay

Xylanase (EC 3.2.1.8) activity was measured by the method of Bailey [15]. Cell-free supernatants were collected after centrifuging for 20 min at 10,000 rpm at 4 °C and analyzed immediately. One milliliter of reaction mixture consisting of 500 μl of 1% birchwood xylan (Sigma) prepared in 100 mM sodium phosphate buffer, pH 7.5 and 3.5% NaCl, 400 μl of the buffer and 100 μl of appropriately diluted enzyme was incubated for 10 min at 60 °C. The amount of reducing sugar released was quantified using 3,5-dinitrosalicylic acid (DNS) with xylose as standard. One unit of xylanase was defined as the amount of enzyme required to liberate 1 μmol of xylose equivalent per min under the specified conditions. The relative xylanase activity was defined as the percentage of the maximum xylanase activity detected in the assay. The above standard assay conditions for xylanase was followed for all further studies.

The assay conditions for xylanase were tested with respect to NaCl, pH and temperature. The effect of NaCl was determined at various salinities (0–30% NaCl) at pH 7.5 and temperature at 60 °C; pH using the following buffers [sodium citrate buffer (pH 5.0–5.5), sodium phosphate buffer (pH 6.0–7.5) TRIS buffer (pH 8.0–8.5) and glycine–NaOH buffer (pH 9.0–10.5)] with 3.5% NaCl and temperature at 60 °C and at various temperatures (10–80 °C) with 3.5% NaCl and pH 7.5.

2.2.4. Enzyme activities in the culture supernatant

β-D-Xylosidase (EC 3.2.1.37) activity was determined by measuring the amount of *p*-nitrophenol released from *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX Sigma) according to Pinphanichakarn et al. [16]. The reaction mixture containing 50 μl of 50 mM *p*NPX, 350 μl of 100 mM sodium phosphate buffer, 3.5% NaCl, pH 7.5 and 100 μl of appropriately diluted enzyme solution was incubated at 60 °C for 10 min. The reaction was stopped by adding 2.5 ml of 0.5 M Na_2CO_3 and absorbance of the released *p*-nitrophenol was measured at 405 nm. For some substrate specificity tests, *p*NPX was replaced by *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) or *p*-nitrophenyl acetate (*p*NPA). Endoglucanase (carboxymethylcellulase, CMCase) (1,4-β-D-glucan 4-glucanohydrolase: E.C. 3.2.1.4), filter paper cellulase (FPase) and Avicelase was assayed as per Saddler and Chan [17]. α-Amylase (EC 3.2.1.1) was assayed by the method of Kiran and Chandra [18] and alkaline protease (EC 3.4.21.19) as per Gessesse and Gashe [19]. Assays were done in triplicates. SD values were less than 0.05%.

2.3. Partial purification of xylanase by acetone, ethanol and ammonium sulfate precipitation

Fifty milliliters of each of cell-free supernatant collected at 60 h of growth was partially purified by precipitating with 2 volumes of cold acetone, 2 volumes of 95% cold ethanol and 26 g of ammonium sulfate (80% saturation) respectively. The precipitate obtained after each precipitation step was centrifuged for 20 min at 10,000 rpm at 4 °C. The pellet so obtained was dissolved in 10 ml of 100 mM phosphate buffer, pH 7.5, containing 3.5% NaCl, and was further tested for xylanase activity and total protein. Ammonium sulfate fractionated xylanase was further dialyzed against the same buffer to remove the ammonium sulfate. Assays were done in triplicates. SD values were less than 0.05%.

2.3.1. Gel electrophoresis and activity staining

Native PAGE (12.5%) was performed as described by Laemmli [20]. Zymogram for xylanase was carried out as described by Breccia et al. [21].

2.3.2. Effect of NaCl, pH and temperature on stability

The stability of the acetone precipitated enzyme was tested by pre-incubating at different NaCl concentrations (0, 1, 3.5, 5, 10, 15, 20, 25 and 30%) at pH 7.5 for 48 h at 4 °C; buffers (pH 5.0–10.5) with 3.5% NaCl for 48 h at 4 °C; and at different temperatures (4, 30, 40, 50, 60, 65 and 70 °C) at pH 7.5 and 3.5% NaCl. The residual activity was determined under the standard assay conditions and activity at 0 h was taken as 100%.

2.3.3. Effect of metal ions

The effect of various metal ions ($CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, $FeSO_4$, $CoCl_2$, $MnSO_4{\cdot}4H_2O$, $ZnSO_4{\cdot}7H_2O$, $HgCl_2$, $NiCl_2$, $Pb(NO_3)_2$ and $CuSO_4$) on the acetone precipitated enzyme was studied by adding 2, 5 or 10 mM concentration of metal ions to the assay mixture. Thereafter, the residual enzyme activities were determined under the standard assay conditions.

2.3.4. Effect of EDTA, surfactants and detergents

Ethylenediamine tetraacetic acid (EDTA), phenylmethylsulfonyl fluoride, *N*-bromosuccinamide, β-mercaptoethanol and dithiothreitol (10 mM), and 0.1% of sodium dodecyl sulfate (SDS), Triton X-100, Tween 20, Tween 40 and Tween 80 were added separately to the acetone precipitated enzyme and pre-incubated at 30 °C for 30 min. Activity in the absence of metal ions and effectors was taken as 100%.

2.3.5. Analysis of hydrolytic products

Partially purified xylanase (5 U) was incubated with 1% (w/v) birchwood xylan in 100 mM sodium phosphate (pH 7.5 and 3.5% NaCl) at 60 °C. At different time intervals the aliquots were removed and filtered (0.2 μm). Monomeric and oligomeric reaction products were analyzed by HPLC with a Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm; Agilent, USA) at 45 °C using 5 mM sulphuric acid as mobile phase at a flow rate of 0.6 ml/min and the products were detected using refractive index detector maintained at 45 °C [22]. Authentic chromatographic grade xylose and XOS (X2–X4) (Megazyme) were used as standards for identification and quantification of the XOS in the reaction mixture.

3. Results and discussion

3.1. Characterization of bacterial strain

Halophilic microorganisms are considered to have biotechnological potential similar to that of other extremophilic organisms [3]. The moderately halophilic and alkali-tolerant xylanolytic bacterium *Gracilibacillus* sp. TSCPVG reported here appears to be unusual, as there are no published reports on the production of halophilic xylanase by *Gracilibacillus* species. The outstanding feature of this strain is that it produced reasonably high levels of extremely halotolerant, alkali-tolerant and moderately thermostable xylanase.

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