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A novel cold-tolerant *Clostridium* strain PXYL1 isolated from a psychrophilic cattle manure digester that secretes thermolabile xylanase and cellulase

G. Akila, T.S. Chandra *

Department of Chemistry, Biochemistry Laboratory, Indian Institute of Technology, Madras, Chennai 600 036, India

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Abstract

A *Clostridium* strain PXYL1 was isolated from a cold-adapted cattle manure biogas digester at 15°C. It could grow at temperatures as low as 5°C up to 50°C with highest specific growth rate at 20°C and is a psychrotroph. It produced extracellular hydrolytic enzymes namely xylanase, endoglucanase, β-xylosidase, β-glucosidase and filter paper cellulase, all of which had maximal activity at 20°C. The induction of xylanase was highest on birch wood xylan (37 IU{mg protein}$^{-1}$) compared with xylose (1.11 IU{mg protein}$^{-1}$), cellobiose (1.43 IU{mg protein}$^{-1}$) and glucose (no activity). The xylanase was thermolabile with a half-life of 30 min at 40°C and 8 min at 50°C but stable for over 2 h at 20°C. The crude enzyme released reducing sugars (1.25 g l^{-1}) from finger millet flour at 20°C, while commercial food-grade xylanases showed no hydrolysis at this temperature. This is the first report of a *Clostridium* strain growing at 20°C and producing an array of xylanolytic and cellulolytic enzymes, possessing low temperature optima of 20°C, which may facilitate degradation of plant fibre under low-temperature conditions.

Keywords: Psychrotroph; *Clostridium*; Xylanolytic; Thermolabile; Xylanase; Cellulase; Birchwood xylan; Finger millet flour; Reducing sugars

1. Introduction

Psychrophilic and psychrotolerant microorganisms are most widely distributed in nature where 80% of the biosphere and 90% of the marine environment have temperatures below 5°C [1]. These organisms can grow at low temperatures, but not at higher temperatures, probably due to the thermolabile nature of certain essential cellular components such as enzymes, regulatory factors, and/or membrane components [2]. Characterisation of enzymes from psychrotrophs has indicated that they are cold-active, exhibit optimal activity towards lower temperatures (10–25°C) and are heat labile, whereas in some psychrotrophs they are cold-active and heat-labile but exhibit optimal activity around 35°C, a temperature at which enzymes from mesophilic organisms exhibit optimal activity [3]

Cold-tolerant organisms have applications in bioremediation, industrial or sewage treatments, wood pulp and paper industries and as environmental biosensors in cold areas [1]. Some of the obvious applications for their psychrophilic enzymes include detergent additives (proteases, lipases and cellulases in cold-wash); use in the textile industry (cellulases for stone-washing and biopolishing) and the food industry as alternatives to existing enzymes for brewing; a source of polyunsaturated fatty acids as food additives [4]. Processes using psychrophilic enzymes can be rapidly and economically terminated by heating [4].

Cold-adapted xylanolytic microorganisms in particular, have not been intensively investigated although a significant part of the hydrolysis of plant cell wall polymer takes place under permanently low temperature conditions such as marine environments, Antarctic climates and hilly regions of tropical and temperate countries [5]. The plant cell wall fibre cellulose and hemicellulose are recalcitrant to microbial attack due to the lignin seal and require synergistic activity of multiple enzymes of cellulase and xylanase. Therefore biodegradation of lignocellulose is slow under mesophilic and thermophilic conditions and more

* Corresponding author. Tel.: +91 (44) 22578245; Fax: +91 (44) 22578241.
E-mail addresses: chandra@iitm.ac.in (T.S. Chandra), tschandra@lycos.com (T.S. Chandra).

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so at temperatures below 20°C. Very few cold-tolerant aerobic xylanolytic and cellulolytic microbes have been isolated and characterised. Information on anaerobic cold-tolerant bacteria is scanty and confined to mycoplasma like bacteria, lactic acid bacteria [3], and methanogens [6] from Antarctic lakes. Psychrophilic anaerobic clostridia are mostly of the food-spoilage and food-poisoning types [7–9] or from marine sediments [10]. To our knowledge, there are no reports of cold-tolerant xylanolytic clostridia or characterisation of their cellulolytic and hemicellulolytic enzyme systems.

While investigating the adaptation of a mesophilic cattle manure digester (37°C) to perform at lower temperatures (15°C) in a tropical Indian climate, we set up enrichments to isolate anaerobic xylanolytic microflora from a 15°C adapted digester. This yielded an unusual clostridial strain having an optimum growth temperature of 20°C and secreting thermolabile extracellular cellulase and xylanase that were maximally active at 20°C. The salient features of the unusual organism and its enzymes are reported here.

2. Materials and methods

2.1. Organism

A mesophilic cattle manure digester was adapted by gradual decrements to 15°C from an initial temperature of 37°C. Enrichments for anaerobic xylanolytic organisms were set up with the digester slurry as inoculum using 0.5% birchwood xylan (Sigma, USA) as carbon source and 0.1% yeast extract as growth supplement in a mineral base medium with trace elements as per Murthy and Chandra [11] in serum vials under anaerobic conditions. Single colonies growing at 20°C in roll tubes, were separated and checked for purity by microscopic examination. One of the purified cultures was identified as *Clostridium* sp. designated as strain PXYL1.

2.2. Growth measurements

Growth of PXYL1 in 0.5% xylan medium was followed by cell protein measurement [12]. Substrate utilisation was followed by determining total sugars (Dubois et al. [13]). To determine the effect of carbon sources on growth, xylan was replaced by appropriate substrates.

2.3. Enzyme assays

Cells were grown at 20°C, centrifuged at $15\,000 \times g$ at 4°C for 30 min and the cell-free supernatant was used as the source of extracellular enzymes. Xylanase (1,4-β-D-xylan xylanohydrolase: E.C. 3.2.1.8) was assayed as reported by Bailey et al. [14], but with a reaction time of 1 min, using 1% birchwood xylan in citrate buffer (50 mM, pH 5.3). Endoglucanase (carboxymethylcellulase, CMCase) (1,4-β-D-glucan 4-glucanohydrolase: E.C. 3.2.1.4) was assayed using carboxymethycellulose (CMC) as substrate (reaction time, 30 min) and filter paper hydrolysis (FPase) using filter paper [15] and a reaction time of 1 h. The liberated reducing sugar was measured by the dinitrosalicylic acid method of Miller [16]. One unit (IU) of enzyme activity was the amount of enzyme that liberated 1 μmol of xylose or glucose per minute under the assay conditions. β-Xylosidase (E.C. 3.2.1.37) and β-Glucosidase (E.C. 3.2.1.21) were assayed using 10 mM *p*-nitrophenyl-β-D-xylopyranoside (reaction time, 20 min) and *p*-nitrophenyl-β-D-glucopyranoside (reaction time, 20 min) as substrates respectively and measuring the *p*-nitrophenol released at 430 nm [17]. Amylase was assayed by the method of Bernfield [18] and protease as per Gessesse and Gashe [19].

2.4. Partial characterisation of the crude xylanase and cellulase

The effect of pH on xylanase and cellulase activity in the cell-free culture broth was determined at 20°C in sodium citrate buffer (50 mM; pH 3–6), sodium phosphate (50 mM, pH 6–8) and glycine–NaOH (50 mM, pH 9–10) The effect of temperature was determined in the range of 10°C-60°C at pH 5.3. Thermostability was determined by incubating the enzyme solution at 20°C to 50°C for different time periods. In all cases, residual activities were determined and expressed as % of the highest activity.

2.5. Hydrolysis of cereal flour

The hydrolysis of whole grain flour of finger millet (*Eleucine coracana*) was carried out at 20°C and 30°C using the crude PXYL1 xylanase. The reaction mixture contained flour 2 g, 75 ml citrate buffer (50 mM, pH 5.3) and 25 ml crude enzyme in cell-free culture broth (47.2 IU ml^{-1}). Reaction was carried out for 6 h; aliquots were drawn at various time intervals and assayed for reducing sugars. Hydrolysis was also carried out using well-known commercial food-grade xylanases – (a) ABCL Xylanase (Advanced Biochemical Company Ltd., Thane, India; 8186 IU g^{-1}) (b) Deerland Pentosanase/Xylanase (Deerland Corporation, 20 000 U g^{-1}) at 20°C and 30°C for comparison after appropriate dilutions such that the enzyme activity in the reaction mixture were (a) 50 IU ml^{-1} and (b) 49.56 IU ml^{-1} respectively.

All data shown are averages of triplicate assays with SD within 10% of the mean value.

3. Results and discussion

The newly isolated anaerobic spore-forming bacillus from the cold-adapted cattle manure digester was identi-

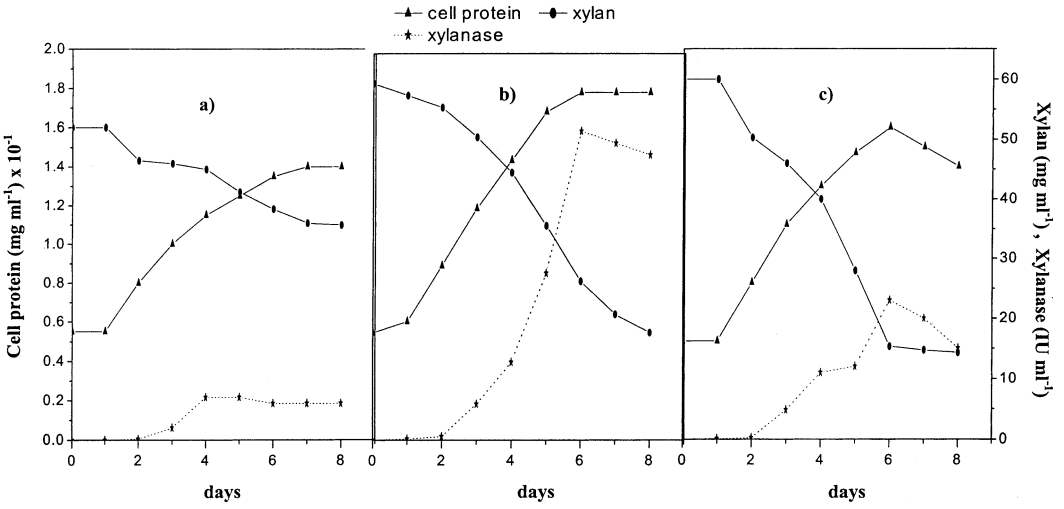

Fig. 1. Time course of growth and xylanase production by PXYL1 at (a) 10°C, (b) 20°C and (c) 30°C.

fied as a *Clostridium* sp. according to Bergey's Manual of Systematic Bacteriology [20] and designated as strain PXYL1.

PXYL1 could grow on xylan in the temperature range of 5–50°C. Growth was observed at as low as 5°C. The growth rate was maximal at 20°C and declined at higher temperatures. The specific growth rates (h^{-1}) were: 0.146 (5°C), 0.155 (10°C), 0.229 (15°C), 0.275 (20°C), 0.225 (25°C), 0.203 (30°C), 0.179(35°C), 0.165 (40°C), 0.095 (45°C) and 0.051(50°C). Fig. 1a–c shows the time course of growth of PXYL1 on xylan at different temperatures. Xylanase production (52 IU ml^{-1} broth) and xylan utilisation were maximal at 20°C. From the growth data, it was evident that xylanase production was growth associated and thermoregulated. The xylanase activity at 10°C and 30°C was only 7 IU ml^{-1} and 23 IU ml^{-1} respectively.

Fig. 2 shows the temperature profile of all the extracellular hydrolytic enzymes of xylan-grown *Clostridium* PXYL1. At 20°C, relatively high levels of extracellular xylanase (37 IU {mg protein}$^{-1}$), endoglucanase (35.75 IU {mg protein}$^{-1}$) and FPase (23.68 IU {mg protein}$^{-1}$) were found, while cell-bound activity of xylanase was nil. In addition, moderate levels of β-xylosidase (0.72 IU {mg protein}$^{-1}$), β-glucosidase (0.89 IU {mg protein}$^{-1}$) and very low levels of amylase (0.053 IU {mg protein}$^{-1}$) and protease (0.0242 IU {mg protein}$^{-1}$) were detected. All the enzymes showed maximal activity at 20°C. This is the first report wherein extracellular multienzyme produced by a *Clostridium* species were all 'psychroactive'. The xylanase enzyme showed 40% of the maximum activity even at 10°C (Fig. 2). The xylanase activity of *Clostridium* strain PXYL1 was found to be appreciably higher than its mesophilic counterparts, viz. xylanolytic *Clostridium* strain SAIV (0.52 IU ml^{-1}) [21] and *C. cellulolyticum* (0.9 IU ml^{-1}) [22]. However, its activity was less than in the thermophilic species, *C. absonum* (422 IU ml^{-1}) [23]. There is no data available in the literature on psychrophilic xylanases from a *Clostridium* species. The FPase and CMCase activities of PXYL1 also decline above 30°C and there was no activity detected at 50°C. This is interesting and suggests that the xylanase and cellulase of this organism are novel, as even the IUPAC-recommended assay temperature for xylanase [14] and cellulase [24] is 50°C.

At 20°C the pH optimum for xylanase was 5.3 (50 IU ml^{-1}). The activities recorded in the pH range of 3–9 were pH 3.0 (14.08 IU ml^{-1}), pH 4 (18.63 IU ml^{-1}), pH 5.3 (50 IU ml^{-1}), pH 6 (22.22 IU ml^{-1}), pH 7 (16.97 IU ml^{-1}), pH 8 (16.8 IU ml^{-1}), pH 9 (no activity). The enzyme activity was seen under both acidic and alkaline conditions (pH 3–8). Similar results were seen only with an alkaliphilic fungus *Acremonium alkalophilum* [25]. However the enzyme appeared to have best activity in the acidic range, making it ideal for food processing. The optimum pH for CMCase and FPase were 5.0 and 6.0 respectively.

Fig. 3 shows the effect of temperature on the thermo-

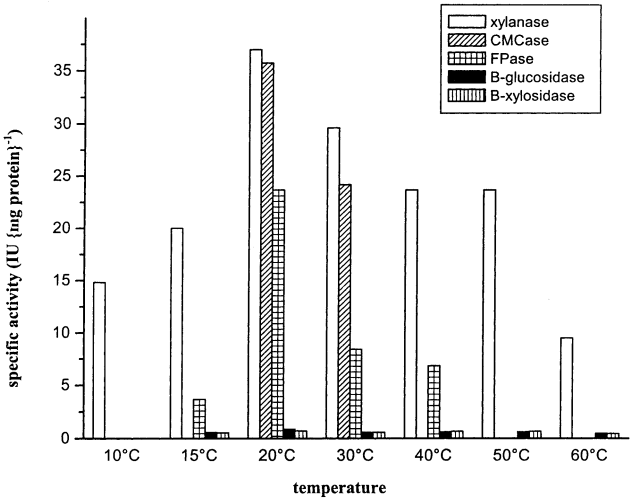

Fig. 2. Temperature profile of xylanase and other hydrolytic enzymes of PXYL1.

stability of the xylanase at different temperatures. The enzyme was thermolabile as it was stable at 20°C (51.8 IU ml^{-1} taken as 100%), and it lost 50% of its activity in 95 min at 30°C, in 30 min at 40°C and in 8 min at 50°C. Similar patterns of thermolability was observed for CMCase and FPase; the enzymes were stable at 20°C, whereas 50% of the activity was lost at 45 min and 30 min respectively at 40°C and within 10 min at 50°C.

The production of xylanase by *Clostridium* PXYL1 was inducible when tested with different carbon sources (1% w/v) at 20°C. After 10 days the organism utilised glucose (80%), xylose (81%), cellobiose (65%). However xylanase activity was nil on glucose and expressed to a low extent of 1.11 and 1.43 IU mg $protein^{-1}$ on xylose and cellobiose respectively.

To explore the advantages of clostridial cold-active enzyme system, the hydrolysis of finger millet flour was investigated, as illustrative of the benefit of enzymatic food processing. Fig. 4 shows the time course of finger millet flour hydrolysis by the PXYL1 xylanase at 20°C. After 6 h of incubation 1.25 g l^{-1} and 0.71 g^{-1} of reducing sugar were released at 20°C and 30°C. Two commercial enzymes popularly used for food processing, did not hydrolyse the flour at 20°C. At 30°C the ABCL enzyme released 2.47 g l^{-1} reducing sugar. Our previous study has shown the benefit of enzymatic hydrolysis for enhanced nutritive potential in finger millet [26]. Finger millet was chosen for this study, as, due to its high dietary fibre content, it easily gels at higher temperature. Hence thermolabile enzymes acting at low temperature appeared suitable to reduce gelling on cooking [26].

The *Clostridium* strain PXYL1 with its ability to degrade plant polysaccharides could have potential use in waste treatment and bioremediation systems operating in places where the temperatures are permanently or cyclically (diurnally) low. Its thermolabile enzymes could be useful for food processing at low temperatures, or as a probiotic inclusion, e.g. as a flour additive. Therefore this new strain of PXYL1 and its unusual enzyme system are of considerable biotechnological interest. The three-dimensional structure of only one xylanase from *Pseudoalteromonas haloplanctis* has been determined so far and further work on purification of the unusual enzyme of PXYL1 and cloning will be of scientific interest for comparing homology and structure with well-known mesophilic and thermophilic xylanases.

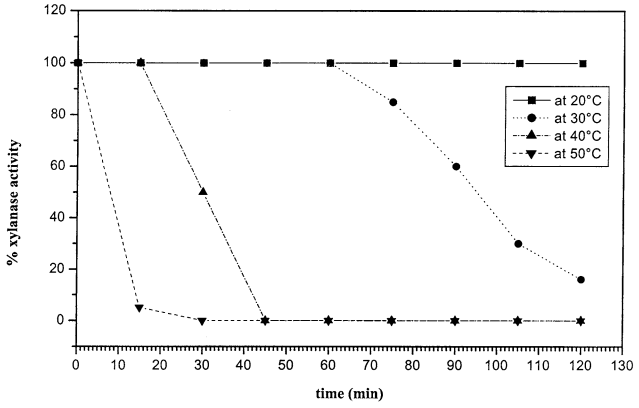

Fig. 3. Thermal deactivation of xylanase at 20°C, 30°C, 40°C and 50°C.

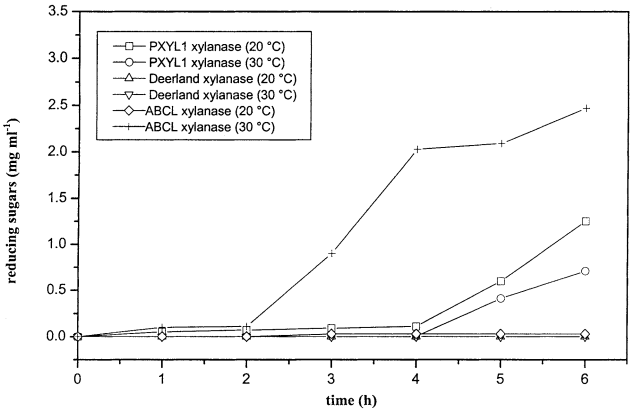

Fig. 4. Finger millet flour hydrolysis by the crude xylanase of *Clostridium* PXYL1 in comparison with commercial food-grade xylanases at 20°C and 30°C.

Acknowledgements

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