

Xylanolytic enzyme systems in *Arthrobacter* sp. MTCC 5214 and *Lactobacillus* sp.

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Abstract

The production of extracellular xylanolytic enzymes such as xylanase, α -L-arabinofuranosidase (α -L-AFase), and acetyl xylan esterase (Axe) by marine *Arthrobacter* sp. and *Lactobacillus* sp. was investigated using different carbon sources. Induction and repression of all these enzymes differed with carbon source and also with the organism. Wheat bran was the best carbon source for the production of xylanase and α -L-AFase, whereas both isolates showed

maximum Axe production when grown on oat bran as a carbon source. Preferential utilization of a carbon source for enzyme production can give us better insights into regulatory mechanism in these marine bacteria. Elution profile as well as zymogram analysis indicated the possibility of bifunctional α -L-AFase–Axes in both marine bacteria. © 2014 International Union of Biochemistry and Molecular Biology, Inc. Volume 00, Number 0, Pages 1–10, 2014

Keywords: xylanase, α -L-arabinofuranosidase, acetyl xylan esterase, marine, bacteria

1. Introduction

Hemicellulose is a complex of polymeric carbohydrates with xylan as its major component. Xylan is a heteropolymer with backbone of β -1,4-D-xylanopyranosyl residues. Because of its complex structure, the complete breakdown of xylan requires the action of several enzymes that are recognized as a xylanolytic complex [1]. Endo- β -1,4-xylanase (β -1,4-xylan xylanohydrolase, EC 3.2.1.8) is the crucial enzyme for xylan depolymerization, which attacks the main chain and releases oligosaccharides; β -xylosidase (β -1,4-xylan xylohydrolase, EC 3.2.1.37) liberates D-xylose from xylobiose and/or xylooligosaccharides; other enzymes responsible for removing xylan substituent's include α -L-arabinofuranosidase (α -L-AFase), α -glucuronidase, acetyl xylan esterases, and feruloyl and *p*-coumaroyl esterases have been referred as auxiliary or accessory xylanolytic enzymes [2]. Xylanolytic enzymes have applications in conversion of lignocellulosic substances to chemicals and fuels, animal feed digestion, in food and textile industries, and as bleaching agents in the pulp and paper processing [3–7].

Abbreviations: α -L-AFase, α -L-arabinofuranosidase; AXE, acetyl xylan esterase.

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Xylanolytic enzymes are mainly used for the hydrolysis of hemicellulosic substrate to produce fermentable sugars. The knowledge gathered on the hydrolysis mechanism of hemicelluloses will greatly promote the rapid application of these enzymes in new areas. Also, the general information already available on individual xylanolytic enzymes will help in the development of future applications. There are many reports on xylanolytic enzymes produced by fungi [8–12] but not much information on the xylanolytic enzyme system in bacteria [13].

Therefore, in this article, an effort is made to study the xylanolytic enzyme system of two marine bacteria: one of which, *Arthrobacter* sp., was previously reported for xylanase and AFase production, whereas the other one is a newly isolated *Lactobacillus* sp. with special reference to isolation, partial purification, and characterization of xylanolytic complex. This study will enable us to understand the behavior of each bacterium with respect to carbon source and its impact on enzyme production. Preferential utilization of carbon source for enzyme production will help us in understanding the regulatory mechanism of these bacteria and their future prospects for biotechnological uses.

2. Materials and Methods

2.1. Microorganism and growth condition

Arthrobacter sp. MTCC 5214 was previously isolated from a sediment sample collected from the Mandovi estuary, on the west coast of India [14]. *Lactobacillus* sp. was isolated from a sediment sample along a mangrove area of the Mandovi estuary, west coast of India. The culture was purified and stored at 4 °C

on ZoBell marine agar slants. The culture was studied for morphological, physiological, and biochemical characteristics following the standard procedures [15]. *Arthrobacter* sp. and *Lactobacillus* sp. cells were grown at room temperature (28 ± 2 °C) at pH 9.0 in 15 mL basal salt solution (BSS) supplemented with peptone (0.03 g), yeast extract (0.2 g), and wheat bran (5 g) as the sole source of carbon (substrate to moisture ratio 1:3) over a period of 6 days. The composition of the BSS medium (w/v) was as follows: NaCl (30.00 g), KCl (0.75 g), MgSO₄ (7.00 g), NH₄Cl (1.00 g), K₂HPO₄ (10%, 7.00 mL), KH₂PO₄ (10%, 3.00 mL), trace metal solution (1.00 mL), and distilled water (1,000 mL). The trace metal solution had the following composition: H₃BO₃ (2.85 g), MnCl₂·7H₂O (1.80 g), FeSO₄·7H₂O (2.49 g), Na-tartrate (1.77 g), CuCl₂ (0.03 g), ZnCl₂ (0.02 g), CoCl₂ (0.04 g), Na₂MoO₄·2H₂O (0.02 g), and distilled water (1,000 mL).

2.2. Effect of carbon sources on xylanase, α -L-AFase, and acetyl xylan esterase activity

The effect of various carbon sources on xylanase, α -L-AFase, and acetyl xylan esterase (Axe) production was assessed by growing both cultures in the BSS medium (pH 9.0) supplemented with peptone (0.03%), yeast extract (0.2%), and 0.5% (w/v) of either pure carbohydrates such as D-galactose, D-xylose, inositol, D-arabinose, D-maltose, D-lactose, sorbitol, D-mannose, cellobiose, D-mannitol, D-fructose, D-glucose, D-sucrose, and oat spelt xylan at room temperature under submerged condition for 48 H, whereas others, which were natural carbon sources such as wheat bran, sugarcane bagasse, oat bran, and beet pulp, were grown under Solid State fermentation (SSF) for 6 days. After growth under submerged condition, broth of each culture was centrifuged at 10,000g and 4 °C for 10 Min, and supernatant was used to estimate enzyme activities as described below. For SSF, 5 mL of 50 mM Na-phosphate buffer, pH 8, was added to the flask containing spent solid substrate and shaken at 200 rpm on a rotary shaker for 30 Min to extract the enzyme. The sample was centrifuged at 10,000g and 4 °C for 10 Min, and the supernatant was analyzed for the enzyme activities as described below.

2.3. Enzymatic assays for xylanase

Xylanase activity was assayed using 1% birchwood xylan as the substrate. Xylan was dissolved in phosphate buffer (50 mM, pH 8.0). The reaction mixture, containing the enzyme and the substrate, was incubated at 80 °C for 10 Min. The amount of reducing sugars liberated was determined by using 3,5-dinitrosalicylic acid method [16]. The absorbance of the reference samples (substrate solution incubated without enzyme and diluted enzyme solution in buffer) was deduced from the values of the test samples. One unit of xylanase activity was expressed as 1 μ mol of reducing sugars (xylose equivalent) released in 1 Min under the above-mentioned conditions.

2.4. Enzymatic assays for α -L-AFase

α -L-AFase activity was assayed by using 2.5 mM *p*-nitrophenyl- α -L-arabinofuranoside, Na-phosphate buffer (50 mM, pH 8),

and an appropriately diluted enzyme solution. After incubating the reaction mixture at 80 °C for 10 Min, the reaction was stopped by adding ice-cold 1 M Na₂CO₃ (1 mL) and the liberated *p*-nitrophenol (pNP) was measured at 420 nm. One unit (U) of α -L-AFase activity was defined as the amount of enzyme that liberated 1 μ mol of pNP at 80 °C in 1 Min [17].

2.5. Enzymatic assays for Axe

Axe activity was determined by measuring the amount of 4-methylumbelliferone released from 4-methyl umbelliferyl acetate [18]. Na-phosphate buffer (pH 8.0; 100 μ L of 100 mM) and 280 μ L of distilled water were preincubated. The enzyme solution was added to the buffer, and the reaction was initiated within 1 Min by adding 4-methyl umbelliferyl acetate dissolved in dimethyl sulfoxide and incubated at 80 °C. After 10 Min, the reaction was stopped using 50 mM citric acid, and the activity was measured at an excitation wavelength of 335 nm and an emission wavelength of 440 nm by using a fluorescence spectrophotometer. An enzyme unit was defined as the amount of enzyme that produced 1 μ mol of product per minute.

2.6. Protein estimation

Protein was analyzed by the bicinchoninic acid method, and bovine serum albumin was used as a standard [19]. The protein content of the chromatographic eluent was measured by monitoring the optical density at 280 nm.

2.7. Isolation of enzyme produced by *Arthrobacter* sp. and *Lactobacillus* sp.

Erlenmeyer flasks (500 mL) each containing 10 g of wheat bran and 30 mL of BSS (1:3) supplemented with peptone and yeast extract at pH 8.0 were sterilized, cooled to room temperature, and inoculated with *Arthrobacter* sp. and *Lactobacillus* sp. culture individually. The flasks were incubated at room temperature (28 ± 2 °C). The culture in stationary growth phase was harvested. Enzyme was isolated by suspending the content of the flask in 100 mL of 50 mM Na-phosphate buffer, pH 8, and mixed carefully on rotary shaker for 1 H. The content of the flask was centrifuged at 10,000g for 10 Min, and the supernatant was collected. The enzyme was precipitated from the culture supernatant by adding ammonium sulfate to 80% saturation. The precipitate obtained was dissolved in 10 mL of Na-phosphate buffer (0.05 M, pH 8.0) and dialyzed against the same buffer for 24 H. Dialysis was carried out with cellulose tubing (molecular weight cutoff 13,000 kDa).

2.8. Ion exchange chromatography

Concentrated enzyme (2 mL) was loaded onto an anion exchange DEAE-Sepharose FF column (Sigma-Aldrich, St. Louis, MO, USA; 15 mm diameter and height 100 mm). Equilibration and elution at 0.5 mL/Min were performed first with 50 mM Na-phosphate buffer to remove unbound proteins, and then with a linear salt gradient from 0 to 3 M NaCl. Fractions (2 mL) were collected and analyzed for xylanase, α -L-AFase, and Axe activity, protein content was also analyzed as described above. Active fractions were pooled and concentrated, and

then purified using cation exchange CM-Sepharose FF column (Sigma–Aldrich Co.) as mentioned above. The active fractions were pooled and concentrated [14].

2.9. Zymogram analysis

The enzyme sample was subjected to electrophoresis on an SDS-PAGE. Gel was divided in two parts. The first part was stained with Coomassie Brilliant Blue, whereas the second part of gel with purified protein was soaked in 25% (v/v) isopropanol with gentle shaking to remove SDS and renature the proteins in the gel. The gel was then washed four times for 30 Min at 4 °C in 50 mM phosphate buffer (pH 7). The washed gel was then laid over a precast 0.5% (w/v) agar gel containing 0.5% (w/v) birchwood xylan in 50 mM phosphate buffer (pH 7.0) for xylanase activity. Further, after incubation for 1 H at 50 °C, the gel was soaked in 0.1% Congo red solution for 30 Min at room temperature and washed with 1 M NaCl until excess dye was removed from the active band [14]. Similarly, after SDS-PAGE, gels were subjected for zymogram analysis for Axe and AFase. For AFase, methylumbelliferyl α -L-arabinofuranoside (Sigma–Aldrich Co.) was used as the substrate, and for acetyl esterase, 4-methylumbelliferyl acetate was used as the substrate in the overlaid gel. The bands of activity were detected using a UV transilluminator.

3. Results and Discussion

3.1. Culture and growth conditions

Arthrobacter sp. MTCC 5214 is reported previously for xylanase as well as α -L-AFase production [14, 20]. New bacterium reported here was isolated locally from a sediment sample collected from the Mandovi estuary, west coast of India. The culture showed good growth at 28 ± 2 °C in BSS medium, pH 8, supplemented with 0.5% birchwood xylan. Its ability to produce xylanase enzyme was further confirmed when it formed orange digestion halos on birchwood xylan plates when treated with Congo red and washed with 1 M NaCl. The isolate is a Gram-positive facultative anaerobic rod-shaped bacterium, which is motile, oxidase positive, catalase negative, and reduced nitrate to nitrite (Table 1).

3.2. Effect of carbon sources on enzyme production

The effect of carbon sources on xylanase, α -L-AFase, and Axe production from *Arthrobacter* sp. and its comparison with new isolate *Lactobacillus* sp. are presented here. All together 18 carbon sources were evaluated for this study. Among the agricultural residue tested, wheat bran showed the maximum xylanase production (Table 2). The xylanase production by *Lactobacillus* sp. on each carbon source was more as compared with *Arthrobacter* sp., with the exception of sugarcane bagassae, where *Arthrobacter* sp. showed higher xylanase production (Table 2). It was interesting to note that the xylanase production by *Arthrobacter* sp. was very poor or almost nil when grown on pure carbohydrates, whereas *Lactobacillus* sp. showed the induction of xylanase with xylose and glucose (Table 3). It has been reported that the fungus

TABLE 1 Morphological, physiological, and biochemical characteristics of *Lactobacillus* sp.

| Test | Result |
|------------------------|--------------------------|
| Color of colony | Cream |
| Shape | Rod |
| Gram stain | Gram +ve |
| Motility | + |
| Oxidase | + |
| Catalase | – |
| Hugh–Leifson | Fermentative |
| Spore staining | No spores |
| Gelatine hydrolysis | + |
| Starch hydrolysis | + |
| Nitrate reduction | + |
| MR | – |
| VP | – |
| Simmons citrate | + |
| Sugar fermentation | |
| Adonitol | – |
| Galactose | A |
| Glucose | A |
| Lactose | A |
| Xylose | A |
| Melibiose | – |
| Sucrose | A |
| Utilization of glucose | + |
| Lactose | + |
| Xylose | + |
| Sucrose | + |
| Mannose | – |
| Fructose | – |
| Mannitol | – |
| Arabinose | + |
| Genus Identified | <i>Lactobacillus</i> sp. |

TABLE 2
Xylanolytic enzymes production by Arthrobacter sp. and Lactobacillus sp. under solid-state fermentation

| Carbon source | <i>Arthrobacter sp.</i> | | | <i>Lactobacillus sp.</i> | | |
|-------------------|-----------------------------------|---|---|-----------------------------------|---|---|
| | Xylanase (IU g ⁻¹) | α -L- Arabinofurano- sidase (IU gL ⁻¹) | Acetyl xylan esterase (IU g ⁻¹) | Xylanase (IU g ⁻¹) | α -L- Arabinofurano- sidase (IU gL ⁻¹) | Acetyl xylan esterase (IU g ⁻¹) |
| Wheat bran | 140.74 ± 1.32 | 0.240 ± 0.01 | 4.82 ± 0.13 | 372.44 ± 2.13 | 0.335 ± 0.04 | 7.16 ± 0.31 |
| Beet pulp | 4.46 ± 0.06 | 0.003 ± 0.00 | 3.18 ± 0.08 | 8.65 ± 0.86 | 0.099 ± 0.00 | 8.92 ± 0.22 |
| Oat bran | 27.31 ± 0.21 | 0.200 ± 0.002 | 4.39 ± 0.07 | 81.32 ± 1.33 | 0.167 ± 0.01 | 11.42 ± 0.18 |
| Sugarcane bagasse | 182.88 ± 2.62 | 0.023 ± 0.00 | 3.64 ± 0.07 | 124.49 ± 2.12 | 0.012 ± 0.00 | 7.48 ± 0.21 |
| Oat xylan | 65.42 ± 1.12 | 0.019 ± 0.00 | 4.31 ± 0.02 | 91.74 ± 0.89 | 0.027 ± 0.00 | 6.26 ± 0.16 |

TABLE 3
Xylanolytic enzymes production by Arthrobacter sp. and Lactobacillus sp. under submerged cultivation

| Carbon source | <i>Arthrobacter sp.</i> | | | <i>Lactobacillus sp.</i> | | |
|---------------|------------------------------------|---|--|------------------------------------|---|--|
| | Xylanase (IU mL ⁻¹) | α -L- Arabinofurano- sidase (IU mL ⁻¹) | Acetyl xylan esterase (IU mL ⁻¹) | Xylanase (IU mL ⁻¹) | α -L- Arabinofurano- sidase (IU mL ⁻¹) | Acetyl xylan esterase (IU mL ⁻¹) |
| Sucrose | 0.45 ± 0.03 | ND | 1.92 ± 0.03 | 1.95 ± 0.05 | 0.052 ± 0.00 | 3.35 ± 0.02 |
| Xylose | ND | 0.070 ± 0.002 | 2.31 ± 0.05 | 38.56 ± 0.12 | 0.046 ± 0.00 | 2.73 ± 0.03 |
| Inositol | 4.47 ± 0.04 | 0.068 ± 0.00 | 2.17 ± 0.02 | 0.93 ± 0.06 | 0.113 ± 0.02 | 3.42 ± 0.02 |
| Sorbitol | 1.19 ± 0.04 | 0.079 ± 0.001 | 2.21 ± 0.03 | 0.85 ± 0.04 | 0.119 ± 0.03 | 3.64 ± 0.01 |
| Glucose | 0.21 ± 0.00 | 0.001 ± 0.00 | 1.87 ± 0.00 | 30.55 ± 0.11 | 0.002 ± 0.00 | 3.21 ± 0.05 |
| Cellobiose | 0.63 ± 0.01 | 0.003 ± 0.00 | 2.09 ± 0.03 | ND | 0.020 ± 0.00 | 2.95 ± 0.04 |
| Mannose | ND | 0.011 ± 0.00 | 2.01 ± 0.04 | 4.51 ± 0.08 | 0.108 ± 0.01 | 3.37 ± 0.02 |
| D-Arabinose | 0.37 ± 0.00 | 0.065 ± 0.003 | 1.97 ± 0.04 | 0.96 ± 0.01 | 0.064 ± 0.00 | 3.08 ± 0.01 |
| Maltose | 0.97 ± 0.01 | 0.063 ± 0.002 | 1.78 ± 0.01 | 3.58 ± 0.03 | 0.119 ± 0.03 | 2.70 ± 0.01 |
| Fructose | 0.31 ± 0.00 | 0.001 ± 0.00 | 2.29 ± 0.01 | 3.22 ± 0.03 | 0.039 ± 0.00 | 3.42 ± 0.04 |
| Galactose | 1.78 ± 0.02 | 0.099 ± 0.002 | 3.07 ± 0.03 | ND | 0.124 ± 0.04 | 2.50 ± 0.01 |
| Mannitol | 0.55 ± 0.01 | 0.003 ± 0.00 | 2.17 ± 0.03 | 6.55 ± 0.04 | 0.002 ± 0.00 | 2.49 ± 0.06 |
| Lactose | 1.02 ± 0.24 | 0.053 ± 0.001 | 2.84 ± 0.02 | 9.82 ± 0.06 | 0.102 ± 0.02 | 2.21 ± 0.02 |

Penicillium janczewskii when grown on different agricultural residues produced highest amounts of xylanase on wheat bran [11], whereas *Myceliophthora* sp., a thermophilic fungus, showed the maximum production of xylanase enzyme on rice straw and a minimum on wheat bran [9]. Andrade et al. [21] reported arabinoxylan from oats to be a good inducer for endoxylanase and β -xylosidase production by an archaeon *Pyrodictium abyssi*. Jung-Han and Choi [13] reported the

production of xylanase, β -xyloxidase, and α -L-AFase from *Bacillus stearothermophilus*, but only three carbon sources were used in this study, so it is difficult to say which carbon source is more effective for the xylanase production.

α -L-AFase production by *Arthrobacter* sp. and *Lactobacillus* sp. was highest when grown on wheat bran. Beet pulp induced the production of α -L-AFase in *Lactobacillus* sp., whereas its production is repressed in *Arthrobacter* sp. (Table 2).

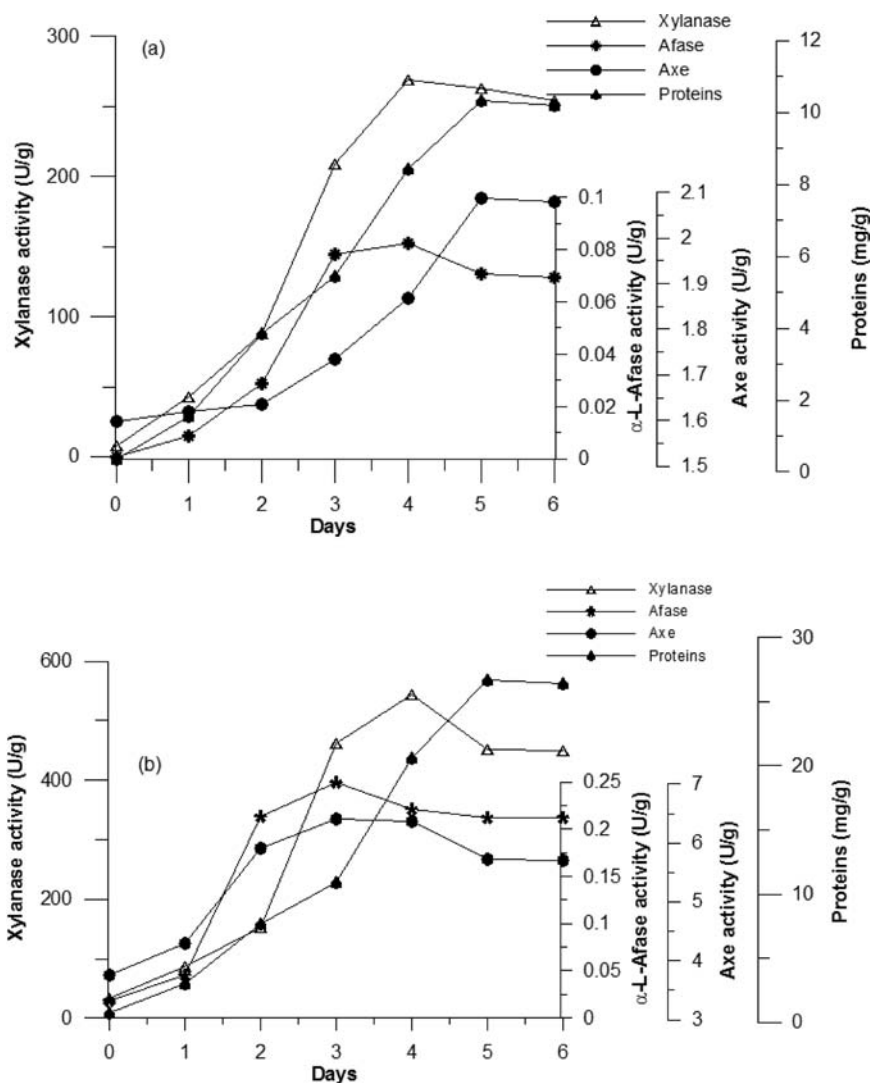


FIG. 1 Growth curve and enzyme production by (a) *Arthrobacter* sp. and (b) *Lactobacillus* sp.

α -L-AFase production by both the cultures was induced by galactose, mannose, sorbitol, inositol, and lactose, whereas glucose, mannitol, and to some extent cellobiose did not show any effect on the α -L-AFase production (Table 3). Orange waste and arabinose showed induced α -L-AFase production in *P. janczewskii*. Xylose showed the least α -L-AFase activity, whereas no activity was detected when glucose, sucrose, maltose, and lactose were used as carbon sources [11].

Lactobacillus sp. showed maximum Axe production when Oat bran was used as a carbon source, whereas Axe production by *Arthrobacter* sp. remained almost same when grown on wheat bran, oat bran, and even on oat xylan (Table 2). Soluble sugars induced Axe production in both bacteria. The affinity of enzymes toward different carbon sources was interesting. For example, xylanase production by *Arthrobacter* sp. and *Lactobacillus* sp. was induced by glucose, whereas glucose inhibited

the α -L-AFase production. Similarly, with exception of glucose and xylose, sugars had no effect on xylanase production. Conversely, Axe production was induced almost by all sugars, in case of both the cultures. α -L-AFase and xylanase were produced in maximum amounts when wheat bran was used as a carbon source, whereas Axe production was highest in the presence of Oat bran in both the cultures. If we compare all carbon sources and the respective enzyme production on each carbon source, we can say that the wheat bran was effective for all three enzymes from *Arthrobacter* sp. and *Lactobacillus* sp., and thus we selected wheat bran as a carbon source for further studies.

3.3. Growth and enzyme production

The time course of growth and the production of extra cellular xylanase, α -L-AFase, and Axe from *Arthrobacter* sp. and *Lactobacillus* sp. were studied using wheat bran as a carbon source. *Arthrobacter* sp. showed the maximum growth on day 5 following incubation and then remained steady on the next consecutive day. *Arthrobacter* sp. showed maximum xylanase

Purification steps of xylanase, α -L-AFase, and Axe isolated from *Arthrobacter* sp. grown on wheat bran
TABLE 4

| Fraction | Total Xylanase | | Specific activity of xy-lanase (IU/mg) | | Purification fold xylanase | | Xylanase yield (%) | | AFase activity (IU) | | Specific activity of AFase (IU/mg) | | Purification fold AFase | | Axe activity (IU) | | Specific activity of Axe (IU/mg) | | Purification fold Axe | | Axe yield (%) | |
|---|----------------|--------|--|---------|----------------------------|------|--------------------|-----|---------------------|------|------------------------------------|---------|-------------------------|------|-------------------|-------|----------------------------------|---------|-----------------------|------|---------------|-----|
| | (mg) | (IU) | (IU/mg) | (IU/mg) | fold | fold | (%) | (%) | (IU) | (IU) | (IU/mg) | (IU/mg) | fold | fold | (IU) | (IU) | (IU/mg) | (IU/mg) | fold | fold | (%) | (%) |
| Filtrate | 719.0 | 33,230 | 46.21 | 46.21 | 1 | 1 | 100 | 100 | 4.66 | 4.66 | 0.006 | 0.006 | 1 | 1 | 33.70 | 33.70 | 0.04 | 0.04 | 1 | 1 | 100 | 100 |
| (NH ₄) ₂ SO ₄ precipitate | 507 | 25,769 | 50.8 | 3.5 | 1.09 | 1 | 77 | 77 | 3.5 | 3.5 | 0.006 | 0.006 | 1 | 1 | 24.46 | 24.46 | 0.04 | 0.04 | 1.03 | 1.03 | 72 | 72 |
| Dialysis | 334 | 18,370 | 55 | 3 | 1.19 | 1.3 | 55 | 55 | 3 | 3 | 0.008 | 0.008 | 1.3 | 1.3 | 20.58 | 20.58 | 0.06 | 0.06 | 1.31 | 1.31 | 61 | 61 |
| DEAE-Sepharose FF (first peak) | 83 | 8,710 | 104.9 | 1.08 | 2.25 | 1.6 | 26 | 26 | 1.08 | 1.08 | 0.01 | 0.01 | 1.6 | 1.6 | 8.54 | 8.54 | 0.1 | 0.1 | 2.19 | 2.19 | 25 | 25 |
| DEAE-Sepharose FF (second peak) | 32 | - | - | 0.76 | - | 3.3 | - | - | 0.76 | 0.76 | 0.02 | 0.02 | 3.3 | 3.3 | 4.26 | 4.26 | 0.13 | 0.13 | 2.84 | 2.84 | 12 | 12 |
| CM-Sepharose (peak A) | 5 | - | - | 0.2 | - | 6.6 | - | - | 0.2 | 0.2 | 0.04 | 0.04 | 6.6 | 6.6 | 1.8 | 1.8 | 0.36 | 0.36 | 4.83 | 4.83 | 5 | 5 |
| CM-Sepharose (peak B) | 5.6 | 3,576 | 638.5 | - | 13.8 | 11 | 11 | 11 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| CM-Sepharose (peak C) | 2.0 | - | - | - | - | - | - | - | - | - | - | - | - | - | 2.4 | 2.4 | 1.2 | 1.2 | 17.3 | 17.3 | 6 | 6 |

Purification steps of xylanase, α -L-AFase, and Axe isolated from *Lactobacillus* sp. grown on wheat bran

TABLE 5

| Fraction | Total protein (mg) | Xylanase activity (IU) | Xylanase activity of xy-lanase (IU/mg) | Purification fold xy-lanase | Xylanase yield (%) | AFase activity (IU) | Specific activity of AFase (IU/mg) | Purification fold AFase | AFase yield (%) | Axe activity (IU) | Specific activity of Axe (IU/mg) | Purification fold Axe | Axe yield (%) |
|---|--------------------|------------------------|--|-----------------------------|--------------------|---------------------|------------------------------------|-------------------------|-----------------|-------------------|----------------------------------|-----------------------|---------------|
| Filtrate | 831 | 55,988 | 67.3 | 1 | 100 | 14.03 | 0.016 | 1 | 100 | 138.2 | 0.16 | 1 | 100 |
| (NH ₄) ₂ SO ₄ precipitate | 598 | 42,061 | 70.3 | 1.04 | 75 | 9.69 | 0.016 | 1 | 69 | 102.4 | 0.17 | 1.06 | 74 |
| Dialysis | 312 | 36,312 | 116.3 | 1.72 | 64.8 | 7.59 | 0.024 | 1.5 | 54 | 69.1 | 0.22 | 1.37 | 50 |
| DEAE-Sepharose FF (first peak) | 48 | 18,123 | 377.5 | 4.80 | 32.3 | 1.23 | 0.025 | 1.56 | 8.7 | 12.8 | 0.26 | 1.62 | 9.2 |
| DEAE-Sepharose FF (second peak) | 3.8 | - | - | - | - | 2.72 | 0.71 | 44 | 19 | - | - | - | - |
| CM-Sepharose (peak A) | 4.2 | - | - | - | - | 0.18 | 0.04 | 2.5 | 1.2 | 5.2 | 1.2 | 7.5 | 3.7 |
| CM-Sepharose (peak B) | 9.1 | 8,825 | 9.69 | 14.3 | 15.7 | - | - | - | - | - | - | - | - |

production on day 3, whereas Axe and α -L-AFase production was highest on day 5 of incubation (Fig. 1a). *Lactobacillus* sp. showed the maximum growth on day 5 following incubation and then remained steady on the next consecutive day. Xylanase production increased in the early stationary phase (day 4) and then remained constant over the period of cultivation. Axe and α -L-AFase reached maximum when the culture was in the exponential growth phase (day 3) and then remained constant or decreased little over the remaining period of cultivation (Fig. 1b).

3.4. Purification of xylanolytic complex

The xylanolytic enzyme complex from *Arthrobacter* sp. and *Lactobacillus* sp. was isolated and partially purified at room temperature (28 ± 2 °C). Ammonium sulfate precipitation was followed by dialysis, and then column chromatography was performed. The culture filtrate was collected by growing the cultures on wheat bran. A summary of purification procedures is presented in Tables 4 and 5. The enzyme complex from *Arthrobacter* sp. when loaded onto the DEAE-Sepharose column showed two peaks (I and II). Peak I showed the presence of xylanase, α -L-AFase, and Axe, whereas peak II showed the enzyme activity for α -L-AFase and Axe (Fig. 2a). Both the peaks were further purified by loading each of them separately onto the CM-Sepharose column. Peak I was separated as three peaks (A, B and C). Peak A showed the presence of α -L-AFase and Axe, peak B exclusively showed xylanase, whereas peak C had only Axe activity (Fig. 2b). Peak II having α -L-AFase and Axe activity was loaded onto the CM-Sepharose column but Axe and α -L-AFase did not separate out and remained as a single peak. The overall level of recovery for the purified xylanase enzyme was 11%, Axe showed 6%, whereas α -L-AFase, which did not elute as a single peak but was always accompanied with Axe, showed 4% yield. Fourteen-fold purification of xylanase was attained with the specific activity of 638 U/mg. Axe showed 17-fold purification with the specific activity of 1.2 U/mg, whereas α -L-AFase showed sevenfold purification with the specific activity of 0.04 U/mg. Purified Axe and α -L-AFase recovered from *Arthrobacter* sp. during column chromatography (Fig. 2) were tested for their activity with natural substrates such as acetylated xylan and arabinoxylan, respectively. Purified AXE showed specific activity of 0.7 IU/mg in the presence of acetylated xylan, whereas α -L-AFase showed 0.16 U/mg of specific activity in the presence of arabinoxylan.

The enzyme complex isolated from *Lactobacillus* sp. showed two peaks (I and II) when loaded onto the DEAE-Sepharose column. Peak I showed the presence of xylanase, α -L-AFase, and Axe. Peak II showed exclusive peak of α -L-AFase (Fig. 2c). Peak I was further purified by loading it onto the CM-Sepharose column. Peak I was again separated as two peaks (A and B). Peak A showed the presence of α -L-AFase and Axe, whereas peak B showed pure xylanase (Fig. 2d). Overall level of recovery for purified xylanase enzyme was 16%, α -L-AFase showed 19%, whereas Axe, which did not elute as a single peak but accompanied with α -L-AFase, showed

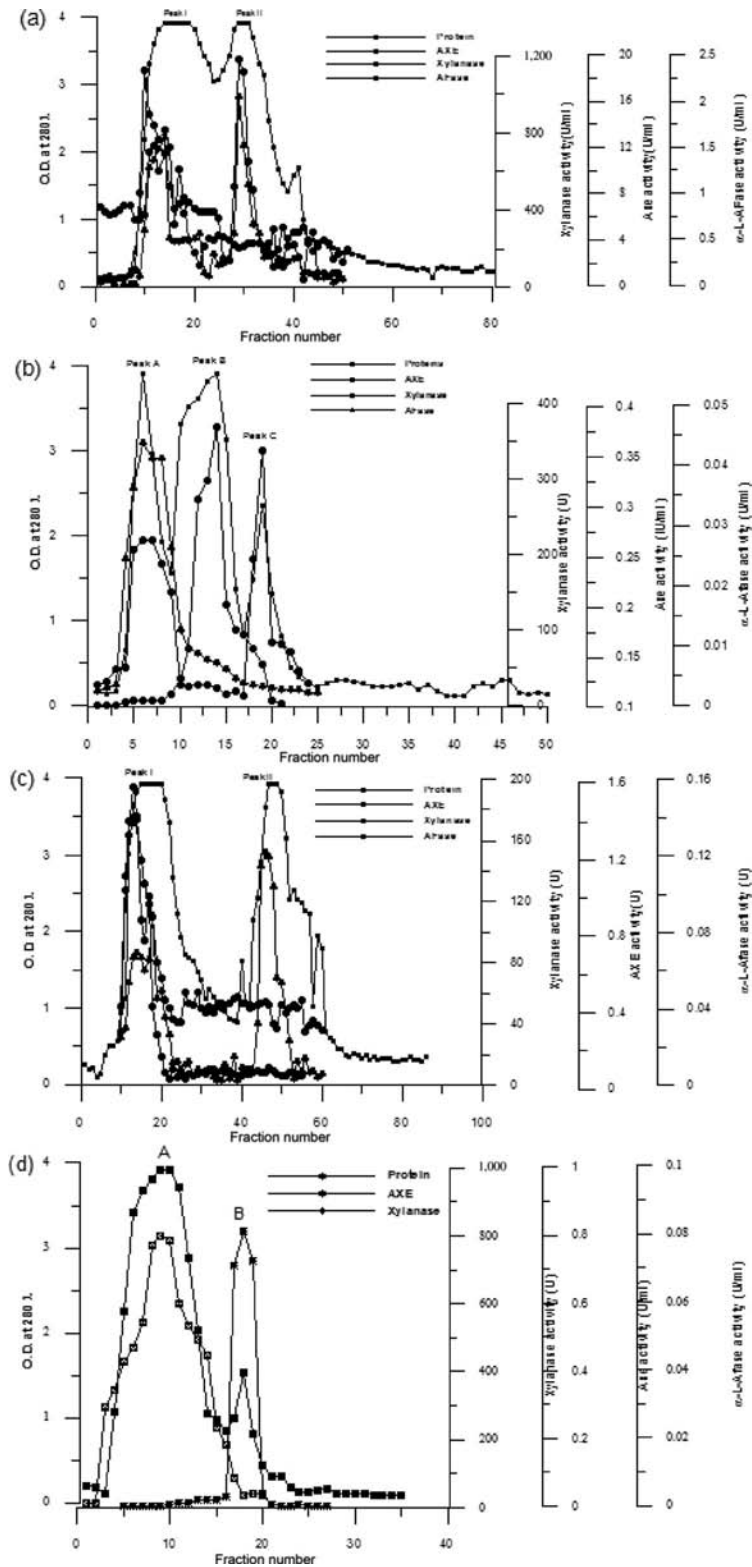


FIG. 2

Elution profile of xylanolytic complex from (a and b) *Arthrobacter sp.* and (c and d) *Lactobacillus sp.* on DEAE- and CM-Sepharose columns, respectively. Fractions containing xylanase (*), Axe (□), and α-L-AFase (♦) activity. (●) Protein profile at 280 nm.

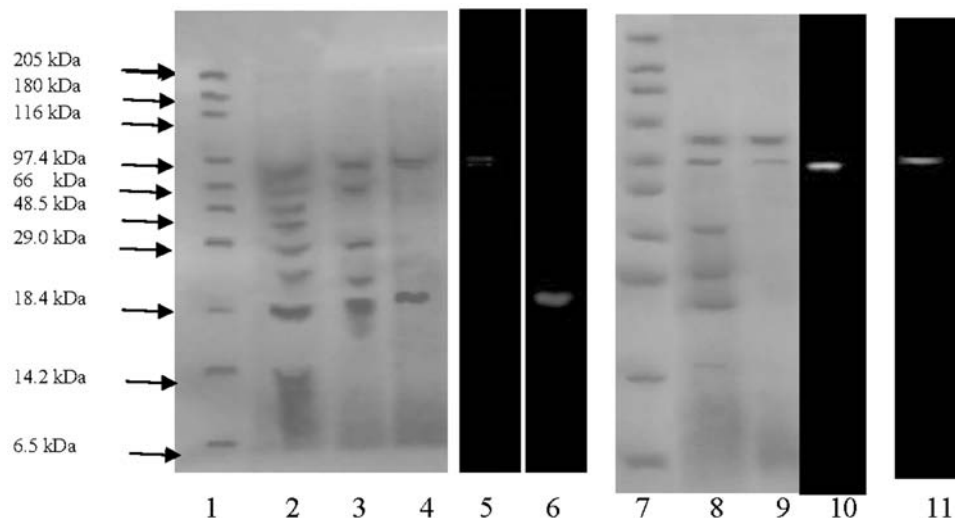


FIG. 3

SDS-PAGE of xylanolytic complex. Lane 1, molecular markers. Lanes 2–6 contain crude enzyme; enzyme after DEAE-Sepharose ion exchange chromatography; after CM-Sepharose ion exchange chromatography containing one band with bifunctional activity of Afase and Axe, second one showing Axe activity, and third band represents xylanase activity; zymogram analysis of AXE activity; zymogram analysis of xylanase activity from *Arthrobacter* sp. enzyme, respectively. Lanes 7–11 contain crude enzyme; enzyme after DEAE-Sepharose ion exchange chromatography; enzyme after CM-Sepharose ion exchange chromatography, containing one peak with bifunctional activity of Afase and AXE, whereas other peak showing xylanase activity; zymogram analysis of Afase activity and AXE activity from *Lactobacillus* sp. enzyme, respectively.

4% yield. Fourteen-fold purification of xylanase was attained with the specific activity of 969 IU/mg. Axe showed eightfold purification with the specific activity of 1.2 IU/mg, whereas α -L-AFase showed 44-fold purification with the specific activity of 0.71 IU/mg.

If we compare elution profile of *Arthrobacter* sp. with *Lactobacillus* sp., it appears that the xylanolytic complex of *Arthrobacter* sp. was more intricate as both the peaks separated by the DEAE-Sepharose column had more than one enzyme activity. α -L-AFase was accompanied by Axe in both bacterial enzyme complexes. It appears that they are bifunctional in nature. The xylanolytic complex of *Arthrobacter* sp. has two types of Axe, the first was completely separated onto the CM-Sepharose column as a single peak, whereas the second one could not be separated from α -L-AFase. In contrast to above-mentioned phenomenon, *Lactobacillus* sp. showed two types of α -L-AFase, where the first could be isolated as a single peak, whereas the second was isolated in association with Axe. Xylanase from both the cultures came out as a single peak. Purified Axe and α -L-AFase recovered during column

chromatography from *Lactobacillus* sp. (Fig. 2) were tested for their activity with natural substrates such as acetylated xylan and arabinoxytan, respectively. Purified AXE showed the specific activity of 1.6 IU/mg in the presence of acetylated xylan, whereas α -L-AFase showed the specific activity of 0.5 U/mg in the presence of arabinoxytan.

There is a possibility that α -L-AFase and Axe exhibit bifunctionality in both of these marine cultures; thus, to visualize the possibility of bifunctionality by enzymes, the enzyme that was purified on ion exchange chromatography was subjected to SDS-PAGE and zymogram analysis (Fig. 3). The SDS-PAGE and zymogram analysis of xylanolytic complex by *Arthrobacter* sp. showed three bands at lane 4 where enzyme from peak one after DEAE ion exchange chromatography (Fig. 2a) was loaded. Same bands when subjected to zymogram analysis. First two bands showed the AXE activity, whereas the third band showed the xylanase activity. The first band of ~97 kDa molecular weight seems to have bifunctional Afase and Axe, whereas the second band of ~90 kDa, which is very close to the first band, had only Axe and the third band with ~20 kDa molecular weight was detected as xylanase band.

The SDS-PAGE and zymogram analysis of xylanolytic complex by *Lactobacillus* sp. (Fig. 3) showed two bands at lane 9, where enzyme from peak one after DEAE ion exchange chromatography (Fig. 2c) was loaded. The first band is xylanase enzyme (results not shown), whereas the second band of ~66 kDa showed Afase (Fig. 3, lane 10) as well as Axe activity (Fig. 3, lane 11), thus giving us a hint that this band may have bifunctional character. As evident from zymogram analysis alone, we cannot conclude that the enzyme is bifunctional, but these evidences allow us to investigate further in this direction. Bifunctional Axe and xylanase are reported from *Neocallimastix patriciarum* [22], there is a report on *Cellulomonas fimi* with bifunctional xylanase-acetylxytan esterase [23]. To the best of our knowledge, there are no reports on bifunctional Axe and α -L-AFase.

4. Conclusions

From the marine environment, which is considered to be a dynamic ecosystem, we look forward to getting the xylanolytic enzyme system from marine bacteria with some unique properties. This is the first report wherein the xylanolytic enzyme system from marine bacteria is studied. Marine bacterial isolates *Arthrobacter* sp. and *Lactobacillus* sp. studied here showed a good potential to produce xylanolytic enzymes when grown on agricultural residues. The information on induction and inhibition of these enzymes by particular carbon source can be further used to understand the insights of regulatory mechanism in these bacteria. Studying these xylanolytic complexes will help us in exploring its potential for industrial applications. As, *Lactobacillus* sp. being the main constituents of probiotics, this study will help us in developing *lactobacilli* with better capacity to digest plant structural carbohydrates by the introduction of heterologous genes from *Arthrobacter*, which is known for its thermoalkalophilic enzyme.

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