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Purification and Characterization of Pectin Lyase Secreted by *Penicillium citrinum*

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Abstract—The importance of various parameters such as sugarcane juice concentration, pH of the medium, and effects of different solid supports for maximum secretion of pectin lyase from *Penicillium citrinum* MTCC 8897 has been studied. The enzyme was purified to homogeneity by Sephadex G-100 and DEAE-cellulose chromatography. The molecular mass determined by SDS-PAGE was 31 kDa. The K_m and k_{cat} values were found to be 1 mg/ml and 76 sec^{-1}, respectively. The optimum pH of the purified pectin lyase was 9.0, though it retains activity in the pH 9.0-12.0 range when exposed for 24 h. The optimum temperature was 50°C, and the pectin lyase was found to be completely stable up to 40°C when exposed for 1 h. The purified pectin lyase was found efficient in retting of *Linum usitatissimum*, *Cannabis sativa*, and *Crotalaria juncea*.

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Key words: pectin lyase, retting, solid state fermentation, *Penicillium citrinum*, *Cannabis sativa*, *Crotalaria juncea*, *Linum usitatissimum*

Pectinases are a group of enzymes responsible for plant tissue maceration and consist of a number of enzymes that are classified according to their mode of action and the substrates they prefer, *viz.* pectin esterases (EC 3.1.1.11), polygalacturonases (EC 3.2.1.15), pectate lyases (EC 4.2.2.2), and pectin lyases (PNL) (EC 4.2.2.10). Amongst these, PNL are of particular interest because they degrade pectin polymers directly by the β-elimination mechanism that results in the formation of 4,5-unsaturated oligogalacturonides, while other pectinases act sequentially to degrade pectin molecule totally. The major source of PNL on industrial scale is fungi belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium*, although a few bacterial PNL are also reported [1]. Solid-state fermentation (SSF) has emerged as preferred technology in industrial fermentation as it offers several practical and economical advantages [2]. Agro-industrial residues and wastes such as wheat bran, rice bran, sugarcane bagasse, corncobs, citrus wastes, apple pomace, and a variety of such byproducts are potentially good substrates for SSF [3]. Acidic PNL have biotechnological potential in fruit juice industries due to the fact, that they degrade pectin without disturbing the ester group, which is responsible for specific aroma of the juice, and also it does not lead to formation of the toxic alcohol methanol.

Though there is a considerable amount of literature on application of acidic PNL in juice clarifying activity [4-6], reports on retting of natural fiber by alkaline PNL are few [7]. Retting is a process of fiber separation from non-fiber parts of plants. The plant fibers are held together by pectin, and pectin plays an important role as a binder between adjacent cells [8]. Since PNL degrades pectin, it is expected that it should play a role in the retting of natural fibers. Fibers present in the stems of many herbaceous dicots, such as flax (*Linum usitatissimum*), Indian hemp (*Cannabis sativa*), and Sunn hemp (*Crotalaria juncea*) are potent source of fibers for textile industries. This communication reports on the purification and characterization of pectin lyase secreted by *P. citrinum* MTCC 8897 and retting of *L. usitatissimum*, *C. sativa*, and *C. juncea* fibers by the purified PNL.

Abbreviations: PNL, pectin lyase; SSF, solid-state fermentation.
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MATERIALS AND METHODS

Chemicals. Citrus pectin, DEAE-cellulose, and Sephadex G-100 were purchased from Sigma (USA). Other chemicals were procured either from Merck (Germany) or Navi Mumbai and S. D. Fine (India) and were used without further purification.

Enzyme assay. Enzyme activity of PNL was assayed by a method reported in the literature [9]. Assay was performed by monitoring the increase in optical density at 235 nm due to formation of unsaturated uronide product using a Hitachi (Japan) model U-2000 UV/vis spectrophotometer, which was fitted with an electronic temperature control unit and had a least count of 0.001 absorbance unit. Enzyme solution (0.2 ml) was added to a reaction mixture containing 0.8 ml citrus pectin (1% w/v) and 2 ml of 100 mM carbonate buffer (pH 9.0) maintained at 37°C. Optical density was measured at zero time and after 20 min, and the steady state velocity was calculated as absorbance change per minute. Enzyme activity was defined in terms of μmoles of unsaturated product released per minute, based on the molar extinction coefficient value of 5500 $M^{-1}\cdot cm^{-1}$ of the unsaturated product.

Protein estimation. Protein was determined by the Lowry method [10] taking bovine serum albumin as the standard.

Screening of PNL producer. A few fungal strains were isolated from soil samples containing decaying vegetation from the backyard of the Department of Biotechnology (D. D. U. Gorakhpur University, Gorakhpur, India) using a serial dilution technique [11]. The isolated and purified fungal strains were screened for the secretion of pectinases by a plate assay method [12]. The fungal strain showing prominent halo zone were further screened for PNL secretion in liquid culture medium containing KH_2PO_4 (0.8%), K_2HPO_4 (0.25%), $MgSO_4\cdot 7H_2O$ (0.11%), $(NH_4)_2SO_4$ (0.1%), and yeast extract (0.03%) and supplemented with 2.0% (v/v) sugarcane juice [13]. The initial pH of the medium was adjusted to 6.3 with dilute NaOH solution. One milliliter of spore suspension (spore density $5\cdot 10^6$ spores/ml) from agar slant was inoculated aseptically into 25 ml of sterilized liquid culture medium contained in 100 ml culture flasks. The flasks were incubated at 25°C in a biological oxygen demand (BOD) incubator (Narang Scientific Works, India), and cultures were allowed to grow under stationary conditions. One milliliter of the culture filtrate was drawn at regular interval of 24 h using a sterile syringe and assayed for the activity of pectin lyase. The PNL producing fungal strain was identified as *P. citrinum* and assigned fungal strain number MTCC 8897 at the Microbial Type Culture Collection and Gene Bank (Institute of Microbial Technology, Chandigarh, India) and deposited there. The culture was maintained by sub-culturing on potato dextrose agar slants at 30°C fortnightly.

Effect of pH of medium on pectin lyase secretion. Different initial pH values (1.0-10.0) were produced using dilute NaOH solution in 10 different sets of duplicate 100 ml culture flasks each containing 25 ml of liquid culture medium (as given in the screening section). One milliliter of spore suspension (spore density $5\cdot 10^6$ spores/ml) from agar slant was inoculated aseptically into each flask. All the flasks were incubated at 25°C in the BOD incubator, and on sixth day (day for maximum secretion of PNL) all flasks were harvested separately and checked for PNL activity by the method given in enzyme assay section.

Effect of sugarcane juice concentration on PNL secretion. Different concentrations of sugarcane juice (v/v) were taken (3-25%) in eight sets of duplicate 100 ml conical flasks, each containing liquid culture medium pH 7.0. One milliliter of spore suspension (spore density $5\cdot 10^6$ spores/ml) from an agar slant was inoculated aseptically into each flask containing 25 ml of liquid culture medium. All the flasks were incubated at 25°C in the BOD incubator for 6 days.

Effect of different solid supports on PNL secretion. For studying the effect of various solid supports on secretion of pectin lyase, three different bagasses, namely sugarcane, carrot, sweet lime, and wheat bran, were taken in range of 2-12% (w/v) in the liquid culture growth medium. Six duplicate sets of 250 ml culture flasks each containing 25 ml of culture medium (pH 7.0) and their respective solid supports were taken. One milliliter of spore suspension (spore density $5\cdot 10^6$ spores/ml) from an agar slant was inoculated aseptically into each flask. All the flasks were incubated at 25°C in the BOD incubator, and on sixth day all flasks were harvested separately by adding 10 ml of cold distilled water.

Culture growth for purification of the enzyme. The fungal strain *P. citrinum* MTCC 8897 was grown for purification of extracellular PNL in SSF medium in six 250-ml culture flasks. Each flask contained 25 ml of liquid culture media (pH 7.0) composed of KH_2PO_4 (0.8%), K_2HPO_4 (0.25%), $MgSO_4\cdot 7H_2O$ (0.11%), $(NH_4)_2SO_4$ (0.1%), yeast extract (0.03%), and 10% (v/v) sugarcane juice, and all the flasks were supplemented with 8% sugarcane bagasse (w/v). One milliliter of spore suspension (spore density $5\cdot 10^6$ spores/ml) from an agar slant was inoculated aseptically into each 250 ml culture flasks containing sterilized SSF medium. The flasks were incubated at 25°C in the BOD incubator, and the cultures were allowed to grow under stationary conditions. On the sixth day, 10 ml of cold distilled water was added to each flask and mixed thoroughly using a glass rod. All the cultures were pooled and filtered using four layers of cheesecloth, and the filtrate was centrifuged at 10,000*g* for 20 min at 4°C. The pellet was discarded, and the supernatant was used as crude enzyme source.

Purification of the enzyme. The clear culture filtrate (100 ml) obtained after centrifugation was concentrated

to 2 ml using an Amicon concentration cell with PM-10 ultrafiltration membrane having 10 kDa molecular weight cut-off value. The concentrated enzyme preparation was dialyzed against 2 liters of 20 mM sodium phosphate buffer (pH 7.0). The dialyzed enzyme was centrifuged at 10,000*g*, and the supernatant was loaded on a Sephadex G-100 column (1.6 × 78.0 cm) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The flow rate was maintained at 7 ml/h, and fractions of 5 ml were collected and analyzed for protein and PNL activity. All the active fractions were pooled and concentrated to 5 ml again using the Amicon concentration cell and PM-10 ultrafiltration membrane. The concentrated enzyme obtained after gel filtration chromatography was dialyzed against 5 liters of 50 mM sodium-phosphate buffer (pH 6.0). The dialyzed enzyme solution was loaded on a DEAE-cellulose column (2.0 × 6.5 cm) that was pre-equilibrated with 50 mM sodium-phosphate buffer (pH 6.0). The protein adsorbed on the column was washed with same buffer and collected as fractions of 5 ml each. The column was eluted by applying a linear gradient to 1 M NaCl in the same buffer (50 ml + 50 ml) at a flow rate of 10 ml/h, and fractions of 5 ml were collected. All the fractions were analyzed for protein and PNL activity.

SDS-PAGE. The enzyme purity and the molecular mass were estimated by 10% SDS-PAGE using phosphorylase B (97.4 kDa), BSA (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa) as standard proteins [14, 15]. The protein bands were located by silver staining.

Characterization of PNL. The K_m and k_{cat} values of the purified enzyme were determined by measuring steady state velocities of the enzyme catalyzed reaction at different concentrations of citrus pectin and drawing double-reciprocal plots [16]. Calculations were made using linear regression analysis. The pH optimum was determined by measuring steady state velocity in buffered reaction solution using 100 mM glycine-HCl (pH 1.0-2.0), citrate-phosphate (pH 3.0-5.0), sodium-phosphate (pH 6.0-8.0), sodium-carbonate (pH 9.0-10.0), and sodium phosphate-NaOH (pH 11.0-12.0). The pH stability of the enzyme was studied by exposing the enzyme to buffers of different pH values for 24 h at 20°C. The optimum temperature for the enzyme activity was determined by assaying the activity of the enzyme at different temperatures in the range 10-100°C. Thermal stability of the enzyme was tested by incubating an enzyme aliquot at a particular temperature for 1 h.

Retting efficiency of purified PNL. The retting of natural fibers of mature stems of *C. sativa* (Indian hemp), *C. juncea* (Sunn hemp), and *L. usitatissimum* (flax) was studied using a reported method [17] with minor modifications. For each natural fiber, two sets of tubes (i.e. sets A and B) were made, and each set had three test tubes. All three tubes of set A in case of the three fibers each contained 10 ml of 100 mM sodium-carbonate buffer (pH 9.0) + 10 mM EDTA and were designated as AE^1, AE^2, and AE^3 containing 0.08, 0.16, and 0.24 IU of the purified enzyme, respectively. Set B was the same except that they did not contain EDTA, and they were designated as BE^1, BE^2, and BE^3. Approximately 10-cm long respective stems were put in each test tube in case of all the three fibers. Simultaneously, one test tube was made as control designated as C containing 0.24 IU of deactivated enzyme with its respective stem in the cases of all the three fibers. All the test tubes were incubated in a water bath at 37°C for 24 h. After 24 h, the stems were shaken vigorously each with 10 ml hot water for 1 min, the hot water was poured off, and the resulting stems were photographed.

RESULTS AND DISCUSSION

Production of PNL. Effect of initial pH of the culture media on secretion of PNL by *P. citrinum* is shown in Fig. 1. Acidic pH ranges (1.0-6.0) seems to be unsuitable for PNL secretion, while neutral pH provided the maximum

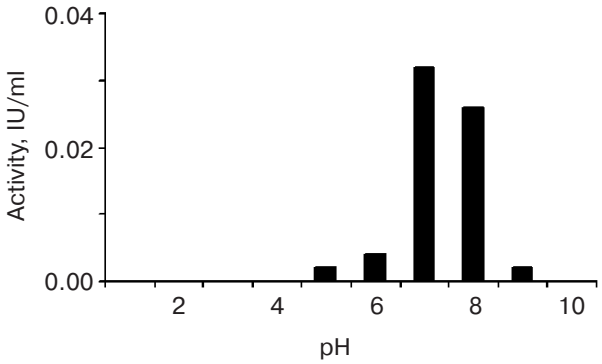

Fig. 1. Effect of initial pH on PNL production by *P. citrinum*.

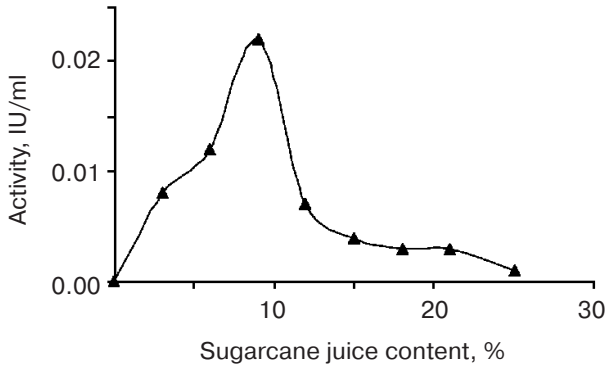

Fig. 2. Effect of sugarcane juice concentration on PNL production by *P. citrinum*.

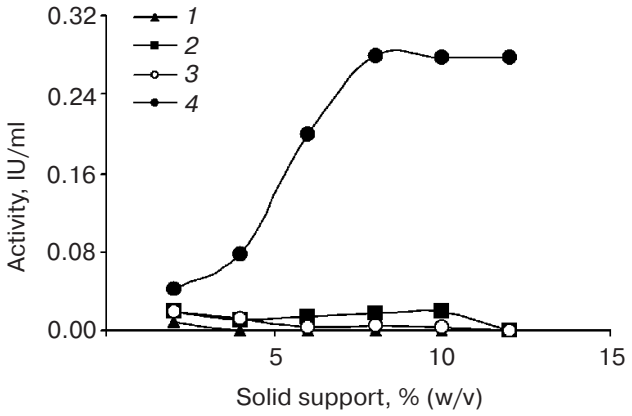

Fig. 3. Effect of different solid supports on PNL production by *P. citrinum*: *1*) carrot bagasse; *2*) sweet lime bagasse; *3*) wheat bran; *4*) sugarcane bagasse.

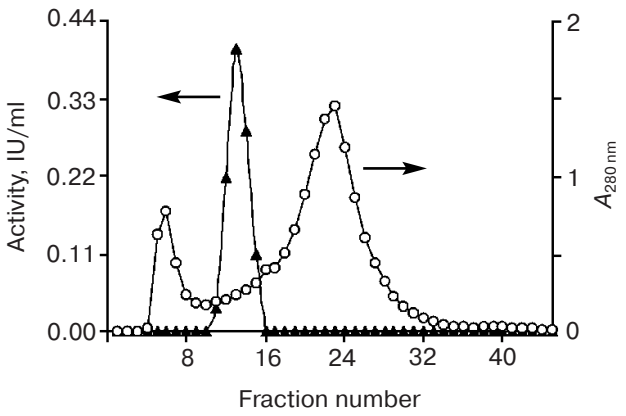

Fig. 4. Gel-filtration chromatography of PNL produced by *P. citrinum*.

secretion. Further increase in pH caused decrease in appearance of PNL activity in the culture medium, and above pH 9.0 there was no sign of PNL activity. This finding is similar to earlier reports [18] in case of *Penicillium expansum*.

The optimization for maximum secretion of PNL was investigated using sugarcane juice as the carbon source. The results given in Fig. 2 show that there is an increase in the secretion of PNL with increasing sugarcane juice up to 10% (v/v), thereafter secretion level decreases, and at 25% (v/v) the secretion is totally inhibited.

The secretion of PNL by *Penicillium* sp. using sugarcane juice as inducers has been reported earlier [13, 19]. Sugarcane juice contains pectic substances, sucrose, and xanthinic compounds. The higher level of the secretion of PNL may be attributed to the synergistic effects of all these chemicals species [13]. SSF using various agricultural wastes as substrates has been reported to be an excellent way to enhance the production of various industrial enzymes from microbes [1]. With this background, an effort was made to investigate the effect of locally available agricultural wastes, namely, sweet lime bagasse, carrot bagasse, sugarcane bagasse, and wheat bran in the range 2-12% (w/v). The results given in Fig. 3 show that sugarcane bagasse is the best solid support for PNL secretion. It is also evident that by increasing the percentage of the sugarcane bagasse, the secretion of PNL increases up to 8% (w/v) and then becomes constant. The results of optimization parameters suggest that liquid culture media (as described in screening section) with initial pH 7.0 containing 10% sugarcane juice (v/v) and 8% sugarcane bagasse gave maximum secretion of PNL in case of *P. citrinum*. This medium was used for growing *P. citrinum* for the purification of PNL.

Purification of PNL. The purification procedure of PNL from the culture filtrate of *P. citrinum* is summarized in Table 1. The elution profile of the enzyme from the Sephadex G-100 gel filtration column is shown in Fig. 4. The activity peak does not coincide with any of the protein peaks, indicating that the enzyme preparation needs further purification. The most active fractions were pooled, concentrated, dialyzed, and loaded on a DEAE-cellulose ion-exchange chromatography column. The protein failed to bind to the matrix, and the activity was recorded in the washing buffer. None of the fractions of the eluted proteins from the DEAE-cellulose column had any activity, and therefore the profile is not shown here. A

Table 1. Purification of PNL secreted by *P. citrinum*

Fraction	Total activity, U	Total protein, mg	Specific activity, U/mg	Yield, %
Crude	27.9	18.7	1.5	100
Concentrated (Amicon)	13.2	3.3	4.0	47.4
Sephadex G-100	9.1	1.1	8.2	32.8
DEAE-cellulose	9.0	0.7	12.9	32.3

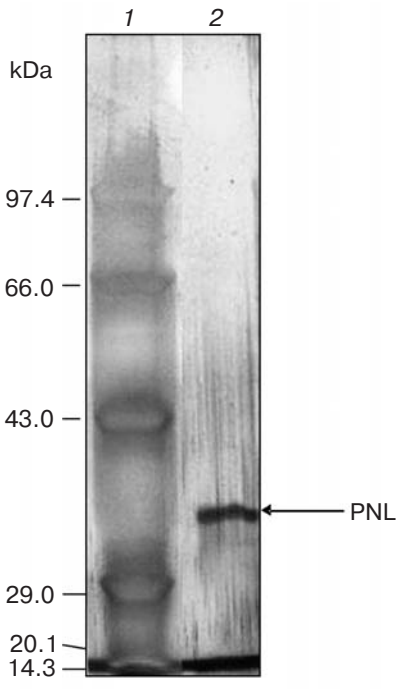

Fig. 5. SDS-PAGE of purified PNL of *P. citrinum*. Lanes: *1*) markers; *2*) purified PNL.

8.6-fold purification with 32.3% yield was achieved. The purification procedure is simpler than the procedures reported for the pectin lyases of *Aspergillus flavus* [7], *P. expansum* [18], *Penicillium italicum*, *Pythium splendens*, and *Lasidioplodia theobromae* [20-22].

SDS-PAGE analysis of the protein, which comes out without binding to the DEAE-cellulose, is shown in Fig. 5. A single band in lane *2* clearly shows that the enzyme is homogeneous. The molecular mass determined by SDS-PAGE was 31 ± 1 kDa, which is in the range of molecular mass (31-38 kDa) reported in literature—*A. ficcum* (31.6 kDa) and *A. flavus* (38.0 kDa) [6, 7], *Aspergillus niger* (38.0 kDa), *Rizopus oryzae* (31.0 kDa), and *Penicillium canescens* (38.0 kDa) [23-25].

Characterization of PNL. The K_m and k_{cat} values were determined to be 1 mg/ml and 76 sec^{-1}, respectively, in the range of substrate concentrations showing no inhibition. The K_m and k_{cat} values are comparable to reported values for PNL from *A. ficcum* [6], *A. flavus* [7], and *P. canescens* [25].

The variation in activity of the purified PNL with pH of the reaction medium is shown in Fig. 6. The pH optimum is 9.0, and the enzyme retains 100% of its activity in pH range 9.0-12.0 if exposed for 24 h. The pH optima of the previously reported PNLs have been found to be acidic for *A. ficcum* (5.0) [6], *Penicillium canescens* (5.5) [25], *Penicillium paxilli* (5.0) [26], and *Aspergillus japonicus* (6.0) [27], neutral for *Rhizoctonia solani* [28] and *P. expansum* [18], and basic for *Penicillium oxalicum* (8.0) [29], *A. flavus* (8.0) [7], and *Fusarium oxysporum* (9.5) [30]. The variation of the activity of the purified pectin lyase with temperature is shown in Fig. 7; the temperature optimum of the enzyme is 50°C. The data on the thermal stability of the purified enzyme are also shown in Fig. 7. The enzyme is fully stable at temperatures up to 40°C, after which it starts loosing activity.

Effects of metal ions and protein inhibitors on the activity of purified PNL. The effects of metal ions like Ag^+, Ca^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, K^+, Mg^{2+}, Zn^{2+}, Na^+, Mn^{2+} and protein inhibitors like EDTA, sodium arsenate, sodium azide, potassium permanganate, and potassium ferrocyanide on the activity of the purified PNL are summarized in Table 2. At 1 mM, Ag^+ totally inhibited the PNL activity, Cu^{2+} and Hg^{2+} were also strong inhibitors, while Ca^{2+} and Co^{2+} had no significant effects. A species of *Aspergillus* has been reported to produce a calcium-dependent PNL [31]. A similar observation for Ca^{2+} (0.2 mM) dependent activity has been reported for PNL produced by *Pythium splendens* [21]. Among protein inhibitors, potassium ferrocyanide and potassium permanganate totally inhibited the activity of the enzyme.

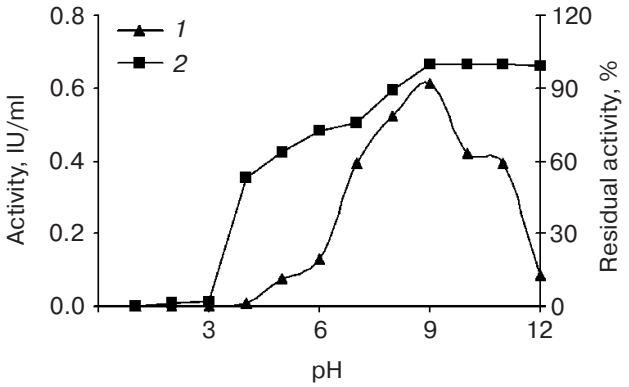

Fig. 6. The pH optimum (*1*) and pH stability (*2*) of the purified PNL produced by *P. citrinum*.

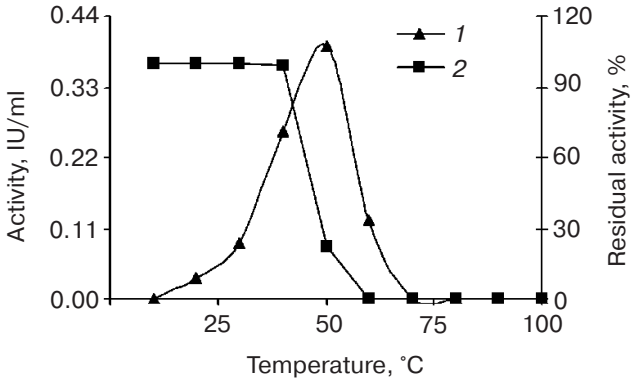

Fig. 7. Optimum temperature (*1*) and thermostability (*2*) of purified PNL produced by *P. citrinum*.

Table 2. Effect of metal ions and protein inhibitors (1 mM) on activity of PNL produced by *P. citrinum*

Addition	Relative activity, %
Control	100
Ag^{+}	0
Ca^{2+}	105
Co^{2+}	114
Cu^{2+}	10
Hg^{2+}	24
K^{+}	91
Mg^{2+}	97
Zn^{2+}	76
Na^{+}	97
Mn^{2+}	76
EDTA	97
Sodium arsenate	122
Sodium azide	91
Potassium permanganate	0
Potassium ferrocyanide	0

While sodium arsenate was found to enhance the PNL activity in the case of this enzyme (Table 2), others have reported a contrasting result showing total inhibition of PNL produced by *Rizopus oryzae* by sodium arsenate [24]. At 1.0 mM concentration, EDTA also had an inhibitory effect on the PNL activity.

Retting efficiency of PNL. Since pectin serves as a glue to hold fibers together in bundles and the bundles to non-fiber tissue [32], pectinases are potential enzymes for retting [33]. Though polygalacturonases [32, 34-37] and pectate lyase [38] have been studied for enzymatic retting, reports on PNL for enzymatic retting are rare [7]. Retting of *C. sativa* (Indian hemp), *C. juncea* (Sunn hemp), and *L. usitatissimum* (flax) were therefore studied to investigate the efficacy of the purified PNL from *P. citrinum* for its possible use in textile industries. The results are shown in Fig. 8. The purified PNL of *P. citrinum* requires EDTA for retting in the case of *L. usitatissimum* stems, whereas EDTA inhibits retting of *C. juncea* and *C. sativa*. In case of *L. usitatissimum*, 0.016 IU of the purified enzyme along with 10 mM EDTA gives complete retting (Fig. 8c, AE^{1}) while the enzyme (0.016 IU) without EDTA gives partial retting (Fig. 8c, BE^{1}). For *C. sativa* (Fig. 8a, AE^{3}) and *C. juncea* (Fig. 8b, AE^{3}), EDTA was found to have an inhibitory effect on retting and gave partial retting when used in combination with purified pectin lyase. With 0.24 IU of the purified enzyme, complete retting of *C. juncea* (Fig. 8a, BE^{3}) and *C. sativa* (Fig. 8b, BE^{3}) was achieved in absence of EDTA.

Recent research has indicated that acidic pectins and Ca^{2+} are located preferentially in the epidermal regions of *L. usitatissimum* (flax) [39], contributing to the structural integrity of the stem and bast fibers. Therefore, chelators such as EDTA have the ability to remove Ca^{2+} and enhance retting of flax [35, 36, 40-42]. This may be the reason for efficient retting of flax fibers in the presence of EDTA. The results of retting of *C. juncea* and *C. sativa* were similar to our earlier report, as in the case of *A. flavus*

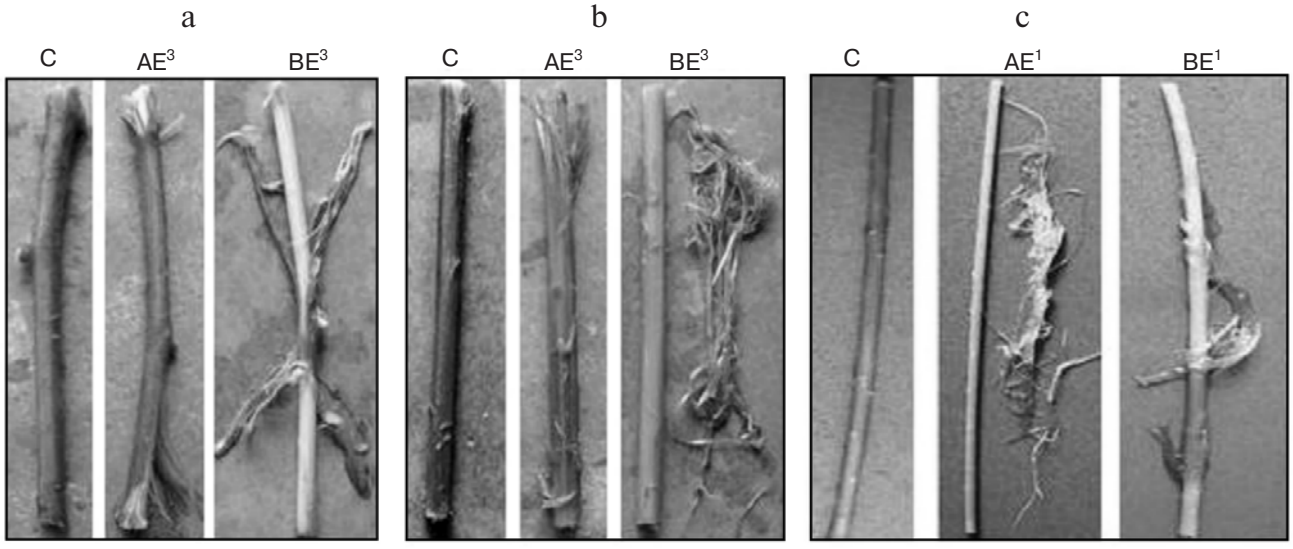

Fig. 8. Effect of purified PNL produced from *P. citrinum* on retting of natural fibers: a) *C. sativa* (Indian hemp); b) *C. juncea* (Sunn hemp); c) *L. usitatissimum* (flax). Stems treated with the enzyme in the presence or absence of 10 mM EDTA are designated as A and B, respectively. The control stem treated with inactivated enzyme is referred as C. E^{1}, E^{2}, and E^{3} contain 0.08, 0.16, and 0.24 IU of the enzyme, respectively, for sets A and B.

[6]. The inhibiting effect of EDTA on retting *C. juncea* and *C. sativa* suggests that their pectin content is not similar to that of *L. usitatissimum*.

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