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ORIGINAL PAPER

Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: production and characterization

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Abstract The enzymatic hydrolysis of cellulose has potential economical and environment-friendly applications. Therefore, discovery of new extremophilic cellulases is essential to meet the requirements of industry. *Penicillium citrinum* (MTCC 6489) that was previously isolated from soil in our laboratory, produced alkali tolerant and thermostable cellulases. Endoglucanase and filter paper activity hydrolase (FPAse) production of *P. citrinum* were studied using wheat bran substrate in solid state and submerged culture. Zymogram analysis of endoglucanase revealed the presence of two isoforms differing in molecular weight. One of them was 90 kDa and other one was 38 kDa. Partially purified endoglucanase showed two different peaks at pH 5.5 and 8.0, respectively, in its pH optima curve. But FPase showed only one peak (at pH 6.5) in its pH optima curve. Cellulase of *P. citrinum* is thermostable in nature. The present work reports for the first time, the alkali stable cellulase from alkali tolerant fungus *Penicillium citrinum.* Thermostable endoglucanase from *P. citrinum* may have potential effectiveness as additives to laundry detergents.

Keywords Alkali tolerant fungus · Extremophilic cellulase · *Penicillium citrinum* · Solid-state fermentation · Wheat bran matrix

Abbreviations

CMC	Carboxymethyl cellulose
DNS	Dinitrosalicylic acid
FPA	Filter paper activity hydrolase
PDA	Potato dextrose agar
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
DTT	Dithiothreitol
FPA	Filter paper activity
EDTA	Ethylenediaminetetraacetic acid
SSF	Solid substrate fermentation
SSC	Solid substrate culture
SF	Submerged fermentation

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Introduction

Plant biomass is mainly composed of cellulose, hemicellulose and lignin. Cellulose, which commonly accounts for up to 40% of plant biomass, is an unbranched linear polymer of glucose molecules with β (1,4) linkages. Cellulose degrading enzymes are widely spread in nature, predominantly produced by microorganisms, such as molds, fungi and bacteria [1, 2]. Enzymatic hydrolysis of cellulose requires a consortium of enzymes, including endo-β-1,4-glucanase (E.C.3.2.1.4), exo-β-1,4-glucanase (E.C.3.2.1.91) and β-glucosidase (E.C.3.2.1.21). There is a considerable interest in cellulases as biocatalysts. However, most of the well-studied cellulases show optimum activity at slightly acidic pH ranges (5.0–6.0) and at temperatures between 40 and 70°C [3].

The majority of cellulases utilized in biotechnology are still derived from well-characterized non-extremophilic bacteria and fungi. Reports show that alkaline thermostable cellulase [4] is required for mild treatment in textile industry as well as for detergent industry [5]. Several reports on cellulase enzymes have been published from

Trichoderma sp. and *Aspergillus* sp., etc., using submerged fermentation as well as solid-state fermentation [6]. However, there is a very little information regarding the extremophilic cellulases. Extremophilic cellulases could be obtained either by protein engineering [7, 8] or by isolating extremophilic microorganisms where such unique properties of extremophilic cellulases may already exist [9]. So to obtain high-titre extremophilic cellulase, alkali tolerant *Penicillium citrinum* MTCC 6489 was isolated from soil. Previous reports showed that *P. citrinum* MTCC 6489 produce high extent of alkaliphilic xylanase [10, 11]. This paper reports for the first time about the production and properties of cellulases from the same *P. citrinum*. An attempt was made in optimizing a low-cost solid-state fermentation medium that could allow enhanced product recovery as well as high-enzyme productivity. Production of cellulase in solid-state fermentation was also compared with the submerged fermentations. In the present study, isozyme pattern, pH optimum, temperature optimum, thermostability and the action of several chemicals on the partially purified enzyme have also been characterized.

Materials and methods

Reagents

All the reagents were of analytical grade and purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise stated. Sodium salt of CM cellulose (medium viscosity) was purchased from Merck (Germany). Wheat bran, rice bran and straw were purchased locally.

Microorganism

Alkali-tolerant filamentous fungi *P. citrinum* MTCC 6489 previously isolated from soil in our laboratory was used for the production of cellulase. The microorganism was maintained over potato dextrose agar and cultivated for the cellulase production in both solid-state and submerged fermentations described below.

Production of cellulase on solid matrix

Spores were collected from PDA slants in Mandel mineral salt solution [12] and spread over the solid bed (10^4–10^6 spores per 5.0 g of wheat bran or rice bran or rice straw) uniformly maintaining the moisture content of the solid bed to 90%. Organisms were then allowed to grow at 30°C. At different time interval, enzyme was extracted from wheat bran bed by agitating it in 20 ml of 50 mM sodium acetate buffer (pH 5.5) for 1 h in a shaker with 150 rpm. Supernatant was collected from the agitated mixture after the centrifugation at 3,000*g* for 30 min. Cellulase activity was determined in the supernatant.

Several natural nitrogen sources (mustered, castor, soybean, almond and sesame cake) were used as the supplement (1.5 g of nitrogen sources + 3.5 g of wheat bran) with wheat bran in SSF to assess the alteration in the production profile of cellulase.

Maintenance of the moisture content of the solid bed

The moisture content of the wheat bran was estimated by drying 10 g of wheat bran to a constant weight at 105°C and the dry weight was recorded. To fix the initial moisture content of the solid medium, wheat bran was soaked with desired quantity of water. After soaking, the sample was again dried as described above and the moisture content was calculated as follows:

$$\text{Percent moisture content of the solid medium} = (\text{wt. of the wheat bran} - \text{dry wt.}) \times 100/\text{dry weight}$$

Production of cellulase in shake flask culture

For submerged fermentation, *P. citrinum* (10^4–10^6 spores per 50 ml liquid culture) was grown in liquid medium [composition: KH_2PO_4 0.5 g, $(NH_4)_2SO_4$ 0.5 g, $CaCl_2$ 0.08 g, $MgSO_4{\cdot}7H_2O$ 0.06 g, $FeSO_4{\cdot}7H_2O$ 5 mg, $MnSO_4{\cdot}7H_2O$ 1.6 mg, $ZnSO_4{\cdot}H_2O$ 1.4 mg, $CoCl_2{\cdot}6H_2O$ 2.0 mg, Tween 80 0.02 ml, wheat bran 2.0 g, H_2O 100 ml] containing wheat bran as the sole carbon source at 30°C for 120 h in shaking condition of 200 rpm. Extra cellular cellulase activity was measured in the culture filtrate of the submerged culture.

Partial purification of cellulase

All purification steps were performed at 4°C unless otherwise stated. The crude enzyme extract was subjected to 0–80% $(NH_4)_2SO_4$ saturation. The precipitated protein collected by centrifugation at 10,000*g*, was dissolved in 50 mM NaOAc buffer (pH 5.5) and dialyzed overnight against the same buffer. Further purification was performed by anion exchange chromatography using FPLC Mono Q Sepharose column (0.7 × 2.5 cm, Amersham Pharmacia, Uppsala, Sweden). Proteins were eluted by a linear gradient of 0–0.5 M NaCl. Elution of proteins was monitored by measuring the absorbance at 280 nm. The active fractions

were pooled and concentrated by ultra filtration on a centriprep 10 system (Amicon Corp., Danvers, MA, USA).

Cellulase assay

All activity assays were carried out in 50 mM sodium acetate buffer (pH 5.5) unless otherwise stated. Endoglucanase activity was determined in accordance with the International Union of Pure and Applied Chemistry recommendations, with a 1% solution of CM cellulose as the substrate. The release of reducing sugars in 30 min at 50°C was measured by the DNS method [13]. FPase activity was assayed [14] in a manner similar to that used to determine endoglucanase activity, by taking 50 mg of Whatman No. 1 filter paper in 50 mM sodium acetate buffer (pH 5.5) as the substrate. Total protein in the crude enzyme extract was determined by Bradford method [15]. One unit of enzyme activity is defined as the amount of enzyme producing 1 μmole of glucose equivalents/min under the given conditions. To study the effect of pH on enzyme activity, substrates (CM cellulose or filter paper) were either dissolved or equilibrated in buffers of different pH for the experimental purpose. The enzyme extract was then added to the substrate and incubated for 30 min at 50°C. Liberated reducing sugar was estimated by DNS reagent. Three different buffer systems were used for the experimental purpose. They were as follows: 50 mM sodium acetate buffer (pH 3.6–5.5), 50 mM sodium phosphate buffer (pH 6.5–7.5) and 50 mM glycine–NaOH buffer (pH 8.5–10.5).

To determine the optimum temperature of endoglucanase and Fpase, the enzyme preparation, containing 5 μg protein, was incubated with 1% of the substrate at different temperature for 30 min in two different buffer solutions of different pH values. Enzyme activity was measured in 50 mM sodium acetate buffer (pH 5.5) and in 50 mM glycine–NaOH buffer (pH 8.5).

Thermostability of endoglucanase and FPase were determined by incubating the enzyme extract in 50 mM sodium acetate buffer (pH 5.5) at 50, 60 and 70°C up to 4 h. At different time interval aliquots, were taken. Denatured protein was precipitated by centrifugation and residual activities of enzymes were measured by DNS reagent.

To investigate the effect of metal ions, detergents and other compounds on enzyme activity, 3 mM or 6 mM concentration of those compounds were used in the assay mixture and the enzyme activities were measured as described previously.

Zymogram analysis of endoglucanase

Zymogram of Endoglucanase was performed according to Coral et al. [16] with little modifications. Enzyme extract was subjected to electrophoresis on 7.5% SDS-PAGE containing 0.1% CM cellulose. After electrophoresis the gel was washed with solution A (50 mM sodium phosphate buffer, pH 7.2, with 40% isopropanol), following the washing with solution B (50 mM sodium phosphate buffer, pH 7.2). After that, the gel was kept overnight in solution C (50 mM sodium phosphate buffer, pH 7.2, containing 5 mM β-mercaptoethanol and 1 mM EDTA) at 4°C. Finally the gel was stained with 0.1% Congo red solution followed by destaining with 1 M NaCl.

Results

Optimization of culture conditions

Fifteen cellulase-producing fungi were isolated from soil using filter paper broth of alkaline pH (pH 10). Of these, *P. citrinum* MTCC 6489 showed highest cellulase activity when grown in CMC agar. High activities were obtained using rice bran, denoting that this agro industrial residue is a good substrate for cellulase production by *P. citrinum* (Table 1). *P. citrinum* showed to be a promising fungus for endoglucanase and FPase production, which produce 1.89 ± 0.12 IU/ml endoglucanase after 168 h and 1.72 ± 0.14 IU/ml FPase after 72 h, respectively, when wheat bran was used as the substrate in SSC. It is worth to mention that fungal protein secretion was highest on wheat bran matrix when compared with the other substrates. Therefore, wheat bran was used as a solid substrate matrix for further enzymatic characterization. Low activities were obtained using rice straw when compared with other data

Table 1 Activity profile of cellulase of *Penicillium citrinum* grown in SSF over different agricultural residues

Raw materials	Protein content (mg/ml)	Endoglucanase		FPase	
		Activity (IU/ml)	Specific activity (μmol/min mg)	Activity (IU/ml)	Specific activity (μmol/min mg)
Wheat bran	0.65 ± 0.021	1.89 ± 0.12	2.99 ± 0.20	1.72 ± 0.14	2.83 ± 0.17
Rice bran	0.17 ± 0.03	2.04 ± 0.13	11.58 ± 1.23	0.64 ± 0.16	3.71 ± 0.96
Rice straw	0.40 ± 0.03	0.93 ± 0.035	2.17 ± 0.08	1.14 ± 0.095	2.66 ± 0.22

The extracted enzymes from SSF bed were subjected to activity measurement of cellulase. The results were expressed in mean ± SD from $n = 5$

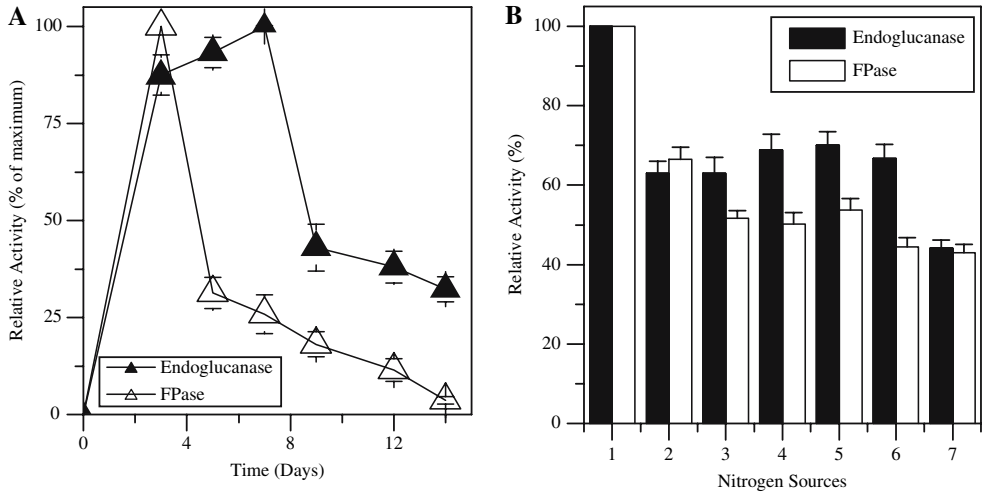

Fig. 1 **a** Time-dependent expression of cellulase of *Penicillium citrinum* in SSF. The relative enzyme activities (% of maximum) were plotted against time. **b** Cellulase production in SSF supplemented with different nitrogen sources: wheat bran (*1*), wheat bran + mustered cake (*2*), wheat bran + sesame cake (*3*), wheat bran + castor cake (*4*), wheat bran + almond cake (*5*), wheat bran + soybean cake (*6*) and wheat bran + neem cake (*7*)

employing wheat bran and rice bran. The low production of protein in rice bran was possibly due to poor growth in rice bran. The kinetic profile of enzymatic production by *P. citrinum* in SSC using wheat bran as the substrate was obtained after cultivation at 28°C for 14 days. Endoglucanase and FPase were differentially expressed with time in solid-state culture (Fig. 1a). This can be explained by the fact that FPase gene may be rapidly expressed followed by induction [17] than endoglucanase gene expression. The highest endoglucanase activity was obtained after a 7-day culture (Fig. 1a). Several natural nitrogen sources were supplemented in the SSF medium (Fig. 1b), but no effective enhancement of cellulase production was observed, implying that cellulase expression is solely regulated by carbon sources.

Comparative analysis of cellulase production in solid state and submerged culture

The comparative analysis (Fig. 2) of cellulase production in solid-state and submerged fermentation revealed that

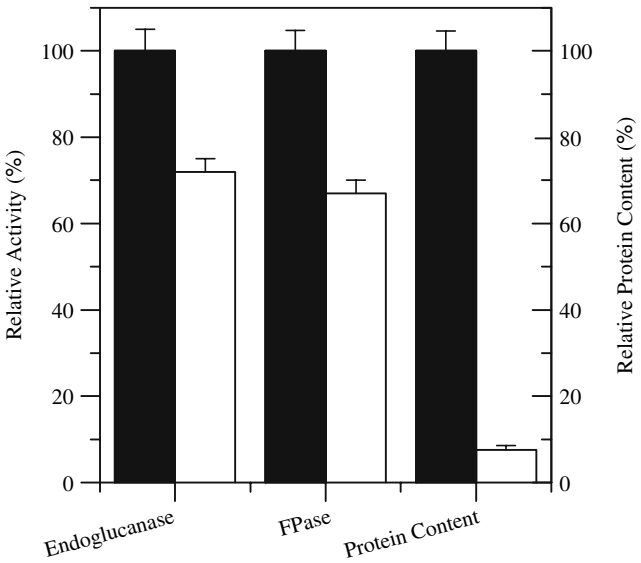

Fig. 2 Comparative analysis of cellulase production and protein secretion in SSF (*filled square*) and SF (*open square*). The results were expressed in mean ± SD from $n = 5$

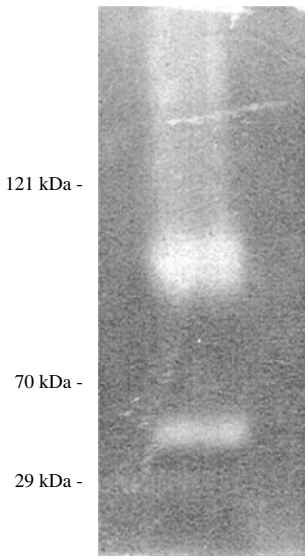

Fig. 3 Zymogram analysis of crude endoglucanase. Enzyme extract (~10 μg protein) was subjected to SDS-PAGE containing corresponding substrate of the enzymes of interest in the running gel. Finally the gel was stained with 0.1% Congo red solution followed by destaining with 1 M NaCl

Table 2 Partial purification profile of endoglucanase and FPase

Purification step	Total protein (mg)	Endoglucanase			FPase		
		Total activity (μmol/min ml)	Specific activity (μmol/min ml)	Purification fold	Total activity (μmol/min ml)	Specific activity (μmol/min ml)	Purification fold
Crude	89.63	268	2.99	1	255.1	2.83	1
80% $(NH_4)_2SO_4$ cut	66.31	262	3.95	1.32	243	3.65	1.29
Mono Q sepharose	12.4	136	10.96	3.66	114.8	9.25	3.27

SSF is much more advantageous in terms of cellulase production and total protein secretion. Nearly 60% of the cellulase activity and only 7% of soluble protein were obtained in case of SF with respect to SSF.

Properties of endoglucanase and FPase produced in SSF

Initial zymogram analysis of the crude enzyme preparation from *P. citrinum*, revealed the presence of two endoglucanase of approximately 38 and 90 kDa (Fig. 3). Multiple forms of cellulolytic enzymes are also a common phenomenon in other organisms [18].

Both endoglucanase and FPase was purified (~threefold) by Mono Q anion exchange chromatography (Table 2). Partially purified endoglucanase from *P. citrinum* showed optimum activity at pH 5.5 with a second peak of activity at pH 8.0 (Fig. 4a). A single peak was observed in the pH profile curve of FPase with optimum pH at 6.5 (Fig 4b). The optimum temperature for endoglucanase was 65°C when measured at pH 5.5 and when assayed at pH 8.5, highest activity was observed at 60°C (Fig. 5a). FPase showed its optimum temperature at 60°C at both the pH

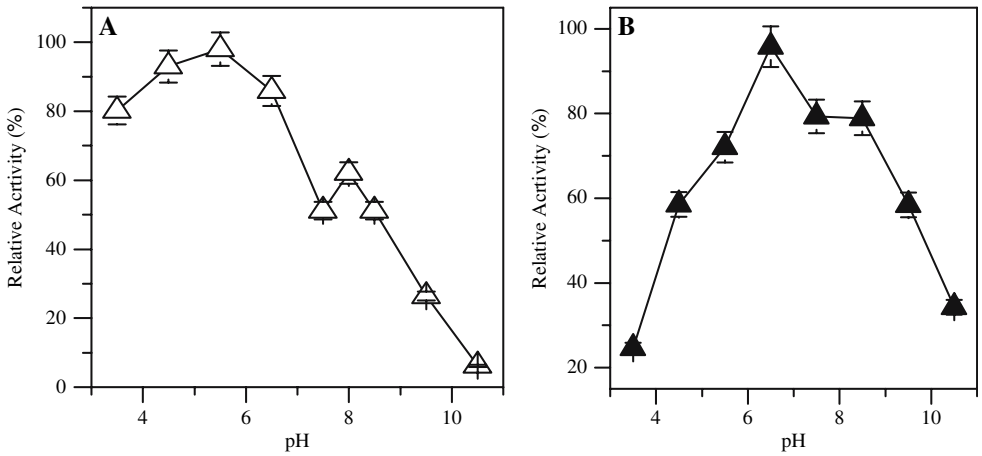

Fig. 4 pH optima curve of **a** endoglucanase and **b** FPase were shown in the figure. Relative enzyme activities (% of maximum) were plotted against pH. All the results were expressed in mean ± SD from $n = 5$

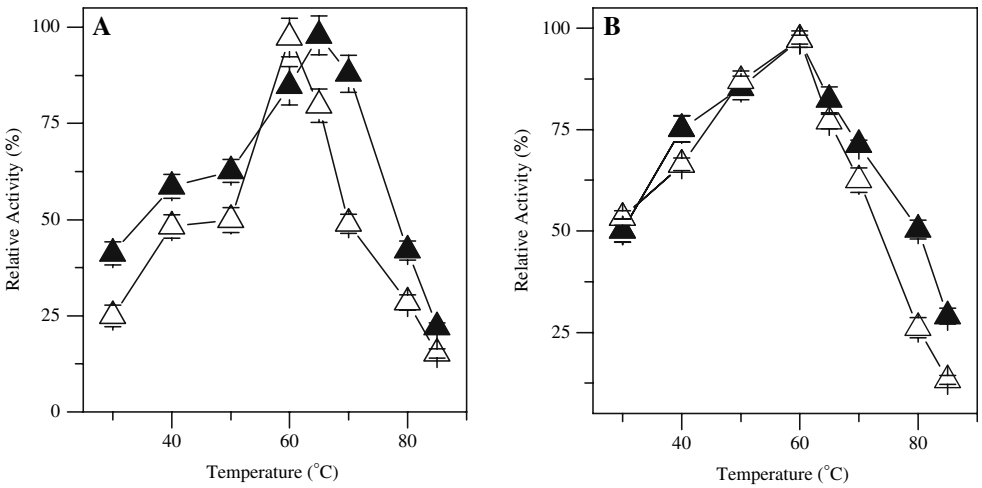

Fig. 5 To determine the optimum temperature of **a** endoglucanase and **b** Fpase, the enzyme preparation, containing 5 μg protein, was incubated with 1% of the substrate at different temperature for 30 min in two different buffer solutions of different pH values. Enzyme activity was measured in 50 mM sodium acetate buffer (pH 5.5) (*filled triangle*) and in 50 mM glycine–NaOH buffer (pH 8.5) (*open triangle*). All the results were expressed in mean ± SD from $n = 5$

(pH 5.5 and 8.5; Fig. 5b). Both the enzymes showed psychrophilic feature, as they showed nearly 30% of their maximum activity when the reaction was carried out at 0°C (data not shown). The psychrophilic feature of the enzyme is quite unusual among the reported fungal cellulases. But this feature was reported in case of blue mussel (*Mytilus edulis*) [19]. Thermal stability studies of endoglucanase (Fig. 6a) showed that the enzyme was fairly stable with in 50–70°C temperature ranges for 2 h of incubation. However, FPase was relatively less thermostable than endoglucanase (Fig. 6b). For most of the microorganisms like *Trichoderma reesei*, *Aspergillus niger*, etc., the optimum temperature for cellulase was within the range of 40–50°C [3], but *P. citrinum* had much higher optimum temperature for cellulase than those microorganisms.

Different metal ions showed varied effects (Table 3) according to the nature of the chemicals; addition of 3 and 6 mM Cu^{2+}, Zn^{2+}, Mg^{2+} or Fe^{3+} (data not shown) caused a slight inhibition of enzyme activity. Strong enzyme inhibition was observed in the presence of Hg^{2+} and Mn^{2+}, which indicated the presence of active site thiol group, in case of both the enzymes namely endoglucanase and FPase. Again the metal ions like Ca^{2+} and Ni^{2+} did not show any effect on endoglucanase and FPase. Nonionic detergents like Triton X100, Tween 20 (data not shown) also showed similar effects. The high stimulation of the enzymatic activity in the presence of thiol group protecting reagents like β-mercaptoethanol, dithiothreitol and cysteine can be explained by preventing the oxidation of sulphydryl groups in presence of the same. Enzyme activity stimulation by such disulfide reducing agents implies that cysteine residue may be the part of the catalytic site of both the enzyme structures [19]. The enzyme activity remained unaltered in presence of EDTA, indicating that endoglucanase and FPase do not contain any metal ion in their active site, which could be chelated by EDTA, thus inactivating the enzymes. On the other hand, NaCl, urea and KCl had slight stimulatory effect on the enzyme activity.

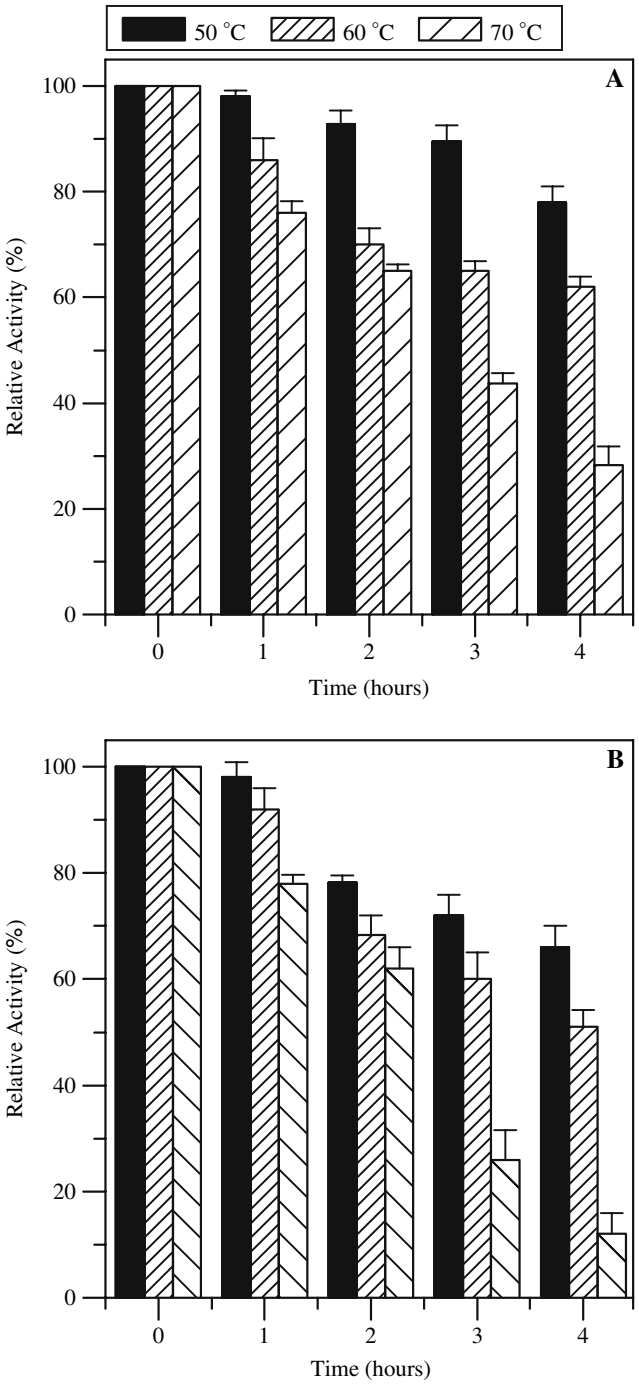

Fig. 6 Thermostability of **a** endoglucanase and **b** FPase were determined by incubating the enzyme extract in 50 mM sodium acetate buffer (pH 5.5) at 50, 60 and 70°C up to 4 h. At different time interval aliquots were taken. Denatured protein was precipitated by centrifugation and residual activities of enzymes were measured by DNS reagent. All the results were expressed in mean ± SD from five separate experiments

Discussion

This is the first report on cellulose degrading enzymes in solid-state culture from a novel alkali-tolerant fungal strain *P. citrinum* MTCC 6489. Apart from the production of industrial enzyme in SSF, the studies on this particular microorganism may also have mycological significance. Nonetheless, cellulose-degrading enzymes produced in solid-state culture were remarkably stable towards temperature, pH, metal ions, etc. Solid substrate culture experiments were carried out for attaining a low-cost medium [20] for cellulase production. There are several advantages of the enzymes produced in solid-state fermentation compared with the produced ones in submerged culture. SSF is relatively resistant to bacterial contamination. The volumetric loading of the substrate is

Table 3 Effect of metal ions and other compounds on cellulase

Compounds	Relative activity of endoglucanase (%)		Relative activity of FPase (%)	
	3 mM	6 mM	3 mM	6 mM
NaCl	106	112	108	121
Urea	114	117	119	123
SDS	104	118	123	128
Ni^{2+}	100	106	101	99
Cu^{2+}	96	90	94	87
Hg^{2+}	–	–	–	–
Mn^{2+}	37	–	42	–
Zn^{2+}	94	86	96	91
Mg^{2+}	87	78	86	74
EDTA	100	103	101	107
Triton X100	101	105	111	109
DTT	125	146	98	114
B Mercaptoethanol	143	159	141	158
L-Cysteine	196	221	187	224
L-Tryptophan	104	119	110	125

The activity is expressed as a percentage of the activity level in the absence of chemicals (control)

much higher in SSF than the submerged fermentation. In case of submerged fermentation, secreted proteins are diluted in a large volume of medium, so for downstream processing, a large volume of solvent is required for solid–liquid extraction or liquid–liquid extraction. On the other hand, SSF requires much less solvent and lower recovery cost than from submerged fermentation. *P. citrinum* MTCC 6489 secrets highest endoglucanase activity (2.04 ± 0.13 IU/ml) in rice bran supplemented solid bed. This value of activity is comparable with those reported for *P. pinophilum* growing in cellulose-supplemented mineral media (1.9 IU/ml) [21], while is lower than those produced by *P. purpurogenum* growing in peptone containing media (24 IU/ml) [22].

Zymogram analysis of the crude enzyme extract of *P. citrinum* MTCC 6489 showed two isoforms for endoglucanase. Among the different sp. of *Penicillium*, *Penicillium pinophilum* showed an extensive multiplicity of cellulase enzymes, which contains an enzyme system with eight endoglucanase components. Similar multiplicity of cellulase was observed in case of *Penicillium brasilinum*, where three different endoglucanases and two cellobiohydrolases have been purified from the same strain [21, 23]. A possible connection between different enzyme isoforms of *P. citrinum* MTCC 6489 and different pH optima could be predicted. Cellulose degrading microorganisms commonly produce multi enzyme systems. Different isoforms of the cellulases function in a distinctive coordinated fashion to hydrolyze the polymeric cellulose to soluble monomeric glucose. The presence of both CMCase and FPAse activity in the crude enzyme extract reflects that *P. citrinum* MTCC 6489 secretes a complex set of cellulose hydrolyzing enzymes, which has distinctive action of both endo- and exo-hydrolysis. Although there is a paucity of studies on the influence of temperature, on the activity of cellulase, the results presented in this paper showed that the cellulase from *P. citrinum* MTCC 6489 has higher optimum temperature and stability than the cellulases from the genus like *Penicillium occitinis*. Endoglucanase from *P. citrinum* MTCC 6489 showed maximum activity at 65°, which was also observed in case of *Penicillium* sp. CR-316 [21].

Except several reported alkaliphilic fungi like *Cephalosporium* sp. (2) [24] and *Aspergillus* sp. [25], generally slightly acidic environment is favorable for the survival of the fungi [1]. *P. citrinum* MTCC 6489 could survive at high-alkaline condition utilizing only cellulosing material as the sole carbon source (pH 10), so it is conceivable that cellulose-hydrolyzing enzymes of *P. citrinum* MTCC 6489 can act under alkaline pH. The explanation for the stimulatory effects may be because of the alteration of enzyme conformation. The enzymes can be modulated by the interaction of cations with the amino acid residues involved in their active sites. This type of interaction can either positively or negatively modulate the enzyme activity.

Reports show that mild treatment of thermostable cellulase is required for the removal of micro fibrils of cotton fabrics generated due to the repeated washing [5]. In that perspective, thermostable endoglucanase from *P. citrinum* MTCC 6489 may have potential effectiveness as additives to laundry detergents. Further studies are required to elucidate the structure-function studies on this new cellulase enzyme.

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