Available online at www.sciencedirect.com
SCIENCE DIRECT®
Enzyme and Microbial Technology 36 (2005) 880–887
ENZYME and MICROBIAL TECHNOLOGY
www.elsevier.com/locate/enzmictec

Production and purification of extracellular chitinases from *Penicillium aculeatum* NRRL 2129 under solid-state fermentation

Parameswaran Binod [a], Tünde Pusztahelyi [b], Viviana Nagy [c], Chandran Sandhya [a], George Szakács [c], István Pócsi [b], Ashok Pandey [a,*]

[a] *Biotechnology Division, Regional Research Laboratory, CSIR, Trivandrum 695019, India*
[b] *Department of Microbiology and Biotechnology, Faculty of Sciences, University of Debrecen, P.O. Box 63, H-4010 Debrecen, Hungary*
[c] *Department of Agricultural Chemical Technology, Technical University of Budapest, 1111 Budapest, Geller ter 4, Hungary*

Received 21 October 2004; received in revised form 29 December 2004; accepted 29 December 2004

Abstract

Fourteen *Penicillium* strains have been screened on wheat bran–crude chitin mixture medium for extracellular chitinase production in solid-state fermentation. Under the experimental conditions tested, *Penicillium aculeatum* NRRL 2129 (=ATCC 10409) was selected as the best enzyme producer. The optimum incubation period for chitinase production by the potent organism was found to be 72 h. Chromatofocusing was performed as the first step in the purification scheme, but high amount of contaminating proteins interfered with the method. Hence, ion-exchange chromatography experiments were carried out followed by gel filtration to separate and isolate chitinase isoenzymes. Four major chitinase peaks of molecular weight 82.7, 44.6, 28.2 and 26.9 kDa were observed after gel filtration chromatography while, on SDS-PAGE, three protein bands of molecular weights 82.6, 33.9 and 29.1 kDa were identified. The purified enzyme showed optimal temperature and pH at 50 and 5.5 °C, respectively.

Keywords: Chitinase; Solid-state fermentation; *Penicillium*; Purification; Chromatography

1. Introduction

Chitin is a homopolymer of *N*-acetyl glucosamine (NAG) and is the major component of the cuticle of insects, shells of crustaceans, and cell walls of most fungi. Chitinases (E.C. 3.2.1.14.) are enzymes capable of hydrolysing chitin to its oligomers and/or monomers. Chitinases from various origins possess versatile enzymological properties and usually are constituents of complex chitinolytic enzyme systems. These enzymes play an important role in nutrition and parasitism in bacteria and fungi and they are also involved in fungal morphogenesis and autolysis [1–5]. While in plants and vertebrates, these enzymes are associated with defence mechanisms [6,7].

Due to various industrial, clinical and pharmaceutical applications of chitin hydrolysing enzymes [8], and because chito-oligosaccharides, the derivatives of chitin hydrolysis, are useful for agrochemical and medicinal purposes [9,10], the potential for industrial application makes the screening for chitinase producing microorganisms an interesting and important task. Among fungi, *Trichoderma harzianum* is currently of major interest [11], however, *Penicillium* species that are versatile organisms and have long been known as producers of a wide variety of extracellular enzymes, some of which are of industrial interest [12–14], had been reported also to be potent producers of chitinases [15,16]. Meanwhile, only very few pieces of information are available on their chitinase production on exogenous chitin substrates.

Since high costs of commercial chitinase production restrict large-scale applications, in recent years, there has been

* Corresponding author. Tel.: +91 471 2495949/2515279; fax: +91 471 2491712.
E-mail addresses: pandey@csrrltrd.ren.nic.in, ashokpandey56@yahoo.co.in (A. Pandey).

0141-0229/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2004.12.031

a lot of interest in enhancing the production of chitinase using fermentation techniques. Both submerged fermentation (SmF) and solid substrate fermentation (SSF) were employed for microbial chitinase production [17–20]. SSF offers a better alternative for cost effective production of enzymes, since it can be carried out using cheaply available agro-industrial residues such as wheat bran, rice husk, sugar cane bagasse, etc. [21–26].

The aim of the present study was to determine the potential of *Penicillium* spp chitinase production in SSF for possible industrial application.

2. Materials and methods

2.1. Fungal strains

Penicillium aculeatum NRRL 2129, *P. chrysogenum* ATCC 10.002 and WA 348, *P. funiculosum* NRRL 1132, NRRL 1768, NRRL 3647 and NRRL 5837, *P. isariiforme* FRR 2639, *P. melinii* NRRL 848, *P. oxalicum* MRL 456, *P. pinophilum* NRRL 1066, NRRL 1142 and NRRL 3503, and *P. verruculosum* WA 30 strains were used in this study. They were obtained from the American Type Culture Collection (ATCC), Manassas, VA, Food Research Laboratory (FRR), CSIRO, North Ryde, NSW, Australia, Structures and Materials, Department of Defence, Materials Research Laboratory (MRL), Ascot Vale, Victoria, Australia, Northern Regional Research Center (NRRL), USDA, Peoria, IL, and Western Australian Department of Agriculture (WA), South Perth, Australia. The cultures were maintained on Potato Dextrose Agar (PDA) slants, sub-cultured regularly at every 2 weeks and stored at 4 °C.

2.2. Screening of fungal strains in SSF

The preliminary screening of the fourteen *Penicillium* strains as above for chitinase production was carried out in cotton-plugged 750 ml Erlenmeyer flasks on solid wheat bran medium containing 10% of purified chitin flakes and moistened with different volume of salt solutions of different compositions (Table 1). Composition of salt solution 1 (w/v) was 0.5% NH_4NO_3, 0.5% KH_2PO_4, 0.1% $MgSO_4 \times 7H_2O$, 0.1% NaCl and 0.1% (v/v) trace element solution, pH 5.0, while the composition of salt solution 2 (w/v) was 0.5% KNO_3, 0.5% $(NH_4)_2HPO_4$, 0.1% $MgSO_4 \times 7H_2O$, 0.1% NaCl and 0.1% (v/v) trace element solution, pH 5.0. The composition of the trace element solution (w/v) was the following: 0.08% $MnSO_4$, 0.17% $ZnSO_4 \times 7H_2O$, 0.25% $FeSO_4 \times 7H_2O$.

Table 1
Composition of SSF media for screening of *Penicillium* strains in chitinase production

Medium	Wheat bran (g)	Crude chitin (g)	Salt solution 1 (ml)	Salt solution 2 (ml)
1	4.5	0.5	10	–
2	4.5	0.5	15	–
3	4.5	0.5	–	10
4	4.5	0.5	–	15

The spore inoculum was prepared by dispersing the spores from fully sporulated slants in 0.1% Tween-80 under aseptic condition and the spore count was adjusted to 5×10^7 spores ml^{-1}. The medium was inoculated with 1 ml of spore inoculum and incubated at 30 °C. The selected chitinase producer *Penicillium* strains were cultured on solid wheat bran medium containing 10% of purified chitin flakes and moistened with 10 ml of salt solution 2 (Table 1).

2.3. Sample preparation for chitinase assay

The extracellular chitinase activity was determined from the culture extract of SSF samples. The content of each Erlenmeyer flask was mixed thoroughly with an appropriate volume (to make a total volume of 100 ml) of 0.1% Tween-80 solution. The SSF material was extracted at room temperature for 2 h with occasional shaking, then centrifuged at $6000 \times g$ for 10 min and the supernatant was used for the assays. All experiments were performed in two sets and average values being reported.

2.4. Measurement of enzyme activities

To determine the chitinase activity from crude SSF extract, the reaction mixture contained 0.5 ml protein extract, 0.5 ml of 1% colloidal chitin in phosphate buffer pH 5.5 and 1 ml distilled water. The solution was incubated at 50 °C for 10 min with continuous shaking. The reaction was terminated by 3 ml 3,5-dinitrosalicylic acid then the solution was mixed and kept in boiling water for 5 min before reading at 575 nm. One unit of chitinase was defined as the amount of enzyme releasing 1 μmol *N*-acetyl-D-glucosamine equivalent per minute from colloidal chitin under the given assay conditions [16].

Chitinase activity in chromatography steps was determined with carboxymethyl-chitin-remazol brilliant violet (CM-chitin-RBV) substrate [22,23]. Protease activity was measured with azocasein substrate and calculated as velocity constant [27]. *N*-acetyl-β-D-glucosaminidase (NAGase) activity was measured by using *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide [28].

2.5. Sample preparation for chitinase purification

Samples were extracted with water containing 0.1% Tween-80, then 2.5% phenyl methyl sulphonyl fluoride (PMSF) was added as protease inhibitor and centrifuged at 10,000 rpm for 30 min at 4 °C before any further purification steps.

2.6. Chromatofocusing

The SSF culture extract was lyophilised and dissolved in minimum quantity of 25 mM imidazole-HCl buffer pH 7.3

containing 3 mM sodium azide and dialysed overnight against the same buffer at 4 °C. About 20 mg protein sample was subjected to Polybuffer Exchanger 94 chromatofocusing column (1 cm × 20 cm). Polybuffer 74 was used as elution buffer and 2 ml fractions were collected at a flow rate of 30 ml h^{-1} [29]. The fractions were analysed for pH, protein (A280) content and chitinase activity.

2.7. Ion-exchange chromatography

DEAE Sephadex A-50 was packed in a 1.6 cm × 20 cm column and pre-equilibrated with 50 mM Tris–HCl buffer pH 7.5 containing 0.1 M NaCl at a flow rate of 30 ml h^{-1} [30]. A linear gradient of 0.1 M to 2 M NaCl concentrations was used for the elution of chitinase activities that was measured from each fraction collected.

2.8. Gel filtration chromatography

Fractions with high chitinase activity from ion-exchange column were applied to Sephacryl S-200 HR column (1.6 cm × 80 cm) that was equilibrated and eluted with 50 mM Na-phosphate buffer pH 7.0 containing 0.2 M NaCl and 3 mM NaN_3 [31]. Calibration of the column was done using different proteins of known molecular weight such as blue dextran (2000 kDa), aldolase (158 kDa), albumin (67 kDa), chymotrypsinogen A (25 kDa), cytochrome C (12.4 kDa) and DNP-L-alanine. The samples were eluted at 60 ml h^{-1} and chitinase activity and protein content was determined.

2.9. Vertical slab gel electrophoresis

Proteins separated during chromatography steps were applied to 12.5% SDS-PAGE [32] and visualised by silver staining method. To determine the relative molecular weights of the protein bands, molecular weight markers (10–200 kDa, MBI Fermentas, Germany) were used.

2.10. Characterisation of crude and purified enzyme

Crude and purified enzymes were used to determine the optimum temperature, pH and thermal stability.

2.11. Determination of optimum temperature and pH

The reaction was carried out at various temperatures ranging from 25 to 80 °C and the enzyme activity at different temperature points were measured in order to find out the temperature optimum of chitinase. Similarly, the enzyme assay was carried out at different pH levels from 2.5 to 7.0 using 0.1 M citrate-phosphate buffer.

2.12. Thermal stability

The crude and purified enzyme was incubated at pH 5.5 (0.1 M citrate-phosphate buffer) at different temperatures (50, 60 and 70 °C) for various time intervals (0, 5, 30, 45 and 60 min). After the heat treatment, samples were cooled and assayed for residual activity at 50 °C for 10 min.

2.13. Measurement of protein content

The protein content of the samples was measured using by Bradford method [33] or A280 nm.

2.14. Chemicals

CM-chitin-RBV was purchased from Loewa Biochemica GmbH, Germany. Azocasein and *p*-nitrophenyl *N*-acetyl glucosaminide were obtained from Sigma Co., USA. Polybuffer 74, Polybuffer Exchanger and Sephacryl S-200HR were obtained from Amersham Bioscience, Sweden. Chitin, crude, from crab shells was obtained from Sigma. All other chemicals were of standard reagent grade and were purchased from Sd Fine Chemicals, India.

3. Results and discussion

3.1. Strain selection and characterisation in SSF

In solid-state fermentation (SSF) experiments, wheat bran and crude chitin flakes were applied to induce the growth and chitinase production of the selected fourteen *Penicillium* strains. Two different salt solutions in two concentrations (Table 1) were applied to moisten the solid dry substrate and to induce the fungal growth in the culture flasks. Chitinase activities were checked 1 day after the visible fungal growth appeared, that meant different culture time of the strains: 2–5 days. The collected results are presented in Table 2. It was worth mentioning that in cultures with Media 1 and 3 with lower phosphate and inorganic nitrate salt concentrations the chitinase activities were much higher than in cultures with higher applied salt concentrations. Application of Media 1 and 3 resulted in comparable chitinase activities in every case with the exception of *P. chrysogenum* ATCC 10.002 strain that has good activities on Medium 2, too. *P. chrysogenum* WA 348 behaved in different way form the other strains considering e.g. the strong activity loss of the cultures, because of the presumably also produced proteolytic activities. Interestingly, high differences were found among various *P. funiculosum* strains, also (Table 2). Activities between 0.1 and 1.86 U/gds were found among these screened fungi. Strains NRRL 1768 and 3647 grew much slower than the other strains. Furthermore, NRRL 3647 produces very low chitinolytic activities (0.1–0.3 U/gds) even after 4 days cultivation. *P. pinophilum* strains were also tested and NRRL 1066 strain was found to produce the highest activities of chitinases (up to 1.55 U/gds) in Medium 3. Furthermore, *P. aculeatum* NRRL 2129 produced quite high activities after 3 days of cultivation, when the best result was gained applying Medium 3. *P. isariiforme* cultures showed very slow growth:

Table 2
Results of screening of *Penicillium* strains for chitinolytic enzyme production in SSF on wheat bran–chitin mixture media

Strains	Medium[a]	Time (days)[b]	pH[c]	Activity (U/gds)[d]
P. aculeatum	1	3	6.6	1.23
NRRL 2129	2		6.6	0.68
	3		6.4	1.65
	4		6.4	0.95
P. chrysogenum	1	3	7.1	1.17
ATCC 10.002	2		7.2	1.08
P. chrysogenum	3	2	6.8	0.63
WA 348	4		6.8	0.49
	1	3	7.4	0.14
	2		7.4	0.14
	3		7.2	0.23
	4		7.2	0.00
P. funiculosum	1	3	6.4	1.33
NRRL 1132	2		6.4	0.75
	3		6.4	1.52
	4		6.4	0.87
P. funiculosum	1	4	6.3	1.04
NRRL 1768	3		6.3	1.86
P. funiculosum	1	4	7.2	0.10
NRRL 3647	3		7.0	0.27
P. funiculosum	1	3	4.6	0.72
NRRL 5837	3		5.5	0.88
P. isariiforme	1	5	5.8	0.34
FRR 2639	3		5.8	0.72
P. melinii	1	2	6.4	0.53
NRRL 848	3		6.4	0.48
P. oxalicum	1	3	7.0	0.85
MRL 456	3		7.0	0.90
P. pinophilum	1	3	6.0	1.21
NRRL1066	3		6.0	1.55
P. pinophilum	1	3	6.4	0.69
NRRL 1142	3		6.4	0.65
P. pinophilum	1	4	6.4	0.53
NRRL 3503	3		6.4	0.40
P. verruculosum	1	3	5.5	0.54
WA 30	3		5.5	0.55

[a] See Table 1.
[b] Culture time.
[c] Final pH of the culture before extraction.
[d] gds: gram dry substrate.

sample could be taken only after 5 days cultivation and although the final pH was quite low, no significant enzyme activities were measurable from the extracts (0.34–0.72 U/gds). In contrast to *P. isariiforme*, *P. melinii* grew rather quickly on Media 1 and 3, however, here high activities were also not visible (0.48–0.53 U/gds). Growth of *P. oxalicum* and *P. verruculosum* strains on Media 1 and 3 were very similar: after 3 days chitinase activities were measured from the cultures. *P. oxalicum* strain produced higher extractable activities (up to 0.9 U/gds).

After 3–5 days incubation time, among the 14 *Penicillium* strains (Table 2) that were screened for chitinase production in SSF, three strains: *P. aculeatum* NRRL 2129, *P. funiculosum* NRRL 1768 and *P. pinophilum* NRRL 1066 were found to be the best chitinase producers under the given conditions, therefore, these strains were selected for the further studies.

In the case of *P. funiculosum* NRRL 1768 strain, the increase of the total protein content together with all enzyme activities was delayed in the SSF in comparison to *P. aculeatum* cultures since the extracted protein samples showed significantly increase in the measured values only after 48 h incubation time (Fig. 1b). Chitinase and NAGase activities increased parallel with protein content while only very low protease activities were detected at acidic pH.

In the case of *P. pinophilum* NRRL 1066 strain, the increase of the total protein was not very intensive in the second 24 h but it became stronger further on (Fig. 1c). Interestingly, the ratio of the chitinase and NAGase enzyme activities in the protein extracts was practically the same in the *P. aculeatum* and *P. pinophilum* cultures while in *P. funiculosum*, it was quite increased in favour of the polymer hydrolysing chitinase activities.

In *P. aculeatum* NRRL 2129 SSF cultures, the total protein content of the fermented samples showed a steady increase during the incubation time while total chitinase activity showed increased levels up to 72 h (1.23 U), however, it was reduced considerably during further incubation (Fig. 1a). Protease and NAGase activities also showed great increase in the extracts up to 96 h. The high increase in the protease activities could explain the decrease in the chitinase yield after an optimum incubation time since the presumable reduced nutrient level in the medium could cause induction of high protease production and also enzyme denaturation of the secreted proteins in the fungal culture. A similar drop in chitinolytic activity was observed by a marine fungus *Beauveria bassiana* after 72 h incubation in a wheat bran based medium [34]. Interestingly, NAGase activity showed greater stability in the medium. In *P. chrysogenum* [28] liquid cultures *N*-acetyl-β-D-hexosaminidase activities were also shown to be stable despite of concomitantly increasing proteolytic activities in the medium.

3.2. Purification of *P. aculeatum* chitinolytic enzymes

From the screening experiment, it was found that *P. aculeatum* NRRL 2129 produced quite high activities after 3 days of cultivation. So this strain was selected for further studies.

3.3. Chromatofocusing

Analysis of the fractions obtained after chromatofocusing showed two minor peaks with low chitinase activity, which was indicative of significant loss of chitinase activity on the PBE matrix and/or during elution. Furthermore, but the presence of huge amount of contaminating other protein made it difficult to proceed the sample for further purification (Fig. 2).

3.4. Ion-exchange chromatography

The elution profile on DEAE Sephadex A-50 was resolved into one major chitinase peak (fraction 29) as revealed in

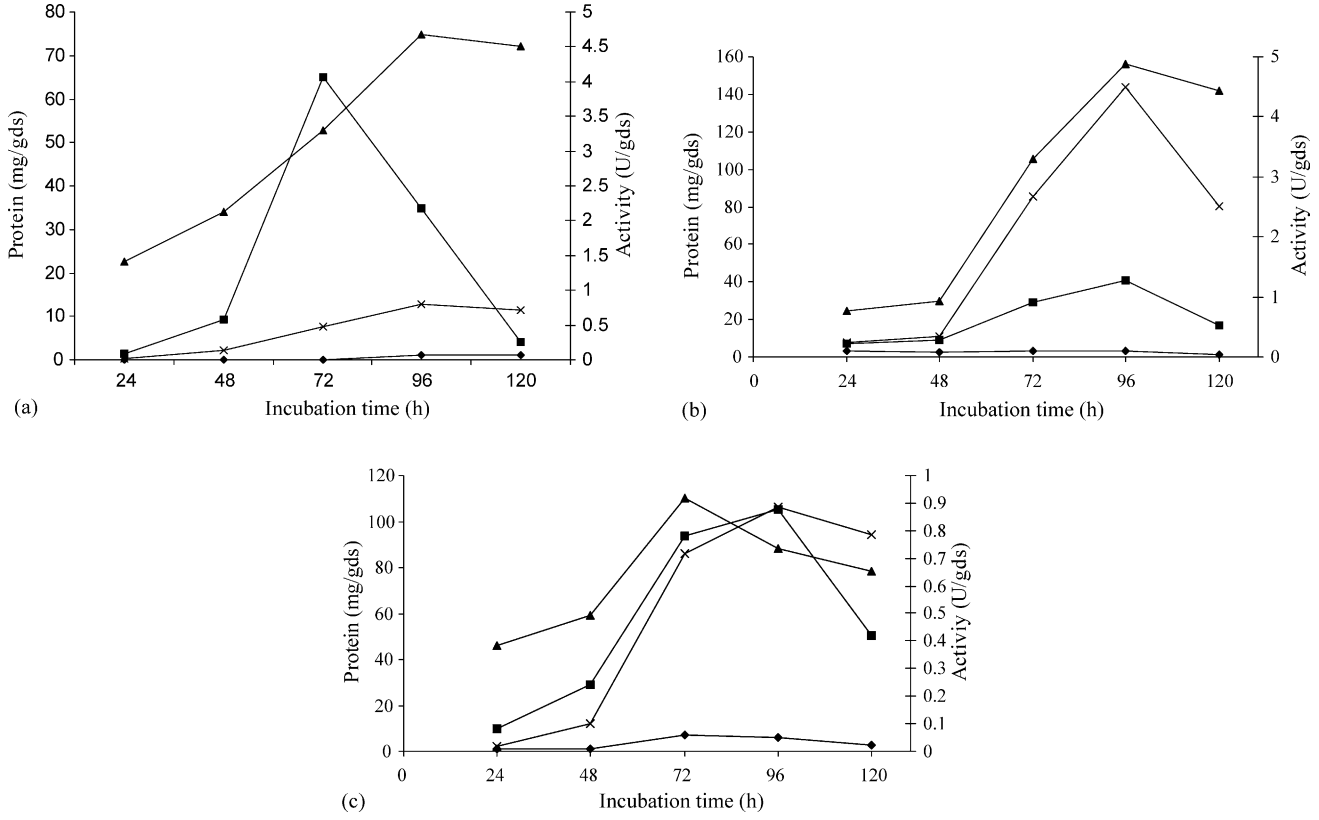

Fig. 1. (a) Extracellular protein production of *P. aculeatum* NRRL 2129 in SSF culture. gds: gram dry fermented substrate. Protein (▲; μg ml^{-1}), chitinase (■; A595), NAGase (×; nkat mg^{-1}10^{-4}), protease (♦; K). (b) Extracellular protein production of *P. funiculosum* NRRL 1768 in SSF culture. gds: gram dry fermented substrate. Protein (▲; μg ml^{-1}), chitinase (■; A595), NAGase (×; nkat mg^{-1}10^{-4}), protease (♦; K). (c) Extracellular protein production of *P. pinophilum* NRRL 1066 in SSF culture. gds: gram dry fermented substrate. Protein (▲; μg ml^{-1}), chitinase (■; A595), NAGase (×; nkat mg^{-1}10^{-4}), protease (♦; K).

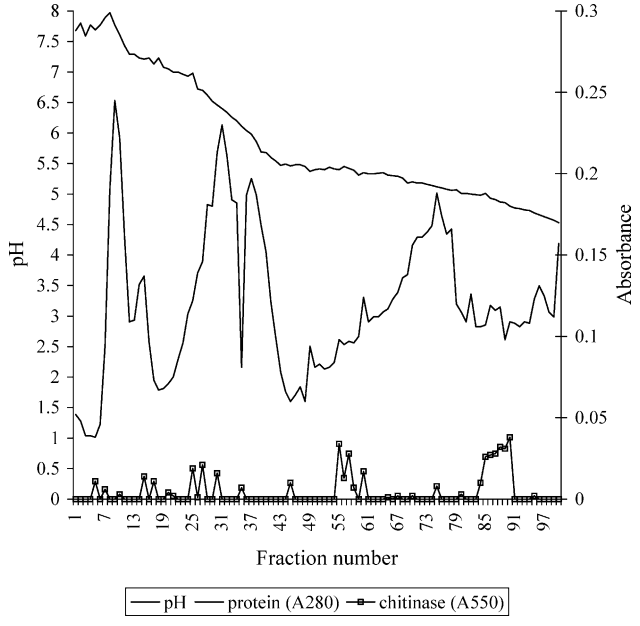

Fig. 2. Chromatofocusing of *P. aculeatum* NRRL 2129 chitinases on Poly-buffer Exchanger 94 between pH 4–7.

Fig. 3. Comparing with the activity of SSF culture filtrate, the yield of chitinase was 60.3% and the purification factor is 2.9 (Table 3). This fraction was then applied for further separation on Sephacryl S-200HR gel filtration column. Sheng et al. observed similar elution pattern during purification of chitinase from *Bacillus brevis* using DEAE Sepharose Fast Flow column [35].

3.5. Gel filtration chromatography

Four major chitinase fractions (F 29, 35, 45, 54) were collected after gel filtration chromatography (Fig. 4). The molecular weight of purified fractions were estimated as 82.7, 44.6, 28.2 and 26.9 kDa. The respective purification factor achieved were 94.1, 7.6, 62.8 and 43.7 (Table 3). Gel filtration of *P. oxalicum* chitinase on Sephacryl S-200 yielded 94-fold purified chitinase of molecular weight 21.5 kDa [15].

3.6. Vertical slab gel electrophoresis

Three protein bands of molecular weights, 82.6, 33.9 and 29.1 kDa could be visualised on SDS-PAGE analysis after Sephacryl S-200HR column separation. No clear band was visualised for fraction number 45, this might be due to the low

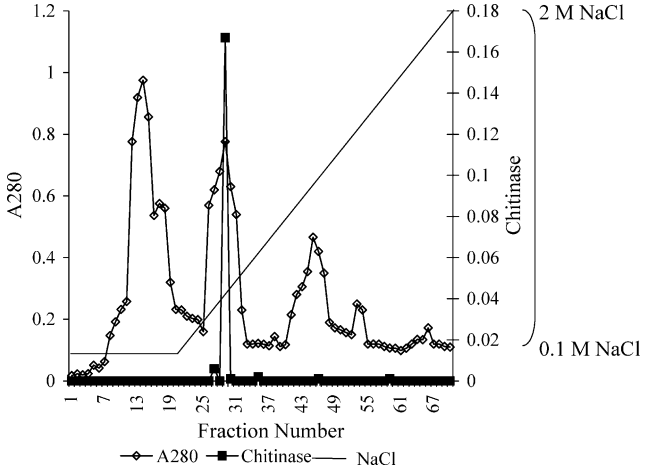

Fig. 3. Fractionation of *P. aculeatum* NRRL 2129 chitinases on a DEAE Sephadex A-50 column (1.6 cm × 20 cm). The column was pre-equilibrated with 50 mM Tris–HCl buffer pH 7.5 containing 0.1 M NaCl and eluted with a linear gradient of 0.1–2 M NaCl in the same buffer. The flow rate was 30 ml/h and the fraction volume was 5 ml.

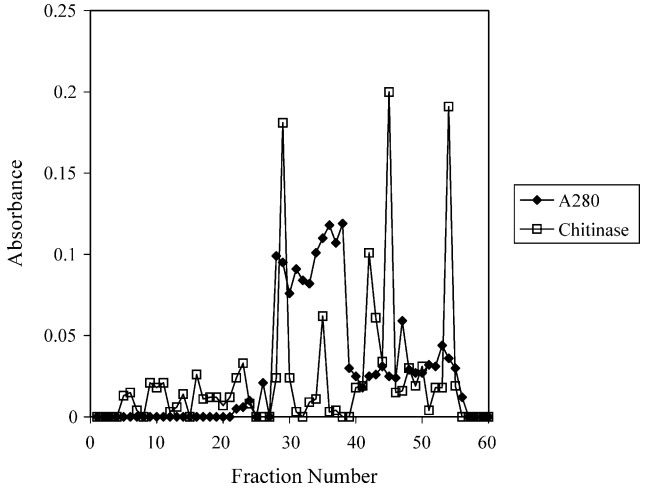

Fig. 4. Further fractionation of *P. aculeatum* NRRL 2129 chitinases from ion-exchange chromatography on Sephacryl S-200HR gel filtration column (1.6 cm × 80 cm) equilibrated and eluted with 50 mM Na-phosphate buffer pH 7.0.

protein content in this fraction (Fig. 5). Comparison of molecular masses of chitinase proteins calculated from SDS-PAGE and Sephacryl S-200 HR gel filtration indicates that these proteins are monomers. Characterisation of purified chitinases from *Trichoderma harzianum* by SDS-PAGE analysis showed three chitinase proteins of molecular masses 33, 37 and 42 kDa respectively [36] while purified chitinase from *Penicillium oxalicum* exhibited a single band of molecular weight 54.9 kDa [15].

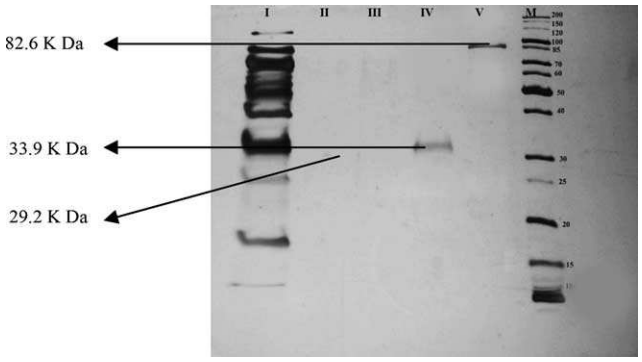

Fig. 5. SDS-PAGE analysis of protein samples obtained after various purification steps. Lane I: fraction containing chitinase activity from DEAE Sephadex A-50 ion-exchange column; Lane II: fraction 54 from Sephacryl S-200HR gel-filtration column; Lane III: fraction 45; Lane IV: fraction 35; Lane V: fraction 29; Lane M: protein marker.

3.7. Characterisation of crude and purified enzymes

Of the four purified fraction obtained, fraction number 29 was used for further characterisation.

3.8. Effect of temperature and pH on chitinase activity

Fig. 6a shows the effect of temperature (at pH 5.5) on chitinase activity of *P aculeatum* NRRL 2129. Highest activity was obtained at 50 °C for both crude and purified enzyme. This result was similar to that observed for chitinase from other microbial sources [36,37].

The optimal pH for crude chitinase, produced by *Penicillium aculeatum* NRRL 2129, was found to be at pH 4.0, but for purified enzyme maximum activity was at pH 5.5 (Fig. 6b). In the crude extract, the combined action of all the chitinases, the pH optimum was found at pH 4.0 but in one of the purified fraction, the optimum pH was at pH 5.5. A fungal chitinase purified by Deane et al. [38] showed optimum pH at 3.5 while endochitinase from *Colletotrichum gleoeosporioides* showed optimum pH as 7.0 [39].

Table 3
Summary of purification steps for chitinase from *P. aculeatum* NRRL 2129

Purification steps	Protein (μg/ml)	Activity (unit/ml)	Specific activity (unit/mg protein)	Recovery (yield), %	Purification fold
Crude extract	3.42	0.290	0.085	100	1.0
Ion-exchange chromatography	0.712	0.175	0.246	60.3	2.9
Gel filtration chromatography					
F 29	0.024	0.188	8.0	64.8	94.1
F 35	0.096	0.062	0.65	21.4	7.6
F 45	0.038	0.203	5.34	70.0	62.8
F 54	0.052	0.193	3.71	66.6	43.7

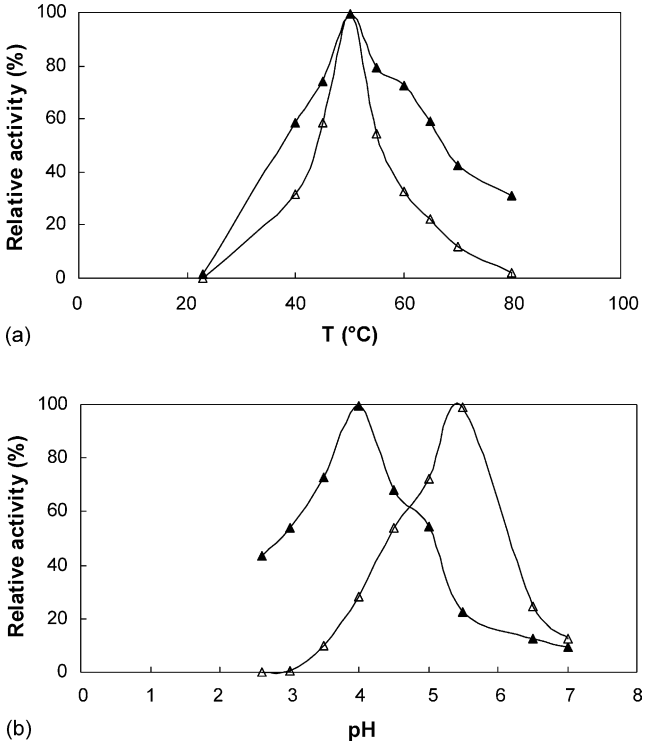

Fig. 6. (a) Effect of temperature on chitinase activity at pH 5.5 (crude extract ▲; purified enzyme △). (b) Effect of pH on chitinase activity at 50 °C (crude extract ▲; purified enzyme △).

3.9. Thermal stability

Thermal stability was investigated at 50, 60 and 70 °C at pH 5.5 (Fig. 7). In the case of crude extract incubation at 50 °C up to 1 h resulted in 25% loss in activity while, with purified enzyme the activity was completely lost. The both crude and purified enzyme completely lost its activity at 70 °C in 60 min. Thompson et al. [40] reported that 90% of activity was retained up to 50 °C by chitinase from *Pseudomonas aeruginosa*. Report shows that chitinase from *Penicillium oxalicum* was thermostable up to 45 °C [41].

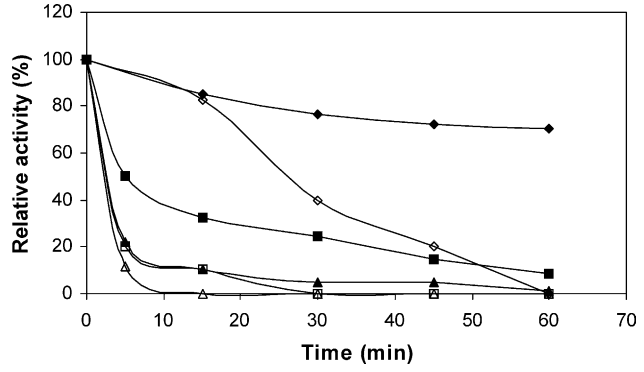

Fig. 7. Thermal stability of chitinase at pH 5.5 (50 °C crude extract ♦; 50 °C purified enzyme ◇; 60 °C crude extract ■; 60 °C purified enzyme □; 70 °C crude extract ▲; 70 °C purified enzyme △).

4. Conclusion

From the above observations it can be concluded that SSF provides an effective means for chitinase production by *Penicillium aculeatum* NRRL 2129. Under the given conditions the chitinase production of the strain was comparable to that of *Trichoderma harzianum* TUBF 781 [17]. The optimum of enzyme induction period was found to 72 h culture time. The purification of the extractable chitinolytic enzymes revealed the multiple enzyme composition that characteristic to other fungal cultures. DEAE Sephadex A-50 chromatography yielded a 2.9-fold purified enzyme. Gel filtration chromatography showed four chitinase fractions of molecular masses ranging from 82 to 27 kDa while SDS-PAGE analysis yielded only three clear protein bands. Optimal temperature and pH of purified enzyme was 50 and 5.5 °C respectively.

Acknowledgements

One of the authors (P.B.) is deeply indebted to Council of Scientific and Industrial Research, New Delhi, India for the award of Junior Research Fellowship for doctoral studies. T.P. was a recipient of a Hungarian Scientific Research Fund postdoctoral fellowship (grant reference number D 034568). I.P. was awarded a Széchenyi István Scholarship by the Hungarian Ministry of Education. The research was a part of Indo-Hungary Intergovernmental S&T collaborative project.

References

[1] Gooday GW, Zhu RW, O'Donnell RW. What are the roles of chitinases in the growing fungus? FEMS Microbiol Lett 1992;100:387–92.
[2] Sahai AS, Manocha MS. Chitinases of fungi and plants: their involvement in morphogenesis and host/parasite interaction. FEMS Microbiol Rev 1993;11:317–38.
[3] Gooday GW. The many uses of chitinases in nature. Chitin Chitosan Res 1997;3:233–43.
[4] Pócsi I, Emri T, Varecza Z, Sámi L, Pusztahelyi T. Allosamidin inhibits the fragmentation and autolysis of *Penicillium chrysogenum*. In: Peter MG, Domard A, Muzzarelli RAA, editors. Advances in chitin science, vol. 4. Potsdam: University of Potsdam; 2000. p. 558–64.
[5] Sámi L, Pusztahelyi T, Emri T, Varecza Z, Fekete A, Grallert Á, et al. Autolysis and aging of *Penicillium chrysogenum* cultures under carbon starvation: chitinase production and antifungal effect of allosamidin. J Gen Appl Microbiol 2001;47:201–11.
[6] Gooday GW. In: Zakaria MB, Wan Muda WM, Abdullah MP, editors. Chitin and chitosan. Malaysia Penerbig Universiti Kebangsaan; 1995. p. 191–202.
[7] Kasprzewska A. Plant chitinases – regulation and function. Cell Mol Biol Lett 2003;8:809–24.
[8] Patil RS, Ghormade V, Deshpande MV. Chitinolytic enzymes: an exploration. Enzyme Microb Technol 2000;26:473–83.
[9] Deshpande MV. Enzymatic degradation of chitin and its biological applications. J Sci Ind Res 1986;45:273–81.
[10] Hirano S. Chitin biotechnology applications. Biotechnol Annu Rev 1996;2:237–58.

[11] Godfrey T, West S. Data index 3–4. In: Godfrey T, West S, editors. Industrial enzymology. London: MacMillan Press; 1996. p. 555–88.
[12] Hamlyn PF, Wales DS, Saga BF. Extracellular enzymes of *Penicillium*. In: Peberdy JF, editor. Penicillium and Acremonium. New York: Plenum Press; 1987. p. 245–84.
[13] Petruccioli M, Federici F, Miller MW. Extracellular enzyme production in species of the genus *Penicillium*. Mycologia 1988;80:726–8.
[14] Wigley RE. Cheese and whey. In: Godfrey T, West S, editors. Industrial enzymology. London: MacMillan Press; 1996. p. 133–54.
[15] Rodriguez J, Copa-Patino JL, Perez-Leblic MI. Purification and properties of a chitinase from *Pencillium oxalicum* autolysates. Lett Appl Microbiol 1995;20:46–9.
[16] Fenice M, Leuba JL, Federici F. Chitinolytic enzyme activity of *Penicillium janthinellum* P9 in bench top bioreactor. J Ferment Bioeng 1998;86:620–3.
[17] Nampoothiri KM, Baiju TV, Sandhya C, Sabu A, Szakacs G, Pandey A. Process optimization for antifungal chitinase production by *Trichoderma harzianum*. Process Biochem 2004;39:1583–90.
[18] Sandhya C, Adapa LK, Nampoothiri KM, Binod P, Szakacs G, Pandey A. Extracellular chitinase production by *Trichoderma harzianum* in submerged fermentation. J Basic Microbiol 2004;44:49–58.
[19] Binod P, Sandhya C, Pandey A. Chitinases. In: Pandey A, Webb C, Soccol CR, Larroche C, editors. Enzyme technology. New Delhi: Asiatech Publishers, Inc; 2004. p. 433–48.
[20] Kapat A, Rakshit SK, Panda T. Optimization of carbon and nitrogen sources in the medium and environmental factors for enhanced production of chitinase by *Trichoderma harzianum*. Bioprocess Eng 1996;15:13–20.
[21] Pandey A. Recent process developments in solid state fermentation. Process Biochem 1992;27:109–17.
[22] Lonsane BK, Ghildyal NP. Exoenzymes. In: Doelle HW, Mitchell DA, Rolz CE, editors. Solid substrate cultivation. Amsterdam: Elsevier; 1992.
[23] Emri T, Molnár Z, Pusztahelyi T, Pócsi I. Physiological and morphological changes in autolyzing *Aspergillus nidulans* cultures. Folia Microbiol 2004;49:277–84.
[24] Pandey A, Soccol CR, Rodriguez-Leon JA, Poonam Nigam. Solid-state fermentation in biotechnology, fundamentals and application. New Delhi: Asiatech Publisher; 2001. p. 221.
[25] Pandey A. Solid-state fermentation. J Biochem Eng 2003;13:81–4.
[26] Larroche C, Theodore M, Gros JB. Growth and sporulation behaviour of *Penicillium roquefortii* in solid substrate fermentation: effect of the hydric parameters of the medium. Appl Microbiol Biotechnol 1992;38:183–7.
[27] Tomarelli RM, Charney J, Harding ML. The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. J Lab Clin Med 1949;34:428–33.
[28] Pusztahelyi T, Pocsi I, Szentirmai A. Aging of *Penicillium chrysogenum* cultures under carbon starvation. II. Protease and *N*-acetyl-β-D-hexosaminidase production. Biotechnol Appl Biochem 1997;25:87–93.
[29] Leiter É, Marx F, Pusztahelyi T, Haas H, Pócsi I. *Penicillium chrysogenum* glucose oxidase – a study on its antifungal effects. J Appl Microbiol 2004;97(6):1201–9.
[30] Pócsi I, Kiss L, Nánási P. Studies on the *N*-acetyl-β-D-hexosaminidase B from germinating *Lupinus luteus* L. seeds. I. Purification and characterization. Biochim Biophys Acta 1990;1039:110–8.
[31] Pócsi I, Kiss L. Kinetic studies on the broad-specificity β-D-glucosidase from pig kidney. Biochem J 1988;256:139–46.
[32] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–8.
[33] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[34] Suresh PV, Chandrasekharan M. Impact of process parameters on chitinase production by an alkalophilic marine *Beauveria bassiana* in solid state fermentation. Process Biochem 1999;34:257–67.
[35] Sheng LI, Zhi-An Z, Ming LI, Zhen-Rong GU, Chen B, Wei-Da H. Purification and characterization of a novel chitinase from *Bacillus brevis*. Acta Biochim Biophys Sinica 2002;34:690–6.
[36] De La Cruz J, Hidalgo-Gallego A, Lora JM, Benitez T, Pinto-Toro J. Isolation and characterization of three chitinases from *Trichoderma harzianum*. Eur J Biochem 1992;206:859–67.
[37] Frandberg E, Schnurer J. Chitinolytic properties of *Bacillus papuli* K1. J Appl Bacteriol 1993;76:361–7.
[38] Deane EE, Whipps JM, Peberdy JF. The purification and characterization of a *Trichoderma harzianum* exochitinase. Biochim Biophys Acta 1998;1383:101–10.
[39] Souza RF, Gomes RC, Coelho RRR, Alviano CS, Soares RMA. Purification and characterization of an endochitinase produced by *Colletotrichum gloeosporioides*. FEMS Microbiol Lett 2003;222: 45–50.
[40] Thompson SE, Smith M, Wilkinson MC, Peek K. Identification and characterization of a chitinase antigen from *Pseudomonas aeruginosa* strain 385. Appl Environ Microbiol 2001;67:4001–8.
[41] Rodriguez J, Copa-Patino JL, Perez-Leblic MI. Purification and properties of a chitinase from *Penicillium oxalicum* autolysates. Lett Appl Microbiol 1995;20:46–9.