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Production of Chitinolytic Enzymes With *Trichoderma longibrachiatum* IMI 92027 in Solid Substrate Fermentation

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Abstract

Thirty *Trichoderma* strains representing 15 species within the genus were screened for extracellular production of chitinolytic enzymes in solid substrate fermentation. *Trichoderma longibrachiatum* IMI 92027 (ATCC 36838) gave the highest yield (5.0 IU/g of dry matter of substrate) after 3 d of fermentation on wheat bran–crude chitin (9:1 mixture) medium. The optimal moisture content (66.7%), chitin content (20%), initial pH of the medium (2.0–5.0), and time course (5 d) of solid substrate fermentation were determined for strain IMI 92027. Cellulase, xylanase, α-amylase, and β-xylosidase activities were also detected. The pH and temperature optima of the chitinase complex of *T. longibrachiatum* IMI 92027 were 4.5 and 55°C, respectively. The enzyme totally lost its activity at 70°C in 5 min in the absence of the substrate but retained about 15% of its initial activity even at 70°C after a 60-min incubation in the presence of solid substrate fermentation solids. Purification of protein extract from the solid substrate fermentation material revealed high chitinolytic activities between p*I* 5.9 and 4.8, where *N*-acetyl-β-D-hexosaminidase and chitinase peaks have been found in the same p*I* range. Two chitinases of 43.5 and 30 kDa were purified at acidic p*I*.

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Index Entries: Chitinase; chitinolytic enzymes; *N*-acetyl-β-D-hexosaminidase; solid substrate fermentation; solid-state fermentation; *Trichoderma longibrachiatum*.

Introduction

Chitin, the β-(1,4)-linked homopolymer of *N*-acetyl-D-glucosamine (NAGA), is one of the most abundant renewable natural resources after cellulose, hemicellulose, and lignin. It is widely distributed in nature as a component of marine invertebrates, insects, fungi, and algae. Chitooligosaccharides and monosaccharides derived as hydrolysis products of chitin are useful materials in the pharmaceutical and food industries *(1,2)*.

Chitinases and *N*-acetyl-β-D-hexosaminidases capable of degrading chitin and chitooligomers directly to NAGA monomers and oligomers have been characterized from various sources such as bacteria, fungi, yeast, and plants *(1,2)*.

Chitinase-producing fungi, especially *Trichoderma* species, can be effective as biocontrol agents against some plant pathogenic fungi *(3)*. An important part of the mechanism involved in the antagonism of *Trichoderma* spp. and fungal pathogens appears to be the production of fungal cell-wall lytic enzymes, including chitinases *(4)*.

The main components of extracellular chitinolytic enzymes of *Trichoderma* sp. are endochitinases ("chitinases") and *N*-acetyl-β-D-hexosaminidases *(5)*. Endochitinases cleave chitin and chitooligomers and release a mixture of soluble low molecular mass end products of different sizes. The main hydrolysis product is diacetylchitobiose. *N*-acetyl-β-D-hexosaminidases split chitooligomers and also chitin progressively from the nonreducing end and release only *N*-acetyl-D-glucosamine (NAGA) monomers *(5)*. *Trichoderma harzianum*—the most studied *Trichoderma* sp. for biocontrol—has been shown to produce 10 different chitinolytic enzymes/isoenzymes *(5)*. Although about 40 *Trichoderma* spp. are currently recognized on the basis of morphologic and molecular characters *(6,7)*, only a few *Trichoderma* spp. have been studied for chitinolytic enzyme production. For example, chitinase production with *T. harzianum* has been reported in submerged fermentation *(8–10)*. In addition, some chitinolytic enzyme components of *Trichoderma viride*, *Trichoderma virens*, *Trichoderma hamatum*, and *Trichoderma reesei* were characterized *(5)*. Chitinase and *N*-acetyl-β-D-hexosaminidase production was also detected in cold-tolerant *Trichoderma aureoviride*, *Trichoderma harzianum*, and *Trichoderma viride* isolates *(11)*. *Trichoderma atroviride* also proved to be a good source of chitinolytic enzymes *(12,13)*. To the best of our knowledge, there are no published studies of chitinase production with *Trichoderma longibrachiatum* isolates.

Solid substrate fermentation holds tremendous potential for the production of microbial enzymes *(14,15)*. Solid substrate fermentation can be of special interest in those processes in which the crude fermented product (whole solid substrate fermentation culture, *in situ* enzyme) may be used directly as the enzyme source *(16)*. Such applications of crude and cheap

Trichoderma enzymes may be forecasted in some agrobiotechnological processes *(17,18)*. Interestingly, we could find no publication on the production of chitinolytic enzymes with *Trichoderma* sp. by solid substrate fermentation.

In the present study, 30 *Trichoderma* strains representing 15 species within the genus were screened in solid substrate fermentation for the production of chitin-degrading enzymes. The objectives were to optimize the medium composition and fermentation parameters with the most promising strain and to examine the properties of chitinolytic enzymes (chitinases and *N*-acetyl-β-D-hexosaminidases) produced by this strain.

Materials and Methods

Microorganisms and Preparation of Inoculum

Trichoderma asperellum TUB F-897, TUB F-1032, and TUB F-1055; *T. atroviride* TUB F-1045 and TUB F-1048; *Trichoderma citrinoviride* ATCC 58843; *Trichoderma erinaceum* TUB F-930 and TUB F-975; *Trichoderma ghanense* DAOM 190.843; *T. hamatum* TUB F-105 (ATCC 62392); *T. harzianum* ATCC 56678, TUB F-691, and TUB F-900; *Trichoderma koningii* TUB F-938 and TUB F-976; *T. longibrachiatum* IMI 92.027 and DAOM 175.956; *Trichoderma pseudokoningii* ATCC 24961; *T. reesei* Rut C30, NRRL 11460, and TUB F-1038; *Trichoderma saturnisporum* ATCC 18903; *Trichoderma spirale* TUB F-893, TUB F-901, and TUB F-970; *T. virens* TUB F-888, TUB F-896, TUB F-926, and TUB F-929; and *T. viride* TUB F-1041 strains were obtained from the American Type Culture Collection (ATCC) (Manassas, VA), the DAOM collection (Ottawa, Canada), the International Mycological Institute (IMI) (Egham, Surrey, UK), the Northern Regional Research Center (NRRL), USDA (Peoria, IL), and the Technical University of Budapest (TUB) collections. TUB isolates have been identified by classic and molecular taxonomy *(7,19,20)*. Although *T. longibrachiatum* IMI 92.027 has been deposited at the IMI and ATCC collections as *T. pseudokoningii*, the latest molecular identification data revealed *(21)* that the strain in fact is *T. longibrachiatum*.

The fungi were grown and maintained on potato dextrose agar (PDA) Petri plates and slants. Viable spores from 7-d-old fully sporulated Petri plates were harvested by washing with 0.1% Tween-80 containing water, and 10^6 CFU (CFU on PDA supplemented with 0.05% Triton X-100) was used for inoculation of 1 g of dry matter (DM) equivalent of solid substrate fermentation substrate.

Substrates

Wheat bran, wheat straw, corncob, canola meal (defatted), oat flakes, and sunflower seed hull were purchased from local sources in Hungary. Wheat straw, corncob, and sunflower seed hull were chopped in a hammer mill into 0.2- to 2-cm particle size. Chitin (practical grade, from crab shells) was obtained from Sigma (St. Louis, MO). Sigmacell Type 20 (Sigma) was used as cellulose powder supplement in some experiments.

Solid Substrate Fermentation

Fermentations were carried out in 750-mL cotton-plugged Erlenmeyer flasks on four solid substrate fermentation media containing 4.5 g of wheat bran, 0.5 g of crude chitin, and 10 or 15 mL of salt solutions for wetting the substrate. Two different salt solutions (I and II) were used. Salt solution I was composed of 5 g/L of NH_4NO_3, 5 g/L of KH_2PO_4, 1 g/L of $MgSO_4 \cdot 7H_2O$, 1 g/L of NaCl, 2 mg/L of $CoCl_2 \cdot 6H_2O$, 1.6 mg/L of $MnSO_4$, 3.45 mg/L of $ZnSO_4 \cdot 7H_2O$, and 5 mg/L of $FeSO_4 \cdot 7H_2O$. Salt solution II contained 5 g/L of KNO_3, 5 g/L of $(NH_4)_2HPO_4$, 1 g/L of NaCl, 1 g/L of $MgSO_4 \cdot 7H_2O$, 2 mg/L of $CoCl_2 \cdot 6H_2O$, 1.6 mg/L of $MnSO_4$, 3.45 mg/L of $ZnSO_4 \cdot 7H_2O$, and 5 mg/L of $FeSO_4 \cdot 7H_2O$. The pH of both salt solutions was adjusted to 5.0. The following salt solutions were used for wetting the mixture of 4.5 g of wheat bran and 0.5 g of crude chitin: medium 1, 10 mL of salt solution I; medium 2, 15 mL of salt solution I; medium 3, 10 mL of salt solution II; medium 4, 15 mL of salt solution II. The wet substrate was sterilized at 121°C for 30 min. The inoculated flasks were incubated at 30°C, and samples were taken for analysis after 3 and 5 d of fermentation. All experiments were carried out in duplicate. The results shown are average values.

Extraction

Enzyme activity was determined from the culture extract of solid substrate fermentation samples. Five grams of dry weight fermented substrate was extracted with 100 mL of water containing 0.1% Tween-80 by shaking for 2 h at room temperature (25°C). At the end of extraction, the suspension was centrifuged (10,300*g*, 10 min). Supernatants were stored at 4°C until the assays were performed.

Enzyme Assays

Chitinase activity was determined as described by Fenice et al. *(22)* using colloidal chitin as substrate and dinitrosalicylic acid reagent *(23)*. Colloidal chitin was prepared according to Roberts and Selitrenikoff *(24)*. One unit of chitinase is defined as the amount of enzyme releasing 1 µmol of NAGA equivalent/min from colloidal chitin under the assay conditions.

Xylanase activity of the supernatant was determined by the method of Bailey et al. *(25)*. Filter paper activity was assayed following the method described by Ghose *(26)*. β-Xylosidase was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-xyloside according to Herr et al. *(27)*. α-Amylase activity was determined as described by Okolo et al. *(28)*.

Effect of Temperature and pH on Chitinase Activity

To determine the optimal temperature of chitinase activity, an assay was performed at 25, 30, 40, 45, 50, 55, 60, 70, and 80°C at pH 5.5 for 10 min using extract of solid substrate fermentation material. To study the optimal

pH of enzyme activity, an assay was performed at 50°C for 10 min in the pH range of 3.0–7.0. Buffers used were 0.1*M* citric acid/sodium citrate (pH 3.0–4.5), 0.1*M* citric acid/disodium phosphate (pH 5.0 to 6.0), and 0.1*M* sodium phosphate/disodium phosphate (pH 7.0).

Thermal Stability of Chitinolytic Enzyme Complex

The thermal stability of *T. longibrachiatum* IMI 92027 chitinolytic enzyme complex was investigated in the temperature range of 50–70°C for different time intervals (5, 15, 30, and 60 min) at pH 4.5. Solid substrate fermentation extract (supernatant) was incubated in the buffer at 50, 60, and 70°C in the absence of chitin or residual solid substrate fermentation material. Similar experiments were carried out in the presence of residual solid substrate fermentation solids (a mixture of undigested substrate and fungal mycelium) to show the stabilizing effect of this material on thermal inactivation of chitinolytic enzymes.

Preparation of Protein Extract and Purification of Chitinolytic Enzymes

Protein extract was prepared from air-dried (28°C, 20 h) solid substrate fermentation culture. One gram of dried material was extracted with 20 mL of water containing 0.1% Tween-80 for 2 h with occasional shaking. Then the slurry was centrifuged (17,300*g*, 10 min) and the supernatant filtered through a 0.2-µm membrane. Extracted proteins (5 mg) were chromatofocused on a PBE94 column (Pharmacia) (1 × 18 cm, 0.5 mL/min) equilibrated with 25 m*M* imidazole-HCl, pH 7.3, buffer. The proteins were eluted using Polybuffer 74 (Sigma-Aldrich). Proteins eluted between p*I* 5.9–5.1 and 5.2–4.8 were dialyzed against distilled water, concentrated, and loaded onto a Sephacryl S200HR column (Pharmacia) (1.6×81 cm, 1 mL/min). Elution was performed using 25 m*M* sodium-phosphate buffer, pH 7.0, containing 0.2*M* NaCl and 3 m*M* NaN_3.

Fractions possessing chitinolytic activities were collected, desalted by dialyzing, concentrated, and analyzed by 12% reducing and nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis *(29)*.

All enzyme assays were performed on microtiter plates. Chitinase activity was assayed using Remazol Brilliant Violet dye-labeled carboxymethylchitin (CM-chitin-RBV; Loewe Biochemica GmbH) by the method of Wirth and Wolf *(30)*. Briefly, 100 µL of enzyme solution was mixed with 100 µL of CM-chitin-RBV (1 mg/mL) in 0.1*M* citric acid–sodium phosphate buffer (pH 6.5) and incubated at room temperature for 10 min. The reaction was stopped by adding 50 µL of 2*N* HCl, cooled on ice for 10 min, and centrifuged, and 175 µL of the supernatant was measured at 550 nm.

For assaying *N*-acetyl-β-D-glucosaminidase and *N*-acetyl-β-D-galactosaminidase activities, 1 m*M* *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNPGlcNAc) and 0.5 m*M* *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide (pNPGalNAc) were used in 0.1 *M* citric acid–sodium citrate buffer (pH 4.5) at 37°C. Higher oligomers such as 0.1 m*M* *p*-nitrophenyl-*N*,*N*'-diacetyl-

chitobiose (pNP[GlcNAc]$_2$) and 0.1 m*M* *p*-nitrophenyl-*N*,*N*',*N*'-triacetylchitotriose (pNP[GlcNAc]$_3$) were also tested for enzyme action. In these assays, 10 µL of enzyme was mixed with 90 µL of substrate in 0.1 *M* citric acid–sodium phosphate buffer (pH 6.5) at 37°C. After incubation for 10 min, 200 µL of 0.2*M* sodium-borate buffer (pH 10.0) was added and the yellow color was read at 405 nm.

Results and Discussion

Comparison of Fungi for Chitinase Production

Thirty *Trichoderma* strains were screened for chitinase production in solid substrate fermentation. The experiments were carried out on four different solid substrate fermentation media (see Materials and Methods). Samples were taken for analysis after 3 and 5 d of fermentation. The results are shown in Fig. 1. *T. longibrachiatum* IMI 92027 (ATCC 36838) strain was found to be the best extracellular chitinase producer on d 3. This isolate was also one of the best producers on d 5, and, therefore, this fungus was selected for further optimization studies. Other good chitinase producers were two *T. atroviride* isolates (F-1045 and F-1048), *T. longibrachiatum* DAOM 175.956, and *T. viride* TUB F-1041. Interestingly, the three tested *T. harzianum* isolates were not among the best chitinolytic enzyme secreters.

Effect of Moisture Content on Chitinase Production

The moisture content of the medium in solid substrate fermentation is very important for the growth of microorganisms, production of enzymes, and enzyme activity. The optimal moisture level must be determined for each system and microorganism *(15)*. To examine the effect of moisture content on chitinase production of *T. longibrachiatum* IMI 92027, moisture levels of 50, 60, 67, 70, 75, 78, and 80% were tested. The highest chitinase production was observed at 67% initial moisture content (Fig. 2). This value was applied throughout the medium optimization experiments.

Effect of Different Additives on Chitinase Production

Different carbon sources (0.5 g of each) were added to 4.5 g of wheat bran, and these mixtures were tested in solid substrate fermentation for chitinase production. The additives were lactose, cellulose powder, ground wheat straw, and crude chitin (Fig. 3). Interestingly, strain IMI 92027 produced chitinase quite well on wheat bran as sole substrate/carrier (Fig. 3). Since—as far as we know—there is no chitin in wheat bran, most of the extracellular chitinase production of strain IMI 92027 in solid substrate fermentation should be constitutive. Carbohydrates containing β-(1,4)-bonds (lactose, cellulose) further enhanced chitinase production. Chitin as additive influenced most positively chitinase yield (Fig. 3).

The effect of different substrates (carriers) at various moisture levels on chitinase production was also investigated. The alternative substrates

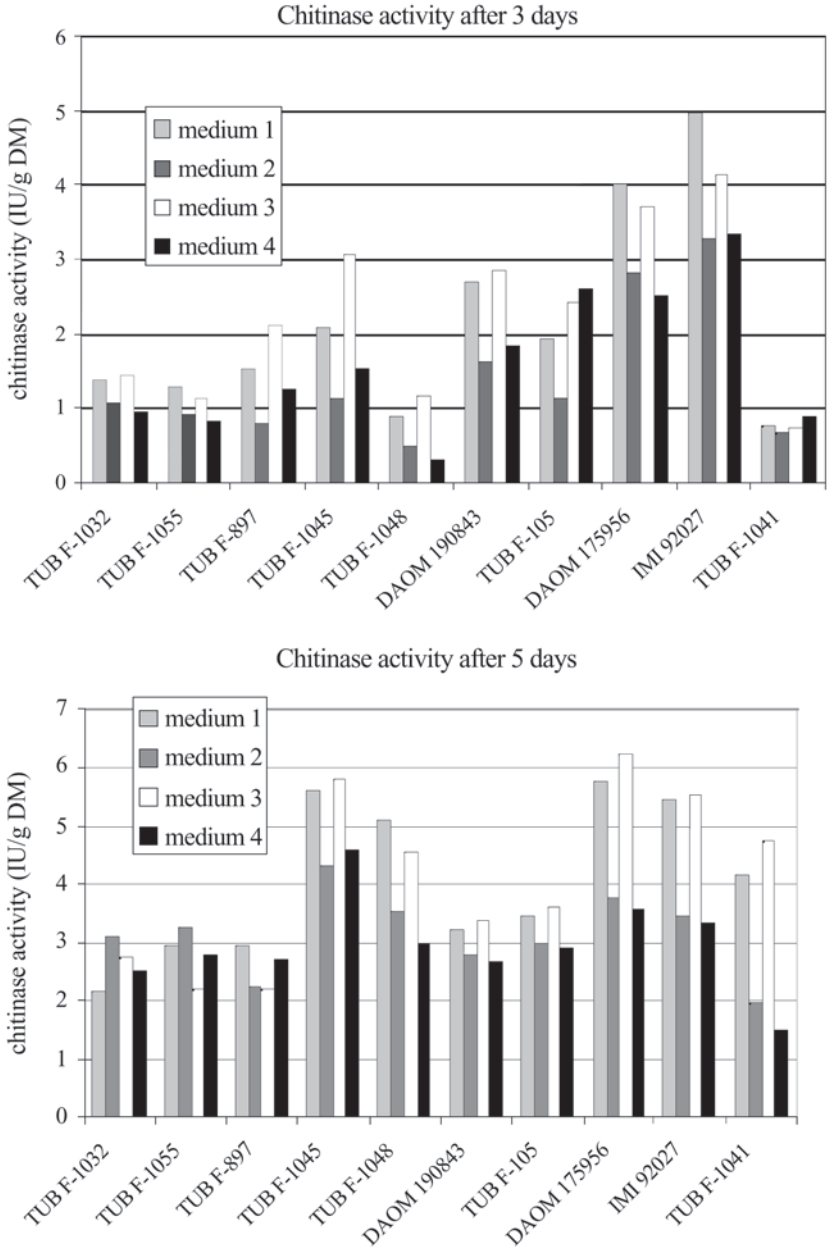

Fig. 1. Chitinase activity in 3- and 5-d solid substrate fermentation with 10 best chitinase producer strains. Substrate: 4.5 g of wheat bran + 0.5 g of crude chitin; fermentation conditions: 30°C, 3 and 5 d.

used were wheat bran, canola meal (defatted), ground corncob, ground wheat straw, oat flakes, and sunflower seed hull (Fig. 4). *T. longibrachiatum* IMI 92027 wheat bran, an excellent substrate used worldwide in many solid substrate fermentation processes, proved to be the best for chitinase production (Fig. 4).

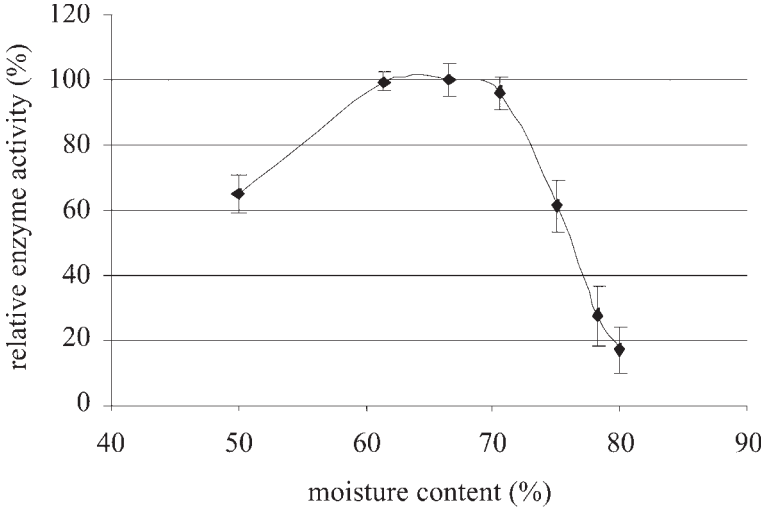

Fig. 2. Effect of moisture content of solid substrate fermentation medium on chitinase production. Strain: *T. longibrachiatum* IMI 92027; substrate: 4.5 g of wheat bran + 0.5 g of crude chitin; fermentation conditions: 30°C, 4 d.

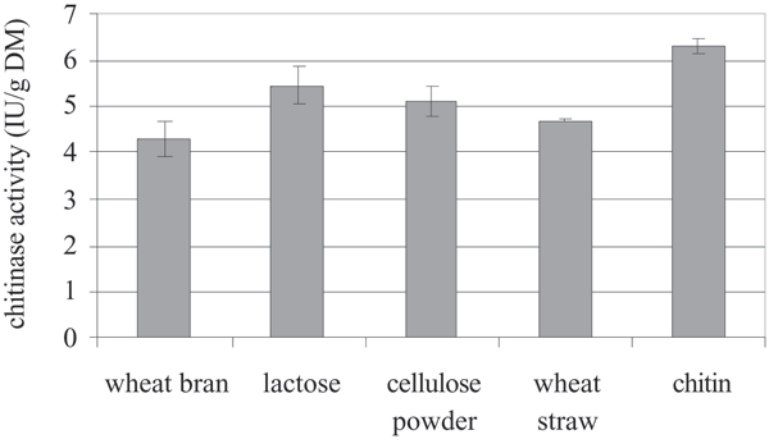

Fig. 3. Effect of additional carbon sources on chitinase production. Strain: *T. longibrachiatum* IMI 92027; substrate: 4.5 g of wheat bran + 0.5 g of additional carbon source; fermentation conditions: 30°C, 4 d, 66.7% moisture content.

The optimal chitin content of the wheat bran–chitin mixture medium was also determined. Chitin content of 20–80% was found to be the best (Fig. 5). Owing to the high cost of chitin, wheat bran medium containing 10 or 20% chitin was used for further studies.

Different N and P additives such as ammonium nitrate, defatted soybean meal, corn steep liquor, urea, ammonium sulfate, potassium nitrate, and potassium dihydrogen phosphate were also tested using the Plackett-Burman experimental design, but no significant effect of these factors was found on chitinase yield (data not shown).

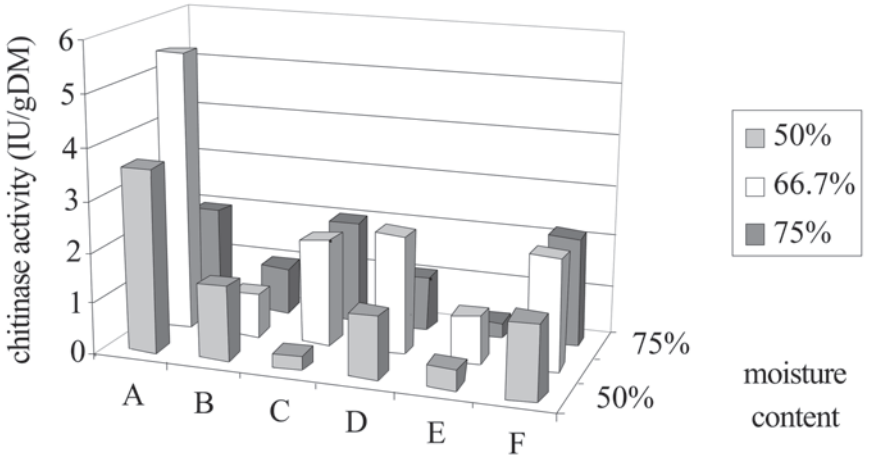

Fig. 4. Effect of different substrates (carriers) at various moisture levels on chitinase production in solid substrate fermentation. Strain: *T. longibrachiatum* IMI 92027; substrate: 4.5 g of alternative substrate + 0.5 g of crude chitin. A, wheat bran; B, canola meal; C, ground corncob; D, wheat straw; E, oat flakes; F, sunflower seed hull. Fermentation conditions: 30°C, 4 d, three different moisture levels (50, 66.7, and 75%).

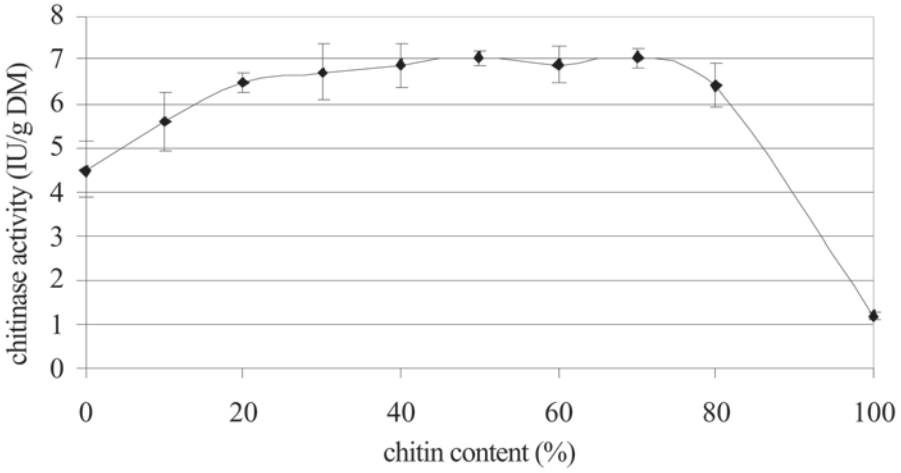

Fig. 5. Effect of chitin content of solid substrate fermentation medium on chitinase production. Strain: *T. longibrachiatum* IMI 92027; substrate: different ratios of wheat bran and crude chitin; fermentation conditions: 30°C, 4 d, 66.7% moisture content.

Effect of Initial pH of Wetting Salt Solution on Chitinase Production

Enzyme production is strongly influenced by the pH of the medium. The effect of initial pH of the wetting salt solution on chitinase production of *T. longibrachiatum* IMI 92027 was tested at the pH range of 0.0–9.0 (Fig. 6). Maximal chitinase activity was measured in the interval of pH 2.0–5.0 (Fig. 6).

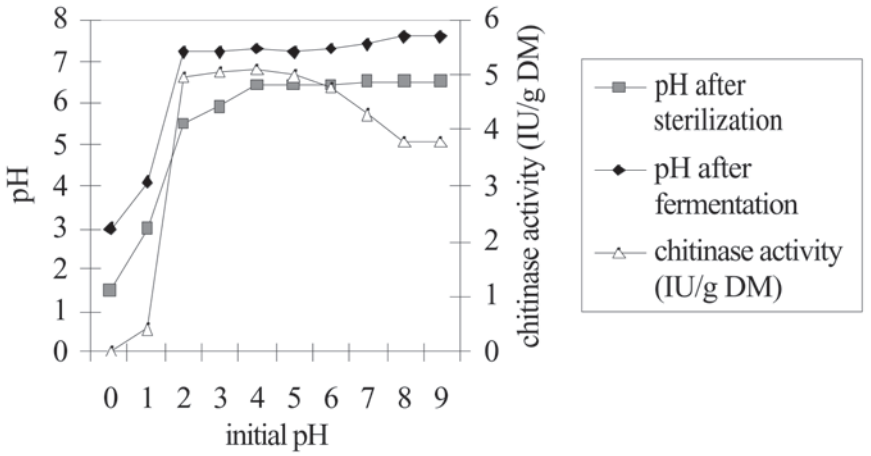

Fig. 6. Effect of initial pH of wetting salt solution on chitinase production. Strain: *T. longibrachiatum* IMI 92027; substrate: 4.5 g of wheat bran + 0.5 g of crude chitin; fermentation conditions: 30°C, 4 d, 66.7% moisture content.

Effect of Temperature and pH on Chitinase Activity

Figure 7 shows the effect of pH and temperature on hydrolysis of colloidal chitin by extracellular chitinase complex of *T. longibrachiatum* IMI 92027. The enzyme's optimal activity was at $T = 55$°C and pH 4.5. These data are rather similar to values published for *T. harzianum (5)*. The *T. harzianum* hexosaminidase CHIT72 shows optimal enzyme activity at pH 5.0–5.5 and 60°C *(31)*, and the optima for CHIT64 are at pH 5.5 and 50°C *(32)*. Maximal enzyme activity of endochitinase CHIT42 was demonstrated at pH 4.0 and 40–45°C *(33)*.

Thermal Stability of Chitinase

The thermal stability of the chitinase complex of *T. longibrachiatum* IMI 92027 was determined in both the absence and presence of residual solid substrate fermentation solids. The enzyme totally lost its activity at 70°C in 5 min in the absence of the substrate (Fig. 8A), but it retained about 15% of its initial activity even at 70°C after 60 min of incubation in the presence of solid substrate fermentation solids (residual substrate and fungal mycelium) (Fig. 8B).

Endochitinases CHIT31, CHIT33, CHIT42, and hexosaminidase CHIT 72 from *T. harzianum* were reported to be heat resistant, whereas endochitinase CHIT 52 and hexosaminidase CHIT 102 were heat sensitive *(5)*.

Time Course of Fermentation

The time course of enzyme production was studied on a medium containing 80% wheat bran and 20% chitin. The moisture content and initial pH were adjusted to 66.7% and 5.0, respectively, by using salt solution I

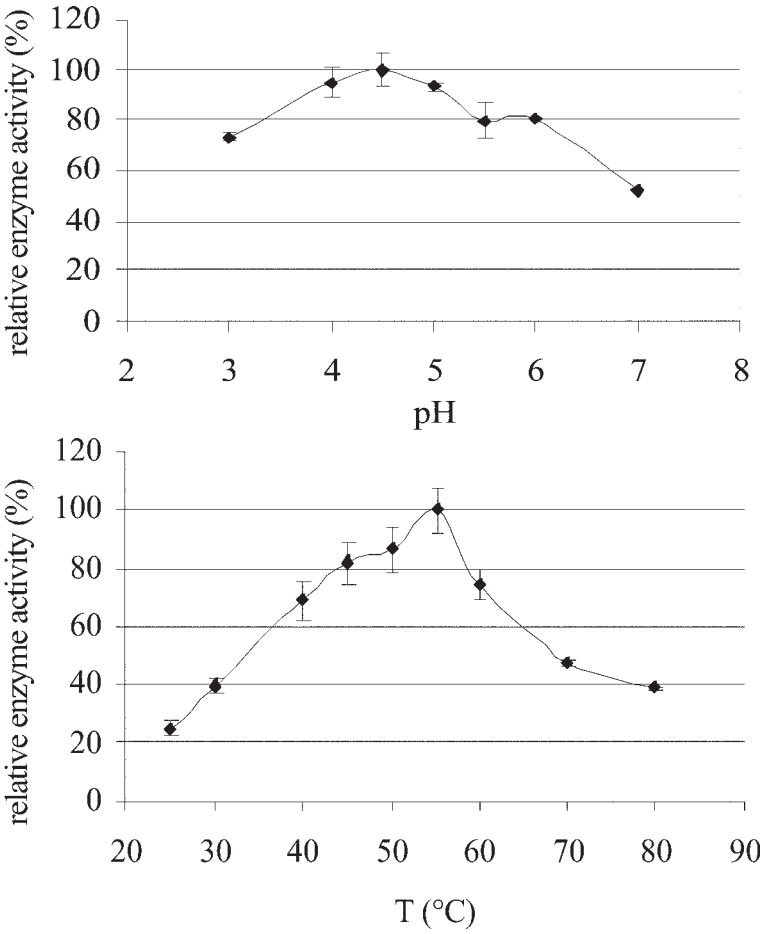

Fig. 7. Influence of pH and temperature on activity of chitinase produced by *T. longibrachiatum* IMI 92027. Incubation time: 10 min; incubation temperature for pH dependence: 50°C; incubation pH for temperature dependence: 5.5.

(see Materials and Methods). Figure 9A shows the time course of chitinase production and pH profile. Maximum chitinase activity (6.45 IU/g of DM) was observed after 5 d of fermentation. The time course of xylanase, cellulase, α-amylase, and β-xylosidase was measured parallel to chitinase production (Fig. 9B,C). Maximal xylanase (919 IU/g of DM), cellulase (1.02 filter paper units [FPU]/g of DM), and β-xylosidase (1.2 IU/g of DM) activities were observed on the second day, and α-amylase reached its maximum level (101.7 IU/g of DM) on the third day. Xylanase activity decreased rapidly after the peak, but the levels of other accessory enzymes remained relatively high even after 7 d of fermentation.

Economic Analysis of Crude Chitinolytic Enzyme Production

There are different technological routes and equipment for scaling up the solid substrate fermentation process, such as, tray (koji) cultivation,

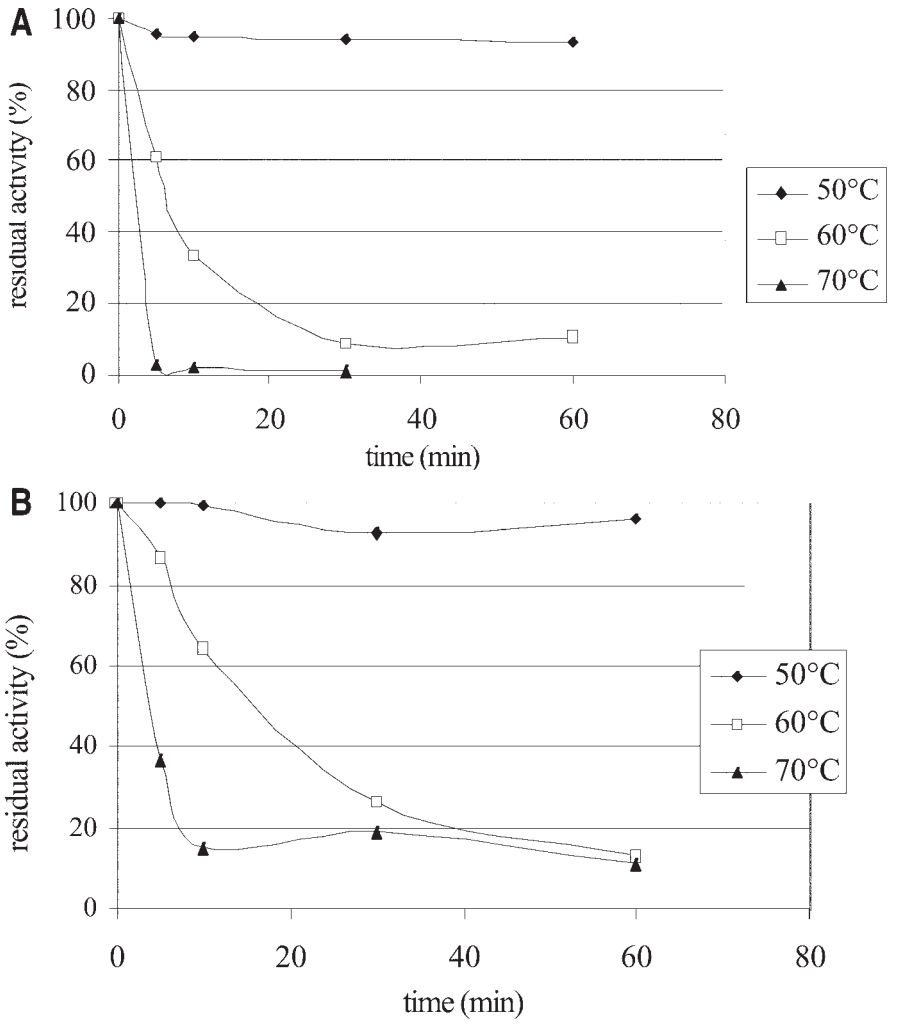

Fig. 8. Heat stability of chitinase produced by *T. longibrachiatum* IMI 92027 in **(A)** absence and **(B)** presence of residual solid substrate fermentation solids. Temperature range: 50–70°C; pH 4.5.

composting bed (pile), rotating drum bioreactor, and packed-bed reactor *(15)*. The tray method is widely used these days to prepare fermented food products such as soy sauce, tempeh, and sake and to produce enzymes. Substrates such as steamed rice, soybean, and wheat bran are fermented in automated tray (koji) fermentors under controlled conditions of aeration, pH, temperature, and humidity. The estimated cost for enzyme production by the solid substrate fermentation tray method is US$150/t or US$0.15/kg of fermented material *(16–18,34)*. Crude chitinolytic enzyme (*in situ* enzyme, whole solid substrate fermentation culture without downstream processing) may be produced at a similar cost by tray fermentors provided cheap chitin-containing byproducts are used as supplements to wheat bran.

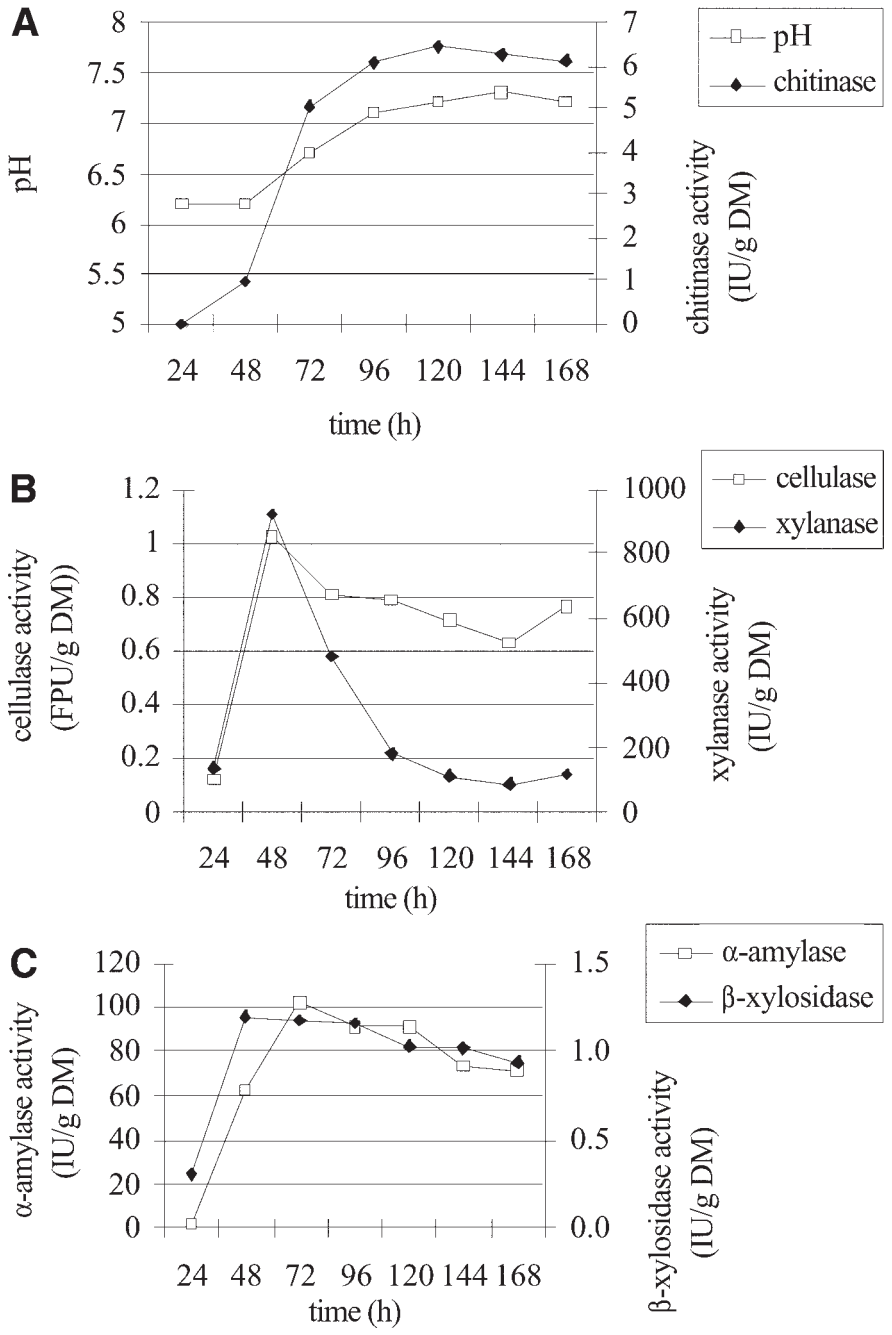

Fig. 9. Time course of different enzyme productions and pH profile of **(A)** cellulase and xylanase production, **(B)** α-amylase and β-xylosidase production, and **(C)** by *T. longibrachiatum* IMI 92027 in solid substrate fermentation. Substrate: 4 g of wheat bran + 1 g of crude chitin; fermentation conditions: 30°C, 66.7% moisture content.

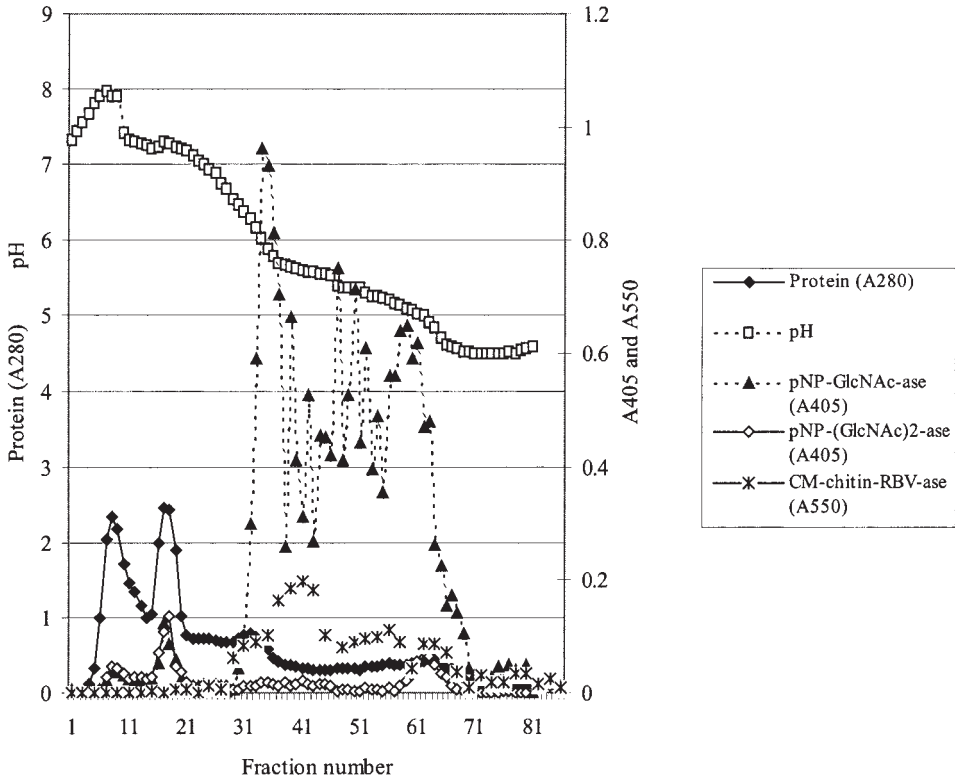

Fig. 10. Chitinolytic activities of chromatofocused protein extract (5 mg).

Purification of Enzyme

Purification of protein extract from the solid substrate fermentation material revealed high chitinolytic activities between p*I* 5.9 and 4.8, where *N*-acetyl-β-D-hexosaminidase and chitinase peaks have been found in the same p*I* range (Fig. 10). Chitinase peaks could be described at least at four p*I* values (5.9, 5.6, 5.3, and 4.8), and at least four main *N*-acetyl-β-D-hexosaminidase peaks could be separated at p*I* 6.0 and 5.1 and at more basic p*I* values of 7.2 and 8.0. Two chitinases of 43.5 and 30 kDa were purified at acidic p*I*. Interestingly, during chromatofocusing at least two protein peaks with p*I* values of 8.0 and 7.2–7.3 showing activity against the dimer and trimer substrates could be separated (Table 1). On the basis of their exclusive activity against these substrates, we can report two *N*-acetyl-β-D-hexosaminidases with p*I* values higher than 7.0. Size-exclusion chromatography revealed that between p*I* 5.9 and 5.1 *N*-acetyl-β-D-hexosaminidases with high molecular weights and chitinases with lower molecular weights could be found. These findings are in good agreement with data published on *T. harzianum* chitinases *(5)* that *N*-acetyl-β-D-hexosaminidases have higher molecular weights than chitinases.

Table 1
Identified Chitinolytic Activities

p*I*	Substrates	Identified M_r (kDa)
8.0	pNPGlcNAc pNPGalNAc pNP(GlcNAc)$_2$	
7.2–7.3	pNPGlcNAc pNPGalNAc pNP(GlcNAc)$_2$	
5.9–5.1	pNPGlcNAc pNPGalNAc	65.5
5.9–5.1	pNP(GlcNAc)$_2$ pNP(GlcNAc)$_3$ CM-chitin-RBV	43.5
5.2–4.8	pNPGlcNAc pNPGalNAc pNP(GlcNAc)$_2$	75 64
4.8	pNP(GlcNAc)$_2$ pNP(GlcNAc)$_3$ CM-chitin-RBV	30

Conclusion

The present study proves that two *T. longibrachiatum* isolates (DAOM 175.956 and IMI 92027) could be effective sources of extracellular chitinolytic enzymes in solid substrate fermentation. Optimization studies with isolate IMI 92027 indicate that the best medium for the enzyme production is a wheat bran–chitin mixture wetted with salt solution to a moisture content of 66.7%. Under optimized conditions, *T. longibrachiatum* IMI 92027 produced 6.45 IU/g of DM chitinase in 5 d. Both endochitinases and *N*-acetyl-β-D-hexosaminidases have been detected by purification methods.

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