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CHITINASE PRODUCTION BY *MYROTHECIUM VERRUCARIA* AND ITS SIGNIFICANCE FOR FUNGAL MYCELIA DEGRADATION[1]

PRANAV VYAS AND MUKUND V. DESHPANDE*

Biochemical Sciences Division, National Chemical Laboratory, Pune 411008, India

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Myrothecium verrucaria produced high levels of chitinases in a medium containing chitin used as a sole carbon source. Adding 0.03% urea increased the enzyme yield 4-fold in 7 days compared to the control. Adding oxgall (0.1%) to the growth medium gave the maximum activity (acid-swollen chitin-degrading activity, 2.0 IU/ml) in 7 days. The biochemical characterization of the chitinase revealed its broader temperature (25–55°C) and pH (4.0–6.5) profiles of activity which showed its potential application in fungal mycelia degradation. Compared to commercial lytic enzyme preparations (NovoZym 234 and Onozuka R-10), *M. verrucaria* culture filtrate had 5–6 times more chitinase activity. And this produced significantly higher levels of *N*-acetyl-D-glucosamine from the fungal mycelia preparations under study.

Chitin, a β-1,4-linked unbranched polymer of *N*-acetyl-D-glucosamine (NAG), occurs particularly in marine invertebrates, insects and fungi. Chitin is completely hydrolyzed to its monomer NAG by an enzymatic chitinolytic system, the action of which is known to be synergistic and consecutive. The endo-chitinase (EC 3.2.1.14) hydrolyzes chitin randomly while chitobiase (EC 3.2.1.30) acts on the dimer, chitobiose. The involvement of exo-chitinase, which hydrolyzes the polymer from the non-reducing end, has also been suggested by a number of researchers (*5*). Microorganisms such as *Serratia*, *Streptomyces*, *Aspergillus* and *Trichoderma* have been studied extensively for enzymes of the chitinolytic complex. The potential uses of chitin-degrading enzymes are in the treatment of chitin-containing wastes produced by the sea food packing industry (*8*), in the biocontrol of soil-borne plant-pathogenic fungi (*15*) and in fungal technological studies (*2*, *7*, *18*).

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* Address reprint requests to: Dr. M. V. Deshpande, Biochemical Sciences Division, National Chemical Laboratory, Pune 411008, India.

Abbreviations: NAG, *N*-acetyl-D-glucosamine; PDA, potato-dextrose agar; MGYP, malt extract-glucose-yeast extract-peptone; IU, international unit.

During the screening programme for chitinase-producing fungi, we have found that *Myrothecium verrucaria* (NCIM 903) is one of the potent chitinase producers. The present study was conducted (i) to optimize the conditions for chitinase production by *M. verrucaria* and (ii) to evaluate the lytic enzyme complex of *M. verrucaria* for fungal mycelia degradation and to compare it with commercially available mycolytic enzyme preparations.

MATERIALS AND METHODS

Organism and cultivation conditions. *Myrothecium verrucaria* NCIM 903, used throughout the studies, was maintained on potato-dextrose agar (2% PDA) slants. The basal medium used for the optimization studies contained (g/*l*): KH_2PO_4, 3.0; K_2HPO_4, 1.0; $MgSO_4$, 0.7; $(NH_4)_2SO_4$, 1.4; NaCl, 0.5; $CaCl_2$, 0.5; yeast extract, 0.5; bacto-peptone, 0.5 and chitin, 5.0. The pH of the medium after autoclaving was between 5.0 and 5.5. For optimization, all the experiments were carried out in shake flasks (100 ml medium/500-ml Erlenmeyer flask) incubated at 28°C with shaking (200 rpm) for 7 days. For further studies, medium optimized for enzyme production was used.

In all the experiments, spore inoculum (10^7/flask) from 7-day-old slants was used.

Enzyme assays. Acid-swollen chitin was used as a substrate to estimate chitinase activity. Phosphoric acid-swollen chitin (*6*) was prepared as described earlier (*5*). The chitin (10 g, purified powder from crabshells, Sigma Chemical Co., U.S.A.) was suspended in *O*-phosphoric acid (88%, w/v) and left at 0°C for 1 h with occasional stirring. The mixture was poured into ice-cold water (4 *l*) and left for 30 min. The swollen chitin was washed several times with cold distilled water, then washed with 1% (w/v) $NaHCO_3$ solution; the swollen chitin was dialyzed at 1°C against water. After a 1-min treatment in a Waring blender, 0.05 M acetate buffer, pH 5.0, was added to the suspension so that 1 ml of suspension contained 7 mg of chitin. A reaction mixture containing 1 ml of 0.7% swollen chitin, 1 ml of 0.05 M acetate buffer, pH 5.0 and 1 ml of enzyme solution was incubated at 50°C for 1 h.

Endo-chitinase and laminarinase activities were determined using ethylene glycol chitin (Sigma) and laminarin (Sigma) respectively, as substrates. An aliquot of enzyme solution (0.5 ml) was mixed with a 1% substrate (0.5 ml) in 0.05 M acetate buffer, pH 5.0, and incubated at 50°C for 30 min. The amount of reducing sugars produced was determined by the Nelson method (*10*), using as standards NAG (for chitinase) and glucose (for laminarinase).

The chitobiase activity was determined by measuring the amount of *p*-nitrophenol released in a reaction mixture containing 0.9 ml of 1 mg/ml *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) in 0.05 M acetate buffer, pH 5.0, and 0.1 ml of enzyme, incubated at 50°C for 30 min.

One international unit was defined as the activity which produced 1 μmol of

the product per min.

Alkaline protease was measured by the method of Sutar et al. (*17*) using Hammersten casein as a substrate. One unit of the activity was equivalent to that amount of enzyme which liberated 1 μmol of tyrosine per min at 35°C.

The release of monosaccharides from lyophilized fungal mycelia was also tested. For dried mycelia as a substrate *M. verrucaria*, *Sclerotium rolfsii* and *Fusarium* sp. were cultured with shaking at 28°C for 4 days on liquid MGYP medium. After incubation, the mycelia were thoroughly washed with distilled water and lyophilized. Dried mycelia, 5 mg, was incubated with 1 mg of enzyme preparation in 1 ml of 0.05 M acetate buffer, pH 5.0, for 1 h at 50°C. The reducing sugars, glucose and NAG were measured by the Nelson method (*10*), the glucose oxidase peroxidase method (*1*) and the method of Reissig et al. (*13*) respectively.

RESULTS AND DISCUSSION

Optimization of chitinase production by M. verrucaria

Chitinase production by *M. verrucaria* was optimized by extracellular acid-swollen chitin degrading activity in culture filtrates. The cultures were examined before, harvesting to ensure that very little, if any, lysis was occurring.

Table 1 shows the influence of inorganic nitrogen sources on the production of chitinase. All of the nitrogen sources examined supported good growth. As no appropriate replacement for $(NH_4)_2SO_4$ (0.14%) in the basal medium was found, it was used in subsequent studies as the inorganic nitrogen source.

Chitinases are produced constitutively (*5*), but adding chitin to culture media greatly enhanced the enzyme production. In the case of *Beauveria bassiana*, Smith and Grula (*16*) reported that glucosamine and NAG released during autoclaving of chitin acted as inducers. While Ridout et al. (*14*) reported that in the mycoparasitic strain *Trichoderma harzianum* the presence of suitable host cell walls induced the chitinase production. Similarly, for *M. verrucaria*, various carbon sources were

Table 1. Effects of various inorganic nitrogen sources on the production of extracellular chitinase by *M. verrucaria*.

Inorganic nitrogen sources[a]	Final pH	IU/ml[b]
Ammonium sulfate, 0.14%	6.3	0.32
Ammonium sulfate, 0.28–0.7%	6.4	0.28
Ammonium chloride, 0.12%	6.2	0.12
Di-ammonium hydrogen phosphate, 0.14–0.7%	6.0	0.15
Sodium nitrate, 0.2%	6.2	0.13
Potassium nitrate, 0.23%	6.4	0.21

Note: Cultures were grown in shake flasks at 28°C for 7 days on basal medium (free of ammonium sulfate) plus the indicated inorganic nitrogen source.

[a] Nitrogen source was added at equivalent nitrogen levels.

[b] Chitinase activity was estimated using acid-swollen chitin as a substrate.

Table 2. Effect of different carbon sources on production of chitinase by *M. verrucaria*.

Carbon source	Final pH	IU/ml[a]
Glucose, 0.5%	6.2	0.08
Glucose, 1%	2.9	0.002
Glucosamine, 0.2–0.5%	5.7	0.02
Glucosamine, 1.0%	3.2	ND
N-Acetyl glucosamine, 0.2%	6.7	0.1
N-Acetyl glucosamine, 1.0%	7.7	0.15
Cellobiose, 0.2%	6.1	0.03
Cellobiose, 1.0%	7.1	0.08
Chitosan, 0.5%	5.4	ND
Chitin, 0.5–1.0%	6.4–6.8	0.33–0.35
Chitin plus chitosan, 0.5% each	6.2	ND
Sclerotium rolfsii mycelium, 10% (wet weight)	6.3	0.24

Note: Cultures were grown in shake flasks at 28°C for 7 days on basal medium containing 0.14% ammonium sulfate as an inorganic nitrogen source plus the indicated carbon source.

[a] See footnote of Table 1.

ND: not detected.

added to the basal medium as a substitute for chitin and their effect on chitinase production was recorded (Table 2). Though the acid-swollen chitin-degrading activity occurred in the culture filtrate of *M. verrucaria* grown in the presence of glucose, glucosamine, NAG and cellobiose, appreciably high level was observed with only chitin (0.35 IU/ml). *S. rolfsii* cell wall is reported to contain almost 60% chitin (*3*). While studying the role of the chitinases of *Serratia marcescens* in biocontrol of *S. rolfsii*, Ordentlich et al. (*11*) reported high chitinase activity in the media containing *S. rolfsii* cell wall as a sole carbon source. *M. verrucaria* also grew luxuriantly on autoclaved *S. rolfsii* mycelium (10%, wet weight) used as the sole carbon source. The activity was of the order of 0.24 IU/ml (Table 2). No activity was detected in culture filtrates grown with lactose, maltose, sucrose, chitosan, chitosan plus chitin, potato starch or cellulose.

The effect of various organic supplements on enzyme production is summarized in Table 3. Adding urea (0.01–0.05%) increased the enzyme yield 4-fold over the control, in 7 days. So in our further optimization studies 0.03% urea was incorporated in the medium.

We studied the effect of various trace metals such as Fe^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+} individually as well as in combination. Trace metal solutions, 1 ml/*l* of growth medium (mg/ml: $FeSO_4$, 5.0; $ZnSO_4$, 1.4; $MnSO_4$, 1.6; $CoCl_2$, 2.0) gave consistent growth and chitinase activity (1.42 IU/ml).

The addition of surfactant has been reported to increase the extracellular appearance of a number of enzymes in a variety of microorganisms (*12*). Incorporation of oxgall in the growth medium gave the highest enzyme activity (2.0 IU/ml) in 7 days in the studied range of the surfactants (Table 4).

The initial pH of the growth medium was also important for the chitinase

Table 3. Effect of various organic supplements of chitinase production by *M. verrucaria*.

Organic supplement	Final pH	IU/ml[a]
None	6.4	0.33
Yeast extract, 0.05%	6.5	0.4
Peptone, 0.05%	6.5	0.4
Urea, 0.01%	7.7	1.3
0.03%	7.8	1.36
0.05%	7.5	1.0
Corn steep liquor, 1.0%	7.6	0.04

Note: Cultures were grown in shake flasks at 28°C for 7 days on basal medium containing 0.5% chitin as a carbon source plus the indicated organic supplement.

[a] See footnote of Table 1.

Table 4. Effect of different surfactants on chitinase production by *M. verrucaria*.

Surfactant	Final pH	IU/ml[a]
None	7.7	1.42
Tween-80, 0.033%	7.5	1.3
0.1%	7.7	1.6
Tween-20, 0.033%	7.7	1.4
Sodium lauryl sulfate, 0.1%	6.2	ND
Oxgall, 0.1%	7.8	2.0

Note: Cultures were grown in shake flasks at 28°C for 7 days on the modified medium (basal medium plus 0.03% urea plus 0.1% (v/v) trace metal solution) in addition to the surfactant indicated.

[a] See footnote of Table 1.

ND: not detected.

production by *M. verrucaria*. At pH 6.0, the activity was consistently high.

The production profiles of acid-swollen chitin-degrading activity, endo-chitinase and chitobiase produced by *M. verrucaria* were studied in the optimized medium. As shown in Fig. 1, all three activities appeared extracellularly within 3 days. The maximum activities occurred in 5 to 7 days, but with further incubation the activities declined. This may be due to increased pH of the culture medium.

Biochemical characterization of the chitinase complex

Some of the biochemical properties of the native enzyme complex were examined using the 7-day-old crude chitinase preparation dialyzed against 0.05 M acetate buffer, pH 5.0 for 16 h at 4°C. The acid-swollen chitin-degrading activity was stable over a broad temperature range, 25–55°C (pH 5.0, 30 min). But when incubated without substrate, at 50°C it was 65% inactiviated in 60 min, and it was 40% inactivated when it was incubated under identical conditions with substrate at assay concentration. The optimum temperature for the activity was 50°C and the E_a (activation energy), calculated from an Arrhenius plot, was 23.9 kJ/mol. The enzyme was active in a pH range of 4.0 to 6.5 (50°C, 30 min); the optimum pH

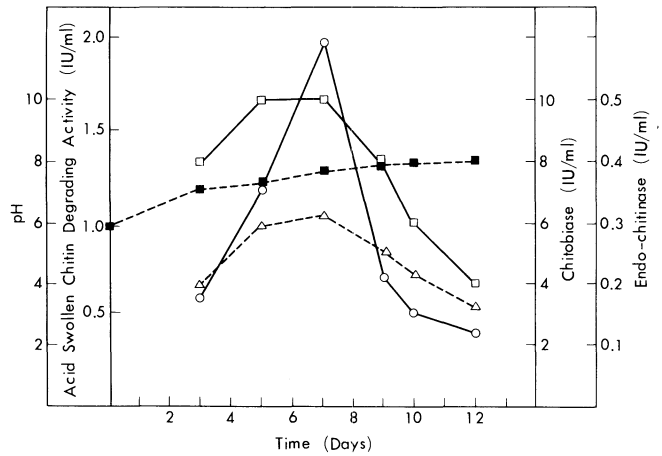

Fig. 1. Production of chitinase and the pH profiles produced by *M. verrucaria* in optimized medium in shake flasks at 28°C. ○, acid-swollen chitin-degrading activity; △, chitobiase; □, endo-chitinase; ■, pH.

was 5.0. The broader temperature-pH profiles of activity have potential application in fungal protoplasting (*9*).

Using acid swollen-chitin as a substrate under standard assay conditions, the apparent K_m, calculated by the Lineweaver-Burk plot, was 5.95 mg/ml; the V_{max} (μmol of NAG equivalents/min/ml culture filtrate) was 2.88.

Enzymatic degradation of fungal mycelia

Use of lytic enzymes to isolate protoplasts from fungi and preparing cell free extracts and organelles for biochemical studies (*7*) and for their role in biocontrol of soil-borne plant pathogenic fungi (*15*) is now a well established technique. Earlier, in the 1960s, Cocking (*4*) reported the use of an *M. verrucaria* cellulase preparation for isolating plant protoplasts. We have evaluated the chitinase complex of *M. verrucaria* along with the commercial preparations NovoZym-234 and Onozuka R-10 for the mycelial degradation of three fungal cultures. The mycelia of *M. verrucaria* were used to evaluate its autolytic activity (*18*), *S. rolfsii* for its high chitin content (*3*) and *Fasarium* sp. because higher sugar liberation values were reported earlier by Beyer and Diekmann (*2*) while studying chitinases from *Streptomyces* sp.

The effectiveness of the enzyme preparations in degrading fungal mycelia was measured by estimating the liberated total reducing sugars (*10*), glucose (*1*) and NAG (*13*). Table 5 shows that more NAG was liberated with the *M. verrucaria* culture filtrate than with NovoZym-234 or Onozuka R-10. The chitinase activity of the *M. verrucaria* was 5 to 6 times higher than NovoZym-234 or Onozuka R-10 activities. Hamlyn et al. (*7*) and Kitamura (*9*) reported that β-D-glucanase (laminarinase) and protease also participated in the effective fungal cell wall degradation. The higher levels of total reducing sugars from all the fungal mycelia

Table 5. Effect of the enzyme systems *M. verrucaria* culture filtrate, NovoZym-234 and Onozuka R-10 on the release of monomers from mycelia of *M. verrucaria, S. rolfsii* and *Fusarium* sp.[a]

Cell wall of	Reducing sugars (μg/ml)			Glucose (μg/ml)			*N*-Acetyl-D-glucosamine (μg/ml)		
	CF	Novo	R-10	CF	Novo	R-10	CF	Novo	R-10
M. verrucaria	240	460	130	50	80	50	79	16	8
S. rolfsii	389	491	460	Trace	340	280	165	65	4
Fusarium sp.	320	400	240	21	50	21	163	19	16

Enzyme activities (IU/mg protein) of: CF, *M. verrucaria* culture filtrate (chitinase, 3.6; laminarinase, 1.8; alkaline protease 0.065); Novo, NovoZym-234 (chitinase, 0.55; laminarinase, 70; alkaline protease, 0.47); R-10, Onozuka R-10 (chitinase, 0.47; laminarinase, 83.7; alkaline protease, 0.025).

[a] As described under MATERIALS AND METHODS.

under study produced by NovoZym could be related to the 5 to 8 times more laminarinase and protease than *M. verrucaria* culture filtrate (footnote of Table 5). Although the Onozuka preparation had the highest laminarinase of the three enzyme preparations, the lowest levels of chitinase and protease hampered its effectiveness compared with NovoZym and *M. verrucaria* culture filtrate.

So the non-commercial enzyme preparation of *M. verrucaria* has high potential for use in fungal technology, singly in combination with other commercially available enzyme preparations. And perhaps it can be inexpensive if produced commercially.

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