Journal of Industrial Microbiology (1995) 15, 397–400

The growth form of the inducing microorganism and chitin addition affect mycolytic enzyme production by *Trichoderma harzianum*

P Žnidaršič[1], A Pavko[1] and R Komel[2]

[1]*Department of Chemistry and Chemical Technology, University of Ljubljana, Ljubljana, Slovenia;* [2]*Institute of Biochemistry, Medical Faculty, Ljubljana, Slovenia*

The effect of the growth form of the inducing microorganism on specific *Trichoderma harzianum* mycolytic enzyme production was studied. The pelleted form of *Rhizopus nigricans* gave a better product concerning protoplast formation ability. The maximum yield of protoplasts from the target fungus *Cochliobolus lunatus* was 1×10^8 ml^{-1}. Analysis of individual specific enzyme activities in *Trichoderma* mycolytic enzyme preparations confirms the importance of high chitinase and low protease activity for high protoplast yields. Supplementation of the production medium with chitin increased the chitinase activity in the *Trichoderma* exoenzyme mixture.

Keywords: mycolytic enzymes; *Trichoderma harzianum*; protoplasts; *Rhizopus nigricans*; induction

Introduction

The most favoured approach for isolation of fungal protoplasts is cell wall digestion with mycolytic enzymes. The great interest in fungal protoplasts in various fields of research has led to the discovery and optimisation of systems for protoplast formation [13]. Our interest was a specific mycolytic enzyme preparation with which high yields of stable protoplasts could be prepared from steroid-transforming *Cochliobolus lunatus*.

Some species of *Trichoderma* are known for their mycoparasitic action and also antagonistic action against plant fungal pathogens [6,14,16]. Numerous isolates of this genus produce hydrolytic enzymes when grown in liquid media supplemented with polysaccharides such as chitin or laminarin [2,7,8,16], purified cell walls [2,6,14,19], or crude mycelial preparations [3,5,10,15,16,20].

Our previous work on *Trichoderma harzianum* enzyme production has revealed that *Cochliobolus lunatus* could merely induce enzymes, lytic towards its own cell walls [3,20]. When *Rhizopus nigricans* from the chitin-chitosan class of fungi [1] was used as the inducing substrate, the preparation obtained was much more efficient regarding protoplast release from *C. lunatus*, presumably because of higher chitinase activity in the induced enzyme mixture. This is in agreement with reports on *Trichoderma* strains having constitutive β-1,3- and β-1,6-glucanases, while chitinase was secreted only in the presence of chitin [2,18]. Therefore, our further experiments were focused on increasing chitinase activity in the specific mycolytic enzyme preparation.

In this paper we describe the effect of different growth forms of the inducing microorganism, filamentous and pelleted, on *Trichoderma harzianum* specific enzyme production. Changes in morphology are associated with changes in the fungal cell wall structure [1,12], which in our case means the change of the substrate for the induction of specific enzymes. In respect to the *Rhizopus arrhizus* cell wall, it was reported that in the pelleted form it contains more crystalline chitin, glucuronan and proteins than in the filamentous one [12]. Similar structural differences in our strain of *Rhizopus* would improve substrate composition for the *Trichoderma harzianum* extracellular enzyme system for protoplasting *Cochliobolus lunatus*.

Furthermore, we tried to improve the protoplast formation ability of induced preparations by supplementation of the production medium with chitin.

Materials and methods

Microorganisms

Trichoderma harzianum 1131 CBS 354-33, *Rhizopus nigricans* ATCC 6227 b and *Cochliobolus lunatus* m118, described previously, were used [20]. *T. harzianum* was maintained on slants according to Dlugonski *et al* [5]. *R. nigricans* and *C. lunatus* were maintained on slants consisting of a malt extract 6 °Blg with 20 g L^{-1} of agar (pH 5.5).

Growth of the inducing microorganism Rhizopus nigricans

The filamentous form of *Rhizopus nigricans* was achieved with an inoculum of 10^6–10^7 14-day-old spores from slants per ml, in 100 ml of liquid medium (LM) containing (g per L): glucose, 20; soya bean peptone, 6; yeast extract, 5.7; NaCl, 4 and K_2HPO_4, 2; the pH adjusted to 6.5. After 24 h of incubation on a rotary shaker (220 rpm at 28° C) the mycelium was collected, washed several times with distilled water, and lyophilised. The same growth form was developed in a 14-L stirred tank fermenter with 8 L of working volume. Cultivation was carried out in LM

Correspondence: P Žnidaršič, Department of Chemistry and Chemical Technology, University of Ljubljana, Aškerčeva 5, 61000 Ljubljana, Slovenia
Received 19 October 1994; accepted 9 June 1995

Table 1 Effect of the growth form of the inducing microorganism *Rhizopus nigricans* on *Trichoderma harzianum* mycolytic enzyme production. The inducer was lyophilised and autoclaved before adding it to the PM medium. Each result is the mean of two replicates. Deviation was less than ±10%

Inducer	No of protopl ($\times 10^{-6}$) ml^{-1}	Specific activities (U mg^{-1} protein)				
		β-1,3-glucanase	Endo-β-1,4-glucanase ($\times 10^2$)	α-1,3-glucanase ($\times 10^2$)	Chitinase ($\times 10^3$)	Protease
FF, SC (24 h)	7.5	1.8	2.66	7.95	0.96	0.23
FF, STR (68 h)	40	1.03	2.45	4.59	4.88	0.07
PF, STR (68 h)	65	0.86	2.80	5.35	1.71	0.15
PF, STR (280 h)	92.5	0.88	3.53	4.09	4.90	0.03
PF, STR (450 h)	100	0.86	2.90	5.34	6.86	0.06
NOVOZYM 234	45	0.22	2.88	6.67	2.02	0.143

FF, filamentous form; PF, pelleted form; SC, shaken cultures; STR, stirred tank reactor

medium with the same inoculum size under the following conditions: temperature 28° C, stirring speed 200–700 rpm, aeration 0.35–1 vvm and incubation time 68 h. The biomass obtained was treated as described above.

For developing *R. nigricans* pellets, spores from slants older than 28 days were used. A 7-L stirred tank reactor with 5 L of working volume was inoculated with 10^4–10^5 spores per ml of LM medium. The cultivation conditions were: starting pH 5.0, temperature 28° C, mixing rate 200 rpm, aeration 1 vvm and incubation time 68 h. Concentrated glucose solution was added several times. The pelleted growth form was also obtained in a 14-L stirred tank reactor with maximal working volume, where pellets (average diameter 1 mm) from shaken cultures were used for the inoculum. The batch fermentation was carried out at 15° C, the mixing rate was 200 rpm and aeration was at 1 vvm. After 120 h, 3 L of the fermentation broth was aseptically transferred into 6 L of fresh medium, while after 280 h of cultivation, 2 L of the culture was used as inoculum for 6 L of LM medium, which was incubated for a further 170 h. The compact pellets were collected, washed several times with distilled water, and lyophilised.

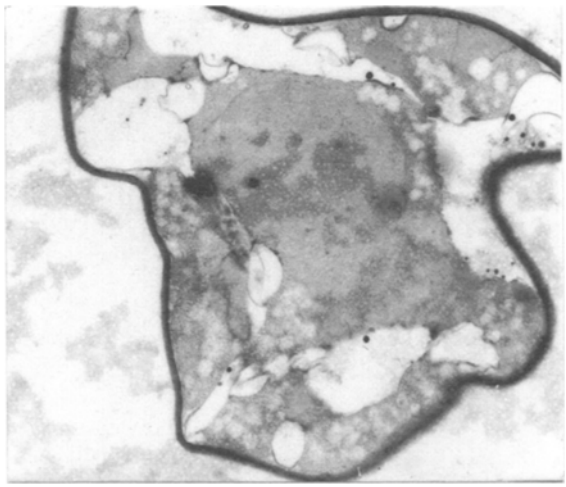

Figure 1 Electron micrograph of cross-section of the hyphae of *Rhizopus nigricans* in a filamentous growth form, obtained with 24-h cultivation on a rotary shaker (magnification × 13000)

Table 2 The effect of carbon source beside glucose in PM medium on *Trichoderma harzianum* specific enzyme activities. The results are the mean of two replicates. Deviation was less than ± 10%

Carbon source	No of protopl ($\times 10^{-6}$) ml^{-1}	Specific enzyme activities (U mg^{-1} protein)				
		β-1,3-glucanase	Endo-β-1,4-glucanase ($\times 10^2$)	α-1,3-glucanase ($\times 10^2$)	Chitinase ($\times 10^3$)	Protease
15 g L^{-1} *R.n.*[a]	7.5	1.8	2.66	7.95	0.96	0.23
10 g L^{-1} *R.n.*[a] + 1 g L^{-1} chitin	1.25	0.67	2.34	5.96	1.45	0.109
10 g L^{-1} *R.n.*[a] + 2 g L^{-1} chitin	6.5	0.91	3.55	6.92	2.53	ND

[a]Dry weight of the lyophilised crude mycelial preparation of the filamentous form of *Rhizopus nigricans*, obtained from 24-h shaken cultures cultivation as described in Materials and methods
ND, not determined

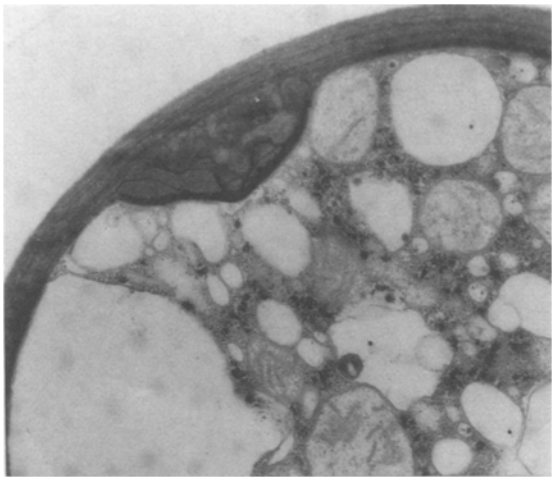

Figure 2 The cross-section of the hyphae of *Rhizopus nigricans* in a pelleted growth form, obtained after 450 h of a repeated batch fermentation process in a laboratory stirred tank reactor (magnification × 13 000)

Both morphological forms of *Rhizopus nigricans* were characterised by transmission electron microscopy. The samples were prepared according to Didek-Brumec *et al* [4].

Preparation of lytic enzymes

For cell wall lytic enzyme production, *Trichoderma harzianum* was grown in a production medium (PM) consisting of 15 g dry weight of lyophilised *R. nigricans* (filamentous or pelleted form), 3 g of glucose and mineral components according to Mandels *et al* [11]; distilled water added up to 1 L. In the case of supplementation of the medium with chitin (1 or 2 g L^{-1}), the concentration of the inducing microorganism in the PM medium was reduced to 10 g L^{-1}.

T. harzianum spores from 7-day-old slants were washed into 500-ml Erlenmeyer flasks with 100 ml of growth medium (GM) containing 4 g L^{-1} of glucose and 4 g L^{-1} of yeast extract in a diluted malt extract (6 °Blg); the pH adjusted to 6.7. After a 24-h incubation at 28° C on a rotary shaker (220 rpm), 10 ml of the mycelium was aseptically transferred to 500-ml Erlenmeyer flasks containing 90 ml of the PM medium. The production of lytic enzymes was carried out for 6 days at 180 rpm and 28° C. Lytic enzymes were isolated from the culture broth according to de Vries and Wessels [19].

Protoplast formation

Protoplasts were isolated from the target fungus *Cochliobolus lunatus* as described previously [20]. The number of protoplasts per ml, released under the same conditions with different mycolytic enzyme preparations, was used to assess the quality of these products. The amount of crude enzyme preparation or Novozym 234 (Novo Nordisk, Denmark), added to the lytic mixture was always 5 mg ml^{-1}.

Protein content

Protein was determined by a modified Folin–Lowry method [17]. The concentrations were expressed in % (mg protein per 100 mg crude enzyme preparation).

Estimation of enzymes

β-1,3 glucanase, endo-β-1,4 glucanase, α-1,3 glucanase, chitinase and protease were determined by incubating enzyme solutions with laminarin, carboxymethylcellulose, nigeran and chitin (all from Sigma, St Louis, MO, USA) and denaturated Hammarsten casein (Kemika, Zagreb, Croatia) as substrates, respectively. After incubating the mixtures for appropriate times in suitable buffers and at appropriate temperatures, the monomers released were measured as described previously [20]. Specific enzyme activities were expressed as units (1 U = 1 μmol glucose, *N*-acetylglucosamine or tyrosine equivalent, liberated per min) per mg of protein.

Results and discussion

The effects of the growth form and the age of the inducing microorganism on *Trichoderma harzianum* extracellular enzyme activities were examined using PM medium. As shown in Table 1, the pelleted forms of *Rhizopus nigricans* induced mycolytic enzymes, which were the most effective in protoplasting the target fungus, also compared with the commercial product Novozym 234 from *Trichoderma harzianum*. The analyses of particular enzyme activities showed the enhancement of chitinase and the reduction of β-1,3 glucanase and protease production in the presence of pellets or older filaments of *Rhizopus nigricans*.

This supports our hypothesis that changes in morphology also could represent alteration of the inducing substrate for lytic enzyme production. In Figures 1 and 2 the filamentous and the pelleted growth form of *Rhizopus nigricans* are represented, showing a thicker wall of the latter. The fungal cell wall consists of 80–90% polysaccharides and chitin and chitosan are among major constituents of the cell wall structure of *Rhizopus* species [1], a thicker wall would most probably include greater amounts of these polysaccharides.

Our results are in accordance with the common conclusion that chitinolytic activity is of great significance in the process of cell wall lysis, whether for the release of protoplasts or for mycoparasitic action [2,8–10,14]. This is also confirmed by the report on a successful release of protoplasts from *Trichoderma reesei* with the addition of chitinase to an ineffective *Trichoderma harzianum* lytic preparation [15]. A detrimental effect of proteases for protoplast stability was also reported [10].

Table 2 shows the effect of chitin addition on the production of *Trichoderma* mycolytic enzymes in comparison to that in the PM medium containing only *Rhizopus nigricans* and glucose as the carbon source. With increasing chitin concentration, the level of chitinase in the mycolytic enzyme preparation increased. This is in agreement with recent reports about the regulation of chitinase synthesis in *Trichoderma harzianum*, indicating induction in a chitin-containing medium [2,18].

However, the partial substitution of *Rhizopus nigricans* crude mycelial preparation with chitin in the PM medium slightly lessened the ability of the *Trichoderma* exoenzyme preparation to digest the cell wall of the target fungus. This is probably due to a lower level of β-1,3- and α-1,3-glucanases, which are also suggested as the key enzymes in fungal cell wall lysis [2,8–10,14,19]. The synergistic effect of

these enzymes is obviously needed for a successful cell wall breakdown.

We suggest that with correct cultivation of inducing microorganisms, the ability to form protoplasts by induced mycolytic preparations could be improved. Thus, the need for the growth of producing microorganisms on mostly expensive polysaccharides, such as chitin and laminarin, is excluded, and also the addition of chitinase or other hydrolases to specific mycolytic mixtures is not required.

Acknowledgements

The support of the Slovenian Ministry of Science, Grant No P4-5318-0103 is gratefully acknowledged. The authors would also like to thank Professor Dr K Jezernik for providing electron micrographs and Mrs B Blaznik for her help in experimental work.

References

1 Bartnicki-Garcia S. 1968. Cell wall chemistry, morphogenesis and taxonomy of fungi. Ann Rev Microbiol 22: 87–109.
2 de la Cruz J, M Rey, JM Lora, A Hidalgo-Gallego, F Domínguez, JA Pintor-Toro, A Llobell and T Benítez. 1993. Carbon source control on β-glucanases, chitobiase and chitinase from *Trichoderma harzianum*. Arch Microbiol 159: 316–322.
3 Dermastia M and R Komel. 1991. Release and regeneration of protoplasts from *Cochliobolus lunatus*. Period Biol 93 (3): 411–415.
4 Didek-Brumec M, K Jezernik, A Puc and H Sočič. 1988. Ultrastructural characteristics of *Claviceps purpurea* seed cultures. J Basic Microbiol 28 (9/10): 589–598.
5 Dlugonski J, L Sedlaczek and A Jaworski. 1984. Protoplast release from fungi capable of steroid transformation. Can J Microbiol 30: 57–62.
6 Elad Y, I Chet, P Boyle and Y Henis. 1983. Parasitism of *Trichoderma* spp on *Rhizoctonia solani* and *Sclerotium rolfsii*—scanning electron microscopy and fluorescence microscopy. Phytopathology 73: 85–88.
7 Fukuda H, M Imura, H Kasahara, K Kirimura and S Usami. 1987. Effects of the addition of laminaran on β-glucosidase production by *Trichoderma viride*. J Ferment Technol 65: 91–93.
8 Hasegawa S, JH Nordin and S Kirkwood. 1969. Enzymes that hydrolyse fungal cell wall polysaccharides. J Biol Chem 244: 5460–5470.
9 Hamlyn PF, RE Bradshaw, FM Mellon, CM Santiago, JM Wilson and JF Peberdy. 1981. Efficient protoplast isolation from fungi using commercial enzymes. Enzyme Microb Technol 3: 321–325.
10 Kitamoto Y, N Mori, M Yamamoto, T Ohiwa and Y Ichikawa. 1988. A simple method for protoplast formation and regeneration from various fungi using an enzyme from *Trichoderma harzianum*. Appl Microbiol Biotechnol 28: 445–450.
11 Mandels M, F Parish and ET Reese. 1962. Sophorose as an inducer of cellulase in *Trichoderma viride*. J Bacteriol 83: 400–408.
12 Morrin M and OP Ward. 1989. Investigation of cell wall composition of different mycelial forms of *Rhizopus arrhizus*. Mycol Res 93: 524–528.
13 Peberdy JF. 1985. Mycolytic enzymes. In: Fungal Protoplasts (Peberdy JF and L Ferenczy, eds), pp 31–40, Marcel Dekker, New York, NY.
14 Ridout CJ, JR Coley-Smith and JM Lynch. 1988. Fractionation of extracellular enzymes from a mycoparasitic strain of *Trichoderma harzianum*. Enzyme Microb Technol 10: 180–187.
15 Sandhu DK, V Wadhwa and PS Bagga. 1989. Use of lytic enzymes for protoplast production in *Trichoderma reesei* QM9414. Enzyme Microb Technol 11: 21–25.
16 Sivan A and I Chet. 1989. Degradation of fungal cell walls by lytic enzymes of *Trichoderma harzianum*. J Gen Microbiol 135: 675–682.
17 Stauf CE. 1975. A linear standard curve for the Folin Lowry determination of protein. Anal Biochem 69: 646–648.
18 Ulhoa CJ and JF Peberdy. 1991. Regulation of chitinase synthesis in *Trichoderma harzianum*. J Gen Microbiol 137: 2163–2169.
19 de Vries OMH and JGH Wessels. 1972. Release of protoplasts from *Schizophyllum commune* by a lytic enzyme preparation from *Trichoderma viride*. J Gen Microbiol 73: 13–22.
20 Žnidaršič P, A Pavko and R Komel. 1992. Laboratory-scale biosynthesis of *Trichoderma* mycolytic enzymes for protoplast release from *Cochliobolus lunatus*. J Ind Microbiol 9: 115–119.