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Laboratory-scale biosynthesis of *Trichoderma* mycolytic enzymes for protoplast release from *Cochliobolus lunatus*

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SUMMARY

Microorganism useful for the induction of enzymes lytic towards walls of filamentous fungus *Cochliobolus lunatus* were studied. Production of specific *Trichoderma viride* mycolytic enzymes was studied in a laboratory fermentor. The product with high chitinase and relatively low protease activity gave better yields of *C. lunatus* protoplasts than commercial Novozym 234.

INTRODUCTION

Cochliobolus lunatus is a filamentous fungus of biotechnological relevance. It is capable of hydroxylating steroids at different positions, with the main reaction at the specific 11-β position [6], which opens the possibility of using this microorganism for the partial synthesis of glucocorticoids [5]. For biochemical investigation of enzymes involved in steroid hydroxylation, and for strain improvement via protoplast fusion or via introduction of in vitro recombined DNA sequences, protoplasts are a prerequisite.

The most favoured approach for the isolation of fungal protoplasts is cell wall digestion with mycolytic enzymes. The great interest in fungal protoplasts in various fields of research has lead to the discovery and optimization of a system for protoplast formation [12]. Although numerous mycolytic enzyme preparations have been reported and some of them are commercially available, our interest was oriented to a specific preparation with which stable protoplasts could be prepared in high yields from steroid bioconverting filamentous fungi such as *C. lunatus*.

The present study deals first with testing of two different fungi as inducers of specific enzymes lytic towards the *C. lunatus* cell wall. Secondly, the production of *T. viride* specific mycolytic enzymes in a laboratory tower reactor was investigated, yielding results which offer possibilities for further development and optimization of the process. We also compared the effectiveness of different concentrations of the *T. viride* enzyme preparation with the commercial enzyme Novozym 234 (Novo Biolabs, Denmark).

MATERIALS AND METHODS

Microorganisms

For production of mycolytic enzymes *Trichoderma viride* Persa ex. Fr., 1131 CBS 3543-33 was used, obtained from the culture collection of the Institute of Microbiology, University of Lodz, Poland. As inducing microorganisms *Rhizopus nigricans* ATCC 6227 b and *Cochliobolus lunatus* m 118 were tested. Both microorganisms are maintained at the Institute for Biochemistry, Medical Faculty in Ljubljana, Slovenia.

Preparation of inducing microorganisms

Cochliobolus lunatus was grown in a liquid medium consisting of malt extract, 6 °Blg, pH 5.5. Spores washed from 5-day cultures on agar slants were inoculated into 50 ml of medium in 500-ml Erlenmeyer flasks and incubated at 28 °C on a rotary shaker. After 24 h 10 vol% of culture was inoculated into 100 ml of fresh medium and growth was continued under same conditions. Mycelium was collected after 16 h and washed several times with distilled water. Before adding it to the production medium, the crude mycelial preparation was suspended in distilled water and autoclaved.

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Spores of 14-day-old *Rhizopus nigricans* were washed from agar slants into 500-ml Erlenmeyer flasks with 50 ml of liquid medium which consisted of (in g per l): glucose, 20; soya bean peptone, 5; yeast extract, 5; NaCl, 5 and $KH_2PO_4 \cdot 3H_2O$, 0.5; pH adjusted with HCl to 5.5. Incubation was performed at 28 °C for 24 h on a rotary shaker. For biomass production in a laboratory fermenter; after 6 h of growth on a rotary shaker 650 ml of culture was inoculated into a 6-l laboratory fermenter with a 4-l working volume. Cultivation in the tower reactor was carried out at 28 °C and aeration of 0.6 vvm for 24 h.

Preparation of lytic enzymes in a laboratory fermenter

Trichoderma viride was grown for cell wall lytic enzyme production in a 9-l bioreactor with a working volume of 6 l. The culture medium consisted of 0.3 g/l glucose, mineral components according to Mandels et al. [10] and 60 g/l wet weight of crude mycelial preparation of *Rhizopus nigricans*. For developing inoculum *T. viride* was incubated for 24 h on a rotary shaker in a liquid medium containing 4 g/l glucose and 4 g/l yeast extract in diluted malt extract (6 °Blg); pH 6.6–7.0. After inoculation of the fermenter with 10 vol% of inoculum, cultivation was carried out under the following conditions: temperature 28 °C, aeration 1–1.5 vvm, for 6 days. Lytic enzymes were isolated from the culture broth according to deVries and Wessels [3].

Protoplast formation

After growth in shaken cultures (see preparation of inducing microorganisms) *C. lunatus* was filtered and washed several times with distilled water and finally with an osmotic buffer (0.05 M succinic acid, 1 M NaCl, pH adjusted with NaOH to 5.0). If not specified otherwise, 100 mg wet weight of mycelium was suspended in 1 ml of osmotic buffer (see above) containing 2.5 mg of the crude enzyme preparation; the mixture was incubated at 28 °C in closed tubes for 6 h and protoplasts were counted using a haemocytometer [6].

Analytical methods

Total dry matter (TDM)

TDM was determined by drying the culture samples for 24 h at 105 °C.

Total sugar concentration (TS)

TS was measured in culture filtrates by a modified Scott's method [1, 15].

pH value

This was measured in withdrawn samples using a pH meter.

In lyophilized crude enzyme preparations (CEP), protein content (PC) was determined by a modified Folin-Lowry method [14].

Estimation of enzymes

β-glucanase (β-1,3(4) glucan glucanohydrolase, EC 3.2.1.6.). The enzyme was assayed using a modification of the method of Huotari et al. [7]. 0.5 ml of suitably diluted enzyme solution was mixed with 0.5 ml substrate solution (0.1% w/v laminarin) in 0.05 M sodium citrate buffer, pH 5.8, and the mixture incubated for 15 min at 37 °C.

α-glucanase (α-1,3(4) glucan glucanohydrolase, EC 3.2.1.61). To 0.5 ml of suitably diluted enzyme solution, 0.5 ml substrate solution containing 0.1% (w/v, in sodium citrate buffer pH 4.8) nigeran was added and the mixture was incubated for 30 min at 37 °C [9].

Endo-β-1,4-glucanase (EC 3.2.1.4). The enzyme was estimated by incubating tubes (at 37 °C for 30 min) containing appropriate dilutions of 0.5 ml of crude enzyme and 0.5 ml of 1% CMC solution in 0.1 M acetate buffer at pH 4.8 [9]. The amount of reducing sugars in each of the above reactions was estimated with DNS reagent according to the method of Miller [11].

Chitinase (EC 3.2.1.14). To suitably diluted 0.5 ml of enzyme sample, 0.5 ml of 0.1% (w/v) suspension of colloidal chitin prepared in 0.05 M sodium citrate buffer (pH 5.8) was added and the mixture was incubated for 2 h at 37 °C. Then the amount of released N-acetylaminoglucosamine was assayed according to the method of Reissig et al. [13].

Protease. To 2.5 ml of 2% casein in 1 M phosphate buffer (pH 7.5) 0.2 ml of suitably diluted enzyme was added and the mixture was incubated for 10 min at 37 °C. Then 5 ml of TCA was added and the mixture was centrifuged after 10 min. Concentration of tyrosine liberated in the supernatant was measured by the method of Lowry et al. [8].

RESULTS AND DISCUSSION

Choice of microorganism for enzyme induction

Two microorganisms were compared as inducers of specific mycolytic enzyme production in *T. viride*: *C. lunatus* which in our investigation is the target strain for protoplast release, and *R. nigricans* which belongs to the chitin-chitosan group of filamentous fungi.This could, therefore, serve as a potential inducer of additional mycolytic activities in *T. viride* which may be necessary for efficient release of stable protoplasts from *C. lunatus*.

As shown in Table 1, *R. nigricans* proved to be a more effective inducer of mycolytic enzymes (designated as RTE) even at lower concentrations. On increasing the concentration of the crude mycelial preparation in a pro-

TABLE 1

Influence of different type and concentrations of inducer on *T. viride* mycolytic enzyme production

Inducer	*C. lunatus*	*R. nigricans*	*R. nigricans*
Inducer conc. (mg/l)	10	2.5	10
Mycolytic enzyme			
Preparation (mg/l)	105.2	86.5	445.7
Protein content (%)	62	39	43
No. protopl./ml	$\leq 10^4$	4×10^4	3×10^5

TABLE 2

Individual specific enzyme activities in mycolytic enzyme preparations RTE and CTE. Comparison with Novozym 234

Specific enzyme activity[a]	Novozym 234[b]	RTE	CTE
β-1,3 glucanase	0.7	0.218	0.115
α-1,3 glucanase	6.57×10^{-2}	2.65×10^{-2}	2.49×10^{-2}
Endo-β-1,4 glucanase		1.61×10^{-2}	1.16×10^{-2}
Chitinase	2.25×10^{-3}	2.04×10^{-3}	0.72×10^{-3}
Protease[c]	300	33.3	61.1

[a] Specific enzyme activity, (μmol/mg protein min).
[b] Novozym 234, data from Hamlyn et al. [15].
[c] Protease, equivalent to μg trypsin/mg enzyme/min.
RTE – *R. nigricans–T. viride* enzyme/min.
CTE – *C. lunatus–T. viride* enzyme.

duction medium, the amount of the final product increased and the effectiveness on the release of protoplasts was improved.

Comparison of the protein content in the mycolytic enzyme preparations shows that *C. lunatus* as an inducer gave a "cleaner" product, while different concentrations of *R. nigricans* had no notable influence in this respect. However, it is obvious (see Table 1) that using CTE, (*C. lunatus-T. viride enzyme*) the release and stability of protoplasts were impaired. The reason for this phenomenon can be found in Table 2. The level of chitinase activity in RTE is much higher than in CTE and is comparable to that in Novozym 234. On the other hand, the level of proteases in RTE is much lower than in CTE and Novozym 234.

Production of lytic enzymes in a laboratory fermenter

Parameters followed during the batch fermentation process in a bubble column are shown in Table 3. Samples were withdrawn at indicated times, and total dry matter (TDM), total sugar concentration (TS), pH value and the amount of crude enzyme preparation (CEP) were determined. The fermentation product-mycolytic enzyme preparation (MEP) is represented as the amount of isolated protein, calculated from the protein content (PC) in the lyophilized crude enzyme preparation. The quantity of *T. viride* mycolytic enzymes gradually increased during the first four days of cultivation, while on the fifth day of production a remarkable enhancement was noticed.

When testing the effectiveness of the products of the first four days, we observed that it was insignificant; the greatest number of protoplasts (NP) was released by application of the final mycolytic enzyme preparation, showing improvement in product quality at the end of the fermentation.

Although there was no important difference among the protein contents of the crude enzyme preparations isolated during production, we can assume that not until the fifth day all enzymes, essential for cell wall lysis, were synthesized. In this respect, it is not excluded that the product contained an additional complex of autolytic enzymes, which in some other cases was observed to be helpful in protoplast release [12].

TABLE 3

Parameters during the production of lytic enzymes in a laboratory fermentor. TDM, total dry matter; TS, total sugars; CEP, crude enzyme preparation; PC, protein content; MEP, mycolytic enzyme preparation; NP, number of protoplasts.

Time (h)	pH	TDM (g/l)	TS (g/l)	CEP (mg/l)	PC (%)	MEP (mg/l)	NP (No./ml)
0.5	5.31	5.00	2.08	45.64	9.6	4.40	0
23	4.43	4.96	0.63	42.66	24.1	10.30	$< 10^4$
47	5.94	3.03	0.35	122.35	27.0	36.14	$\leq 10^4$
73	7.59	2.79	0.30	168.23	26.9	45.36	$\leq 10^4$
97	8.13	2.76	0.28	244.71	22.2	54.40	$\leq 10^4$
121	8.30	3.11	0.29	543.12	23.1	123.65	10^4–10^5
145	8.41	2.66	0.37	349.9[a]	22.2		3×10^6

[a] Practical data (all others are calculated from sample volumes).

The growth of *T. viride* was moderate, as evident by the slow decrease of total dry matter, which represents the sum of *T. viride* and *R. nigricans* cell mass. Also indicative of the moderate mycelial growth is almost constant total sugar concentration during the major phase of the fermentation. The pH value slightly decreased at the beginning, while after the first day it showed an atypical tendency to increase. Stagnation of the pH curve at the end of fermentation indicated a significant rise of enzyme production phase and was concordant with moderate growth and sugar consumption.

Effect of enzyme concentration

Considering the low protein content in the isolated product, we tested higher concentrations of the mycolytic enzyme preparation RTE. Commercial Novozym 234 was used as a comparative substance. After enzyme treatment protoplasts were separated from mycelial debris by filtration through sintered glass (porosity 1). Protoplasts were pelleted (1000 rpm, 10 min) and counted in the resuspended pellet and in the supernatant [2]. The results are summarized in Table 4.

Within the range tested, the protoplasts yield increased with increasing enzyme concentration. However, a lot of protoplasts were present in the supernatant (more than 10^7 per ml by using *T. viride* enzyme) and they were apparently bigger than those collected in the pellet. This may be due to vacuolisation of protoplasts which prevents sedimentation.

DeVries and Wessels [3] reported that the relative density of the protoplasts depends on the osmotic stabilizer; in further investigations we shall follow our mycolytic mixtures in this respect.

When comparing the effectiveness, crude *T. viride* mycolytic enzyme preparation and Novozym 234 were used in concentrations at which the protein concentration was nearly at the same level. The yield of protoplasts obtained with RTE (1.7×10^7/ml) was about 4.6 times that of the

TABLE 4

Influence of different concentrations of RTE (*R. nigricans–T. viride* enzyme) on number of released protoplasts and comparison with commercial product

Enzyme	RTE	RTE	RTE	Novozym 234
Enzyme concentration (mg/ml)	5	10	20	5
Protein concentration (mg/l)	1.1	2.2	4.45	4.15
No protoplast $\times 10^{-6}$/ml	0.2	5.2	17	3.7

commercial enzyme, which in our opinion is very favourable for preparation of large amounts of protoplasts from *C. lunatus*, necessary for biochemical investigations of intracellular steroid hydroxylating enzymes. This mycolytic enzyme preparation, derived from inducer of which the cell wall composition was different and more complex than that of target strain, contained high level of chitinase and some other activities, essential for efficient cell wall degradation and protoplast release from *C. lunatus*. Its relative low protease activity was sufficient to contribute in cell wall degradation, but low enough not to impair the stability of resulting protoplasts. The protoplasts were stable enough to be useful in genetic manipulations in potential experiments of strain improvement. Additionally, our preparation was, besides its main purpose, also effective in releasing protoplasts from some other biotechnologically important fungi, such as *Claviceps* sp. and in lower yields from *Aspergillus* sp. (Valinger R., Pliva, YU,-personal communication).

As a result, we consider the present study as a possible introduction to prepare larger amounts of specific mycolytic enzyme mixtures in laboratory fermenters, which should be comparable to other broad range commercial preparations like Novozym 234, but more effective in particular cases with specific filamentous fungi such as *C. lunatus*.

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