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Improvement of Cell Wall Degrading Enzymes Production by Alginate Encapsulated *Trichoderma* spp.

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Summary

Conidia of three *Trichoderma* isolates were formulated to make alginate pellets with or without 0.5 % chitin or dried fungal mycelium of *Fusarium oxysporum* as carbon source. The formulations were compared for their ability of *in vitro* chitinase and β-1,3-glucanase production with free fungal spore suspensions. Conidia entrapped in alginate with or without adjuvant showed high production of enzymes (especially for chitinase) even when repeated 4 times. The addition of chitin or dried fungal mycelium as adjuvant enhanced the enzyme production up to 5 and 2-fold for chitinase and β-1,3-glucanase, respectively. Alginate concentration and surface area of the beads affected the enzyme production. The optimum initial pH, incubation time and temperature were pH=6, 12 days and 40 °C for chitinase, and pH=7, 10 days and 35 °C for β-1,3-glucanase production. The improvement of cell wall degrading enzyme production by alginate encapsulated *Trichoderma* could explain the *in vivo* inhibitory effect of such formulations on the target phytopathogenic fungi.

Key words: Trichoderma spp., biocontrol, chitinase, glucanase, immobilization, Ca-alginate

Introduction

Biological control of soil-borne plant pathogens is a potential alternative to the use of chemical pesticides, which have already been proved harmful to the environment. *Trichoderma* spp. have been described as fungal biocontrol agents, acting by either the production of antimicrobial compounds or parasitism of fungal plant pathogens (*1*). Thus, the necessity to achieve large-scale, cost-effective production of active preparations of this biopesticide has been increased (*2*).

Immobilization of microbial cells and enzymes has become one of the most valuable tools in the field of biotechnology (*3*). Moreover, microbial entrapment gives prolonged metabolic activity when microbial cells are reused and protects the organism from inhibitory compounds or metabolites (*4,5*).

Alginate formulations of conidia and/or mycelia or ascospores of several biocontrol agents (*e.g. Trichoderma* spp., *Talaromyces* spp., *Gliocladium* spp.) resulted in rapid fungal increase and proliferation in the soil, enabling successful biological control of several plant diseases (*6–9*).

Our research group is interested in studying the survival and proliferation of the beneficial microorganisms such as the nitrogen fixing *Herbaspirillum* spp. (*10*) and *Trichoderma* spp. (*9,11*) in the soil. The latter investigation reported that alginate formulations of *Trichoderma* spp. were better for the proliferation and survival than conidia added directly to the same soil after a 3-month incubation period. Moreover, these formulations had inhibitory effect on some pathogenic (*Fusarium* spp., *Rhizoctonia* spp. and *Sclerotium* spp.) and nonpathogenic

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(*Aspergillus terreus* and *Alternaria* spp.) fungi species (*9,12*).

The objective of this study was to investigate the *in vitro* production of chitinase and β-1,3-glucanase by alginate encapsulated *Trichoderma* spp. and to optimize the conditions required for improving the production of these enzymes by the immobilized fungi spores.

Materials and Methods

Fungal isolates and cultivation

Three local isolates of *Trichoderma* spp. used in this study were isolated by Shaban from soil samples collected from El-Minia Governorate, Egypt (*13*). Two isolates (T3 and T24) were identified as *T. harzianum* Rifai by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), while (T1) is referred to as *Trichoderma* sp. A *Fusarium oxysporum* isolate obtained from the culture collection of the Botany Dept. of El-Minia University was applied for the preparation of dried fungal mycelium. Fungal cultures were maintained on potato dextrose agar (PDA) at 4 °C.

Production of inocula and microencapsulation

Methods used for the production of inocula and microencapsulation were described earlier (*9*). Microencapsulation was performed using different alginate concentrations of 1, 2, 3 and 4 %. In some other experiments, carbon source (0.5 % chitin or dried mycelium) was added to the alginate-spore suspension mixture. Nozzles with different diameters were also used to obtain beads with different surface areas (2, 2.5 and 3.5 cm^2). The fresh beads were either used directly, or kept at 4–5 °C in sealed flasks for several days. The viable population size of *Trichoderma* was determined in the pellets before its use in the batch culture fermentation.

Chitinase and β-1,3-glucanase production by immobilized or free Trichoderma isolates in batch culture fermentation

Batch culture fermentation was carried out in Erlenmeyer flasks (50 mL), each containing 10 mL of minimal synthetic medium (MSM) with the following composition (in g/L): $MgSO_4 7H_2O$ 0.2; K_2HPO_4 0.9; KCl 0.2; NH_4NO_3 1.0; $FeSO_4 7H_2O$ 0.002; $MnSO_4$ 0.002 and $ZnSO_4$ 0.002. The appropriate carbon source (0.5 % chitin or dried mycelium of *F. oxysporum*) was supplied and the pH was adjusted to 5.5 with 50 mM acetate buffer. Flasks were inoculated with either 1 mL of fungal spore suspension or 3 g of fresh beads containing ($3 \cdot 10^6$ CFU/flask).

Dried fungal mycelium of *Fusarium oxysporum* was prepared as described by El-Katatny (*14*). Flasks were incubated in a shaker incubator (125 rpm and 30 °C) for 7 days. At the end of the incubation period, cultures were separated by filtration and centrifuged at 6000 rpm for 10 min in a cooling centrifuge at 4 °C and the clear supernatant was used as crude enzyme preparations. The effects of chitin concentration (0.1–1.5 %), initial pH (3–8) and temperature (20–45 °C) on the production of enzymes were tested.

Repeated batch fermentation

The reusability of the immobilized cultures was tested in batch cultures by replacing the culture broth with a fresh sterile one every 7 days. Cultivation conditions were as previously described for each set.

Enzyme activity assays

Chitinase activity was assayed using the colorimetric method described by Molano *et al.* (*15*) with minor modifications (*16*). The assay mixture contained 1 mL of 0.5 % pure chitin (Sigma) suspended in 50 mM acetate buffer (pH=5.2) and 1 mL of crude enzyme preparation. The reaction mixture was incubated for 7 h at 37 °C by shaking, then the reaction was stopped by centrifugation (5000 rpm for 10 min) and 1 mL of dinitrosalicylate (DNS) reagent was added (*17*). The amount of reducing sugars released was calculated using a standard curve recorded for GlcNAc. One picokatal (pkat) of enzyme activity releases one pmol s^{-1} of GlcNAc equivalent at 37 °C.

β-1,3-glucanase was assayed based on the release of reducing sugars from laminarin as a substrate. The activity was routinely assayed by incubating 500 μL of 0.5 % laminarin in 50 mM acetate buffer (pH=4.8) with 200 μL of crude enzyme preparation at 45 °C for 30 min, and the reducing sugars produced were determined by the method by Miller (*17*) using DNS reagent. One nanokatal (nkat) of β-1,3-glucanase activity was defined as the amount of enzyme that releases one nmol s^{-1} of glucose equivalent under the given conditions. All the values of enzyme activity are the mean values of at least two replicates.

Results

Screening of Trichoderma isolates for enzyme production using free or immobilized cells

Results presented in Table 1 showed that *Trichoderma harzianum* isolate No. 24 (T24) was the most potent isolate for chitinase and β-1,3-glucanase production when used as free or immobilized spores. Therefore, it has been selected for further investigations. In both free and immobilized cultures, dry mycelium of *F. oxysporum* as a carbon source stimulated enzyme production for all tested isolates more than chitin.

Reusability of immobilized spores

The reusability of the immobilized fungi for enzyme production was studied. Beads entrapping *Trichoderma* spores were successfully used in 4 repetitions in the presence of chitin or dried mycelium as carbon source (Fig. 1). When chitin was used as a carbon source, the activity of β-1,3-glucanase increased slightly with the recycled use of beads. However, recycling had no significant effect on chitinase activity. Furthermore, the isolate *T. harzianum* (T24) was superior to the other isolates for enzyme production (Fig. 1A).

Table 1. Screening for β-1,3-glucanase (nkat/mL) and chitinase (pkat/mL) production by free or immobilized *Trichoderma* spp.

Isolate	Free spores		Immobilized spores	
	Chitin C-source	Dry-mycelium of *F. oxysporum* C-source	Chitin C-source	Dry-mycelium of *F. oxysporum* C-source
Activity of β-1,3-glucanase/(nkat/mL)				
Trichoderma sp. (T1)	0.53	4.9	0.36	3.85
T. harzianum (T3)	0.62	6.45	0.38	3.6
T. harzianum (T24)	1.25	6.8	1.2	4.4
Activity of chitinase/ (pkat/mL)				
Trichoderma sp. (T1)	7.9	22.7	10.0	19.2
T. harzianum (T3)	7.35	11.14	10.5	18.8
T. harzianum (T24)	8.5	23.1	10.8	19.7

Table 2. Consecutive improvement of *T. harzianum* culture conditions* for chitinase and β-1,3-glucanase production by immobilized *T. harzianum*

Parameters	β-1,3-Glucanase		Chitinase	
	Activity (nkat/mL)	Fraction of the highest enzyme activity**/%	Activity (pkat/mL)	Fraction of the highest enzyme activity**/%
Alginate fraction:				
- 1 %	0.50	87.7	2.71	70.4
- 2 %	0.51	90.0	3.85	100
- 3 %	0.56	98.3	3.39	88.1
- 4 %	0.57	100	2.26	58.7
Inoculum:				
- Spores	0.51	100	3.85	100
- Spores + Mycelium	0.51	99.4	2.60	67.5
Bead area:				
- Small	0.48	83.2	3.35	64.4
- Medium	0.50	86.7	3.90	75.0
- Large	0.58	100	5.20	100
Adjuvant addition:				
- Without	0.51	68.9	3.85	19.3
- Chitin	0.74	100	20.0	100
- Dry mycelium	0.59	79.7	10.4	52.0
Shaking:				
- No shaking	0.22	81.5	1.95	72
- Shaking (125 rpm)	0.27	100	2.71	100

* For other conditions, see Figs 2-5.

** The highest enzyme activity in each separate experiments was given as 100 %

When dried mycelium was used as a carbon source, the activities of chitinase and β-1,3-glucanase produced by T3 and T24 decreased slightly up to the 2nd reuse followed by a sharp increase in the last cycle. However, the isolate T1 showed an increase in the activities of both enzymes when recycling. Generally, T1 showed higher enzyme activities than T3 and T24 (Fig. 1B).

Culture conditions for chitinase and β-1,3-glucanase production by immobilized T. harzianum (T24)

Alginate concentration (1–4 %) affected the enzyme production by immobilized *T. harzianum* (Table 2). The optimum alginate concentrations for β-1,3-glucanase and chitinase were 4 and 2 %, respectively. Enzyme activities increased with the increase of bead surface area (Table 2). Beads containing fungal spores showed higher enzyme chitinase production than those containing both spores and mycelium. The addition of chitin or dried mycelium as adjuvant significantly improved enzyme production (Table 2). Furthermore, chitin has a greater effect on β-1,3-glucanase and chitinase production, which was increased 1.5 and 5.2-fold, respectively. Enzyme production using immobilized fungal spores (2 % alginate) in the presence of chitin as a carbon source was investigated at different time intervals (Fig. 2). The data indicated that β-1,3-glucanase production increased slightly by increasing the incubation time up to 12 d. Chitinase production was the highest after 12 d of incubation.

The data presented in Fig. 3 showed that chitinase production had a wide optimum pH range of 4–7. β-1,3-glucanase exhibited its maximum production at pH=7. The optimal temperatures for chitinase and β-1,3-glucanase production by immobilized *Trichoderma* were 40 and 35 °C, respectively (Fig. 4). The chitinase and β-1,3-glucanase production by immobilized *T.*

Fig. 1. Repeated use of immobilized *Trichoderma* spp. for enzyme production in the presence of chitin (A) and dried mycelium (B) as a carbon source; T 1 : *Trichoderma* sp., T 3 : *T. harzianum* Rifai, T 24 : *T. harzianum* Rifai

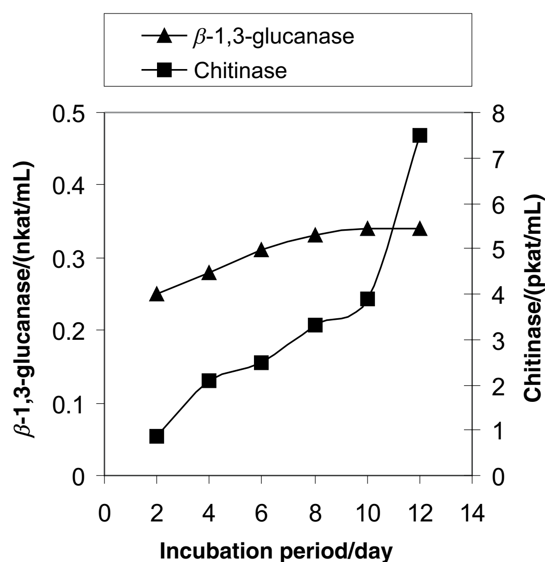

Fig. 2. Effect of incubation period on chitinase and β-1,3-glucanase production by immobilized cells of *T. harzianum*

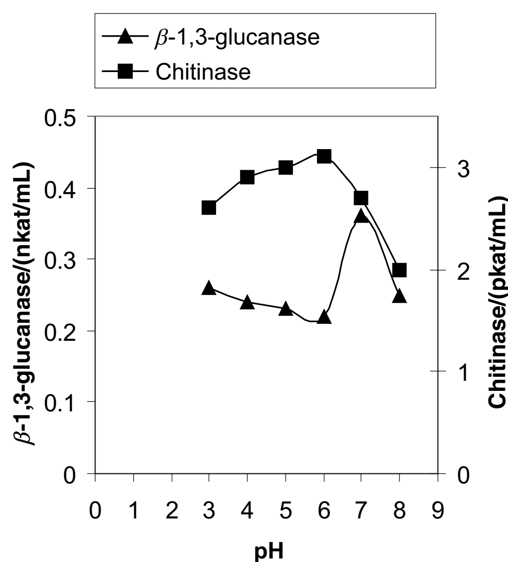

Fig. 3. Effect of initial pH of culture medium on chitinase and β-1,3-glucanase production by immobilized cells of *T. harzianum*

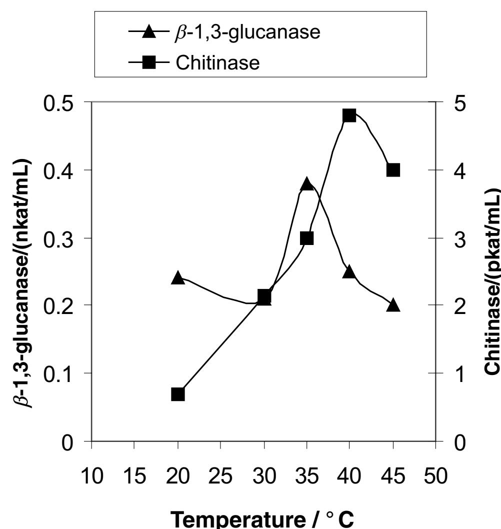

Fig. 4. Effect of incubation temperature on chitinase and β-1,3-glucanase production by immobilized cells of *T. harzianum*

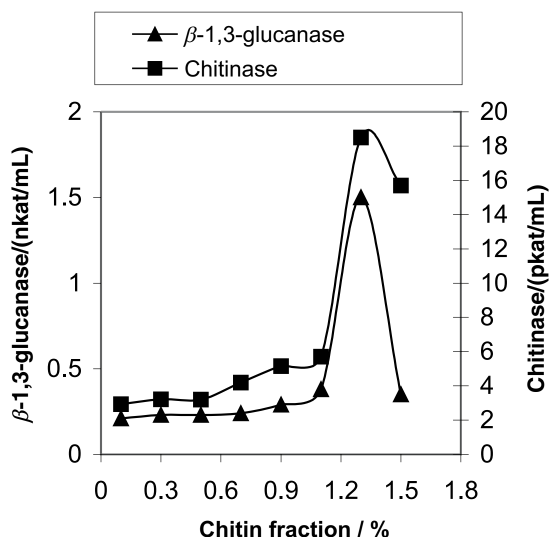

Fig. 5. Effect of chitin fraction on chitinase and β-1,3-glucanase production by immobilized cells of *T. harzianum*

harzianum was affected by the concentration of chitin. The highest enzyme production was obtained at 1.3 % chitin, while further increase in chitin concentration reduced enzyme production (Fig. 5).

Discussion

The direct mycoparasitic activity of *Trichoderma* species has been proposed as one of the major mechanisms for their antagonistic activity against phytopathogenic fungi (*18,19*). Chitinase and β-1,3-glucanase produced by some *Trichoderma* species are the key enzymes in the lysis of cell walls during their mycoparasitic action against phytopathogenic fungi (*20,21*). These enzymes may be important in the destruction of plant pathogens and could be used as the basis of screening for potential biocontrol agents (*22*).

Entrapment of microbial cells has been reported to improve the production of proteolytic enzymes (*23–25*). The aim of this work is to improve chitinase and β-1,3-glucanase production by three alginate encapsulated *Trichoderma* spp. The results indicated that *T. harzianum* Rifai (T24) was the most potent isolate for chitinase and β-1,3-glucanase production when used either as free or immobilized culture. Previous studies of El-Katatny *et al.* (*26*) indicated that *T. harzianum* Rifai (T24) was the most suitable isolate for the production of both enzymes among 24 tested isolates when used as free spore suspension.

Results also showed that immobilized *Trichoderma* improved chitinase production compared with free spore suspension especially when chitin was used as a carbon source. However, the production of β-1,3-glucanase by the immobilized culture was lower than that by free culture. These results are not surprising, since no consistent pattern has been reported for the effect of encapsulation process on enzyme production. High level of alkaline protease enzyme production was observed in the immobilized cultures of *Conidiobolus* spp. and *Aspergillus flavus*, while alginate immobilized cultures of *Streptomyces craterifer* produced lower levels of mannanase than those of free cultures (*25,27*).

Alginate encapsulation of *Trichoderma* prolonged the durability of the inoculum and retained or in some cases even increased the enzyme production during 4 repetitions. It was observed that the beads became weak and breakable (fragile) before the last cycle of reuse, especially when dried mycelium was used as a carbon source. This might explain why the enzyme production increased in the last cycle, since the fragile beads allowed the release of more conidia supporting higher growth and enzyme production. The degradation of pellets has been reported to be due to the presence of certain ions in the medium affecting the stability of the gel (*28*).

Alginate concentration for chitinase production was optimal at 2 %. However, the production of β-1,3-glucanase increased slightly with the increase of alginate concentration up to 4 %. It was reported that 2 % was the optimal alginate concentration for alkaline protease production by immobilized *A. flavus* (*25*) as well as for the survival of *Trichoderma* spp. and *Gliocladium* spp. in soil (*29*). Higher alginate concentration (5 %) reduced microbial growth and enzyme production as a result of limited diffusion of nutrients and oxygen (*30*).

Encapsulation technique was further refined by incorporation of nutrient carriers (adjuvant), *e.g.* wheat bran, milled chitin, corn cobs, fish meal, soy fibers and peanut hulls into the biopolymers (*e.g.* alginate) to provide a food base necessary for proliferation of the microorganisms (*10,31*). The results of this study indicated that when 0.5 % of chitin was added as an adjuvant, chitinase production was increased by 80 % compared to the beads prepared without chitin. Previous studies reported that the addition of 10 % cellulose as food base material increased the survival and proliferation of the entrapped conidia of *Trichoderma* spp. more than those prepared without cellulose (*9*). Moreover, alginate formulations of *Trichoderma* and *Gliocladium* spp. containing wheat bran as a carbon source carrier reduced the survival of *Rhizoctonia solani* from 34–78 % in the in-

fested soil (*7*). Since chitinase is an inducible enzyme, the presence of chitin as an adjuvant has led to a sharp increase in its production. Such effect was not observed with the constitutive β-1,3-glucanase.

Results of this study showed that pH=6 and 7 were the optimum initial pH values for the production of chitinase and β-1,3-glucanase, respectively. These results are in accordance with previous studies of El-Katatny *et al.* (*26*) for chitinase, however, the optimum initial pH for β-1,3-glucanase production in the case of immobilized *Trichoderma* was higher than that recorded previously (pH=5.5) for the free culture of *Trichoderma* of the same isolate (*26*).

Our results also indicated that optimum incubation temperatures for chitinase and β-1,3-glucanase from immobilized cultures were 40 and 35 °C, respectively. In previous studies, where free culture was used, the optimal incubation temperature was 30 °C for both enzymes and the increase of the temperature to 40 °C resulted in sharp reduction (85 % for β-1,3-glucanase and 92 % for chitinase) in the enzyme production (*26*). The presented results suggest that immobilization of *Trichoderma* extended the range of incubation temperature for enzyme production up to 40 °C. This could be explained on the basis that microbial immobilization changes the physiological state of entrapped microorganisms as reported by Van Elsas *et al.* (*32*) and El-Komy (*10*).

In conclusion, alginate encapsulation of *Trichoderma* successfully improved chitinase and β-1,3-glucanase production (*in vitro*) for a prolonged time even at higher temperature (40 °C). This could explain the *in vivo* inhibitory effect of such formulations on the target fungi recorded by many investigations (*7–9*). Further studies are needed to clarify the role of enzyme production by alginate formulations of *Trichoderma* in the soil to control soil-borne plant diseases.

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Poboljšanje proizvodnje enzima za razgradnju staničnoga zida pomoću *Trichoderma* spp. obavijene alginatom

Sažetak

Za proizvodnju alginatskih zrnaca upotrijebljene su konidije triju izolata *Trichoderma* s 0,5 % hitina ili bez njega, odnosno s osušenim micelijem *Fusarium oxysporum* kao izvorima ugljika potrebnih za rast. Pripravljena zrnca (peleti) uspoređena su, prema svojim sposobnostima proizvodnje hitinaze i β-1,3-glukanaze *in vitro*, sa suspenzijom slobodnih spora. Konidije uklopljene u alginat s dodacima ili bez njih imale su veliku sposobnost proizvodnje enzima (osobito hitinaze), čak i nakon što su 4 puta upotrijebljene. Dodatak hitina ili osušenog micelija povećao je enzimsku proizvodnju hitinaze 5 puta, a β-1,3-glukanaze 2 puta. Koncentracija alginata i ukupna površina zrnaca utječe na proizvodnju enzima. Optimalna početna pH-vrijednost, vrijeme inkubacije i temperatura iznosili su: pH=6, 12 dana i 40 °C za hitinazu, a pH=7, 10 dana i 35 °C za proizvodnju β-1,3-glukanaze. Poboljšanje proizvodnje enzima za razgradnju staničnoga zida pomoću vrste *Trichoderma* obavijene alginatom objašnjava inhibitorski učinak *in vivo* takvih pripravaka na fitopatogene gljivice.