ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 9 (4): 529-532, 2010
ISSN 1682-296X

Improvement of Carboxymethyl Cellulase and Xylanase Production by Alginate Immobilized *Trichoderma harzianum*

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Abstract: Conidia of two isolates of *Trichoderma harzianum* (T7 and T8) (Rifai) were formulated to make alginate pellets with or without xylan or CMC as a food-base material. The formulations were compared for their ability of *in vitro* carboxymethycellulase and xylanase production with free fungal spore suspensions. Conidia entrapped in alginate with or without adjuvant showed high production of enzymes even when repeated 4 times. The addition of adjuvant significantly enhanced the enzyme production. Alginate concentration and surface area of the beads affected the enzyme production. The optimum initial pH and incubation temperature were pH = 5-7 and 35°C for CMC-ase and pH = 5 and 30°C for xylanase. Alginate encapsulated *Trichoderma* not only prolonged the metabolic activity of the entrapped organism, but also it promotes slow release of microbial spores into the medium for successful enzyme production.

Key words: *Trichoderma harzianum*, biocontrol, immobilization, Ca-alginate, carboxymethyl cellulase and xylanase production

INTRODUCTION

Trichoderma is a fungal genus widely distributed all over the world. In addition to its biological control activities, different species belonging to the genus *Trichoderma* have been reported to produce cellulases (Chaudhary and Tauro, 1982). Cellulases produced by *T. harzianum*, is the most efficient enzyme system for the complete hydrolysis of cellulosic substrates into its monomeric glucose, which is a fermentable sugar (Ahmed *et al*., 2009). Cellulolytic enzyme production is one of the key steps in any process involving enzymatic hydrolysis of lignocellulosics into sugar monomers (Warzywoda *et al*., 1982). The use of such enzymes to convert wastes for the production of sugars, syrups, alcohol and single cell protein for food and feed has been investigated (Rye and Mandels, 1980). Thus, the necessity to achieve large-scale, cost-effective production of active preparations of *Trichoderma* has been increased (El-Katatny *et al*., 2003, 2004).

Immobilization of microbial cells and enzymes has become one of the most valuable tools in the field of biotechnology (EL-Komy, 2005). Moreover, microbial entrapment gives prolonged metabolic activities when microbial cells are reused and protects the organisms from inhibitory compounds or metabolites (Shaban and El-Komy, 2000). Alginate formulations of conidia and mycelia or ascospores of several fungi resulted in rapid fungal increase and proliferation in the soil (Bashan *et al*., 2002).

The objective of this study was to investigate the *in vitro* production of CMC-ase and xylanse by alginate encapsulated *Trichoderma* sp. and to optimize the conditions required for improving the production of these enzymes by the immobilized fungi species.

MATERIALS AND METHODS

Fungal isolates and cultivation condition: Two local isolates of *Trichoderma harzianum* (T7 and T8) used in this study were isolated from soil samples collected from El Faidia and El Mansora cities, El Bayda, Libya, respectively. 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 (Bashan *et al*., 2002). Microencapsulation was performed using different alginate concentrations: 1, 2, 3, 5%. In some other experiments 0.5% Carboxy Methyl Cellulose (CMC) was added to the alginate spore suspension mixture as adjuvant. Nozzles with different diameters were also used to obtain beads with different surface areas (3, 3.5 and

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4 cm^3). 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.

CMC-ase and xylanase production by immobilized or free *Trichoderma* isolates in batch culture fermentation: Batch culture fermentation was carried out in Erlenmyer flasks (50 mL) each containing 10 mL of a medium containing (g L^{-1}): $NaNO_3$, 2; KPO_4, 1; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.001. The appropriate carbon source (0.5% CMC or xylan) was supplied and the pH was adjusted to 5.5 with 50 mM acetate buffer. Flaks were inoculated with either 1 mL of fungal spore suspension or 3 g of fresh beads containing 3×10^6 CFU per flasks. Flasks were incubated for 7 days at 30°C. After incubation, cultures were separated by filtration for enzyme assays. The effects of initial pH (3-8) and temperature (20-45°C) on the production of enzymes were tested.

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

Enzyme assays: CMC-ase and xylanase activities were assayed using the viscometric method described by El-Naghy *et al.* (1991) using CMC and xylan as substrates. Reaction mixture containing 10 mL of 1% substrate dissolved in 0.1 M acetate buffer pH 4.5, 3 mL of 0.1 M acetate buffer pH 4.5 and 2 mL of enzyme preparation and incubated at 30°C for 1 h. Activity was estimated as percentage in reduction of viscosity during specific period of incubation as the following equation:

$$REA = \frac{T_0 - T_1}{T_0 - T_w} \times 100$$

where, REA is relative enzyme activity; T_0 is flow time immediately after the addition of enzyme filtrate and T_1 is flow time after incubation and flow time of water.

RESULTS

Results presented in Table 1 showed that *Trichoderma harzianum* isolate (T7) showed higher CMC-ase and xylanase production than isolate (T8) in both free and immobilized cultures. Therefore it has been selected for further investigations.

The reusability of the immobilized fungus (T7) for enzyme production was studied. Entrapped spores of *Trichoderma* were successfully used in 4 repetitions for both CMC-ase and xylanase production (Fig. 1). However, enzymes activities were at the maximum at the 4 th reuse. Optimum alginate concentration for CMC-ase and xylanase was 3%. Enzyme activities increased with the increase of bead surface area (Table 2). Beads entrapping fungal spores showed higher CMC-ase activity, however, beads entrapping spores and mycelium showed higher xylanase activity. The addition of CMC or xylan as adjuvant significantly improved enzyme production.

Data in Fig. 2 showed that CMC-ase had a wide optimum pH range of 5-7, xylanase exhibited its maximum production at pH = 5. The optimum temperatures for

Table 1: CMC-ase and xylanase production* by free and alginate encapsulated *Trichoderma* isolates

	Free spores		Immobilized spores	
Isolates	CMC-ase	Xylanase	CMC-ase	Xylanase
Trichoderma (T7)	18	12	23	13
Trichoderma (T8)	16	11	19	16

*As percentage of reduction in viscosity

Table 2: Consecutive improvement of immobilized *Trichoderma* (T7) for CMC-ase and xylanase production*

Parameters	CMC-ase	Xylanase
Alginate fraction		
1%	15	11
2%	20	14
3%	22	16
5%	16	12
Inoculum		
Spores	21	13
Spores+Mycelium	18	14
Bead area		
Small	18	11
Medium	19	14
Large	21	13
Adjuvant addition		
Without	17	12
Xylan	-	17
CMC	23	-
Shaking		
No shaking	16	11
Shaking (125 rpm)	18	14

*As percentage of reduction in viscosity

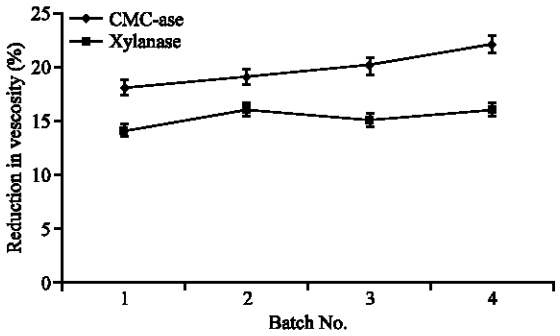

Fig. 1: Repeated use of immobilized *Trichoderma* (T7) for CMC-ase and xylanase production

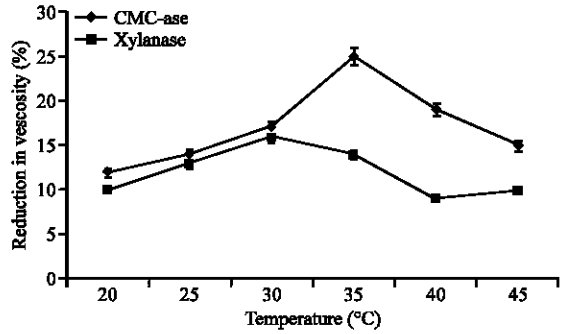

Fig. 2: Effect of incubation temperature on CMC-ase and xylanase production by immobilized *Trichoderma* (T7)

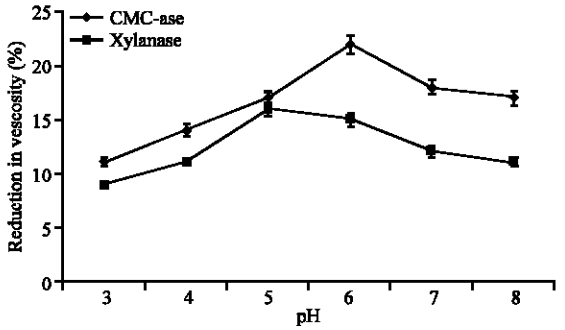

Fig. 3: Effect of initial pH of culture medium on CMC-ase and xylanase production by immobilized *Trichoderma* (T7)

CMC-ase and xylanase production by immobilized *Trichoderma* were 35 and 30°C, respectively (Fig. 3).

DISCUSSION

Cellulose is the most abundant renewable organic compound on earth and has received a great attention as a potential substrate for the production of alcohol fuel, chemicals and single cell protein via enzymatic degradation by microbial enzymes (El-Naghy *et al*., 1991). Entrapment of microbial cells has been reported to improve the production of proteolytic enzymes (Woodward, 1988). Results showed that immobilized *Trichoderma* improved CMC-ase and xylanase production compared with free spore suspension especially when CMC and xylan were used as adjuvant. Previous studies indicated that encapsulation technique was further refined by incorporation of nutrient carriers (adjuvant) e.g. wheat bran, milled chitin, corn cobs, soy fibers and peanut hulls into the biopolymers (e.g., alginate) to provide a food base necessarily for proliferation of the entrapped microorganisms (El-Komy, 2001, 2005).

Alginate encapsulation of *Trichoderma* prolonged the durability of the inoculum and increased in some cases the enzyme production during 4 repetitions. It was observed that the beads became weak and fragile before the last cycle of reuse. 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 the pellets has been reported to be due to the presence of certain ions in the medium affecting the stability of the gel (Kennedy and Cabral, 1985).

Optimum alginate concentration for CMC-ase and xylanase production was 2%. It was reported that 3% was the optimal alginate concentration for alkaline proteases produced by *Aspergillus flavus* ((Woodward, 1988), as well as for the survival of *Trichoderma* spp. in soil (Lewis *et al*., 1991). Higher alginate concentration (5%) may reduce microbial growth and enzyme production as a result of limited diffusion of nutrients and oxygen (Van Elsas *et al*., 1986).

Results of this study showed that pH = 5-7 and pH = 5 were the optimum initial pH values for the production o f CMC-ase and xylanase, respectively. These results are in accordance with previous studies of Ali and Akhand (1992) who reported that pH = 4 was the optimum for cellulase produced by some isolates of *Trichoderma*. (Gupta *et al*., 2009) found that xylanase production by *Fusarium solani* F7 was optimum between pH = 5.0 and 5.5, but the best at pH 5.5 below and above this pH production of xylanase was significantly lower. Moreover, Monti *et al*. (1991) reported that the pH optimum for *Humicola grisea* was 5.5 which was close to other microbial xylanases, which were between pH4.0 and 6.0 (Dekker and Richards, 1976).

Data also indicated that optimum temperature for the studied enzymes were 30-35°C. Such results are in agreement with many studies on mesophilic strains of *Penicillium* spp. and Actinomycetes, which was ranged from 20-40°C (Van Zyl, 1985). Also, Ali and Akhand (1992) reported that maximum cellulase production by *Trichoderma* isolates was obtained at 28-30°C.

In conclusion alginate immobilization of *Trichoderma* not only prolonged the metabolic activity of the entrapped organism, but also it promotes slow release of microbial spores into the medium for successful enzyme production. Further studies are needed to clarify the importance of fungal immobilization for the production of such important enzymes in a large scale.

ACKNOWLEDGMENTS

We gratefully acknowledge Professor Lennart Johnsson (MASE laboratory, Uppsala, Sweden), for help

with the statistical analyses, helpful comments and discussion; and Dr. Paul Johnson for assistance in interpreting the data, for critical reading and refining of the manuscript; Universiti Sains Malaysia (Malaysia) for inviting me as a scientific visitor and Omar Al-Mukhtar University, Al-Bayda, (Libya) for the financial support.

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