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EFFECT OF BENOMYL ON CARBOXYMETHYL CELLULASE (CMC-ASE) PRODUCTION BY TRICHODERMA KONINGIIAS FREE OR ALGINATE IMMOBILIZEDCELLS

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Abstract:-
The Screening of three isolates of Trichoderma (T. ghanense, T. harzianum and T. koningii) for Carboxymethyl cellulase (CMC-ase) production. Whereas, T. koningii was the most potent isolates for enzyme activity. Trichoderma, koningii produced CMCase at all the tested levels of benomyl ($C_{14}H_{18}N_4O_3$) and enzyme production with entrapped fungal species was not affected by the addition of benomyl at concentrations 5, 10, 15 and 20 µg a.i / ml. Enzyme production was improved by the alginate encapsulation as compared with conidia suspension. Alginate entrapped conidia of Trichoderma koningii was successfully used in 3-repition for CMC-ase production.

Key words:-*Benomyl - Carboxymethyl cellulase - Immobilization - conidia*

INTRODUCTION

Trichoderma has been covered to produce Cellulases[1]. Cellulases produced by *T. Harzianum*, is the most efficient enzyme system for the complete hydrolysis of cellulosic substrate into its monomeric glucose, which is fermentable sugars [2]. The purpose of such enzymes to convert wastes for the production of sugars, syrups, alcohol and single cell protein for food and feed has been investigated [3]. Therefore, the necessity to achieve large-scale, costeffective production of active preparations of *Trichoderma* has been increased[4, 5].

Immobilization of microbial cells and enzymes has become one of the most important tools in the field of biotechnology[5]. Moreover, microbial entrapment not only prolonged the survival and the metabolic activity of *Trichoderma* in the soil and in culture – medium, but also prevent the organism from inhibitory compounds[6].

Conidia of *T.harzianum* and *T. pseudokoningii* entrapped in alginate with or without cellulose and introduced into the soil, they survived better than conidia added directly to the same soil after three months of incubation [6]. The immobilization of *T. harzianum* in alginate pellets improved chitinase and ß-1,3 Glucanase production at low benomyl levels [4].

MATERIALS AND METHODS

Fungal isolates and cultivation

Three localisolates of *Trichoderma (T. ghanense, T. harzianum* and *T. koningii*)used in this study were given from Department of Botany, Faculty of Science, Omar El Mukhtar University, Libya, *Trichoderma* isolates were grown in Petri plates on PDA for 1 week at 28ºC.

Screening of *Trichoderma* isolates for Carboxymethyl cellulase (CMC-ase) production:

Qualitative determination of cellulase enzymes:

Using qualitative methods, established on the visualization of zones of CMC-hydrolysis. Conidia of *Trichoderma* were produced by transferring agar disks (7mm in diameter) from PDA cultures in petri dishes containing minimal synthetic medium (MSM) with the following composition (g/l): MgSO4.7H2O 0.2; K2HPO4 0.9; KCL 0.2; NH4NO3 1.0; FeSO4.7H2O 0.002; MnSO4 0.002 and ZnSO4 0.002. The PH was 5 and for the cellulolytic test (1% CMC) and 1.5% agar. After incubation at 28Cº for 3days. Petri dishes were flooded with aqueous solution of Congored (2%) and shaking for 15 min. The Congo-red was then poured off, plates were further flooded with NaCl as to destine for another 15 min. The radial growth (colony diameter) of the fungus colony was measured daily from the reverse side] 7[.

Quantitative assays of cellulase activity

Quantitative assays were obtained by measuring the accumulation of soluble sugars using the phenol sulfuric acid [8] as follows: Conidia of *Trichoderma* were produced by transferring agar discs (7mm in diameter) from PDA culture to flasks each containing 20 ml of minimal synthetic medium (MSM) supplemented with 1% CMC and incubated at 30Co for 7 days. 0.5 ml of the diluted glucose standard was added to each tube along with 0.5 ml at 5% phenol solution. Likewise 0.5 ml of the samples supernanet was added to each respective tubes with 0.5 ml of 5% phenol solution. All tubes were vortexes to thoroughly mix the contain 2.5 ml of Conc. H2SO4 was added to each tube. The tubes were sealed and were incubated for 30 min at room temperature before reading the absorbance at 485 nm.

The dry weight method

The tested fungal species were grown in MSM liquid medium in 100 ml volume flasks containing 20 ml of the liquid media. After 4 day incubation period, the fungal mycelia were filtrated dried at 80Co for constant weight. The fungal growth was reported as mg/flask.

Production of incula and microencapsulation

The method used for the production of inocula and microencapsulation was described earlier[6].

Effect of benomyl on enzyme activities

The effect of benomyl on the activity of free or immobilized Carboxy methyl cellulase - CMC preparations were investigated. Crude enzyme was entrapped in calcium alginate using the same method described earlier. Immobilized enzyme was incubated in the presence of different concentrations of benomyl (5, 10, 15, 20 µg a.i / ml)

The activities of CMC - ase were determined by the viscometric method according to] 9[. The reaction mixture consisted 8 ml of 0.6% CMC buffered of pH 4.5 with 0.05M citrate plus 2 ml each of enzyme preparation (fungal filtrate) and incubation at 30 for 1hour. Activity was estimated as a percentage in reduction of viscosity during specific period of incubation as the following equation:

$$\%REA=\frac{To-Tt}{To-Tw}\times 100$$

Where:

REA = Relative enzyme activity.

To = Flow time immediately after the addition of enzyme filtrate.

Tt = Flow time after incubation Tw = Flow time of water.

Repeated batch fermentation

The reusability of the immobilized fungal isolates was tested in batch cultures by replacing the culture broth with a fresh sterile one every 7 days. Cultivation conditions were as previously for each set] 4[.

RESILTS

Fig. (1) Was shown the results of screening of three *Trichoderma* isolates for Carboxymethyl cellulose (CMC-ase) production using qualitative methods (visualization of zones of CMChydrolysis) and quantitative methods (the accumulation of soluble sugars and dry weight). Results indicate that maximum diameters of hydrolysis were obtained by Trichoderma koningii recording 4.7 cm. Quantitative assays indicated that maximum concentrations of soluble sugars were 11.4 mg/ml for *Trichoderma koningii*. Moreover, *Trichoderma koningii* gave the highest values of mycelail dry weight and recorded 39.1 mg/flask. So, *T. koningii* has been selected for further investigations in this study.

The effect of different benomyl concentrations (0, 5, 10, 15, 20 µg a.i / ml) on CMC-ase activity (REA %) of *Trichoderma koningii* as free or immobilized cells was studied. Results presented in Figure 2 was shown that *T. koningii* produced CMC-ase at all the tested levels of benomyl with the highest enzyme production at 20 µg a.i / ml. Enzyme production of CMC-ase was improved significantly by alginate encapsulation. The reusability of the immobilized fungi for enzyme production was studied. Results in Figure 3 indicated that the entrapped fungi were successfully used in 3 repetitions for CMC-ase production. Moreover, enzyme activities were at the maximum at the 3rd reuse.

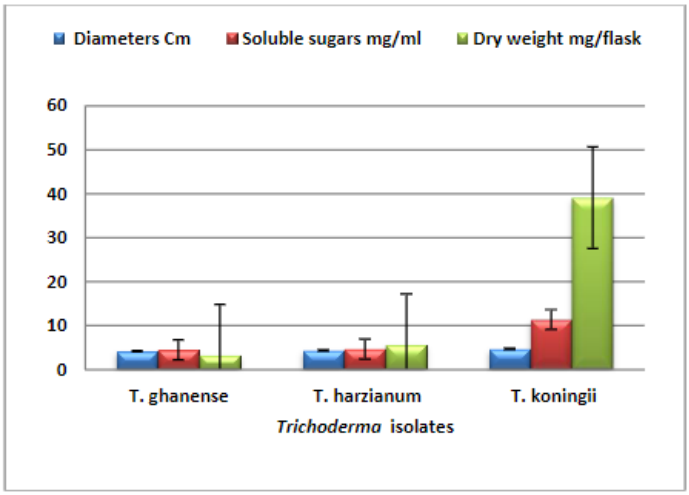

Fig. (1) : Screening of Trichoderma isolates for Carboxymethyl cellulase (CMC-ase) production by using of method are Diameters of hydrolysis zone, Soluble sugars and Dry weight).

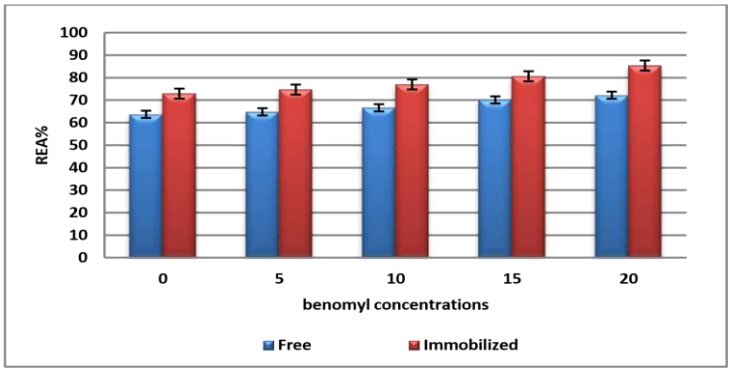

Fig. (2): The Effect of benomyl concentrations (µg a.i / ml) on CMC-ase activity (REA %) by Trichoderma koningii as free or immobilized cells.

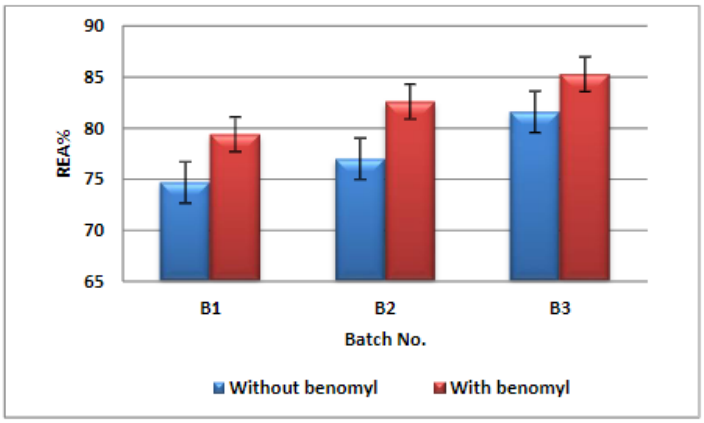

Fig. (3): Repeated batch fermentation of immobilized Trichoderma koningii for CMC-ase activity (REA %). (Each batch was 72 h incubation period (10 µg a.i / ml)

DISSCION

The direct mycoparasitic activity of *Trichoderma* species has been proposed as one of the major mechanisms for their antagonistic activity against phytopathologenic fungi[10]. Chitnase and β-1, 3-glucanase produced by some *Trichoderma* species is the key enzymes in the lyses of cell walls during their mycoparasitic action against phytopathogenic fungi[11, 12].

Entrapment of microbial cells has been reported to improve the production of proteolytic enzymes[13,14]. Results showed that immobilized *Trichoderma* improved CMC-ase production compared with free spore suspension especially when CMC was used as an adjuvant.

The enhancement of enzyme production by immobilization could be justified on the basis that fungal immobilization in calcium-alginate may have gained resistance to benomyl either by detoxification or lack of intake. Moreover, it provides physical protection, and beads may have the advantage of promoting a slow release of spores into the medium[15].

The results of this study also indicate that Alginate encapsulation of *Trichoderma* prolonged the durability of the inoculum and increased in some cases the enzyme production during 3 repetitions of reuse. It was found that the beads became weak and breakable (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 liberation of more conidia supporting higher growth and enzyme production.

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