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GLUCOSE PRODUCTION USING IMMOBILIZED MYCELIAL-ASSOCIATED β-GLUCOSIDASE OF TRICHODERMA E58

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SUMMARY

The immobilization of the mycelial-associated β-glucosidase of Trichoderma E-58 has been carried out by encapsulating, in calcium alginate beads, the fungal mycelium obtained during liquid culture. The activity of this immobilized β-glucosidase was found to vary with culture age and to be more thermally stable than the extracellular β-glucosidase produced by this organism. The activity of the immobilized enzyme was successfully demonstrated in both static and shake-flask batch reaction mixtures at 50°C using both cellobiose and salicin as substrates.

INTRODUCTION

The enzymatic hydrolysis of cellulose is a multi-step process which includes the conversion of the intermediate cellobiose to glucose by β-glucosidase. The presence of glucose results in an inhibition of β-glucosidase activity, whereas the presence of cellobiose inhibits both endoglucanase and cellobiohydrolase activities. One way to circumvent these inhibitory mechanisms is to use constitutive cellulase producers (Montenecourt and Eveleigh, 1979). Another possible approach, which does not involve the use of mutants, is to introduce some means of removing the cellobiose and/or glucose as soon as they are produced in the enzyme reactor. Such methods of simultaneous removal permit organisms with high cellulase-producing capacity to be used in a continuous reactor with little inhibition of the enzyme production or activity. Such systems include simultaneous saccharification and fermentation (SSF) processes (Hagerdal, 1980; Emert, 1980) or immobilized β-glucosidase as reported by Sundstrom et al (1981). The latter was used with Trichoderma reesei cellulase to hydrolyze cellulosic materials such as Solka Floc, corn stover and exploded wood. This latter group found that the use of supplemental immobilized β-glucosidase significantly increased the yields of glucose.

We wish to report the immobilization of whole fungal mycelium by encapsulation in calcium alginate beads in order to effectively immobilize the β-glucosidase activity. These immobilized whole cells were tested at 50°C in both static and shake-flask cultures and were found to be effective in hydrolyzing low molecular weight substrates (salicin, cellobiose).

MATERIALS AND METHODS

Organism and Immobilization Technique: The fungus selected for this study, Trichoderma sp. E-58, is maintained in Forintek's culture collection of microorganisms. The fungal pellets used for the immobilization procedure were obtained from a fresh culture of Trichoderma E-58 grown in 300 ml flasks in Vogel's medium using 2% Solka Floc as a carbon source (Saddler et al., 1982). The washed, filtered fungal pellets obtained from a 100 ml culture were resuspended in 300 ml of 0.05 M citrate buffer (pH 4.8). To this was added 300 ml of 4% (W/V) solution of sodium alginate (BDH Chemicals). The resulting thick sodium alginate/mycelium suspension was pumped drop by drop into a stirred 1% (W/V) calcium chloride solution. The peristaltic pump tubing size was chosen so as to obtain beads which were approximately 6 mm in diameter. The formed beads were then filtered, washed, suspended in a 0.05 M citrate buffer (pH 4.8) and stored at 4°C. This procedure resulted in beads having a displacement of 0.111 ml or 9 beads per ml (average of about 500 beads). No noticeable decrease in the activity of these beads could be detected after 60 days storage.

Enzyme Activity: The immobilized β-glucosidase activity was determined by adding a counted number of the mycelium-containing calcium alginate beads into 1 ml of citrate buffer (0.05 M, pH 4.8). To this mixture was then added 1 ml of 1% salicin. Quadruplicate samples were then incubated, without shaking, for 30 minutes at 50°C in a constant temperature water bath. The reaction was quenched by the addition of 3 ml of the dinitrosalicylic acid (DNS) reagent (Miller, 1959). The measured reducing sugars are reported as μmoles glucose produced per hour.

Time Course of Hydrolysis: The time course of the hydrolysis of salicin and cellobiose was carried out in shaken 50 ml erlenmeyer flasks containing 30 ml of citrate buffer (0.05 M, pH 4.8), 50, 100 or 150 mg of salicin or cellobiose and 135 of the mycelium-containing calcium alginate beads. Duplicate samples were removed intermittently and analyzed for liberated glucose. The glucose produced as a result of the hydrolysis of salicin was measured using DNS while that from the cellobiose hydrolysis was measured using the glucostat enzyme assay (Raabo and Terkildsen, 1960). The data have been presented as total glucose (mmoles) found in the reaction mixture as a function of time.

Extracellular Enzyme Activity: The extracellular enzyme activity of the original culture supernate was determined using the methods suggested by Mandels et al (1976) using carboxymethylcellulose (CMC) and salicin.

Temperature Stability: The thermal stability of the encapsulated mycelial-associated β-glucosidase activity was compared to that of β-glucosidase activity found in the crude culture filtrate. Samples of both enzyme systems, in a sodium acetate buffer (0.02 M, pH 4.8) containing calcium chloride (2.5 $g.\ell^{-1}$), were incubated at 50°C in a constant temperature water bath. Quadruplicate samples were removed and analyzed for β-glucosidase activity over a period of 11 days. The average activity of each fraction is reported relative to quadruplicate samples of identical fractions which were kept at 4°C and analyzed at the same time.

Protein: The protein content of the mycelium was determined using the Biuret method (Herbert et al., 1971) with bovine serum albumin (BSA) as the standard.

RESULTS

Enzyme Concentration: A five-day liquid culture of Trichoderma E-58 was filtered to separate the mycelium from the filtrate containing soluble enzymes. The filtrate was assayed for both carboxymethylcellulase (CMCase) activity and β-glucosidase (salicinase) activity. The former was found to be 6 I.U. ($\mu mol\ glucose.ml^{-1}.min^{-1}$) and the latter was 0.3 I.U. The washed solids had a dry weight of 7.8 $mg.ml^{-1}$ of original culture and contained 47% protein by weight. Microscopic examination showed the presence of a substantial amount of unreacted cellulose. Based on the dry weight measurement, the concentration of solids (mycelium plus unreacted cellulose) in the calcium alginate beads was calculated to be approximately 140 μg/bead.

Enzyme Deactivation Resulting from Immobilization: The decrease in activity which resulted from the encapsulation of the five-day liquid culture of mycelium was determined by comparing the β-glucosidase activity of an equivalent volume of the sodium alginate/mycelia suspension with that of the formed calcium alginate beads in static test-tubes at 50°C. Under these conditions the decrease in activity was found to be approximately 33%.

Temperature Stability: The effect of the incubation at 50°C on the relative activities of both the immobilized mycelial-associated and the extracellular β-glucosidase from Trichoderma E-58 is shown in Figure 1. The short half-life (~1 day) of the crude culture β-glucosidase activity is typical of that found by other researchers working with Trichoderma (Bissett and Sternberg, 1978; Barker et al., 1971). The mycelial-associated immobilized β-glucosidase, on the other hand, shows very good activity retention-90% after 11 days at 50°C.

Effect of Culture Age at Time of Immobilization: The β-glucosidase activity data for calcium alginate immobilized mycelium from five, seven and nine-day old liquid cultures of Trichoderma E-58 at various levels of enzyme (bead) concentration are presented in Table 1. The results obtained in static test-tubes suggest that the β-glucosidase activity associated with the mycelium of Trichoderma E-58 varies with the age of the culture. Similar results have been recently reported for the mycelial-associated β-glucosidase from Aspergillus phoenicis (Allen and Sternberg, 1980) and for Trichoderma reesei QM 9414 (Kubicek, 1981).

Time Course of Hydrolysis: The time course of the hydrolyses of various initial concentrations of cellobiose and salicin are presented in Figures 2a and 2b. All hydrolyses proceeded in an initial rapid fashion followed by a decrease in the rate of hydrolysis. The initial kinetics (3 hours), show that the specific activities of the immobilized mycelia ranged from 0.2-0.4 $\mu moles\ glucose.bead^{-1}.h^{-1}$ with increasing salicin concentration and from 0.36 to 0.67 $\mu moles\ glucose.bead^{-1}.h^{-1}$ with increasing cellobiose concentration.

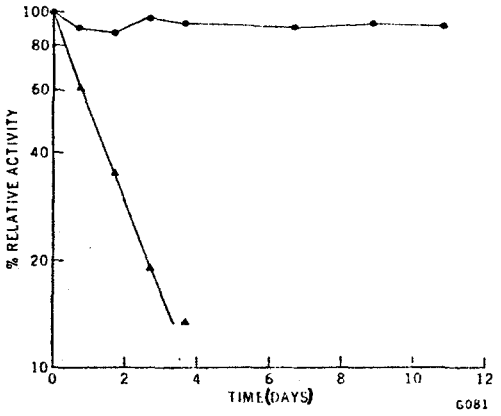

Figure 1: Thermal stability of soluble (▲—▲) and encapsulated mycelium associated (●——●) β-glucosidase activity.

Figure 2a

Figure 2b

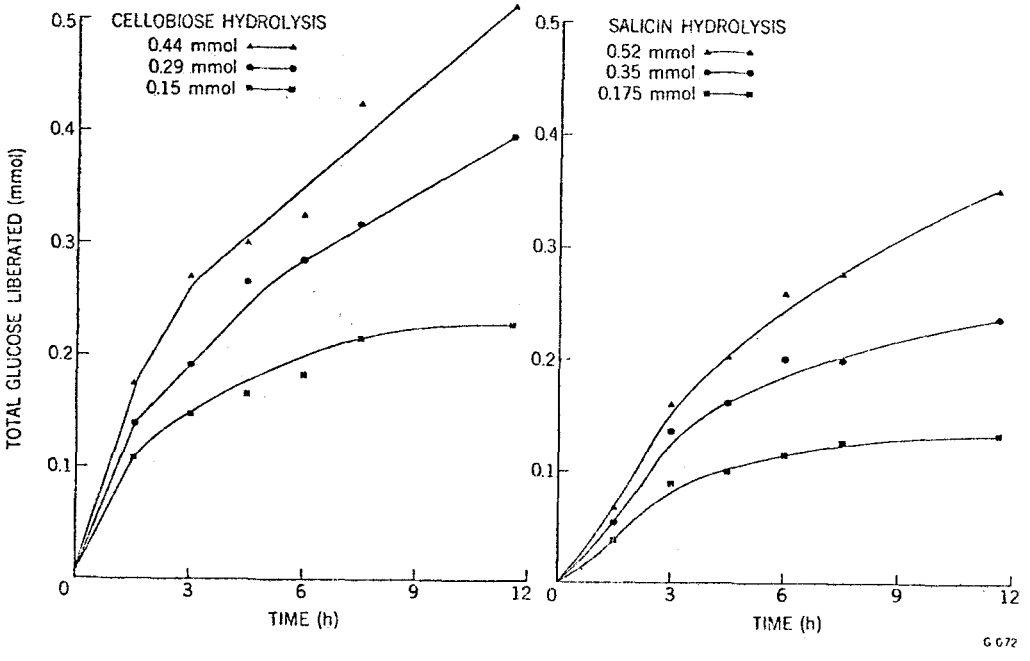

Figure 2: Time course of the hydrolysis of various initial substrate levels of cellobiose (2a) and salicin (2b). The hydrolyses were carried out using 135 calcium alginate beads in 30 ml of citrate buffer (pH 4.8). Initial substrate levels are as indicated.

TABLE 1

Effect of Liquid Culture Age on Immobilized Trichoderma E58 Mycelial-Associated β-Glucosidase Activity

Age of Culture at time of Immobilization	Salicinase Activity of Specified Number of Calcium Alginate Beads (μmol glucose.h^{-1})			
	4 beads	8 beads	12 beads	16 beads
5	3.4	5.1	5.9	8.0
7	3.1	4.2	5.4	5.2
9	1.8	2.5	3.8	5.4

DISCUSSION

The use of immobilized reuseable enzymes for cellulose degradation will become important because a substantial part of the cost of producing ethanol from cellulose is taken up by enzyme production and subsequent hydrolysis (Allan, 1976). For example, the Gulf Oil/University of Arkansas Process (Emert, 1980) for the conversion of solid municipal wastes to ethanol requires that a significant fraction of the cellulase enzymes be discarded. This suggests the advantage of a stable reuseable cellulase enzyme system. Because the endo- and exo-cellulase complexes must be in a soluble form in order to react with the insoluble cellulose complex, the majority of work in this area has focused on the immobilization of the less stable soluble extracellular β-glucosidase on a variety of supports (Sundstrum et al., 1981; Hagerdal, 1980; Vernardos et al., 1980; Bissett and Sternberg, 1979; Barker et al., 1971). Each of these have reported varying degrees of success with the immobilized purified β-glucosidase from a variety of sources.

The use of immobilized whole cells as heterogeneous catalysts offer several advantages over the immobilization of purified enzymes (Larsson et al., 1979). The data presented here suggest that, by encapsulating the mycelium of Trichoderma E-58, it is possible to isolate and obtain stable β-glucosidase. The data obtained suggest that the time of immobilization of the mycelia is critical to obtaining high immobilized activity. This is consistent with the results recently reported by others (Kubicek, 1981) which suggest that extracellular β-glucosidase is a result of lysis of the cell wall of Trichoderma.

Although the data presented here indicate that specific bead β-glucosidase activity is low (approximately 0.2-0.4 μmoles substrate. $bead.^{-1}.h^{-1}$) it should be pointed out that a substantial dilution of the original culture has occurred as a result of the formation of the calcium alginate beads. Use of the immobilized mycelium in a continuous packed-bed reactor should more fully demonstrate their value. These experiments and others designed to fully characterize the immobilized mycelial-associated β-glucosidase are in progress. It is thus hoped to determine the advantages and drawbacks of using such a system when incorporating it into a complete cellulose to glucose hydrolysis process.

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