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Glucose oxidase and catalase activities of *Penicillium variabile* P16 immobilized in polyurethane sponge

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Conidia of *Penicillium variabile* P16 were immobilized in polyurethane sponge and used in repeated-batch processes in a fluidized-bed reactor. Optimal conditions for production of glucose oxidase and catalase were: inoculum size, 10%; glucose concentration, 80 g L^{-1}; Ca-carbonate concentration, 15 g L^{-1}; temperature, 28°C and aeration rate, 4 V V^{-1} min^{-1}. In an extended repeated-batch process, glucose oxidase activity was highest after the fourth batch and catalase activity was highest after the fifth batch. Scanning electron microscopy showed that the fungus grew only in the interior of carrier particles.

Keywords: glucose oxidase; catalase; *Penicillium variabile*; immobilization; polyurethane sponge

Introduction

Glucose oxidase (EC 1.1.3.4), and catalase (EC 1.11.1.6) have considerable commercial applications, the former in food technology, as an oxygen and/or sugar scavanger and as an analytical tool [1,10,16], and the latter in food processing to remove hydrogen peroxide formed as a consequence of the glucose oxidase action or added as an antimicrobial agent, and in the textile industry [10,17].

Commercially, the two enzymes are produced mainly from selected strains of *Aspergillus niger* as by-products in the industrial fermentation of gluconic acid [1,10]. However, both are intracellular enzymes; thus, their recovery requires cell disruption which makes semicontinuous or repeated-batch processes difficult. The two enzymes can also be produced extracellularly by selected strains of *Penicillium* spp [1,8]; in this case, continuous or repeated-batch processes are possible, including with immobilized cells. Immobilized cell systems are of great potential interest since they offer several advantages over suspended-cell cultures, such as increased cell productivity, faster reaction rates, reduced cell wash-out and easier product separation from biomass [2,18].

In a previous paper [15], the production of glucose oxidase in shaken cultures by *Penicillium variabile* P16 immobilized in/on different carriers, such as Ca-alginate, agar, Perlite, activated carbon and polyurethane sponge was studied. Polyurethane sponge was the most promising carrier with respect to both mechanical strength and chemical stability.

This paper deals with the production of glucose oxidase and catalase by this fungal strain immobilized in polyurethane sponge and used in repeated batch processes in a fluidized-bed reactor. The fermentation process was optimized after studying the effects of some physico-chemical and biological parameters such as glucose and $CaCO_3$ concentrations, temperature, aeration rate and inocolum size. The morphological development of the immobilized fungus was followed by scanning electron microscopy.

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Materials and methods

Organism

Penicillium variabile P16, which was selected in a previous screening [13] for its ability to release high levels of glucose oxidase and catalase activities in culture broths, is stocked in the culture collection of the Dipartimento di Agrobiologia e Agrochimica, University of Tuscia, Viterbo, Italy. During the study, the culture was maintained on Malt Extract Agar (Oxoid, Unipath Ltd, Basingstoke, UK) at 4–6°C and subcultured every month.

Culture medium

The basal culture medium contained (g L^{-1} of distilled water): $NaNO_3$ 5.0, KH_2PO_4 1.0, KCl 0.5, $FeSO_4 \cdot 7H_2O$ 0.01 and Mycological Peptone (Oxoid) 3.0. After sterilization (121°C for 20 min) and unless indicated otherwise, $CaCO_3$ (sterilized separately at 121°C for 20 min) and filter-sterilized glucose (Millipore, Bedford, USA, Membrane HA, 0.45 μm pore size, 47 mm diameter) were added to a final concentration of 30 and 80 g L^{-1}, respectively.

Immobilization procedure

Spores from 6-day-old Malt Extract Agar (Oxoid) slants were suspended in sterile 0.05 M phosphate buffer (pH 6.8) containing 0.1% Tween 80. Immobilization in polyurethane sponge (approximately 5.0 mm^3 particles; pore size 0.4–0.6 mm; 8–10 particles per cc) was carried out by the procedure of Fiedurek and Ilczuk [6] modified as follows: 100 ml of the spore suspension (about 1×10^7 spores ml^{-1}) were poured into a 1000-ml Erlenmeyer flask, containing, unless indicated otherwise, carrier particles for a total of 25 cc (0.5 g), and incubated at 28°C for 24 h on a rotary shaker at 80 rpm. After this time the non-adsorbed spores were removed with the liquid.

Culture conditions

The inoculated carrier, washed twice with sterile distilled water, was transferred into a sterilized air-bubble column bioreactor [4]; the culture medium was then added to fill the bioreactor to the working volume (250 ml). Repeated batch fermentations were carried out under the following conditions: temperature 28°C, aeration rate 4 V V^{-1} min^{-1} (the minimum air flow to give a fluidized-bed configuration), duration of cycle 48 h for the first cycle and 24 h for subsequent ones. After each batch, the immobilized mycelium was washed with sterile distilled water to remove any free spores and loose mycelial fragments, and fresh medium was added. Broth samples were taken every 6–12 h and used for the various tests.

The effect of culture conditions on production of glucose oxidase and catalase were tested. They included polyurethane sponge concentration (5, 10, 15 and 20%, v/v), glucose concentration (60, 80 and 100 g L^{-1}), $CaCO_3$ concentration (0, 7.5, 15 and 30 g L^{-1}), temperature (25, 28, 31 and 34°C) and aeration rate (4, 6 and 8 V $V^{-1}min^{-1}$). When calcium carbonate was not present in the medium, or present at a concentration of only 7.5 g L^{-1}, the medium pH was kept constant at 6.0 by automatically controlled addition of 4 M KOH.

Analytical methods

Glucose oxidase and catalase activities were determined spectrophotometrically as described previously [13]. One unit (U) of activity was defined as the amount of enzyme that reduced 1.0 μmol of substrate (benzoquinone and hydrogen peroxide, respectively) per ml per min.

Gluconic acid production was measured spectrophotometrically (340 nm) by the D-Gluconic acid/D-glucono-δ-lactone test-combination kit (Boehringer, Mannheim, Germany). Residual glucose was measured by the DNS method [9].

Scanning electron microscopy

The polyurethane sponge pieces were rinsed free of the growth medium with deionized water and, where needed, halved with a sharp lancet. After dehydration in an ethanol series (50, 70, 80, 90% and absolute), the beads were critical-point dried and coated with gold. A JEOL JSM 5200 (Tokyo, Japan) scanning electron microscope was used to examine the preparations.

Results and discussion

Effect of different culture conditions

The production of glucose oxidase and catalase by *Penicillium variabile* P16 immobilized in polyurethane sponge and cultivated under various culture conditions was studied in repeated-batch processes (five cycles each) in a fluidized-bed reactor. Results are reported in Table 1.

With a carrier concentration of 10% (standard condition), the glucose oxidase mean volumetric productivity (183.2 U L^{-1} h^{-1}) was significantly higher than those obtained with particle concentrations of 5, 15 or 20%. However, while the low productivity at 5% inoculum was likely due to the limited surface of the biocatalyst [7,11], those obtained at 15 and 20% polyurethane particles could be explained by insufficient medium agitation caused by the high number of carrier particles [7]. Catalase productivity reached its maximum (1352 U L^{-1} h^{-1}) at a polyurethane particle concentration of 20% (Table 1).

Glucose oxidase activity and productivity were maximum at a glucose concentration of 80 g L^{-1} (standard condition); the concentration of 60 g L^{-1} appeared to be limiting: glucose oxidase, in fact, was only 3.46 U ml^{-1} and the productivity only 65.5 U L^{-1} h^{-1}. At a sugar concentration of 100 g L^{-1}, glucose oxidase activity and productivity were both low, confirming previous observations with free cells of *P. variabile* P16 in shaken culture [12]. Catalase productivity (1327 U L^{-1} h^{-1}) and activity (53.4 U ml^{-1}) were highest at 80 and 100 g L^{-1} of glucose, respectively (Table 1).

Glucose oxidase activity and productivity of the immobilized cells were influenced greatly by the concentration of calcium carbonate: both enzyme activity (7.04 U ml^{-1}) and mean volumetric productivity (183.2 U L^{-1} h^{-1}), were maximum at 30 g L^{-1} of calcium carbonate; the enzyme volumetric productivity, however, was not significantly different from that obtained at 15 g L^{-1} (168.7 U L^{-1} h^{-1}) (Table 1). For this reason and to minimize foaming caused by the presence of calcium carbonate [3], the concentration of 15 g L^{-1} was taken as optimal. Catalase was also influenced by the concentration of calcium carbonate: both enzyme activity and productivity were maximum when 30 g L^{-1} of salt were added to the medium.

The minimum air flow necessary to create satisfactory particle turbulence was 4 V V^{-1} min^{-1}; at this air flow, the activities and productivities of both enzymes were maximum (Table 1). Increasing aeration rates resulted in a decrease of maximum activity and mean volumetric productivity of both enzymes. Similar results were reported by Petruccioli *et al* [14] with free cells of *P. variabile* P16 cultured in a bench-top fermenter and by Fiederuk *et al* [5] with immobilized cells of *A. niger* cultivated in shaken culture. In both cases, however, agitation rather than direct aeration was investigated.

Of the four different temperatures (25, 28, 31 and 34°C) studied, 28°C was optimal: at this temperature, both glucose oxidase activity (7.04 U ml^{-1}) and mean volumetric productivity (183.2 U L^{-1} h^{-1}) were significantly higher than those obtained at 25 and 31°C (Table 1). The results at 34°C are not shown; at this temperature only traces of glucose oxidase and catalase activities were detected throughout the whole repeated-batch process.

Extended repeated-batch process

On the basis of the above results, an extended repeated-batch process (eight batches over 216 h) was conducted. Figure 1 shows the time course of glucose oxidase and catalase activities (a) and of glucose consumption and gluconate production (b) by *P. variabile* P16 immobilized in polyurethane sponge particles and cultured in a fluidized-bed reactor under the standard conditions; the concentration of $CaCO_3$, however, was 15 instead of 30 g L^{-1}. After the first batch (activation batch), activities of glucose oxidase and catalase increased and reached their maximum (7.17 and 44.86 U ml^{-1}, respectively) at the end of the fourth and fifth batch, respectively.

Table 1 Effect of culture conditions on glucose oxidase and catalase production by *Penicillium variabile* P16 immobilized in polyurethane sponge[a]

	Maximum enzyme activity (U ml^{-1})		Mean volumetric productivity (U L^{-1} h^{-1})[b]	
	Glucose oxidase	Catalase	Glucose oxidase	Catalase
Control[c]	7.04 ± 0.22	47.4 ± 4.9	183.2 ± 15.0	1327 ± 82
Inoculum size (%,v/v)				
5.0	3.08 ± 0.55	57.4 ± 7.7	82.8 ± 10.2	1280 ± 180
15.0	5.54 ± 0.22	51.3 ± 2.3	96.1 ± 13.9	1245 ± 87
20.0	2.82 ± 0.17	46.8 ± 4.2	60.6 ± 6.7	1352 ± 93
Glucose concentration (g L^{-1})				
60.0	3.46 ± 0.65	40.1 ± 5.8	65.5 ± 11.2	829 ± 167
100.0	4.48 ± 0.68	53.4 ± 4.9	110.2 ± 14.9	918 ± 38
$CaCO_3$ concentration (g L^{-1})				
0.0	1.17 ± 0.24	16.3 ± 3.3	31.0 ± 6.7	471 ± 6
7.5	3.34 ± 0.48	28.3 ± 3.8	81.2 ± 8.8	674 ± 24
15.0	6.65 ± 0.77	39.1 ± 7.2	168.7 ± 9.7	861 ± 23
Aeration rate (V V^{-1} min^{-1})				
6.0	6.92 ± 0.74	45.3 ± 6.1	168.1 ± 10.1	978 ± 50
8.0	3.30 ± 0.46	30.3 ± 1.9	56.5 ± 5.1	970 ± 42
Temperature (°C)				
25	1.80 ± 0.84	49.6 ± 6.5	29.9 ± 6.9	859 ± 48
31	1.84 ± 0.07	34.1 ± 2.9	42.2 ± 5.9	923 ± 102

[a]Values represent means of at least two experiments ± standard deviation.
[b]Mean volumetric productivity of the whole process (five cycles, 144 h).
[c]Control: standard conditions (inoculum size, 10%; glucose, 80 g L^{-1}; $CaCO_3$, 30 g L^{-1}; aeration rate, 4 V V^{-1} min^{-1}; temperature, 28°C).

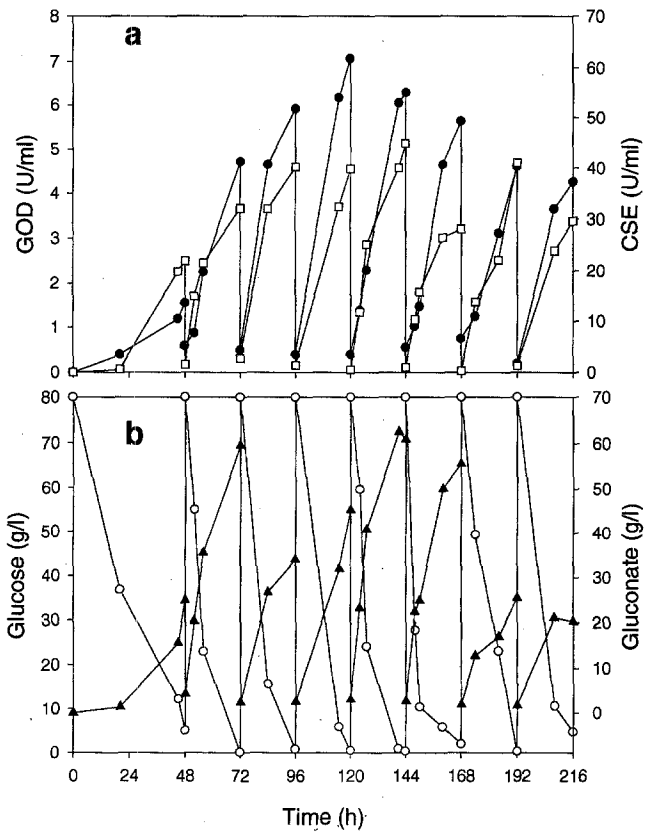

Figure 1 Time course of glucose oxidase (a: GOD, ●) and catalase (a: CSE, □) activities of *Penicillium variabile* P16 immobilized in polyurethane sponge and cultivated in repeated-batch process. Glucose consumption (b: ○); gluconate accumulation (b: ▲).

The whole process mean volumetric productivities were higher than those obtained using the same bioreactor but under the initial standard conditions and with a duration of only five cycles, and those obtained in shaken culture with both free and immobilized cells [15] (Table 2).

With the exceptions of the first and the last batches, glucose disappeared from the medium rapidly and completely, mainly due to its conversion to gluconic acid, the production of which reached its maximum in the course of the fifth batch (Figure 1b).

Morphological development of the immobilized fungus

The carrier colonization and the morphological development of the immobilized *P. variabile* P16 (inoculum size, 10%) was followed by scanning electron microscopy (Figure 2). After inoculation (Figure 2a), the immobilized fungus began to grow immediately; however, its growth was limited to the particle interior (Figure 2b). The hyphal growth was slow; at the end of the eighth batch the fungus had not yet completely colonized the polyurethane particle (Figure 2c).

The fungus appeared to cling to the carrier fibers twisting around them strongly, allowing great biomass retention (Figure 2d). Similar observations were made by Vassilev *et al* [19] with *A. niger* immobilized in polyurethane foam and cultivated in shake culture, repeated batch processes, for the production of gluconic acid, and also by Petruccioli *et al* [12] with *Rhizopus arrhizus* immobilized on the same carrier and cultivated in a fluidized-bed reactor, repeated batch processes, for fumaric acid production.

The results reported in this and a previous paper [15] indicate the possibility of producing extracellular glucose oxidase and catalase using immobilized mycelium of *P.*

Table 2 Glucose oxidase and catalase mean volumetric productivities of free and immobilized cells of *Pencillium variabile* P16 cultivated in shaken culture and in a fluidized-bed reactor in repeated batch processes[a]

	Number of batches	Mean volumetric productivity ($U\ L^{-1}\ h^{-1}$)[b]		Reference
		Glucose oxidase	Catalase	
Free cells, shaken culture	3	102.2 ± 4.7	n.d.[c]	[9]
Immobilized cells, shaken culture	7	76.3 ± 2.2	866 ± 37	[9]
Immobilized cells, bioreactor, standard conditions	5	183.2 ± 15.0	1327 ± 82	This work
Immobilized cells, bioreactor, optimized conditions	8	204.6 ± 8.0	1389 ± 51	This work

[a]Values represent means of at least two experiments ± standard deviation.
[b]Mean volumetric productivity of the whole process.
[c]n.d. = not detected.

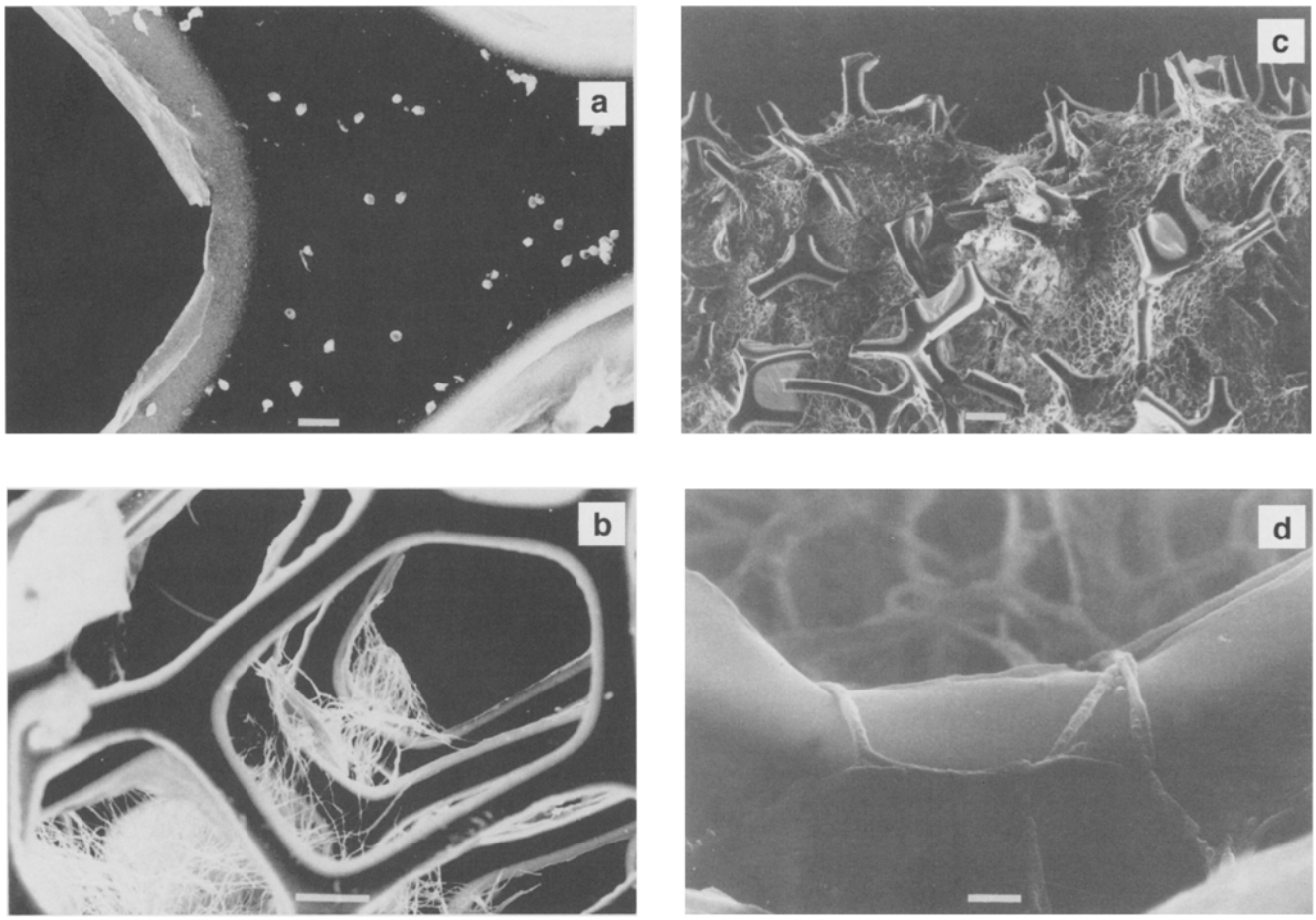

Figure 2 Morphology of *Penicillium variabile* P16 immobilized in polyurethane sponge and cultivated in repeated-batch process (inoculum size, 10%). (a) Detail of a particle after spore immobilization (scale bar, 10 μm). (b) Detail of a particle at the first batch (scale bar, 100 μm). (c) Detail of a particle surface at the end of the eighth batch (scale bar, 100 μm). (d) Detail of a particle structure at the first batch (scale bar, 10 μm).

variabile P16. Due to the stability of the biocatalyst enzyme activity, extended repeated-batch production processes appear to be possible. Further scale-up studies, however, are necessary to evaluate the techno-economic feasibility of the proposed process.

Acknowledgements

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