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EFFECTS OF IMMOBILIZATION IN Ba-ALGINATE ON NITRILE-DEPENDENT OXYGEN UPTAKE RATES OF *CANDIDA GUILLIERMONDII*

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ABSTRACT

Yeast cells immobilized by entrapment in Ba-alginate gel were investigated for growth pattern and respiratory activity. The oxygen uptake rates (OUR) of cells entrapped in gels with 4% alginate were 5.2 and 23% lower than the OUR of 2% alginate and free cells, respectively. The mass-transfer resistance offered by the matrix and growth of the entrapped cells determine a gradient of nutrients throughout the gel which is responsible for both a lower specific growth rate of immobilized cells with respect to that of free ones, and a heterogeneous biomass distribution, with progressively increasing cellular density from the inside to the outside of the matrix. Gel-matrix polymer concentration affected the maximum oxygen uptake of immobilized growing yeast cells.

Key words: Nitriles, *Candida guilliermondii*, Ba-alginate, respiration

INTRODUCTION

Industrial use of nitrile may result in discharge of synthetic nitriles into marine, freshwater and soil environments. Increasing accumulation of such compounds in these ecosystems may cause deleterious effects since most of them are highly toxic, mutagenic and carcinogenic (7).

In the practical utilization of living cells entrapped in alginate gel, diffusion of essential nutrients, oxygen transfer, physical and chemical properties of the gel and immobilization procedure are the important factors affecting microbial metabolism and the efficiency of the system. Although immobilized cells have received a lot of attention (2), it is not possible to make a general statement about the behavior of microorganisms in alginate. Literature results are not uniform, but vary according to the type of microorganism, of immobilizing matrix and of productive system.

In the present study *Candida guilliermondii* UFMG-Y65 cells immobilized by encapsulated in Ba-alginate, were investigated for pattern of growth and the attainment of a spatially organized micro-environment which leads to the formation of a biofilm that controls cell respiratory activity. Freely suspended cells growing under the same culture conditions were also studied in parallel. We also investigated factors affecting the maximum oxygen uptake of immobilized growing yeast cells such as gel-matrix polymer concentration and length of the period of incubation in $BaCl_2$ solution.

MATERIALS AND METHODS

Organism

The yeast was isolated from the gold extraction circuit liquid and identified as *Candida guilliermondii* UFMG-Y65 (4). The strain was maintained on GYMP agar slant medium (2% glucose, 0.5% yeast extract, 1% malt extract, 0.2% NaH_2PO_4, and 2% agar) under a mineral oil layer and stored at 4ºC or in liquid nitrogen.

Cell mass production

C. guilliermondii UFMG-Y65 was inoculated into 250 ml Erlermeyer flasks containing 50 ml of Yeast Carbon Base (YCB, Difco, Detroit, Mi, USA) with 6% acetonitrile as the sole nitrogen source, and incubated under shaking at 120 rpm for 120 hours at 25ºC.

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Yeast cells encapsulated by Ba-alginate

2 or 4% Alginate (w/v) was dissolved in boiling water and autoclaved at 121ºC for 15 min. Six ml of yeast cell suspension (10^9/ml) was added to 18 ml sterilized alginate solution and mixed by stirring with a magnetic stirrer. This mixture was extruded drop by drop through hypodermic needles into a cold 0.1 M $BaCl_2$ solution. Gel beads approximately 2 mm in diameter were obtained. The beads were solidified by resuspending into a fresh $BaCl_2$ solution for 24 h with gentle shaking at 5ºC. Finally the beads were washed with distilled water and used for experimentation. All experimental steps were performed under aseptic conditions (3).

Culture conditions

250 ml Erlenmeyer flasks containing 40 ml of minimum medium (1% glucose, 0.1% K_2HPO_4; 0.02% $MgSO_4$ $7H_2O$; 0.01% NaCl) plus containing 1000 mM of: acetonitrile as the sole nitrogen source were inoculated with 10 ml of gel capsules containing *C. guilliermondii* UFMG-Y65 cells. The flasks were incubated with shaking for 120 hours at 25ºC. A parallel experiment with free cells in solution was also carried out.

Determination of cell concentration

The samples of free cells were centrifuged for 10 minutes at 5000 rpm, and washed twice in deionized water. The cells were then resuspended in 1 ml deionized water and dried for 48 hours at 80ºC. The dry weight of gel capsules and cells, was determined as described by Dias *et al*. (3). One mL of capsules was washed with 50 mL of deionized water and dried by the same procedure as described for free cells.

Measurement of oxygen uptake rates (OUR)

The rates of cell maximum oxygen uptake were measured at 30ºC using an Yellow Springs Instrument Co. oxygraph, model 53. The samples with free and immobilized cells were added to 100 mM nitrile or amide solutions, in 3 ml final volume, previously saturated with oxygen. Oxygen consumption was measured immediately after cell immobilization and during the stage of gel bead formation.

Electron microscopy

Gel beads containing yeast cells were observed by electron microscopy. The capsules containing yeast cells were fixed using a 2.5% glutaraldeide solution (SIGMA) in 0.1 M phosphate buffer pH 7.3, for 5 hours at 5ºC. After fixation, the samples were washed twice in ice-cold phosphate (0.1 M, pH 7.3) for 10 minutes. The material was dehydrated in an ethanol gradient (50, 60, 70, 80, 90, 95 and 100%), being left for 10 minutes in each solution. The dehydration was repeated twice in 100% alcohol. The samples were dried adding 0.5 ml hexamethyldisilazane to the material for 30 seconds and the process was repeated twice. The samples were sputter coated and observed under a Zeiss scanning electron microscope (model DSM - 906) at 20 kV.

All experiments were repeated three times and the mean values obtained are reported. Data were subjected to analysis of variance. When the main effects were significant ($P < 0.005$) differences between means were evaluated by the Duncan multiple range test.

RESULTS AND DISCUSSION

Fig. 1 clearly shows the yeast growth in the peripheral regions of the gel, with the formation of small colonies after 72 hours of incubation with shaking. This behavior was observed by several authors (10,12,13), and is due to better nutrient availability, mainly oxygen, in this area. Thus, in the central parts of the beads, the development of colonies was not as robust as found near the surface of the capsule. The reduction of the diffusion coefficient of different substances towards the interior of the gel capsules was studied by some investigators (5,9,11), who observed that the phenomenon is enhanced by the polymer concentration limiting the substrate and product diffusion.

C. guilliermondii UFMG-Y65 grown in acetonitrile showed high oxygen consumption compared to cells grown in the presence of other nitriles (Table 1). According to Nawaz *et al*.

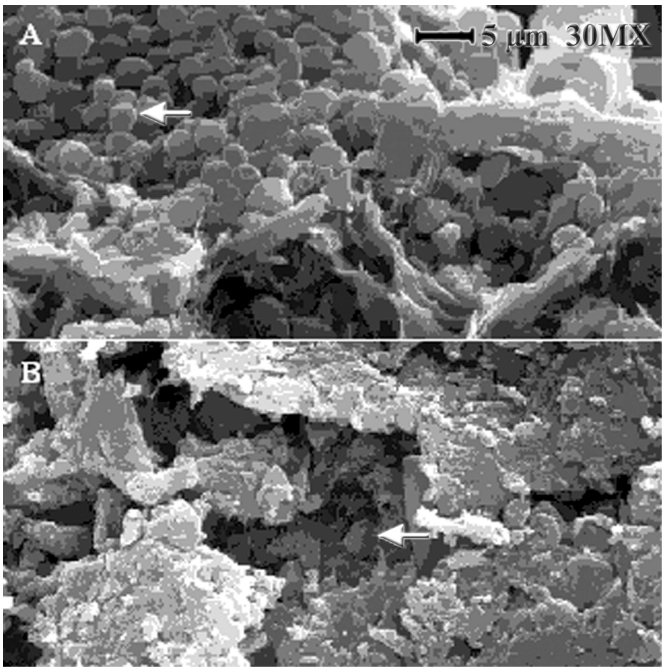

Figure 1. Scanning electron micrograph of immobilized *C. guilliermondii* UFMG-Y65 cells growing in 1000 mM acetonitrile for 72 h. Transverse cut of a gel capsule containing 4% alginate, cross linked in a 0.1 M $BaCl_2$ solution for 24 hours. Detail of the presence of colonies unequally distributed among the outlying area (A) and the center of the capsule (B) after 72 hours of grow.

Table 1. Maximum specific oxidation rates (OUR) of compounds (nitriles and amide) by free and immobilized of *C. guilliermondii* UFMG-Y65 cells in phosphate buffer, pH 7, at 30ºC.

Substrate	OUR_{max} (nmol O_2. mg^{-1} dry mass. min^{-1})	
Nitriles	**Free**	**Immobilized**
Acetonitrile	$12.65^{a} \pm 0.35$	$11.03^{a} \pm 0.57$
Acrylonitrile	$2.57^{d} \pm 0.14$	$1.45^{e} \pm 0.41$
Adiponitrile	$3.42^{d} \pm 0.26$	$2.87^{d} \pm 0.33$
Benzonitrile	N.C	$0.76^{f} \pm 0.93$
Butyronitrile	$5.39^{c} \pm 0.18$	$4.56^{c} \pm 0.24$
2-cyanopyridine	$3.55^{d} \pm 0.39$	$2.32^{d} \pm 0.35$
3-cyanopyridine	$5.32^{c} \pm 0.23$	$4.85^{c} \pm 0.32$
4-cyanopyridine	$3.41^{d} \pm 0.37$	$2.98^{d} \pm 0.55$
Cyclopentanecarbonitrile	N.C	$0.65^{f} \pm 0.47$
Glutaronitrile	$3.54^{d} \pm 0.08$	$2.75^{d} \pm 0.11$
Isobutyronitrile	$5.67^{c} \pm 0.53$	$4.45^{c} \pm 0.56$
Methacrylonitrile	$6.89^{c} \pm 0.39$	$5.39^{c} \pm 0.49$
Propionitrile	$8.73^{b} \pm 0.21$	$7.21^{b} \pm 0.43$
Succinonitrile	$7.48^{b} \pm 0.34$	$6.34^{b} \pm 0.21$
Amides		
Acetamide	$7.32^{a} \pm 0.28$	$6.49^{a} \pm 0.38$
Adipamide	$3.40^{cd} \pm 0.09$	$2.78^{c} \pm 0.53$
Acrylamide	$3.69^{cd} \pm 0.54$	$2.04^{c} \pm 0.45$
Benzamide	N.C	$0.71^{d} \pm 0.33$
Isobutyramide	$5.92^{b} \pm 0.43$	$4.23^{b} \pm 0.47$
Glutaramide	$3.25^{d} \pm 0.35$	$2.79^{c} \pm 0.26$
Succinamide	$4.36^{c} \pm 0.38$	$3.58^{b} \pm 0.19$

Immobilization in matrices containing 2% alginate, cross linked in a 0.1M $BaCl_2$ solution for 24 hours. Means followed by the same letter are not significantly different ($P < 0.005$) as determined by Duncan's multiple range test; N.C. Not consumed.

(8), oxygen consumption may be considered an approximate measure of enzymatic activity. Oxygen consumption by free cells was not observed in the presence of benzonitrile, benzamide or cyclopentanocarbonitrile, a fact possibly related to the high toxicity of these substances to *C. guilliermondii* UFMG-Y65. Bettmant and Rehm (1) and Goldstein *et al*. (6) have suggested that immobilization confers greater protection against the toxicants present in the environment. This is probably due to the change in the cell membrane, with a high protein/lipid concentration in the membranes of the immobilized cells. The effect of the immobilization on the maximum specific oxygen uptake rate of *C. guilliermondii* UFMG-Y65 was evaluated in terms of the more important variables in the process of cell immobilization in Ba-alginate gels: $BaCl_2$, polymer concentration and time needed for bead formation.

The oxygen consumption rates of free and immobilized cells were found to be linear during the first 25 minutes (Fig. 2). The assays with immobilized cells presented yielded slightly higher

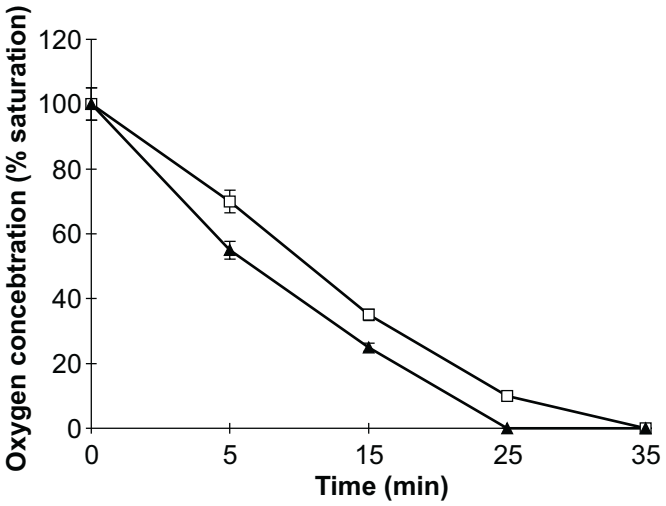

Figure 2. Oxygen consumption of *C. guilliermondii* UFMG-Y65 free (σ) and immobilized (| |) cells in phosphate buffer, pH 7.0, at 30ºC, in the presence of 100 mM acetonitrile. Immobilization in 2% alginate, cross linked in a 0.1 M $BaCl_2$ solution for 24 hours.

compared with free cells, which also shows linear oxygen consumption rates indicating a limitation in the oxygen supply in addition to lower initial rates of microbial oxygen uptake. Scott *et al*. (11) obtained similar results with *Streptomyces clavuligerus* cells immobilized in K-carragenan gels. The authors suggested that the rate of external oxygen diffusion from the medium to the capsule, together with the intracapsular diffusion rate, cannot be so high, in order to supply the cells with a sufficient oxygen concentration to allow a maximum rate of cellular oxygen uptake.

Alginate concentrations were varied to evaluate the effect of the different gel bead formation processes on cell oxygen uptake (Table 2). In spite of the restricted number of points, it was observed that maximum oxygen uptake rate of *C. guilliermondii* UFMG-Y65 cells was reduced as the alginate content increased. The OUR of cells entrapped in gels with 4% alginate were 5.2 and 23% lower than the OUR of 2% alginate and free cells, respectively (Table 2). These reductions were probably due to problems of oxygen diffusion and to deleterious effect of the immobilization process. In general, it has been found that oxygen supply in alginate decreases with increased cell loading, bead diameter, and polymer concentration. The differences in oxygen permeability between beds formed with solution containing concentrations of 0.1 and 1 M of the cross linked agent (barium) and 10 min and 24 hours times of gel bead formation were not statistically significant. A 42% reduction of oxygen consumption rate was observed for free cells in aqueous solution of 1.0 M $BaCl_2$ compared to cells suspended in distilled water under the same conditions (data

not shown). Similar results were reported by Scott *et al*. (11). This reduction can also be due to a decrease in the level of available oxygen for cells in solution containing barium chloride because oxygen solubility in 1.0 M KCl solutions is 74% of that obtained in distilled water (11). The present data show that free and immobilized yeast cells present different rates of consumption with respect to nitriles and amides. Gel-matrix polymer concentration affected the maximum oxygen uptake of growing immobilized yeast cells.

Table 2. Specific oxygen uptake rates of. free and immobilized of *C. guilliermondii* UFMG-Y65 cells, in phosphate buffer, pH 7.0 at 30ºC, in the presence of 100 mM acetonitrile, under different conditions.

Test	Alginate Concentration (%)	Conditions of gel bead formation	OUR_{max} (nmol O_2. mg^{-1}dry mass. min^1)
1	2	C_{BaCl} = 1.0 M tg = 10 min	$10.28^{cb} \pm 0.25$
2	2	C_{BaCl} = 0.1 M tg = 24 hours	$11.03^{b} \pm 0.43$
3	4	C_{BaCl} = 1.0 M tg = 10 min	$9.74^{c} \pm 0.33$
4	Free cells	–	$12.65^{a} \pm 0.35$

Means followed by the same letter are not significantly different ($P < 0.005$) as determined by Duncan's multiple range test; C_{BaCl} - Concentration of $BaCl_2$ solution; tg = - Time of gel bead formation.

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RESUMO

Efeitos da imobilização em Ba-alginato na taxa de remoção de oxigênio nitrila-dependente pelas células de *Candida guilliermondii*

Foram estudados os efeitos da imobilização no crescimento e na atividade respiratória das células de *Candida guilliermondii* UFMG-Y65 imobilizadas por encapsulamento, utilizando-se gel de Ba-alginato. As taxas máximas de utilização de oxigênio apresentadas pelas células imobilizadas em matriz contendo 4% de alginato foram 5,2 e 23% inferiores às taxas apresentadas pelas células imobilizadas em 2% de alginato e livres, respectivamente A resistência à transferência de massa oferecida pela matriz e o crescimento das células foram responsáveis pela baixa taxa de crescimento. Como conseqüência, observou-se uma distribuição heterogênea da biomassa, com aumento da densidade celular progressivamente do interior para regiões periféricas da matriz. A concentração do polímero afetou a taxa de utilização máxima de oxigênio pelas células da levedura imobilizada.

Palavras-chaves: Nitrilas, *Candida guilliermondii*, Ba-alginato, respiração

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