Brazilian Journal of Microbiology (2000) 31:61-66
ISSN 1517-8382

BIODEGRADATION OF ACETONITRILE BY CELLS OF *CANDIDA GUILLIERMONDII* UFMG-Y65 IMMOBILIZED IN ALGINATE, κ-CARRAGEENAN AND CITRIC PECTIN

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Submitted: October 08, 1999; Returned to authors for corrections: December 06, 1999; Approved: January 11, 2000

ABSTRACT

Different encapsulation matrices were tested for immobilized cells of *Candida guilliermondii* UFMG-Y65 used for acetonitrile degradation. Acetonitrile degradation by free cells and cells immobilized in Ba-alginate, κ-carrageenan and citric pectin was studied. The rate of acetonitrile degradation was monitored for 120 h by measuring yeast growth and ammonia concentration. Different alginate concentrations did not affect cell viability, but the period of incubation in $BaCl_2$ solution reduced the number of viable cells. Likewise, the gel nature and the matrix structure of the support resulting from the cell immobilization conditions were of fundamental importance for biocatalyst activity and performance, affecting substantially the patterns of microbial growth and enzymatic activity. Alginate-immobilized cells degraded acetonitrile more efficiently than κ-carrageenan or citric pectin-immobilized cells.

Key words: *Candida guilliermondii*, acetonitrile, biodegradation, immobilization

INTRODUCTION

The biological degradation of nitriles proceeds through two enzymatic routes. Nitrilase (E.C. 3.5.5.1) catalyses the direct cleavage of nitriles to yield the corresponding acids plus ammonia, whereas nitrile hydratase (E.C. 4.2.1.1.84) catalyses the hydration of nitriles to amides, which are subsequently hydrolyzed to acids and ammonia by amidase (E.C.3.5.1.4). The hydrolysis of nitrile by microbial nitrile hydratase has been exploited for the commercial production of acrylamide (12). Recently attention has been directed towards regio-and/or stereo-selective nitrile hydrolysis by, for example, *Brevibacterium imperiale* B222 (4), *Pseudomonas* spp. (15).

The potential of using immobilized cells in industrial processes is regarded as a valuable application (9, 5). Cells at different stages (viable, resting, dead etc.) have been successfully encapsulated in various matrices (17, 6, 21). Bioremediation using cells has been widely investigated for numerous toxic chemicals such as phenol (3), pentachlorophenol (20), acetonitrile (8) and acrylamide (18). In the practical utilization of living cells encapsulated in hydrophilic gels, important factors affect microbial metabolism and the efficiency of the system such as diffusion of essential nutrients, oxygen transfer, physical and chemical properties of the gel and immobilization procedure. Immobilized cells in hydrophilic gels have received a lot of attention in environmental applications. Although it is not possible to make a general statement about the behavior of microorganisms in hydrophilic gels. The literature data are not uniform, but vary according to the type of microorganism, immobilizing matrix and productive system (7). In the present study, biodegradation of acetonitrile using immobilized *Candida guilliermondii* UFMG-Y65 in various matrices was investigated and compared with that obtained with freely suspended cells. The influence of time of incubation in $BaCl_2$ solution on cell viability, and of alginate concentration was also investigated.

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MATERIALS AND METHODS

Microorganism. The yeast was isolated from water samples collected from gold extraction circuit (Mineração Morro Velho, Nova Lima, Brazil) according to Linardi *et al.* (16). The strain was characterized by standard methods, and identified as *Candida guilliermondii* by keys reported by Kreger-van Rij (13) and Barnett *et al.* (2) and by molecular methods performed as described by Lachance *et al.* (14). The strain was maintained on GYMP slant medium (- w/v - 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 preparation. In order to obtain microbial suspensions of high cellular density the *C. guilliermondii* UFMG-Y65 strain was inoculated into 250 ml Erlenmeyer flasks containing 50 ml Yeast-Carbon-Base (YCB-Difco) plus 6 % acetonitrile as sole nitrogen source. The flasks were incubated under shaking at 120 rpm, for 120 hours at 25ºC.

Determination of cell concentration. Cell growth was determined by 2 different methods. In the first case, an appropriately 0.1 ml aliquot of the sample was distributed on the surface of Sabouraud agar. After 72 h of incubation at 30ºC, the number of colonies grown was determined and, the results were expressed as colony forming units/ml solution (CFU/ml)**.** In the second method, sample dry weight was determined by two different procedures. 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 by the methods of Wada *et al*. (22), 1 ml of capsules was washed with 50 ml of deionized water and dried by the same procedure as described for free cells.

Effect of barium chloride and potassium chloride on the growth of *C. guilliermondii* UFMG-Y65. To 250 ml Erlenmeyer flasks containing 50 ml of minimum medium (- w/v - 0.1 % K_2HPO_4; 0.02 % $MgSO_4$ 7 H_2O and, 0.01 % NaCl) enriched with 1.0 M acetonitrile, we added either $BaCl_2$ or KCl at concentrations of 5, 25, 50, 100 or 200 mM and inoculated with 1.0 ml of a cell suspension of *C. guilliermondii* UFMG-Y65 (A_{480}=0.155). Cultures were incubated at 25ºC in a rotary shaker at 120 rpm for 72 h. In each experiment, the growth was estimated by measuring CFU/ml, and the ammonia concentration in the supernatant was measured according to Fawcett and Scott (11).

Immobilization of yeast cells by encapsulation on different support materials. Alginate (4.25 %, w/w) (Vetec Fine Chemistry Ltda), 2.13 % κ-carrageenan (Sigma Chemical Co.) and 2.13 % citric pectin, low methoxy (Braspectina - Citrus Colloids S/A), suspensions of polymers of similar viscosity, were dissolved in 18 ml of distilled water. After sterilization at 120ºC for 15 min, the polymer suspensions were added to aliquots of 6 ml containing 10^8 cell/ml at 40ºC for κ-carrageenan gel and at 30ºC for alginate and pectin gels. The suspensions were forced out with the aid of a hypodermic syringe of 1 mm of inner diameter, and dropped into 200 ml of sterile solution of cross-linking salt at concentrations of 0.1 up to 1.0 M. KCl was used for the κ-carrageenan matrix and $BaCl_2$ for citric pectin and alginate. The capsules, measuring approximately 2 mm in diameter, were maintained in the cross-linking solution for 10 min to 24 h at 10ºC. Before use, the capsules were washed with 200 ml distilled water at 10ºC to remove excess salt.

Influence of different polymer types. 250 ml Erlenmeyer flasks containing 40 ml 1.0 M acetonitrile in minimum medium (0.1 % K_2HPO_4; 0.02 % $MgSO_4$ 7 H_20; and 0.01% NaCl) were inoculated with 10 ml of *C. guilliermondii* UFMG-Y65 encapsulated in different matrices (alginate, κ-carrageenan, citric pectin). The assays were maintained under shaking at 120 rpm at 25ºC for 120 h. After this period, the ammonia concentrations and cell growth were determined. The experiments were carried out with free cells solution using the same cell concentration as in the assays involving cells immobilization.

Influence of polymer concentration, gel bead formation time and cross-linking salt solution concentration on the process of polymeric matrix formation and on biocatalyzer activity. With the objective of evaluating the effects of these variables, we tested different gel bead formation conditions on ammonia generation, stability of matrices and growth of *C. guilliermondii*-UFMG-Y65 after 120h of cultivation, as shown in Table 1.

All experiments were repeat three times and the mean values obtained are reported.

Table 1. Influence of polymer concentration, gel bead formation time and cross-linking salt solution concentration in the process of formation of the polymeric matrix

Assay	Alginate concentration (%)	$BaCl_2$ concentration (M)	gel bead formation time
1	2.0	1.0	10 min
2	2.0	1.0	60 min
3	2.0	0.1	14 h
4	2.0	0.1	24 h
5	4.0	1.0	10 min
6	4.0	1.0	60 min
7	4.0	0.1	14 h
8	4.0	0.1	24 h

RESULTS AND DISCUSSION

Barium chloride and potassium chloride are cross-linking salts for the κ-carrageenan and pectin supports, respectively. Bajpai *et al.* (1) suggested improvement of the culture medium with the cross-linking salt used in the manufacture of beads. According to these authors, the addition of the salt guarantees chemical stability and mechanical integrity of the gel. However, the concentrations vary according to the authors. Fig. 1 demonstrates the variations in ammonia production and in the cell growth caused by different $BaCl_2$ and KCl concentrations added to the culture medium. The results indicate that the inhibitory effect of potassium ions was lower than that barium ions on ammonia generation and cell growth. Increasing concentrations of $BaCl_2$ blocked the action of nitrilases. Nilson *et al*.(19) observed that bi-and trivalent cations at low concentrations are extremely toxic for the growth of microorganisms.

The applicability of several polymeric natural or synthetic polymers as matrices for immobilization of viable cells motivated the study of the use of hydrophilic gels such as alginate, pectin and κ-carrageenan. The effects of these types of polymer supports used for immobilization on the acetonitrile biodegradation by free and immobilized cells are demonstrated in Fig. 2 (A and B). The κ-carrageenan gel presented lower biodegradation efficiency than the alginate and pectin gels, a fact that can explained by the differences in the porous structures of the matrices, probably permitting a better growth of the cells in the alginate and pectin matrices and, as a consequence higher ammonia generation (Fig. 2 B). Besides κ-carrageenan was the one that shown the higher cell release rate into the culture medium when compared to the alginate and pectin gels (Fig. 3)

Cheetham *et al.* (10), immobilizing cells of *Saccharomyces uvarum* in calcium alginate gel, observed the influence of polysaccharide concentration and the existence of a relationship between gel bead formation time and cross-linking salt solution

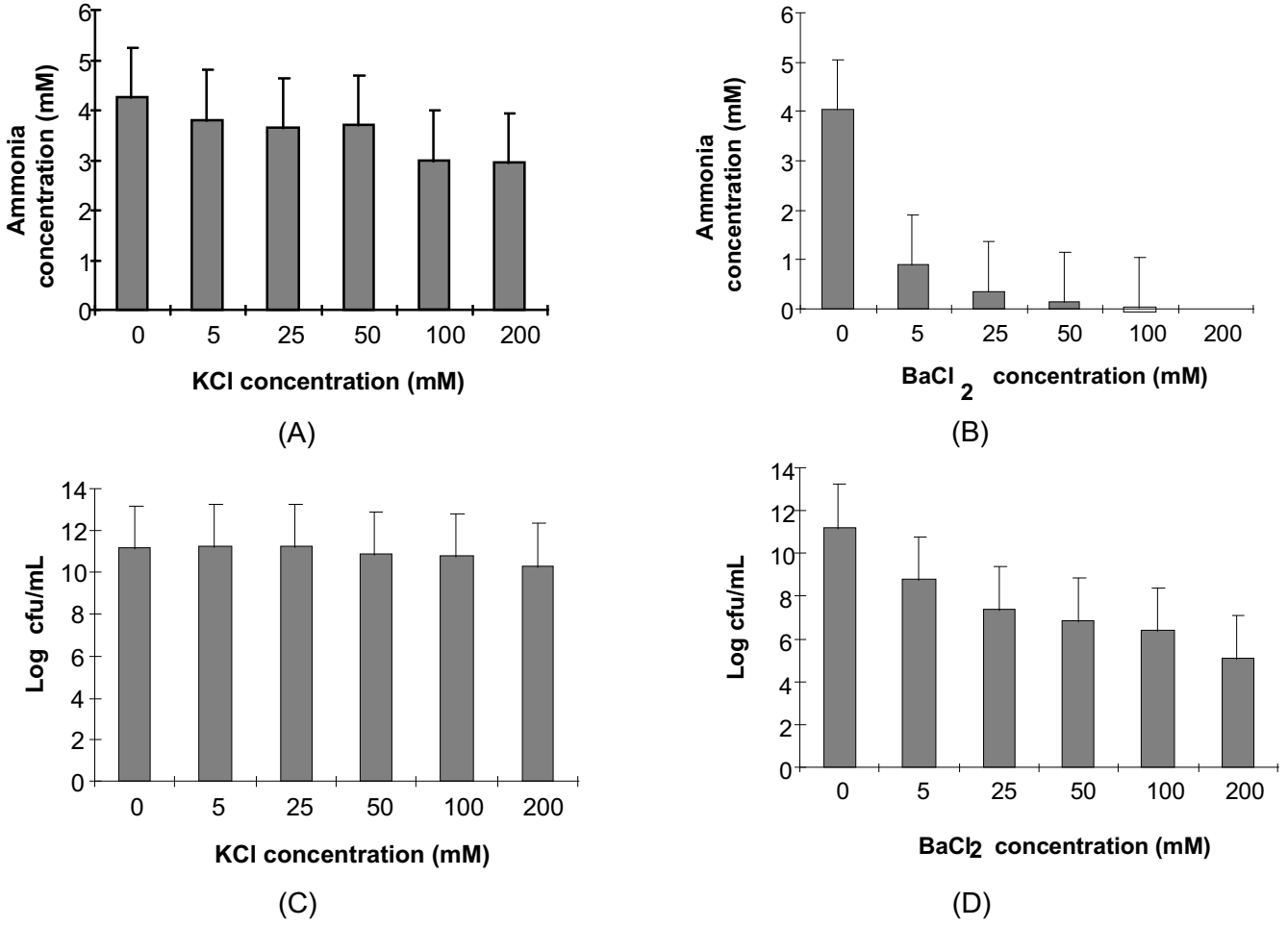

Figure 1. Influence of potassium chloride and barium chloride on ammonia generation (A and B) and growth (C and D) of *C. guilliermondii* UFMG-Y65 in medium containing 1.0 M acetonitrile under shaking at 120 rpm at 30°C, for 120 hours.

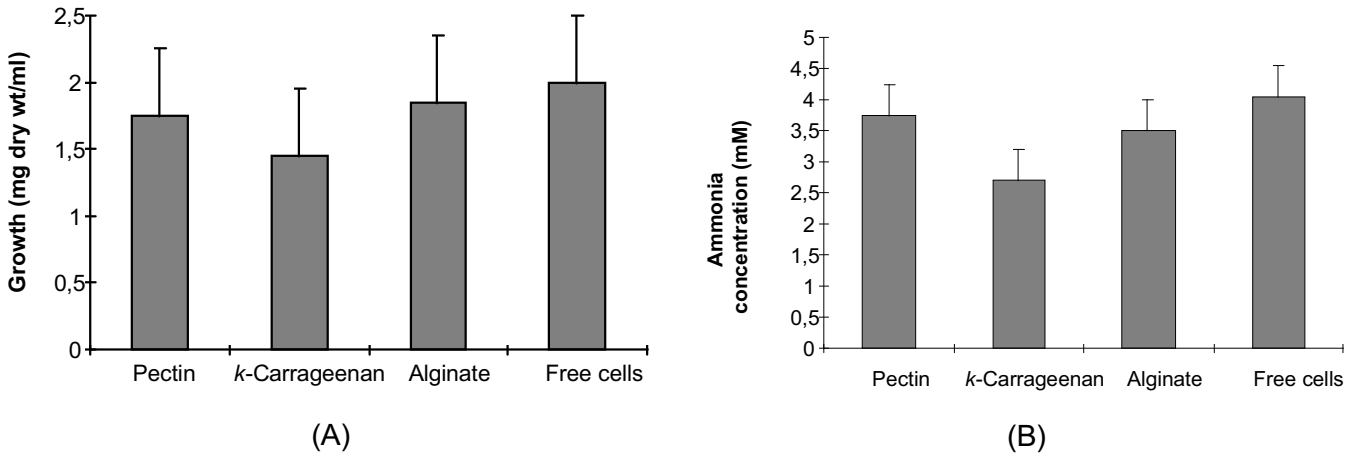

Figure 2. Influence of different types of supports on mass production (A) and ammonia generation (B), by *C. guilliermondii* UFMG-Y65 in 1.0 M acetonitrile, after 120 h, under shaking at 120 rpm at 30ºC.

concentration in the process of formation of the polymeric matrix. Fast gel bead formation processes, of the order of minutes, involve high concentrations of $BaCl_2$. In diluted $BaCl_2$ more time was necessary to complete the reaction. When gel bead formation processes occurs only partially, the central area of the capsule tends to stay more fluid than its surface.

Fig. 4 (A and B) shows the ammonia conversion and the concentration of cells that were released from the gel at the end of the process. For the same salt concentration and same gel bead formation time the profiles of ammonia production did not differ significantly at different polymer concentrations. After 120 hours of cultivation, practically no variation was observed in the ammonia concentration with the same polymer concentration. Cell release into the medium (Fig. 4B), however was more marked for gels with greater mechanical resistance.

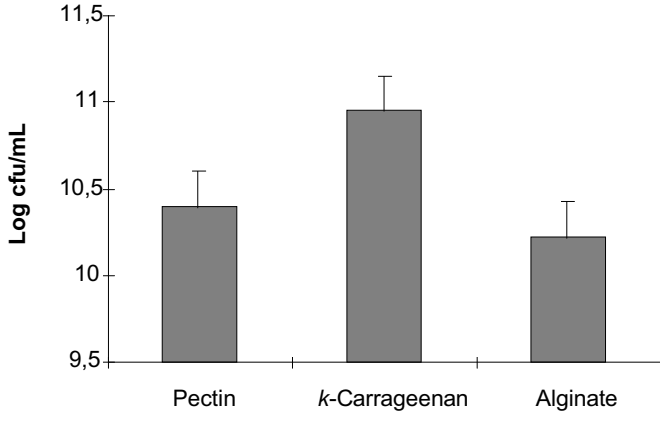

Figure 3. Influence of different types of gels on the cell release of *C. guilliermondii* UFMG-Y65, in flasks containing 1.0 M acetonitrile after 120 h, under shaking of 120 rpm at 30ºC.

In the evaluation of effects of different gel bead formation types on ammonia production (Fig. 4 A), the matrices that were cross-linking quickly presented lower values, possibly due to the structural difference between them and the matrices that cross-linking with different size, distribution and amounts of pores. Although there was a difference in ammonia production between fast and slow, with a fixes polymer concentration, no appreciable variations in ammonia production were observed with different gel bead formation time, for the same concentration of $BaCl_2$. For the slowly cross-linking biocatalyser after 120 hours of incubation, a high level of cell release was observed when compared to that obtained with rapidly cross-linking matrices. This fact could be due to a smaller mechanical resistance of these matrices which reduced the effects of mass transfer. Acetonitrile conversion in ammonia (Fig. 4 A) has little effected by the increase of polymer percentage. Probably, the $BaCl_2$ concentration is the limiting factor in such conversion. Fig. 4 (C). shows that the capacity of retention of cells inside the gel was higher for matrices with 4% polymer. The variation of polymer concentration directly affects the final structure of the matrices, but not cell viability. The salt concentration, however, significantly affects the two parameters. The liberation of cells into the culture medium, on the other hand, was quite marked for gel containing 2% alginate (Fig. 4 B).

The study reveals slight increase of acetonitrile degradation by free cells of *C. guilliermondii* UFMG-Y65. Even with limited performance increase the use of immobilized cells can be considered an advantageous alternative, because it avoids the bioreactor obstruction resulting from the low resistance and small size of biomass. This culture is also capable of degrading other aromatic and aliphatic nitriles (data not shown). Thus there is a potential for the development of microbial technology for the treatment of effluents containing nitriles.

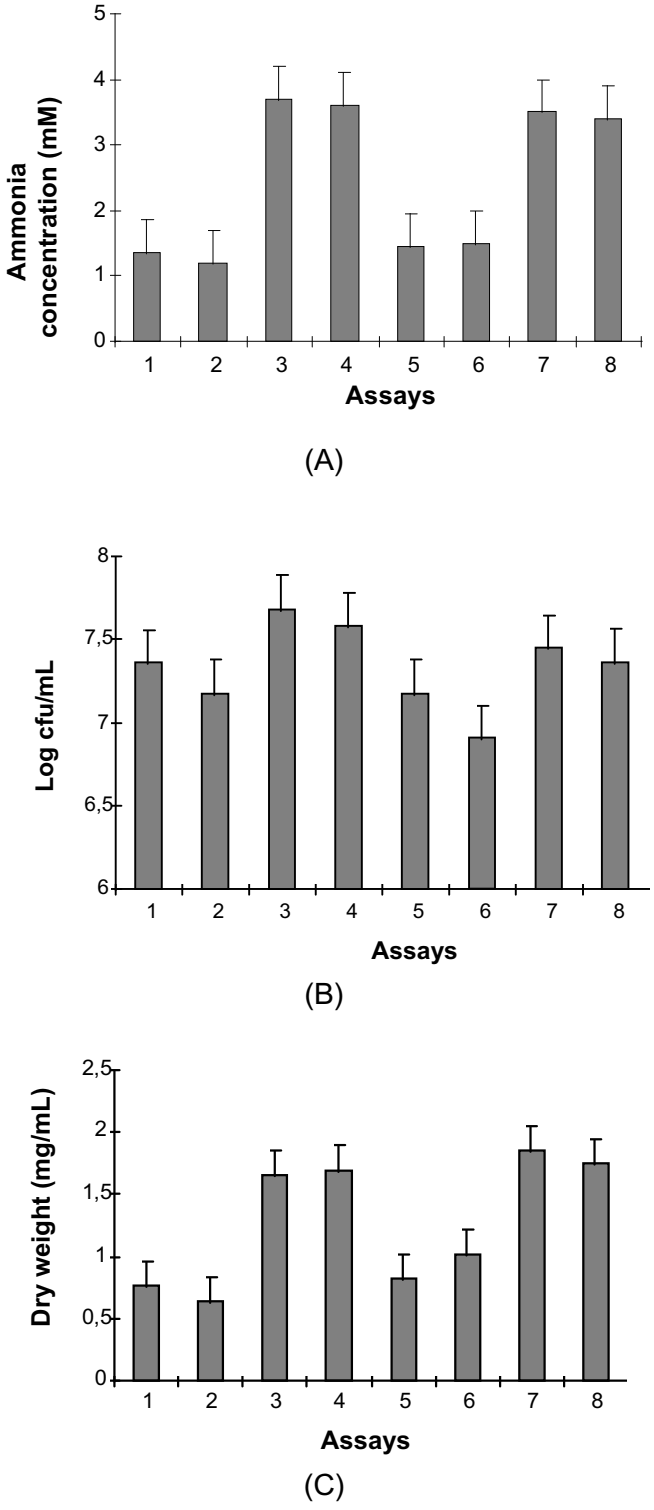

Figure 4. Influence of alginate concentration, gel bead formation time and $BaCl_2$ concentration on ammonia production (A), stability of the matrices (B) and growth of immobilized *C. guilliermondii* UFMG-Y65 (C), after 120 hours of incubation in flasks containing 1.0 M acetonitrile with shaking at 120 rpm at 30°C.

ACKNOWLEDGMENTS

The authors would like to thank FAPEMIG for financial support (Proc.CBS-1001/97), CNPq for fellowships (Proc. 523158/96) and Braspectina - Citrus Colloids S/A for providing citric pectin.

RESUMO

Biodegradação de acetonitrilas por células de *Candida guilliermondii* UFMG-Y65 imobilizadas em alginato, κ-carrageno e pectina cítrica

Na degradação de acetonitrila, foram testadas células livres de *Candida guilliermondii* UFMG-Y65 e imobilizadas em diferentes suportes, quais sejam, Ba-alginato, κ-carrageno e pectina cítrica. A velocidade de degradação da acetonitrila foi monitorada por 120 h, mediante o crescimento da levedura e geração de amônia. Diferentes concentrações de alginato não afetam a viabilidade das células; mas o período de incubação, em solução de $BaCl_2$, reduziu o número de células vivas. Da mesma forma, a natureza do gel e a estrutura da matriz do suporte, ambas resultantes das condições de imobilização das células, foram de fundamental importância para a atividade catalisadora e sua performance; afetando assim, os padrões de crescimento microbiano e a de atividade enzimática. As células imobilizadas em alginato degradaram acetonitrila com maior eficácia do que as imobilizadas em κ-carrageno ou as células imobilizadas em pectina cítrica.

Palavras-chave: *Candida guilliermondii,* Acetonitrila, biodegradação, imobilização

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