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Production of High Hydroxytyrosol Yields via Tyrosol Conversion by *Pseudomonas aeruginosa* Immobilized Resting Cells

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An immobilized whole cell system was successfully performed to produce the most powerful antioxidant, hydroxytyrosol. Bioconversion of tyrosol into hydroxytyrosol was achieved via the immobilization of *Pseudomonas aeruginosa* resting cells in calcium alginate beads. Immobilization was advantageous as it allows immobilized cells to tolerate a greater tyrosol concentration than free cells. The bioconversion yield reached 86% in the presence of 5 g L^{-1} of tyrosol when cells immobilized in alginate beads were carried out in single batches. Evaluation of kinetic parameters showed the maintenance of the same catalytic efficiency expressed as K_{cat}/K_m for both free and immobilized cells. The use of immobilized cells in repeated batches demonstrated a notable activity stabilization since the biocatalyst reusability was extended for at least four batches with a molar yield greater than 85%.

KEYWORDS: Hydroxytyrosol; bioconversion; *Pseudomonas aeruginosa*; alginate beads; repeated batch

INTRODUCTION

Many epidemiological studies have reported the mutual correlation between human health and diet composition (*1*, *2*). Dietary factors could influence the development of different phenotypic and molecular deficiencies (*3*). Nowadays, more attention has been given to polyphenolic compounds. These substances are evident constituents of daily nutritional intake, particularly in the Mediterranean basin, where olive oil is well-represented (*4*). In this respect, hydroxytyrosol, a naturally occurring ortho-diphenol, has been reported to exert different biological properties such as antioxidant (*5*, 6, *7*), antimicrobial (*8*), and anticarcinogenic activities (*9*, *10*).

Owing to its application in foods as well as in cosmetic and pharmacological industries, more interest has been given to the production of hydroxytyrosol even from natural sources or by synthetic procedures. The first synthetic procedure goes back to 1949 (*11*). This method was based on the reduction of 3,4-dihydroxyphenylacetic acid in the presence of $LiBH_4$. Then, hydroxytyrosol was recovered by bench scale purification from olive mill wastewaters (*12*, *13*). Recently, Espin et al. discussed the production of hydroxytyrosol using mushroom tyrosinase as a biocatalyst and ascorbic acid as a reducing agent (*14*). Another enzymatic method was also described, and it is founded on oleuropein hydrolysis by β-glucosidase and destabilization of the aglycone structure under defined temperatures and pH conditions (*15*). Enzymatic strategies have opened the channel for the bioconverting way, using whole cells for hydroxytyrosol production (*16*).

Microorganisms have already been successfully applied for the selective hydroxylation of organic compounds. Such biotransformations could be performed on a preparative scale. The immobilization of microorganisms can strongly enhance the stability of enzymatic activities and simplify biocatalyst recovery (*17*). Whole cell immobilization was defined as ''the physical confinement or localization of intact cells to a certain region of space with preservation of some desired catalytic activity" (*18*). Immobilization often mimics what occurs naturally when cells grow on surfaces or within natural structures (*19*). Artificial immobilization is commonly accomplished using a high hydrophilic gel. The use of immobilized whole microbial cells eliminates the often tedious, time-consuming, and expensive steps involved in the isolation and purification of intracellular enzymes (*20*). The most extensively studied method in cell immobilization is their entrapment in polymeric matrices. The application of immobilized cells for bioconversion reactions has been described for several particular cases (*17*, *21*). Some of these exhibited higher conversion rates and a longer half-life (*22*).

In this study, an immobilized cell system was adopted to perform the bioconversion of tyrosol to its ortho-diphenolic corresponding compound, hydroxytyrosol. This process could be scaled up to industrial applications especially for pharmaceutical and cosmetic products.

MATERIALS AND METHODS

Chemicals. Tyrosol, *p*-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, and alginate (powder) were purchased from Fluka (Switzerland). Ethyl acetate, acetonitrile, and orthophosphoric acid were HPLC-grade solvents and purchased from Prolabo (France). Hydroxy-

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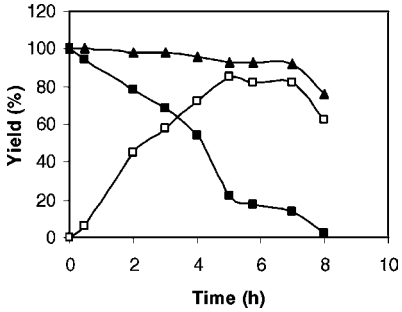

Figure 1. Time course bioconversion of tyrosol by free cells: (▲) aromatic compounds; (□) hydroxytyrosol; and (■) tyrosol.

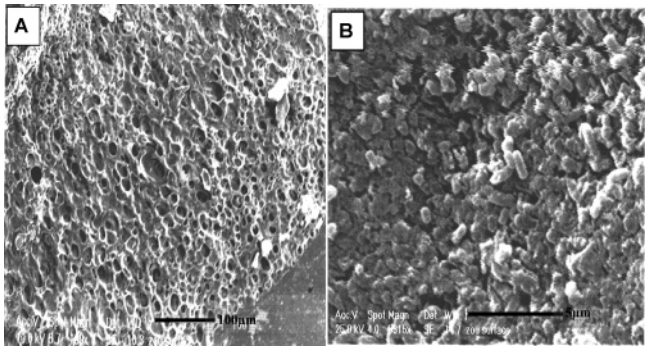

Figure 2. Scanning electron micrographs of immobilized *P. aeruginosa* cells in alginate beads. (**A**) Surface of alginate beads with immobilized bacterial cells below the surface and in crater-type structures (bar indicates 100 μm) and (**B**) magnification of cell agglomeration in the bead surface (bar indicates 5 μm). Magnifications: (**A**) ×189 and (**B**) ×5315.

tyrosol, used as a standard, was purified from olive mill wastewater in our laboratory (*23*).

Biological Material. *Pseudomonas aeruginosa* was isolated, by enrichment on a minimal medium, from soil regularly irrigated for 5 years with olive mill wastewater (100 m^3 ha^{-1}). Cells were grown on tyrosol as the sole source of carbon and energy as previously described (*16*).

Culture Media. In addition to the classic Lauria−Bertani (LB) medium, a minimal medium was also used for bacteria growth. It consists of (g/L): Na_2HPO_4, 2.44; KH_2PO_4, 1.52; $(NH_4)_2SO_4$, 1.5; $MgSO_4 \cdot 7H_2O$, 0.2; and $CaCl_2 \cdot H_2O$, 0.05 and 10 mL of a trace-element solution that contains (% w/v): EDTA, 5; $ZnSO_4 \cdot 7H_2O$, 2.2; $CaCl_2$, 0.55; $MnCl_2 \cdot 5H_2O$, 0.5; $Na_2MoO_4 \cdot 2H_2O$, 0.11; $CuCl_2 \cdot 6H_2O$, 0.16; and $CoCl_2 \cdot 6H_2O$, 0.16. The pH of the medium was adjusted to 7.2. Solid media were prepared by the addition of 1.5% agar.

Bacteria were grown in Lauria−Bertani (LB) medium at 30 °C in an orbital shaker at 180 rpm. Tyrosol was supplemented in the rich medium as an inducer of hydroxylating activity. Mineral media were inoculated with Lauria−Bertani cultures, and tyrosol was added as a sole carbon source.

Entrapment of *P. aeruginosa* Cells in Alginate Beads. *P. aeruginosa* cells used for immobilization were cultured in minimal medium containing 1 g L^{-1} of tyrosol at 30 °C for 7 h (end of the exponential growth phase). Biomass production was conducted in 1 L Erlenmeyer flasks in an orbital shaker at 180 rpm. Cultures were inoculated with 9% (v/v) active culture in LB medium. Cells were harvested aseptically by centrifugation at 6000 rpm for 10 min at 4 °C, resuspended in 2 mL of PBS, and mixed with 3% (w/v) sodium alginate solution in the proportion of 1:2 (v/v) cell suspension and alginate solution, respectively. The mixture was extruded dropping into a 0.1 M sterile cold solution of calcium chloride, through a hypodermic needle using a peristaltic pump to obtain uniform-sized particles. Formed beads were kept to harden overnight at 4 °C. Before being used in the bioconversion medium, beads were washed twice with distilled water to remove excess calcium ions and unentrapped cells.

Cell Enumeration. Free cells were quantified by drying 25 mL of minimal medium culture at 105 °C overnight. The immobilized biomass

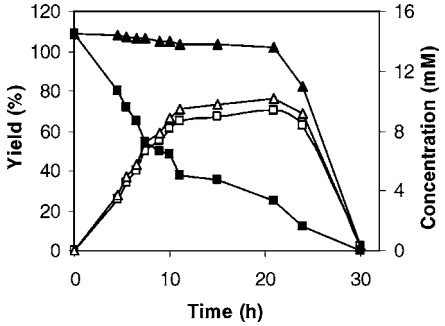

Figure 3. Time course bioconversion of tyrosol by immobilized cells: (Δ) hydroxytyrosol (mM); (■) tyrosol; (▲) aromatic compounds; and (□) hydroxytyrosol yield (%).

was also estimated by drying at 105 °C overnight after dissolution of 10 alginate beads in 1 M sodium pyrophosphate solution and centrifugation of the cell suspension for 10 min at 6000 rpm. Free and immobilized cells were quantified as dry cell weight (DCW). All experiments were repeated 3 times, and the mean values are reported.

Biotransformation in Batch Conditions. In our working conditions, bioconversion reactions were conducted in 250 mL Erlenmeyer flasks with a 25 mL working volume. Cells cultured in minimal medium and collected by centrifugation (6000 rpm, 10 min) during the late log phase were either directly used as free cells or immobilized as previously described and suspended in the bioconversion medium.

Evaluation of Kinetic Parameters. To study the effect of immobilization on kinetic parameters and particularly the catalytic efficiency of bioconversion reaction, free and immobilized *P. aeruginosa* cells were assayed at increasing tyrosol concentrations in the PBS buffer. The reaction was performed at 30 °C, shaking at 200 rpm. Free and immobilized cells were tried at concentrations of 1.2 g_{DCW} L^{-1} and 12.5 g_{DCW} L^{-1}, respectively. Individual experiments were performed using fresh biocatalysts for each substrate concentration. Kinetic parameters were determined using the Lineweaver−Burk plot method.

Repeated Use of Free and Immobilized Cells. A distinguished characteristic of immobilized cells is that they can be used repeatedly in successive batches. Therefore, reusability and stability of both free and calcium alginate immobilized cells were examined. Free cells were assayed at a concentration of 1.2 g L^{-1} of dry weight in the presence of 4 g L^{-1} of tyrosol, whereas immobilized cells were entrapped in calcium alginate beads and resuspended in the reaction medium at 12.5 g L^{-1} of biomass concentration and tested in repeated batches for the bioconversion of 5 g L^{-1} of tyrosol. After each batch, the biocatalysts were washed with PBS and transferred to fresh media.

Quantitative Analyses by HPLC. HPLC was performed on a Shimadzu C-R6A liquid chromatograph. The separation was carried out in a C18 column (250 mm × 4.6 mm; Waters Chromatography). Compounds were eluted with a gradient, acetonitrile (70%)−H_3PO_4 (0.1%), where the acetonitrile concentration was varied as follows: 0 min, 10%; 0−20 min, increases to 50%; 20−25 min, 50%; and 25−30 min, decreases to 10%. The column temperature was maintained at 40 °C, and the flow rate was 0.5 mL min^{-1}. Sample detection was achieved at 280 nm with a Shimadzu SPD 6AUV detector connected to a Shimadzu C-R6A integrator. The injection volume was 20 μL. Compounds were identified and quantified by comparison of retention times and peak areas with those of authentic samples.

Samples were withdrawn periodically from the biotransformation medium and filtered through a 0.22 μm filter. The filtrate was analyzed directly by HPLC for substrate and intermediary metabolite quantification. The bioconversion yields of produced hydroxytyrosol were calculated as follows: hydroxytyrosol molar yield = hydroxytyrosol produced concentration (mol/L)/initial tyrosol concentration (mol/L) × 100.

Liquid Chromatography-Tandem Mass Spectrometry. Liquid chromatography consisted of an Agilent Series 1100 system (Agilent, Waldbronn, Germany) equipped with an autosampler, degasser, a quaternary pump, and a reversed phase C^{18} analytical column of 150 mm × 4.6 mm and 5 μm particle size eluted with the same gradient as

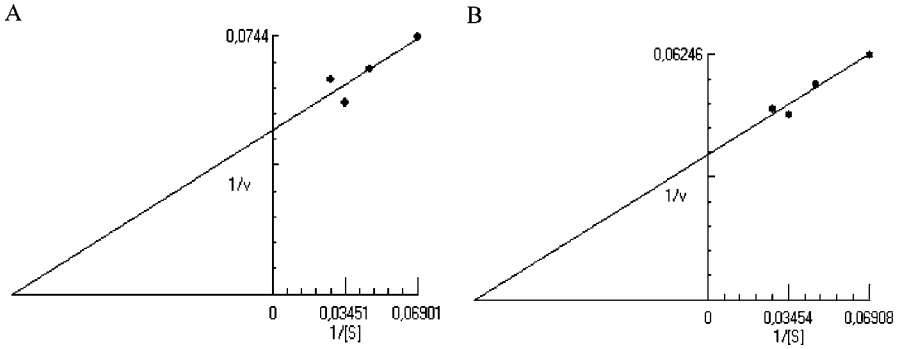

Figure 4. Lineweaver–Burk kinetics representations for free (**A**) and immobilized (**B**) cells, plotted using Hyper32 program. Substrate concentration is expressed as millimolar.

Table 1. Comparison of Kinetic Parameters Obtained for Free and Immobilized Cells

	free cells	*immobilized cells*
K_m (mM)	8.37	10.04
K_{cat} (min^{-1})	8.00×10^{-9}	11.00×10^{-9}
K_{cat}/K_m (min^{-1} mM^{-1})	10^{-9}	1.09×10^{-9}

described in the previous section. This HPLC system was connected to an Agilent series LC/MSD trap XCT instrument (Agilent Technologies, Waldbronn, Germany) where ionization was performed in positive mode.

Scanning Electron Microscopy. The alginate gel beads were prepared as described in the immobilization section. The scanning electron microscopy method was adapted to that described by Dias et al. (*24*). Beads were separated from the immobilization medium by filtration and then resuspended in 6.25% buffered glutaraldehyde 0.1 M Tris−HCl buffer, pH 7.0 with 0.1 M $CaCl_2$. After 24 h at 4 °C, beads were washed with buffer and attached to coverslips and stepwise dehydrated in an increased ethanol gradient (50, 60, 70, 80, 90, 95, and 100%) with an exposition time of 10 min in each concentration. The dehydration was repeated twice in 100% alcohol. Finally, beads were dried using the CO_2 critical-point drying technique and coated with gold for examination by scanning electron microscopy in a Philips XL-30 microscope attached to an EDX unit.

Statistical Analysis. Analysis of variance was carried out with Data Analysis Tools of Microsoft Excel. Significance of repeated batch results was tested with the *t*-test.

RESULTS

Tyrosol Biotransformation by Free Resting Cells. Tyrosol hydroxylation was done by cells previously grown on tyrosol to the late log phase in minimal medium. Cells were collected by centrifugation and suspended in PBS buffer. the optimal biomass concentration was assessed to be 1.2 g_{DCW} L^{-1}. For the tyrosol concentration, the production of hydroxytyrosol was proportional to increased substrate concentration up to 4 g L^{-1}. Under these optimal conditions, the maximal hydroxytyrosol yield was about 86% after 7 h (**Figure 1**). Hydroxytyrosol accumulation was coupled to tyrosol disappearance. Bacterial growth was initiated when tyrosol was totally removed from the medium.

Production of Hydroxytyrosol by Immobilized Cells. Calcium alginate was chosen because cells immobilized in alginate beads maintained a good viability during long-term culture due to the mild environment of the gel network.

In a preliminary study, different sodium alginate concentrations (2−3.5%) were tried. High hydroxytyrosol yields were obtained at low alginate concentrations (2 and 2.5%), whereas beads were relatively soft and showed a rapid leakage of cells.

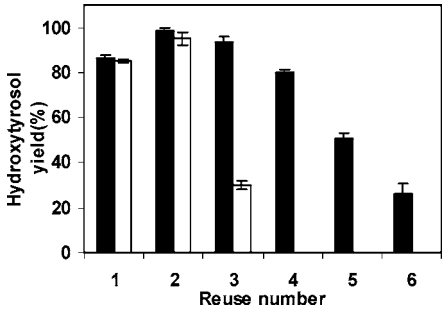

Figure 5. Reusability of free (□) and immobilized (■) cells in repeated batches for hydroxytyrosol production (bars indicate standard deviation).

A concentration of 3% alginate had been revealed to be optimal as it gives consistent beads with satisfying hydroxytyrosol yields. This concentration had been used for the optimization of the biotransformation with immobilized cells. **Figure 2** shows the microporous structure of alginate beads (**A**) and the random distribution of bacterial cells through these beads (**B**).

Using 10 g_{DCW} L^{-1} of *P. aeruginosa* cells, grown on 1 g L^{-1} of tyrosol concentration to the late log phase and immobilized in 3% calcium alginate beads, the hydroxytyrosol yield reached 70% after a reaction time of 20 h in the presence of 2 g L^{-1} of tyrosol under 200 rpm magnetic agitation at 30 °C. During this time, the aromatic compound degradation had not occurred yet. This could be explained by a toxicity phenomenon. **Figure 3** shows the time course of tyrosol running down coupled to the hydroxytyrosol transient accumulation.

To improve the bioconversion yield with immobilized cells, we have attempted to optimize the immobilized biomass, bead inoculum size, and maximal tyrosol concentration that can be tolerated by immobilized cells.

The immobilized biomass was quantified to choose the optimal microbial concentration. For this reason, four concentrations were tested (7.5, 10, 12.5, and 15 g_{DCW} L^{-1}). The hydroxytyrosol molar yield increased with the biomass concentration until 12.5 g_{DCW} L^{-1}. Higher biomass concentrations were disadvantageous as they resulted in inhibition by biomass excess and therefore a decreased hydroxytyrosol yield. On the contrary, the activity stability level was not affected by the cell concentration, and major inhibition problems could essentially be attributed to mass transfer limitations.

To examine the effect of the inoculum size on the formation of hydroxytyrosol, calcium alginate beads were introduced in the bioconversion medium at different percentages (10, 20, and 30% v/v). Results had shown that the hydroxytyrosol yield is closely related to the bead inoculum size. Maximum hydroxytyrosol production was obtained with 20% (v/v) inoculum size.

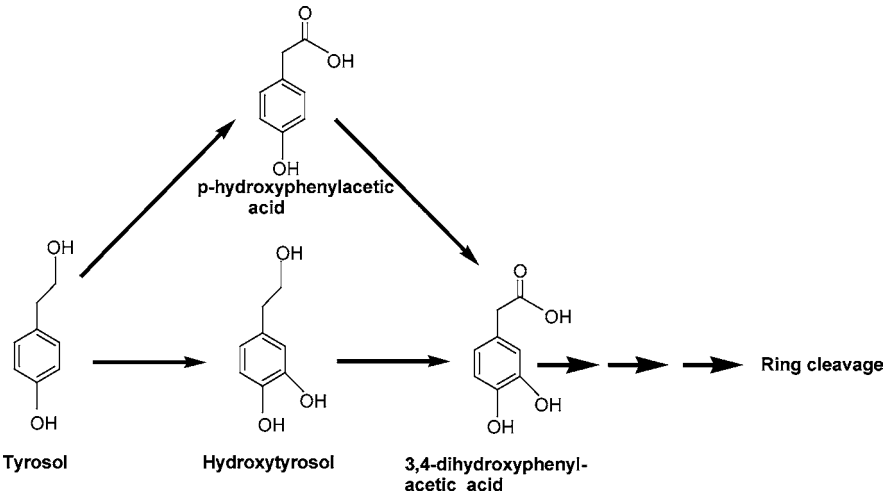

Figure 6. Proposed pathway for tyrosol metabolism by *P. aeruginosa*.

The increase of hydroxytyrosol production is proportional to the exchange surface leading to the facility of mass transfer.

The relationship between hydroxytyrosol production and tyrosol concentration was also examined. In this respect, a series of batch experiments was conducted. Tyrosol concentrations varied in the range of 2−6 g L^{-1}. As the tyrosol concentration increased in the bioconversion medium, the hydroxytyrosol yield increased as well. Thus, the higher hydroxytyrosol yield (86%) obtained for immobilized cells was observed at 5 g L^{-1} of tyrosol concentration. Over this concentration, hydroxytyrosol decreased drastically. These results mention the advantageous application of immobilized cells with toxic substrates.

Evaluation of Kinetic Parameters. After optimizing different parameters of the bioconversion reaction, we focused on the evaluation of kinetic parameters in batch conditions. Kinetic studies, performed within the substrate concentration range of 2−5 g L^{-1}, revealed that the Michaelis−Menten equation holds, and the kinetic parameters were determined. The Lineweaver−Burk plot of kinetic runs (**Figure 4**) carried out at 30 °C allowed the evaluation of V_{max}, K_m, K_{cat}, and catalytic efficiency (**Table 1**). The comparison of V_{max} and K_m under experimental conditions was not allowed as the biocatalyst quantities were not the same (1.2 g L^{-1} of free cells and 12.5 g L^{-1} of immobilized cells). Values obtained for the catalytic efficiency were very encouraging. No relevant effects on catalytic efficiency were observed when immobilized *P. aeruginosa* cells were tested under previously optimized conditions and compared to free cells.

Repeated Batch Bioconversion. Repeated batch hydroxylation experiments were performed by placing free and immobilized cells in the presence of 4 and 5 g L^{-1}, respectively. When the bioconversion yield reaches its maximum, the biocatalysts are washed and resuspended in fresh media. The reuse of both biocatalyst forms is shown in **Figure 5**. Immobilization enhanced the biocatalyst stability, and by this way, it allowed the retention of cell activity for more than five cycles. Hydroxytyrosol production had increased in the second batch, with both free and immobilized cells, suggesting an activation of resting cells (*25*). After a third batch, free cells lost more than 60% of their activity, and only immobilized ones could perform excessive batches.

DISCUSSION

In a previous study, Allouche et al. studied the relationships between the production of hydroxytyrosol and (i) the growth state of the culture utilized for biomass production, (ii) the carbon source on which the biomass was grown, (iii) the concentration of the biomass, and (iv) the amount of tyrosol that was treated, for bioconversion with free *P. aeruginosa* cells. In this paper, we have examined the production of hydroxytyrosol using an immobilized cell system.

The employment of polysaccharides for gel entrapment or encapsulation has become a challenging method where alginate stands out as the most promising polymer. Calcium alginate gels form rapidly in very mild conditions and provide suitable media for the immobilization by entrapment of whole microbial cells. A low diffusion coefficient has been shown already to be a powerful characteristic of alginate immobilization systems due to its high degree of cross-linking that limits the permeation rate of substrate and product.

Here, both free and immobilized whole cells were used for the production of hydroxytyrosol via the bioconversion of tyrosol. Hydroxytyrosol has been identified as an intermediate of a tyrosol degrading pathway. Indeed, we found that under experimental conditions, hydroxytyrosol was further oxidized to give 3,4-dihydroxyphenylacetic acid that can be engaged in the metabolic pathway of aromatic compounds by *P. aeruginosa*. But, when the reaction was supplemented with oxygen, tyrosol was converted to *p*-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid, which can be metabolized more as described by Kevin et al. (*26*) (**Figure 6**). The presence of *p*-hydroxyphenylacetic acid was confirmed by using an LC-MS apparatus. Positive ionization spectrum exhibited a molecular ion at *m*/*z* 153 with a fragment at *m*/*z* 107. These data were compared with those of available authentic standards.

During tyrosol bioconversion, no degradation of aromatic compounds was observed. This fact led to the hydroxytyrosol accumulation in the medium. Oxidation of hydroxytyrosol was inhibited by tyrosol, and it was not allowed until a drastic decrease of the latter's concentration.

No relevant changes were observed when comparing the catalytic efficiency for both free and immobilized cells used in single batch. The increase in the K_m value after immobilization may be partially due to mass transfer resistance of the substrate into the immobilization medium. The difference worth noting is the fact that the immobilized cells tolerate more tyrosol concentration than the free cell system. The resistance to environmental stresses, in particular, exposure to toxic substrates, is considered to be a major characteristic of immobilized cells (*27*). To explain the tolerance of increased substrate concentra-

tions, one hypothesis, based on the creation of a decreasing gradient of tyrosol concentration from the surface to the inner bead, was proposed.

A recent work dealing with the bioconversion of tyrosol to hydroxytyrosol using an immobilized cell extract of *P. putida F6* has been reported by Brooks et al. (*28*). The application of cell extract is mostly advantageous due to lower diffusion problems as compared to whole cells. However, these authors have obtained weak bioconversion yields as compared to the present work or the previously published one (*16*). Moreover, the step of extract preparation and the addition of ascorbic acid would considerably affect the economic efficiency of the reaction.

Repeated batch bioconversion of tyrosol using calcium-alginate entrapped *P. aeruginosa* cells demonstrated the retention of the bioconverting activity for a long time. Decreased activity within free cells used in repeated batches could be attributed on one hand to the toxicity of tyrosol and on the other hand to the washout of cells during recovery after each batch. However, in the case of immobilized cells, this phenomenon resulted, especially, from cell leakage caused by mechanic shocks due to the magnetic agitation (data not shown). Nevertheless, the immobilized biomass has already been sufficient for the performance of at least five consecutive batches with convenient yields.

For the statistical analysis, repeated batch experiments were performed in triplicate. The average values and standard deviations were calculated using Microsoft Excel software. The data were analyzed by the Student's *t*-test ($P < 0.05$). Collected information was significant in favor of immobilized cell bioconversion. Then, biocatalysts could resist the phenomenon of tyrosol toxicity and by this way retain activity for a long time. Consequently, the evaluation of operational stability of immobilized cells indicated that the hydroxylating activity was durable under repeated use.

As a scope, choosing an appropriate agitation system and microencapsulating beads with thin layers of alginate gel could minimize cell loss and retain high bioconversion yields for longer times.

Finally, the production of hydroxytyrosol via bacterial synthesis was performed in environmentally friendly conditions that can be scaled up for industrial applications. Hydroxytyrosol accumulated in the reaction medium can be recovered by simple liquid−liquid extraction by ethyl acetate followed by purification on reversed phase chromatography.

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