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Bioconversion of Vanillin into Vanillic Acid by *Pseudomonas fluorescens* Strain BTP9

Reactor Design and Parameters Optimization

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ABSTRACT

Pseudomonas fluorescens strain BTP9 is used as biocatalyst to produce vanillic acid from vanillin. Several two-phase reactors were investigated and compared to the corresponding one-phase systems in order to optimize this bioconversion.

A water–dodecanol system was set up. High cell density and entrapment of the cells in alginate beads are two characteristics of this reactor. With this kind of reactor, vanillic acid productivity was increased (3.4 g/L/d) compared to the one-phase reactor, with a conversion rate near 80%. Vanillic acid yield and productivity strongly depended on pH, stirring rate, cell immobilization, and substrate concentration.

Index Entries: Vanillin; vanillic acid; *Pseudomonas fluorescens;* immobilized cell reactor; two-phase reactor.

INTRODUCTION

For the last 15 yr, synthetic organic chemistry has closely evaluated biochemical reactions performed by microorganisms or catalyzed by enzymes. Their high potential both in theoretical and practical applications in synthetic chemistry has been demonstrated (*1*).

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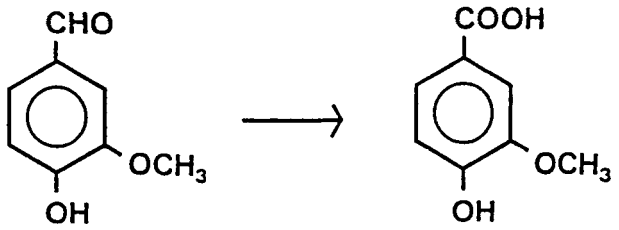

Fig. 1. Diagram of the bioconversion of vanillin into vanillic acid.

Among biochemical reactions, oxidoreduction reactions in particular are of great interest for organic synthesis. Moreover, they are important steps in living cell metabolism and energy conversion (*2*).

In order to produce chemicals at a low cost, we investigated, in our laboratories, several redox reactions carried out by microorganisms, such as reduction of xylose to xylitol by *Pachysolen tannophilus* (*3*), reduction of vanillin to vanillyl alcohol by *Saccharomyces cerevisiae* (*4,5*), and stereoselective reduction of a β-keto ester to β-hydroxy ester by *Saccharomyces cerevisiae* to produce L-carnitin, an important physiological agent (*6,7*).

We recently reported that (*8*) *Pseudomonas fluorescens* strain BTP9 could convert vanillin into vanillic acid (Fig. 1). Vanillic acid is a phenolic compound of high added value compared to vanillin (*9*). It is used as an antibacterial agent (*10*) in dental prophylaxis, and as a specialty chemical and a valuable synthon in chemistry (*11*).

In this article, optimization of vanillin bioconversion to vanillic acid is presented at two levels: (1) physico-chemical parameters and (2) reactor design. Bioconversion often strongly depends on the choice of cultivation conditions, such as pH, substrate concentration, and cell density. In order to increase yield and productivity of vanillin bioconversion, cell immobilization in alginate beads and addition of an organic phase or a second aqueous phase to the reactor ("two-phase systems") are also investigated.

Immobilization techniques often allow cell activity stabilization; another advantage is an easier biocatalyst recycling. Both those reactor features are necessary to set up a continuous process. Two-phase systems are an additional way to optimize vanillin bioconversion. Such systems are often used to improve product or substrate solubilization, reduce inhibition by substrate or product, and remove product in cell-free phase during bioconversion. This article is the second step in setting up a reactor for the production of vanillic acid from vanillin.

MATERIALS AND METHODS

Strain

Pseudomonas fluorescens strain BTP9 was chosen as previously described (*8*) for the bioconversion of vanillin.

Culture Medium

The feeding medium contained 1% $Na_2HPO_4 \cdot 12H_2O$, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% NaCl, 0.005% $CaCl_2 \cdot 2H_2O$, 0.6% yeast extract, and 0.01% vanillin. This medium was used to produce biomass for bioconversion assays.

Growth Assay

Cell concentration was measured by turbidimetry. One unit of optical density (OD) at 540 nm equaled 5×10^8 cells/mL.

Biomass Production

The biomass of the reactors was obtained by incubating the newly inoculated culture medium we previously described, at 29°C, on an orbital shaker Infors AG (190 rpm) for 16 h. Cells were harvested by centrifugation for 30 min at 4470 g (Sorvall RC-3) and then washed before being centrifuged again. Cell concentration in the reactors was expressed in OD at 540 nm from spectrometric measurements carried out on the cultures after incubation (the removed cell concentration was related to the volume of the unique liquid phase [in one-phase reactors] or the two liquid phases [in two-phase reactors]).

Cell Immobilization

Centrifuged cells were suspended in 50 mL of distilled water. The cell suspension was mixed with an equal volume of 4% sodium alginate. The cells–alginate mixture was dropped—with the aid of a pump (Gilson Minipuls 2) and a rubber tubing linked to a glass pipet—into a 1% $CaCl_2$ solution. The beads were left for 1 h in the solution and suspended again for 5 min in a 0.8% $BaCl_2$ solution to make them harder. The beads were then filtered to be used in the reactor.

One-Phase Reactors

In the case of free-cell reactors, cells collected by centrifugation were suspended in 100 mL of a 0.05*M* acetate buffer (pH 5) or a 0.05*M* phosphate buffer (pH 6 or 7), containing 0.1–0.5% of vanillin. In the case of immobilized cell reactors, 60 g of alginate beads were placed in 100 mL of a 0.028*M* acetate buffer (pH 6) containing 0.028*M* $CaCl_2$ and 0.1–0.5% vanillin. The reactors (500-mL flasks) were incubated at 29°C on an orbital shaker Infors AG (stirring rate: 80–320 rpm).

Two-Phase Reactors

Aqueous Two-Phase Reactors

Cells removed by centrifugation were suspended in 250 mL of a 0.2*M* phosphate buffer (pH 6) with 17% polyethylene glycol (PEG) 10,000. 750

mL of a 18% KH_2PO_4 solution ("continuous phase"), buffered at pH 6 with NaOH pellets and containing 0.05% vanillin, were added to the PEG phase ("dispersed phase"). The reactors (3-L flasks) were incubated at 29°C on an orbital shaker Infors AG (200 rpm).

Water-Organic Solvent Reactors

In the case of free-cell reactors, centrifuged cells were suspended in 50 mL of a 0.028*M* acetate buffer (pH 6) containing 0.028*M* $CaCl_2$. 50 mL of dodecanol with 0.3% vanillin were added to the aqueous phase. In the case of immobilized-cell reactors, 60 g of alginate beads were placed in 50 mL of the same buffer as the one used for free-cell reactors. 50 mL of dodecanol with 0.2–1.2% of vanillin were added to the aqueous phase. The reactors (500-mL flasks) were incubated at 29°C on an orbital shaker Infors AG (160 rpm).

Vanillin and Vanillic Acid Concentration Measurement

The HPLC method using a reversed-phase technique (C8) has been described previously (*8*). For the PEG phase, the HPLC column was washed with 100% methanol after each analysis to avoid polarization of the reversed-phase by PEG.

RESULTS

Bioconversion optimization of vanillin to vanillic acid by the strain BTP9 consists of the use of this microorganism under drastic experimental conditions—as opposed to the natural conditions—both to increase yield, productivity, and process stability, and to minimize its cost. We have investigated the effects of several culture parameters on bioconversion yield and productivity, in one- and two-phase reactors in order to optimize the microbial transformation of vanillin.

One-Phase Reactors

Bioconversion with Free Cells

The first studied parameter is pH. pH often plays a very important part in oxidoreduction reactions, in conventional chemistry. In biology, on the other hand, pH variation tolerance is usually rather restricted. Figure 2 shows that bioconversion yield strongly decreases with a pH value lower than 6 (initial concentration of vanillin: 1 g/L; cell density of each reactor, expressed in OD at 540 nm: 9.3 ± 0.1).

A good oxidation of vanillin to vanillic acid can also be linked to the microorganism's increased oxygen requirement. The influence of the medium's oxygen concentration on bioconversion can initially be studied by testing different stirring rates (Fig. 3). Initial concentration of vanillin is 1 g/L, and cell density of each reactor is 4.4 ± 0.2 U of OD.

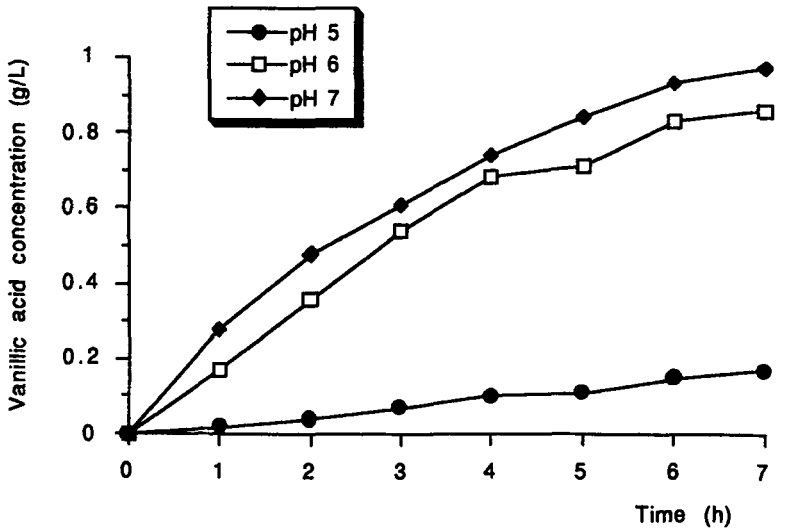

Fig. 2. Evolution of vanillin bioconversion in free-cell one-phase reactors buffered at different pH values.

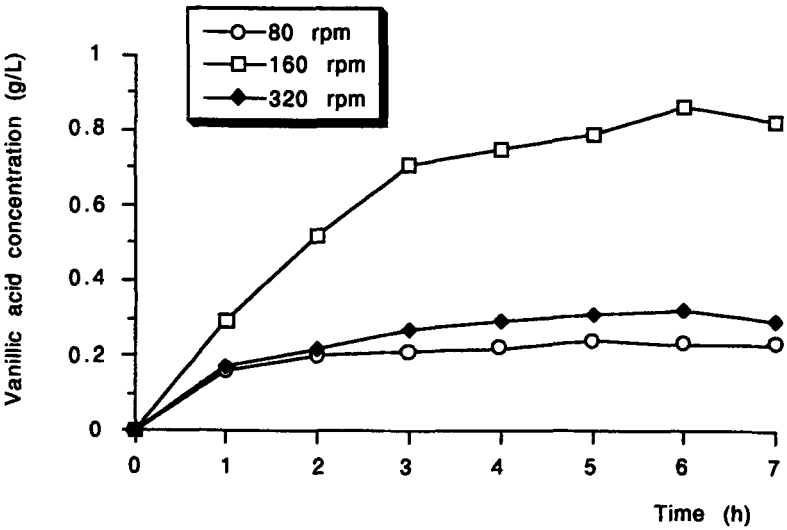

Fig. 3. Evolution of vanillin bioconversion in free-cell one-phase reactors at different stirring rates.

An intermediate stirring rate appears to be better for an optimal bioconversion. At 320 rpm, vanillic acid is chemically unstable probably because of further oxidation as observed with the same stirring conditions without microorganisms (data not shown). The influence of different initial concentrations of vanillin on the bioconversion yield and productivity was also examined. Figure 4 shows vanillic acid production is optimal with the initial vanillin concentration of 3.14 g/L (cell density of each reactor is 4.6 ± 0.1 U of OD and their stirring rate is 160 rpm). However, the

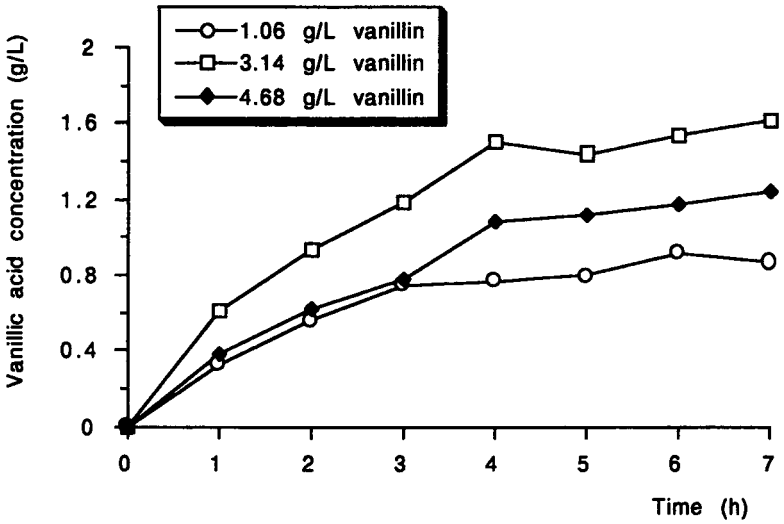

Fig. 4. Evolution of vanillin bioconversion in free-cell one-phase reactors with different initial concentrations of vanillin.

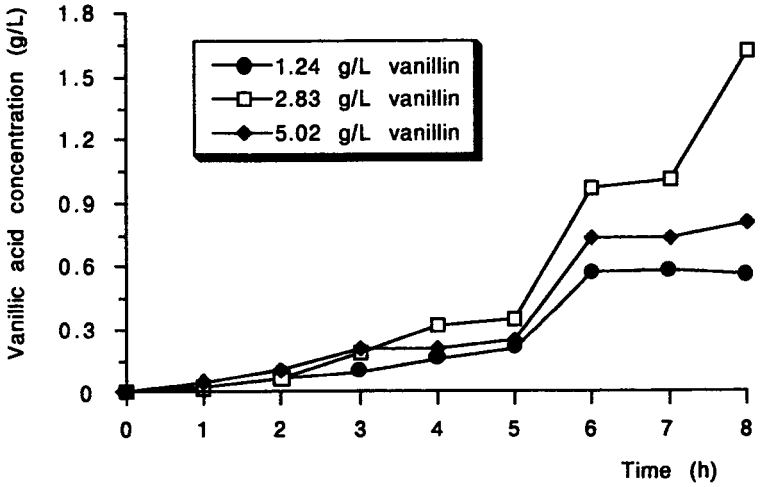

Fig. 5. Evolution of vanillin bioconversion in immobilized-cell one-phase reactors with different initial concentrations of vanillin.

higher the initial concentration of vanillin, the lower the bioconversion yield: after a reaction time of 7 h, yields for increasing initial concentrations of vanillin are, 74, 47, and 24%, respectively.

Bioconversion with Immobilized Cells

Cell immobilization in alginate beads often offers easy recycling and a better biocatalyst stability. The influence of initial concentration of vanillin on bioconversion is investigated with immobilized cells. Cell density of the reactors is 4.8 ± 0.1 U of OD, and stirring rate is 160 rpm. As shown in Fig. 5, vanillic acid production is higher with an initial vanillin concentration of 2.83 g/L.

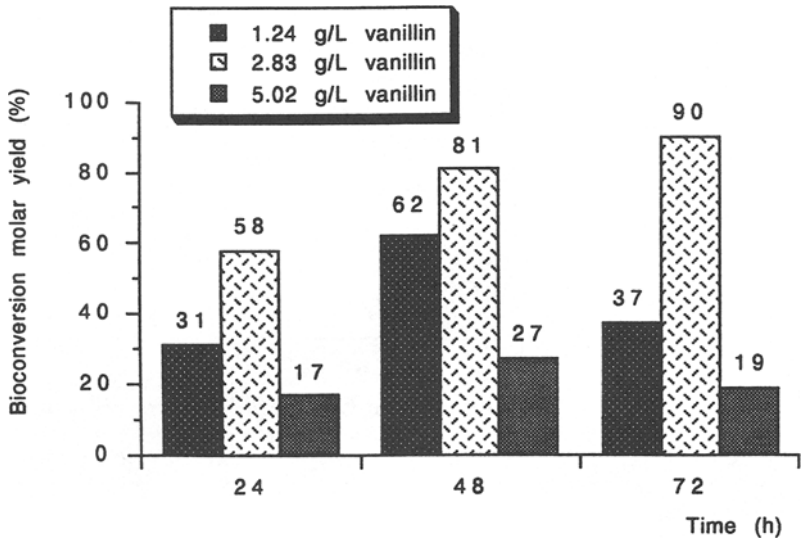

Fig. 6. Molar yield of vanillic acid after 24, 48, and 72 h of bioconversion for three different initial concentrations of vanillin (immobilized-cell one-phase reactors).

After 24 h of bioconversion, liquid phases are removed and replaced by new phases with the same initial concentrations of vanillin. The process is repeated after 48 h of microbial transformation. Figure 6 shows molar yield of vanillic acid for the three reactors in relation to time.

The second experiment confirms the better results obtained with a vanillin concentration of 2.83 g/L: from the second day, bioconversion yield is maintained between 81 and 90%, corresponding to a daily productivity of 2.53–2.81 g/L.

Two-Phase Reactors

Aqueous Two-Phase System

An aqueous two-phase system is obtained by mixing together either two aqueous solutions of incompatible polymers (e.g., polyethylene glycol and dextran) or one polymer solution and one adequate saline solution (e.g., phosphate salt or sulfate salt). The aqueous two-phase system studied belongs to the second type. It is prepared as described in Materials and Methods. With this system, the number of cells in the noncellular continuous phase (KH_2PO_4 phase) markedly increases—increase equal to or higher than 10% of the total reactor load—for a cell density higher than 1.5 U of OD. Another important factor is the phase-separation process: centrifuging the emulsion at 4000 g for 10 min is a good way to separate the phases completely; the volumes of the PEG-rich top phase and the KH_2PO_4-rich bottom phase are then in the ratio 1:7. Table 1 shows, after different bioconversion times, the reactor's vanillic acid concentration of both phases (cell density: 1.0 ± 0.1 U of OD).

Table 1
Vanillic Acid Concentration in the Two Aqueous Phases of the PEG-KH_2PO_4 Reactor as a Function of Time

Time, h	PEG phase, g/L	KH_2PO_4 phase, g/L
0	0	0
6	0.25	0.04
14	0.39	0.04
19	0.45	0.04
24	0.48	0.04

Table 2
Characteristics of the Three Water–Dodecanol Reactors Studied

Reactor	Immobilized cells	Cell density, OD_{540}
1 (Control)	No	4.4 ± 0.1
2	No	9.0 ± 0.1
3	Yes	4.4 ± 0.1

Water-Organic Solvent System

A water-organic solvent system is composed of an aqueous phase containing the biocatalyst and of an adequate immiscible organic solvent, in which substrate and/or product was preferentially made soluble. The chosen organic solvent is dodecanol. The partition coefficient of a determined substance in a water–dodecanol mixture can be defined as the ratio of the substance concentration in dodecanol over the substance concentration in water; partition coefficients for vanillin and vanillic acid are 8 ± 1 and 0.62 ± 0.06, respectively. Three water–dodecanol reactors were prepared in order to investigate the influence of cell density and cell immobilization on the bioconversion in a water-dodecanol system. Those reactors are described in Table 2.

Initial concentration of vanillin in the three reactors is 1.5 g/L (aqueous phase + organic solvent). Both phases of each reactor are renewed after 24 and 48 h of bioconversion. Figure 7 shows for each reactor vanillic acid concentration in both phases after 24, 48, and 72 h.

The best conversion is obtained with the immobilized-cell reactor (reactor 3). Daily productivity of vanillic acid for that reactor is between 1.17 and 1.40 g/L (aqueous phase + organic solvent), which is equivalent to bioconversion molar yields of 53 and 63%, respectively. Finally in order to increase productivity again, the effect of various initial concentrations of vanillin on bioconversion is investigated with immobilized-cell water–dodecanol reactors. Cell density of those reactors is 4.9 ± 0.1. Both phases of each reactor are renewed after 24 and 48 h of microbial transformation. Figure 8 shows for the three reactors vanillic acid concentration in both phases (averages of the initial vanillin concentrations of both phases: 2, 4, and 6 g/L) after 24, 48, and 72 h.

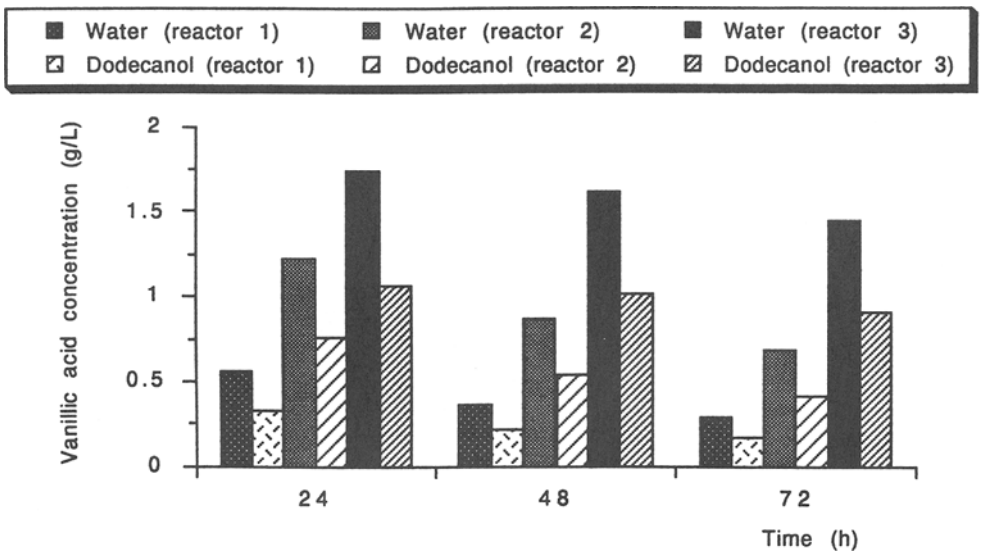

Fig. 7. Vanillic acid concentration of both phases of the three water–dodecanol reactors studied (Table 2) vs time.

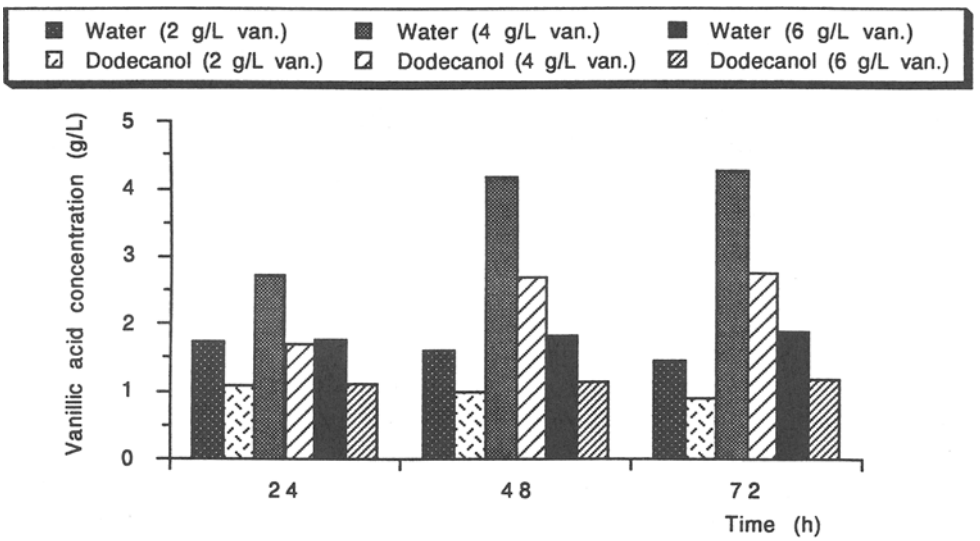

Fig. 8. Vanillic acid concentration of both phases of the three water–dodecanol reactors studied (averages of the initial vanillin concentrations for both phases: 2, 4, and 6 g/L) vs time.

DISCUSSION

Use of soil bacteria to generate useful low-molecular-weight phenolic compounds from lignin and lignin model dimers has been reported by several authors (*12–15*). Among those low-molecular-weight phenolic compounds, vanillic acid appears to be a commercially interesting chemical.

Pometto and Crawford (*9*) and Perestelo et al. (*16*) have shown the possibility of converting vanillin to vanillic acid from resting-cell suspensions of *Streptomyces viridosporus* and *Serratia marcescens*, respectively. More recently, we have reported the ability of *Pseudomonas fluorescens* strain BTP9 to perform this bioconversion (*8*). In this article, emphasis has been put on the choice of culture parameters and on reactor design to optimize the microbial transformation of vanillin.

Thus, with one-phase reactors, although the optimal values of pH (6 or 7) and of stirring rate (160 rpm) clearly appear in Figs. 2 and 3, the vanillin concentrations in the reactor favor either yield (low concentrations) or productivity (high concentrations). It is important to notice that for vanillin concentrations of 3–5 g/L, vanillic acid production rapidly stops owing to substrate toxicity and not owing to vanillin disappearance. This toxicity has been observed by several authors (*9,17*) for vanillin concentrations higher than 1.5 g/L.

When entrapped in alginate beads, cells are protected against substrate toxicity for concentrations close to 3 g/L, and vanillic acid production is considerably increased. This cell protection *vis-a-vis* phenolic compound toxicity by alginate gel has been demonstrated by other authors (*18*).

Two-phase reactors were also investigated. The low yield and productivity reached with the aqueous two-phase system can be explained by the fact that cells and products (vanillin and vanillic acid) are almost exclusively present in the dispersed phase. This situation is detrimental to the primary objective of extractive fermentation since the reactor acts then like a one-phase system.

With the water–dodecanol systems, immobilizing the cells in alginate beads appears to have a stronger positive effect on the catalytic activity of the reactor than increasing twofold the cell density itself, probably because of dodecanol toxicity toward free cells. On this subject, Bar (*19*) makes a distinction between solvent toxicity exerted on the microorganism at the molecular level (enzyme inhibition, membrane modification) and that at the phase level (depletion of essential nutrients from the broth as a result of extraction by the organic liquid, blockage of nutrient diffusion from broth to cells because of solvent coating, cell-wall disruption as a result of extraction of some outer cellular components). The author mentions that *Lactobacillus* cells can be protected against the latter kind of toxicity by their immobilization in carrageenan gel beads. It is thus reasonable to think that the dodecanol toxicity toward *Pseudomonas fluorescens* cells occurs at the phase level.

With immobilized cells, the best productivity in water–dodecanol reactors is obtained with an initial vanillin concentration of 4 g/L. Table 3 summarizes the performances of various kinds of reactor investigated in this work.

The water–dodecanol reactor with immobilized cells offers four major advantages:

Table 3
Performances of Various Kinds of Reactors Investigated in This Work, for the Bioconversion of Vanillin into Vanillic Acid

Reactor design	Concentration of vanillin, g/L	OD_{540}	Daily productivity of vanillic acid, g/L/d	Mean molar yield, %
One-phase free cells	1.1	4.6	-	74
One-phase immobilized cells	2.8	4.8	2.5–2.8 (from the 2nd day)	85 (from the 2nd day)
Two aqueous phases free cells	1.0	1.0	-	24
Water–docecanol free cells	1.5	4.4	0.2–0.4	14
Water–dodecanol immobilized cells	4	4.9	3.4–3.5 (from the 2nd day)	79 (from the 2nd day)

1. High daily productivity owing to high vanillin concentrations in the reactor;
2. Easy biocatalyst recycling;
3. Stable activity; and
4. Possibility of a continuous process with separation of the substrate and the product at the phases level.

This reactor thus appears to be a technology alternative to the conventional chemical methods (*20*) for the production of vanillic acid.

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