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

Cell Reactors and Mutants Study

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ABSTRACT

The ability of a fluorescent *Pseudomonas* to bioconvert vanillin, a phenolic compound, into vanillic acid was investigated. Free and immobilized cell reactors were tested. With free cells, the optimal yield reaches 98% after 6 h and 30 min of bioconversion. With cells immobilized in alginate beads, transformation rate is only 47% after 13 h of conversion. Nevertheless, a continuous immobilized cell reactor was used for 76 h. With this one, the optimal yield is higher than 80%.

The influence of the residence time and cell concentration of the alginate beads in the reactor over the reactor's productivity has also been studied. Catabolically blocked mutants for vanillic acid degradation were searched. To screen these mutants, a new and very sensitive method was reported. The results of our mutant screenings were discussed.

Index Entries: Vanillin, vanillic acid; *Pseudomonas fluorescens;* immobilized cell reactor; mutant.

INTRODUCTION

Oxidoreduction reactions constitute indisputably the most important class of the universal chemical reactions and are consequently of great importance in chemical synthesis. Although some chemical syntheses require numerous reaction and purification steps that are very expensive,

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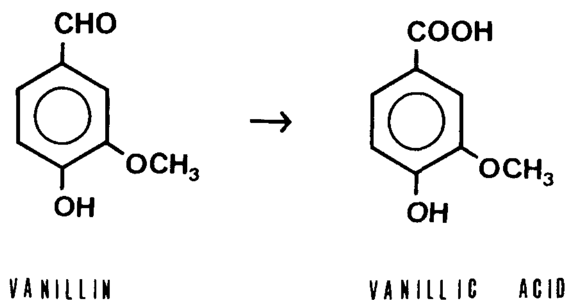

Fig. 1. Schema of bioconversion of vanillin into vanillic acid.

the oxidoreductions catalyzed by enzymes or cells are particularly interesting, because these reactions are generally quick, highly specific, and in certain cases regioselective. Moreover these bioreactions take place in conditions (pH, temperature, and so on) compatible with environment and life. In our laboratories, cell reactors able to achieve this type of reaction are studied in order to produce new chemicals at a low cost (*1*).

By using living microorganisms that possess the enzymatic capabilities, we have investigated reduction of xylose to xylitol by *Pachysolens tannophilus* (*2*), reduction of vanillin to vanillyl alcohol by *Saccharomyces cerevisiae* (*3,4*), and stereoselective reduction of a β-ketoester to β-hydro xyester by *Saccharomyces cerevisiae* to produce L-carnitin, an important physiological agent (*1,5*). These reductions have been studied from the point of view of strain selection, culture conditions, enzyme system, and choice of the reactor.

Among the soil microorganisms, numerous bacteria are known to degrade lignin. One reaction step is the oxidation of vanillin, a phenolic compound. For example, *Pseudomonas sp.* (*6–9*), *Rhodococcus erythropolis* (*10*), *Corynebacterium sp.* (*11*), *Bacillus subtilis* (*12*), *Xanthomonas sp.* (*13*), and *Streptomyces sp.* (*14*) are able to oxidize vanillin into vanillic acid.

Vanillic acid (and it derivatives) is used in the pharmaceutical industry, for its antibacterial activity (*15*), as an analeptic drug (*16*), and also as a monomer in the polyester synthesis (*17*). More important, however, is its relatively high cost, compared to vanillin. In this article, we will report the oxidation of vanillin to vanillic acid (Fig. 1) by a fluorescent *Pseudomonas.*

MATERIALS AND METHODS

Strain

BTP9 strain was chosen among 62 soil bacteria isolated in the laboratory or in other research departments (Agrostar) for its special ability to grow on a minimum medium with vanillin (concentration of 0.1, 0.2, or 0.3%) as sole source of carbon. These bacteria belonged to the *Streptomyces, Xanthomonas, Bacillus,* and *Pseudomonas* genera. This strain was identified as a *Pseudomonas fluorescens* by the API test.

Feeding Media

Culture Media

The culture 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.

Screening Media

The media used for mutant selection were mineral media (MM) containing 0.2% $(NH_4)_2SO_4$, 0.05% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, and variable concentrations of vanillin, vanillic acid, protocatechuic acid, or glycerol as substrates. In addition, solid media contained 2% agar-agar. pH was adjusted to 7.

All media were autoclaved. (For the screening media, the source of carbon was sterilized separately.)

Growth Assay

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

Mutagenesis

Mutants were isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG): Log phase cells were suspended at a titer of 5×10^7 cells/mL in distilled water with 0.1% bactopeptone, 0.5% NaCl, and 0.2% tween 80. One drop of a NTG-saturated solution was added to this cell suspension (vol: 10 mL). The suspension was then incubated for 20 min at 29°C on an orbital shaker (Infors AG). The mutagenized cells and a nonmutagenized control culture were washed three times and spread at different dilutions on mineral medium with 0.5% glycerol (MMG5). After incubation for 48 h at 29°C, the death rate was calculated.

Screening Methods

For the search for vanillin hypertransforming mutants, the mutagenized cells, after washing, were spread on mineral medium with 0.4% vanillin and incubated for 1 wk at 29°C. For the search for blocked mutants for vanillic acid degradation, the bacterial colonies isolated on MMG5 (solid medium) were subcultured one by one with the help of sterilized wooden toothpicks on "microtitration plates" (plates with 96 wells used in ELISA assays) containing 150 μL (per well) of MMG5 (liquid medium). These microtitration plates constituted the mutant collection. The mutant strains were subcultured in turn with the help of a homemade replication tool—this consisted of a block, embedded with 48 metal rods (8×6). The first experiment was performed on solid mineral media (Petri dishes) containing either 0.3% vanillic acid or 0.3% protocatechuic

acid. Glycerol (0.3%) was substituted for the control. The second experiment was performed on microtitration plates: Each well contained 150 μL of mineral medium with 0.1% glycerol added. The plates were then incubated for 72 h at 29°C on an orbital shaker (Infors AG). Fifty microliters of 0.02% vanillic acid solution were added to each culture. The microtitration plates were incubated again for 72 h at 29°C on the orbital shaker. The wells in which the mutants did not grow remained clear (first examination of the microtitration plates). Then, 50 μL of a 3% $FeCl_3$ solution and 50 μL of a 0.1% $K_3(Fe[CN]_6)$ solution (both solutions were newly prepared) were added to each well. A dark blue color appeared after 6–10 min, (second examination of the microtitration plates) in the wells where the mutant strains had not grown or were unable to degrade vanillic acid, whereas the other wells remained light green (vanillic acid dissimilation). Protocatechuic acid was never detected.

Biomass Production

The biomass of the reactors was obtained from the culture medium described previously, and the cells were centrifugated for 30 min at 4470*g* (Sorvall RC-3). The cell concentration of the reactors was expressed in OD at 540 nm from the spectrometric measurements carried out on the cultures after incubation.

Free Cell Reactors

The centrifugated cells were suspended in 100 mL of a 0.028*M* phosphate buffer (pH=6) containing about 0.1% vanillin ("bioconversion medium"). The reactors (250-mL flasks) were then incubated at 29°C on an orbital shaker (Infors AG).

Immobilized Cell Reactors

The centrifugated cells were suspended in 50 mL of distilled water and mixed with an equal volume of 4% sodium alginate. These cells-alginate mixtures were dropped into 1% $CaCl_2$ solutions (250-mL flasks). The beads formed were left for 1 h in these solutions and suspended again for 5 min in 0.8% $BaCl_2$ solutions in order to make them harder. These solutions were replaced by 100 mL of 0.028*M* acetate buffer (pH=6) containing about 0.1% vanillin ("bioconversion medium"). The immobilized cells were then incubated at 29°C on an orbital shaker (Infors AG). In other experiments, the alginate beads were recycled. At the end of each cycle of bioconversion, the vanillin and vanillic acid concentrations were measured, and the media were replaced by a new acetate buffer containing the same vanillin concentration.

Analyses of the Conversions Statement

Disappearance of vanillin and appearance of vanillic acid were monitored by aseptically removing 1-mL samples at different periods of time. The samples of the free-cell reactors have been centrifugated for 10 min at 14,926*g* (Biofuge A Heraeus Sepatech). All samples were then filtered (0.2 μm Spartan filter). For qualitative analyses, measurements of ultraviolet (UV) absorption spectra between 200–400 nm were obtained from the samples diluted 100 times (absorption maxima for vanillin: 200, 227, 279, and 309 nm; absorption maxima for vanillic acid: 200, 251, and 284 nm). Quantitative analysis was performed by liquid chromatography, using a Pharmacia HPLC (LCC-500 PLUS model) equipped with a Series 1050 Hewlett-Packard autosampler. The compounds were separated by reversed-phase chromatography (Chromspher C8 as stationary phase) with a 800:200:2 ratio of water:methanol:phosphoric acid as eluant. The flow rate was 0.6 mL/min, and the phenolic substrates were detected at 254 nm. Standards of vanillin and vanillic acid were regularly used for the calibration. Their retention times were 14.4 and 10.0 min, respectively. All samples were injected twice on the HPLC column (the precision specification of the autosampler is $<0.5\%$ RSD (relative standard deviation) of peak areas).

RESULTS

As BTP9 grows on a medium containing vanillin as substrate, the ability of this strain to convert vanillin into vanillic acid is investigated. Two types of reactor were compared: those with free cells and those with immobilized cells. The influences of residence time and cell concentration of the alginate beads on the bioconversion were examined. Mutants were then selected and tested.

Nature of the Cell Reactor

Two kinds of cell reactors are investigated: a free-cell reactor and an immobilized-cell reactor (alginate beads). The cell concentrations of both reactors are very similar: 9.6 for the first one and 9.0 for the second one, i.e., 4.8×10^9 and 4.5×10^9 cells/mL of bioconversion medium, respectively.

For the free-cell reactor (Fig. 2), vanillin (initial concentration: 0.88 g/L) disappears from medium after 4 h and 30 min, whereas vanillic acid reaches after 7 h a concentration of 0.95 g/L, which corresponds to a bioconversion molar yield of 98%.

For the immobilized-cell reactor (Fig. 3), the vanillic acid concentration in the medium reaches only 0.56 g/L after 13 h (for a vanillin initial concentration of 1.09 g/L), which is equivalent to a bioconversion rate of 47%. This therefore appears to be the optimal yield of this reactor.

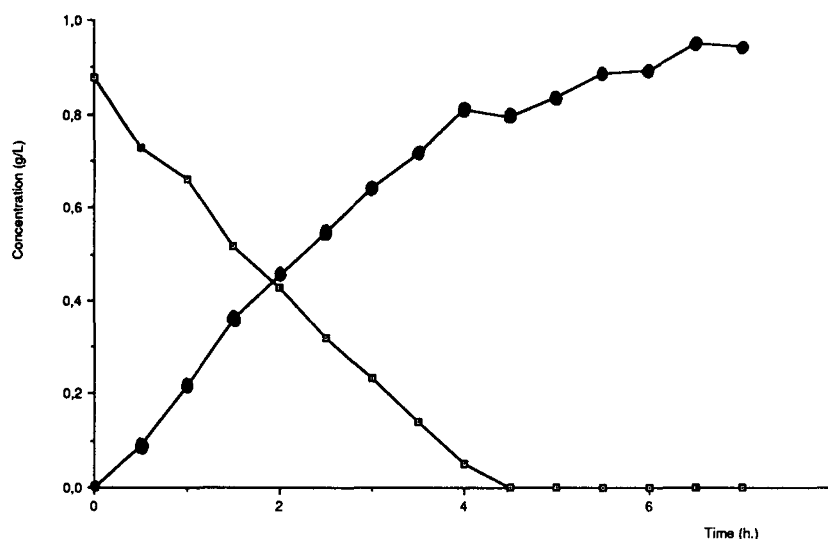

Fig. 2. Evolution of the vanillin bioconversion into vanillic acid in a free-cell reactor (OD_{540}=9.6). —⊡— Vanillin; —●—vanillic acid.

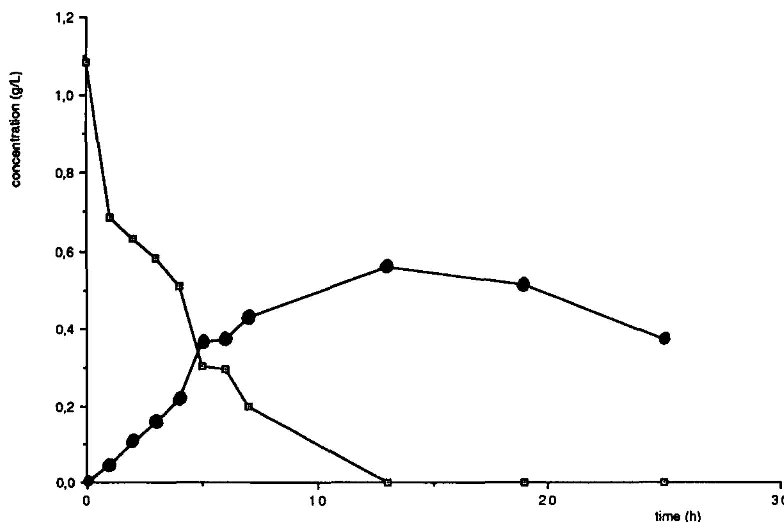

Fig. 3. Evolution of the vanillin bioconversion into vanillic acid in an immobilized-cell reactor (OD_{540}=9.0). —⊡—Vanillin; —●—vanillic acid.

Resident Time and Cell Concentration of the Alginate Beads in the Reactors

In order to increase the efficiency of the immobilized-cell reactor, the alginate beads are recycled, and various cell concentrations are tested. The cell concentrations of the reactors, expressed as optical densities, are in the ratio of 1:2:4, and are 4.5, 9, and 18, respectively. The bioconversion media are replaced successively after 25, 16, 13, 15, and 7.5 h. The vanillin and vanillic acid concentrations (vanillin initial concentration:

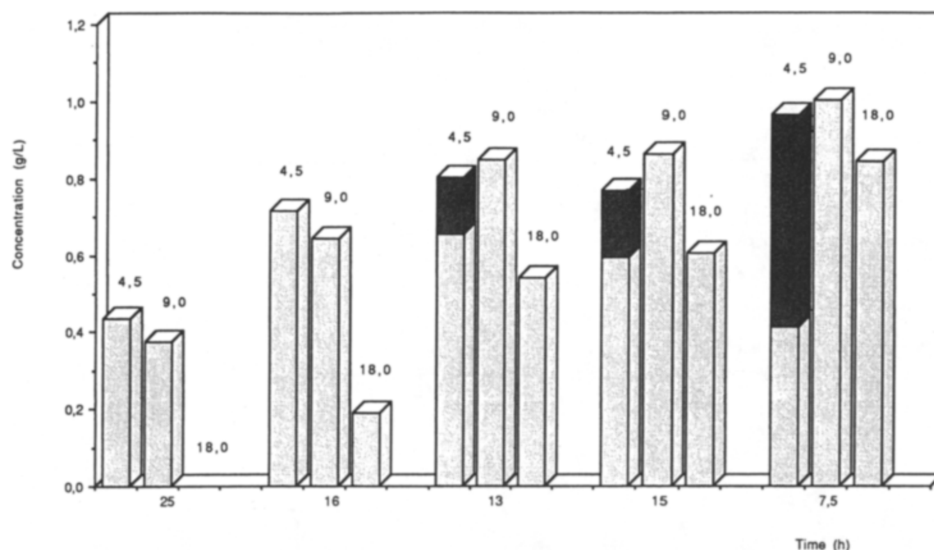

Fig. 4. Vanillin and vanillic acid concentrations as a function of the bioconversion times (25, 16, 13, 15, and 7.5 h) and the residence times of the beads (25 h + 16 h + 13 h + 15 h + 7.5 h = 76.5 h) in each of the three cell reactors (OD_{540}: 4.5, 9.0, and 18.0). ■ Vanillin; ▨ vanillic acid.

1.09 g/L) of the three reactors are measured at the end of each cycle of bioconversion (Fig. 4).

This experiment shows that the vanillic acid concentration increases, after the first cycle, to a maximum of 1.00 g/L for the intermediary cell concentration during the last cycle. This is equivalent to a conversion rate of 83%.

Furthermore, it is interesting to note that, in these reactors, vanillic acid is still degraded by the cells. The extent of this degradation is directly proportional to both residence time of the beads in the bioconversion medium and cell concentration of the reactors. Indeed, in the last three cycles, vanillic acid is in the consumption phase for the highest cell concentration, whereas it is in the production phase for the lowest cell concentration.

Mutants

De Wulf (*18*) and Pometto (*19*) reported that vanillin was toxic for cells at a concentration higher than 1.5–2 g/L. Thus, to improve the productivity of the reactors and to prevent the bioconversion product from disappearing, mutants able to grow with a vanillin concentration of 0.4% (growth substrate) and mutants unable to degrade vanillic acid are searched.

For the search for vanillin hypertransforming mutants, no mutagenized cells are able to grow on mineral medium with vanillin (concentration: 0.4%) as sole source of carbon. For the search for blocked mutants for vanillic acid degradation, 1920 mutagenized cells (death rate after mutagenesis: 99%) ranked in collection are tested on solid mineral medium

Table 1
Growth of Mutants on Vanillic Acid,
Protocatechuic Acid or Glycerol ("Control")*

Mutant	Vanillic	Protocatechuic	Glycerol
10D3	−	−	+
10F11	−	+	+
15H2	+	−	+
16D4	+	−	+
17D9	+	−	+
20H11	+	−	+

* +: Growth; −: No growth.

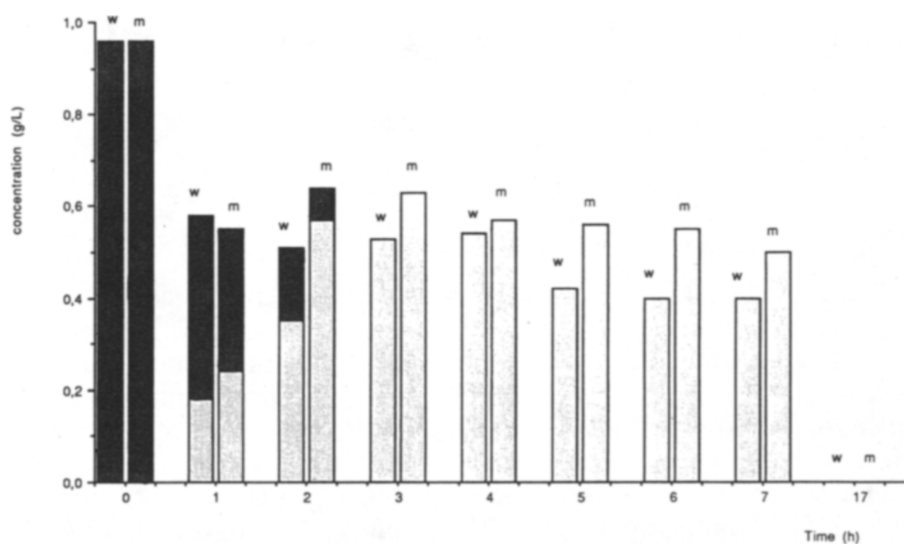

Fig. 5. Conversion yields of vanillin into vanillic acid in immobilized-cell reactors with the wild strain (w) and the mutant 10F11 (m) (OD_{540} of both reactors: 28.0). ■ Vanillin; ▨ vanillic acid.

containing vanillic acid, protocatechuic acid (the first degradation product of vanillic acid with *Pseudomonas* (*6,7*), or glycerol (control) as growth substrates. Six mutants are affected in growth on vanillic acid and/or protocatechuic acid (Table 1). Since the mutants 10D3 and 10F11 do not grow on vanillic acid, these mutants and the wild strain are produced, and cells are harvested by centrifugation and used in experiments of vanillin bioconversion.

Figure 5 shows both conversion yields of the mutant 10F11 comparatively to the wild strain in g/L. The ODs at 540 nm for both these immobilized cell reactors are identical, namely 28, which corresponds to 1.4×10^{10} cells/mL of bioconversion medium. After 3–4 h of conversion, the vanillic acid concentrations of both reactors (mutant and wild strain) reach max-

imal values, namely 0.63 and 0.54 g/L, respectively, which is equivalent to conversion molar yields of 51 and 59%. In both cases, vanillic acid is assimilated by the cells.

The mutant 10D3 is tested in a free-cell reactor and compared with the wild strain. The latter does not degrade vanillic acid immediately, whereas the mutant 10D3 does, without lag (data not shown). However, this mutant was not able to grow with vanillic acid as its sole source of carbon.

In order to find a mutant really unable to use vanillic acid, a new and original screening test is made (*see* second experiment of screening in Screening Methods). At first, the 1920 cells mutagenized and ranked in collection are tested with this new screening method. As expected, these 1920 mutants degrade vanillic acid.

Then, in order to deprive completely the cells of the function of vanillic acid dissimilation, other mutants are prepared by mutagenesis, on the one hand, of the mutant 10D3 and, on the other hand, of the mutant 10F11. Lastly, 1920 mutagenized cell colonies of each mutagenesis (death rate: 99% in both cases) are treated as the first 1920 mutants ranked in collection. The wells occupied by three mutants of the first mutagenesis (5C1, 5E1, and 5H1) and by four mutants of the second mutagenesis (12A6, 13B4, 14G4 and 19A5) assume a dark blue color after the coloring test.

In order to check if these seven mutants convert vanillin into vanillic acid and do not degrade the last one, these mutants, like both mutants of the first mutagenesis (10D3 and 10F11) and the wild strain, are used in an experiment of free-cell bioconversion. The results of these vanillin transformations are shown in Table 2. Although the mutants 5C1, 5E1, 5H1, 12A6, and 13B4 convert vanillin more slowly than the mutants 10D3 and 10F11 and the wild strain, the mutants 14G4 and 19A5 use vanillic acid only after a lag of 4–5 d. When immobilized in alginate beads, both these mutants degrade vanillic acid more slowly than the wild strain.

DISCUSSION

The strain BTP9 has been used in this work, because it is particularly capable of converting vanillin (a readily available phenolic compound often used in the food processing industry) into vanillic acid. To improve this bioconversion, we have compared free-cell reactors with immobilized-cell reactors, studied the influence of residence time and cell concentration of the alginate beads in the reactors on the conversion yield, and searched for mutants catabolically blocked for the degradation of vanillic acid.

In our work conditions, the optimal transformation rate of vanillin to vanillic acid reaches 98% for a free-cell reactor against 47% for a reactor of cells immobilized in alginate beads. Furthermore, a disappearance of vanillic acid is noticed for the immobilized cell reactor.

Table 2
Bioconversion Results (g/L) of Vanillin into Vanillic Acid as a Function of Time, with the Wild Strain and Various Mutants in Free-Cell Reactors*

	1 d		2 d		3 d		4 d		5 d		6 d		7 d	
Strain	Vanillin	Vanillic acid	Vanillin	Vanillic acid	Vanillin	Vanillic acid	Vanillin	Vanillic acid	Vanillin	Vanillic acid	Vanillin	Vanillic acid	Vanillin	Vanillic acid
Wild strain	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5C1	0.79	0.1	0.8	0.2	0.87	0.32	0.84	0.41	0.86	0.44	0.65	0.46	0	0.93
5E1	0.49	0.54	0.42	0.62	0.05	1.07	0	1.13	0	0.84	0	0	0	0
5H1	0.79	0.15	0.8	0.21	0.86	0.29	0.88	0.39	0	1.17	0	0.95	0	0.48
10D3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12A6	0	0.6	0	0.72	0	0.78	0	0	0	0	0	0	0	0
13B4	0	0.62	0	1.14	0	0.71	0	0	0	0	0	0	0	0
14G4	0	0.95	0	0.98	0	1.14	0	1.14	0	1.13	0	0.55	0	0
19A5	0	1.1	0	1.11	0	1.16	0	1.09	0	0.66	0	0	0	0
10F11	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* OD_{540} of the reactors: 2.8.

The higher vanillic acid percentages after replacing the bioconversion media (cell recycling) in the immobilized cell reactors attest an improvement of the production process owing to a lower vanillic acid dissimilation or a delayed release of vanillic acid from the alginate beads (polar environment) by comparison with the free cells or both together. Nevertheless, Fig. 4 shows vanillic acid is still degraded by the cells. This degradation increases with both residence time of the beads in the bioconversion medium and cell concentration of the reactors.

Thus, the main limiting factor of this reactor is the vanillic acid degradation. Another limiting factor is the low productivity owing to a bioconversion inhibition by the substrate. To overcome these limitations, mutants were searched.

The inability to select a mutant that can grow on a mineral medium with 0.4% vanillin as sole growth substrate leads us to think that, at this concentration, vanillin inhibits some vital cellular activities. On the other hand, six mutants unable to grow on vanillic acid and/or protocatechuic acid as sole sources of carbon, but growing on glycerol were isolated. Four mutants grew on vanillic acid, but did not on protocatechuic acid. This result is, at first sight, surprising because vanillic acid is the catabolic precursor of protocatechuic acid (*20*). That can be explained by the fact vanillic acid when transformed to protocatechuic acid releases formaldehyde. It is known that *Pseudomonas* are able to grown on monocarbon compounds as substrates (*21*).

The mutants 10D3 and 10F11, although unable to grow on vanillic acid, still metabolized vanillic acid. Because the mutant 10D3 uses vanillic acid without lag comparatively to the wild strain, it can be said that it is probably a gene regulating the lignolytic pathway, which is affected.

To select a mutant suitably affected in the degradation of vanillic acid, we have proposed a new and original screening method. The objective of this test has been to draw nearer to the conditions of bioconversion. This selection has been done with the mutagenized cells obtained from a new mutagenesis of the mutants 10D3 and 10F11. This method has excellent reproductivity and very high sensitivity. This test on microtitration plates could be extended to other mutant screenings in liquid medium (good sensitivity) via the suitable coloring tests. It could, for example, allow the screening in liquid medium of a good many mutants with regard to their ability to oxidize—and not specially to grow on—other aromatic substances.

The mutagenesis of the mutants 10D3 and 10F11 results in only two mutants able to convert vanillin into vanillic acid, like the wild type. These are affected in their ability to degrade vanillic acid, yet do not lose it completely.

These results suggest that, in the strain BTP9, several enzymes coded by distinct genes are involved in the degradation of vanillic acid. Indeed, although the mutants 14G4 and 19A5 are unable to grow on vanillic acid

and their ability to degrade this phenolic compound is impaired, these two mutations do not prevent the vanillic acid degradation. This problem could be solved by finding other kinds of reactors (e.g., "extractive bioconversion"); vanillic acid could then be extracted from the bioconversion medium as it forms, yet before it dissimilates.

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