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Bioconversion of Vanillin to Vanillyl Alcohol in a Two-Phase Reactor

I. Parameters Improvement

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ABSTRACT

One of the next challenges in the use of biocatalysts (enzyme or microbial cells) is the upgrading of biological reactions of oxidoreduction. The oxidoreductases need cofactors that must be regenerated. Practical experience shows that this is most readily achieved by using living cells of microorganisms.

Living cells of *Saccharomyces cerevisiae* are able to bioconvert vanillin to vanillyl alcohol (*1*). By working with a two-phase reactor (dodecanol—feeding medium) it has been possible to use higher vanillin concentrations without inhibiting the bioconversion (*2*).

Several parameters, such as, volume ratio of aqueous over organic phase, pH, vanillin concentration seem to influence the bioconversion greatly. Bearing this is mind, two-phase reactors have been set up. Productivities exceeded 5000 g/m3/d.

On the other hand, *Saccharomyces cerevisiae* mutants have been selected as vanillyl alcohol hyperproducers: vanillyl alcohol productivity of the best selected mutant is twice as high as wild-type strain productivity. Their specific behavior has been studied.

Index Entries: Vanillin; vanillyl alcohol; two-phase reactor; *Saccharomyces cerevisiae;* reduction.

INTRODUCTION

Oxidoreduction reactions are of great importance in chemical synthesis today. For example, reduction of ketones and double-bond compounds

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gives, in particular conditions, stereospecific compounds (*3*). Moreover, various pheromones and biological active molecules are chiral.

Their chemical synthesis requires numerous reaction and purification steps, which are very expensive. The enzymatic synthesis gives the required molecule in one step because of enzyme specific activity. However, oxidoreductases require particular cofactors, such as NAD(P)H, which are expensive to regenerate. Living cell utilization can solve this problem: The general metabolism of the microorganism is diverted to the cofactor regeneration, especially in immobilized cell reactors, where growth and bioconversion steps are independent (*4*).

Several chiral chemicals can be produced with living cells of *Saccharomyces cerevisiae*, such as α-tocopherol (*5*), steroids (*6*), polyoxoantibiotics (*7*), L-olivomycose (*8*), pheromones like S(+)sulcatol (*9*), L-carnitin (*10*), chiral synthons (*11*), or β-ketoesters (*12–14*).

On the other hand, most enzymatic reactions are inhibited by the substrate and/or the product of the reaction. Two-phase reactor (extractive fermentation) utilization avoids this by controlling substrate diffusion and product recovery (*15–19*).

In this context, we have studied the reduction of vanillin to vanillyl alcohol by living cells of *Saccharomyces cerevisiae* (*1*) in two-phase reactor (dodecanol—feeding medium).

This reduction reactor design has several useful characteristics: utilization of living cells (NADPH regeneration), selection of hyperproducing mutants (bioconversion improvement), alginate beads inclusion (growth and bioconversion separation), and two-phase reactor techniques (which avoid bioconversion inhibition by the substrate vanillin).

We have studied in this paper the influence of cellular concentration and volume ratio of alginate beads over total volume and the influence of solvent choice, pH, vanillin concentration, and volume ratio of aqueous over organic phase influence. Optimized reactors productivities are also reported and illustrate the improvement that we have achieved.

This paper is the first step in setting up a continuous two-phase reactor for vanillin bioconversion.

MATERIALS AND METHODS

Strains

Saccharomyces cerevisiae 1278b as wild type strain (*20*). Selected mutant of the wild type strain (mutant 13).

Chemicals

Chemicals are of analytical grade.

Culture Media

Bactopeptone 1%, yeast extract 1%, glucose 2%, agar 1.5% was used as Petri dish medium (868 medium). Yeast nitrogen base (DIFCO) 0.15%, ammonium Sulfate 0.5%, and glucose 2% was used as a basic medium (SC medium). This medium was buffered in a pH range of 3.5–7.0 (acetate buffer or imidazole chloride buffer).

Mutagenesis and Screening Method

The ethyl methane sulfonate mutagenesis was conducted according to Thonart (*21*). Mutants were spread on petri dishes of 868 and of 868 0.25% vanillin. After 48 h of incubation, petri dishes of 868 0.25% vanillin were revealed with a saturated solution of thiobarbituric acid (in HCl 0.1 *N*). After 30 min, vanillin gives a red complex with thiobarbituric acid. The size of the uncolored halo (where vanillin is bioconverted) surrounding the yeast colonies is characteristic of the bioconversion yield of the screened mutants.

Immobilization Techniques

Induced yeast cell suspensions (various cell concentrations) were mixed with 4% sodium alginate in equal volume. This mixture was dropped in a $CaCl_2$ solution. The globular mixture, which is like beads remained 1 h in the solution before being filtrated. Afterward, the beads were placed in reactors.

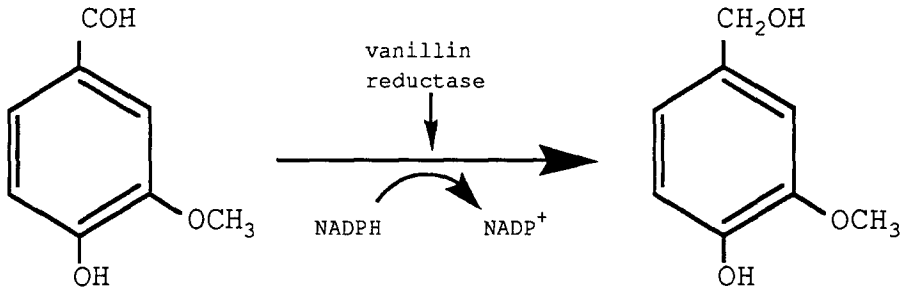
Noncontinuous Reactors

Alginate beads were placed in one or two-phase reactors. One-phase reactors contained 0 to 200 mL alginate beads, 100 mL SC medium and 0–0.05% vanillin.

Two-phase reactors contained 30 mL alginate beads, SC medium and dodecanol to attain 100 mL. (Volume ratio of aqueous over dodecanolic phase varied from 1/0 to 1/3. Total vanillin concentrations varied from 0 to 6 g/L.) Reactors were incubated at 29°C in an orbital shaker (Gallenkamp).

Continuous Reactors

One-phase reactors contained 150 mL beads (OD 10 at 540 nm or 3.9 mg dry matter per mL)) and 250 mL medium. Fresh feeding medium (SC) was added continuously while an equal volume of used medium was removed. Vanillin concentration was 4 g/L in the feeding medium. Reactors were incubated at 29°C in an orbital shaker (Gallenkamp).



vanillin vanillyl alcohol

Fig. 1. Sketch of the reduction of vanillin to vanillyl alcohol by vanillin reductase (intracellular enzyme of *Saccharomyces cerevisiae*).

Vanillin, Vanillyl Alcohol, and Vanillic Acid Assay

This HPLC method using a reverse phase technique (C8) has been described previously (*1*).

Cell Concentration Assay

Cell concentrations are measured by turbidimetry. 1 U of Optical Density (OD) at 540 nm equals 0.39 mg/mL dry matter.

RESULTS

Saccharomyces cerevisiae is able to bioconvert vanillin to vanillyl alcohol. This reduction is catalyzed by an inducible enzyme, vanillin reductase. This enzyme requires a specific cofactor NADPH (Fig. 1) (*1*).

To improve this bioconversion, we have selected hyperproducing mutants; optimized bioconversion reactors; and observed the effects of these improvements in optimized reactors.

Mutants Screening

Thanks to the screening method that we have set up in our laboratories, coupled with a chemical mutagenesis (ethyl methane sulfonate), several interesting mutants have been screened.

This screening method is based on the specific coloring of vanillin by thiobarbituric acid (they form together a red complex). The size of the uncolored halo surrounding the colonies (where vanillin was bioconverted) is a function of the bioconversion ability of the mutants.

One of these mutants, mutant 13, shows a bioconversion ability twice as high as the one of the wild strain (Fig. 2). Moreover, this mutant shows a lower bioconversion inhibition by the substrate vanillin and a faster

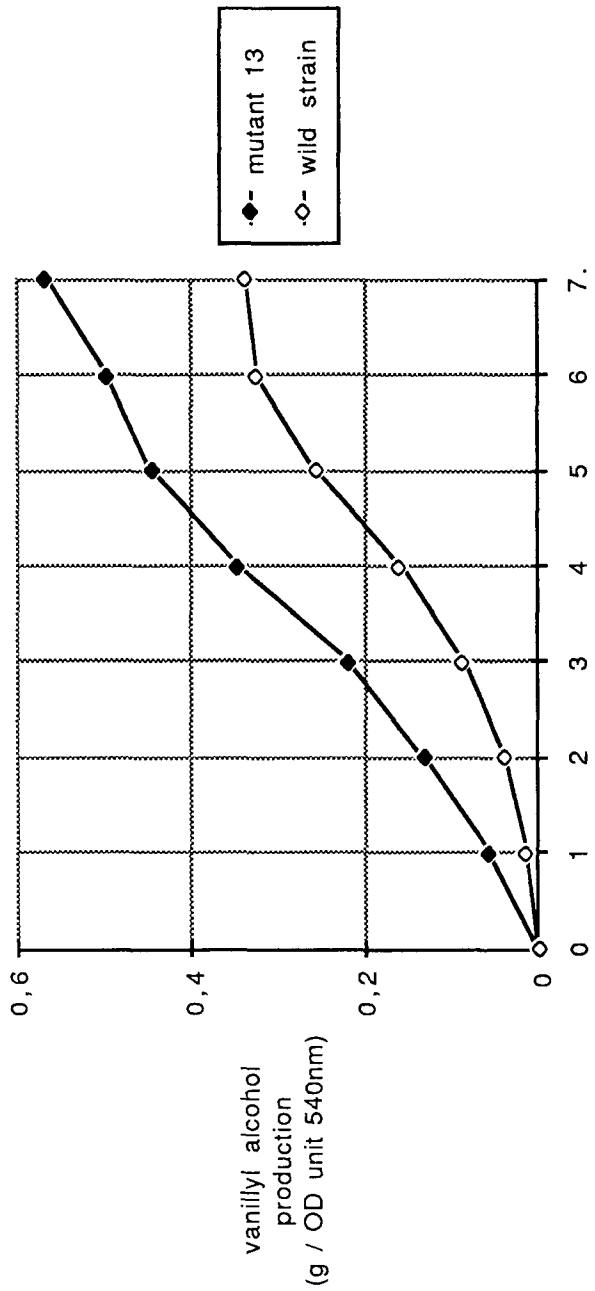


Fig. 2. Bioconversion of vanillin to vanillyl alcohol by *Saccharomyces cerevisiae* (wild type strain and mutant 13). Production (g vanillyl alcohol per optical density 540 nm) during the first 7 h of culture (free-cell reactor, 29°C, vanillin 0.5 g/L).

enzyme induction (data not shown). It can bioconvert vanillin concentrations as high as 6 g/L and, 3 h of induction are sufficient to attain the maximum level of enzymatic activity (compared to 6 h for the wild type strain).

Two-Phase Reactor Optimization

Immobilization Improvement

In order not to be inhibited by the solvent, *Saccharomyces cerevisiae* needs to be immobilized (especially by inclusion in alginate beads). Cellular concentration in beads and volume ratio of solid (alginate beads) over solid and liquid phases are the two most important parameters. Both of them have been studied. We can see that a cellular concentration of 10 (expressed in optical density at 540 nm), i.e., 3.9 mg dry matter per mL and a volume ratio of solid over solid and aqueous phases of 0.55 give an optimal bioconversion yield (Figs. 3 and 4). We shall use these values in the optimized reactor.

Solvent Screening

Several solvents have been screened for toxicity and bioconversion inhibition assessment. Among them (dibutyl phtalate, 2-ethyl 1,3-hexanediol, methyl crotonate, isoamyl acetate, ethyl butyrate, dodecanol), only one, dodecanol, combines good cell viability and good bioconversion yield (lowest inhibition by the substrate and the solvent). That is why we have studied the behavior of two-phase reactors with dodecanol as organic solvent.

Influence of Temperature, pH, Vanillin Concentration, and Volume Ratio of Aqueous over Organic Phase

We have optimized the following parameters in two-phase (dodecanol—feeding medium) reactors: temperature, pH, vanillin concentration, and volume ratio of aqueous over organic phase.

Temperature did not influence significantly the bioconversion yield (whereas in one-phase reactor an optimum was observed at 29°C). However, experiments were carried out at 29°C, because the melting point of dodecanol is around 25°C.

Figure 5 expresses the influence of pH, vanillin concentration, and volume ratio of aqueous over organic phase. For every vanillin concentration and for every volume ratio, we have only reported the highest productivities that were reached and the pH at which they were attained.

The optimum was at pH 7, vanillin 4 g/L, and a volume ratio of aqueous over organic phase of 1/1. It should be noted, too, that because the distribution coefficient (which represents the ratio of the product concentration in the aqueous phase over the product concentration in the organic phase) of vanillin is reduced with a pH decrease (Fig. 6), an increase in the

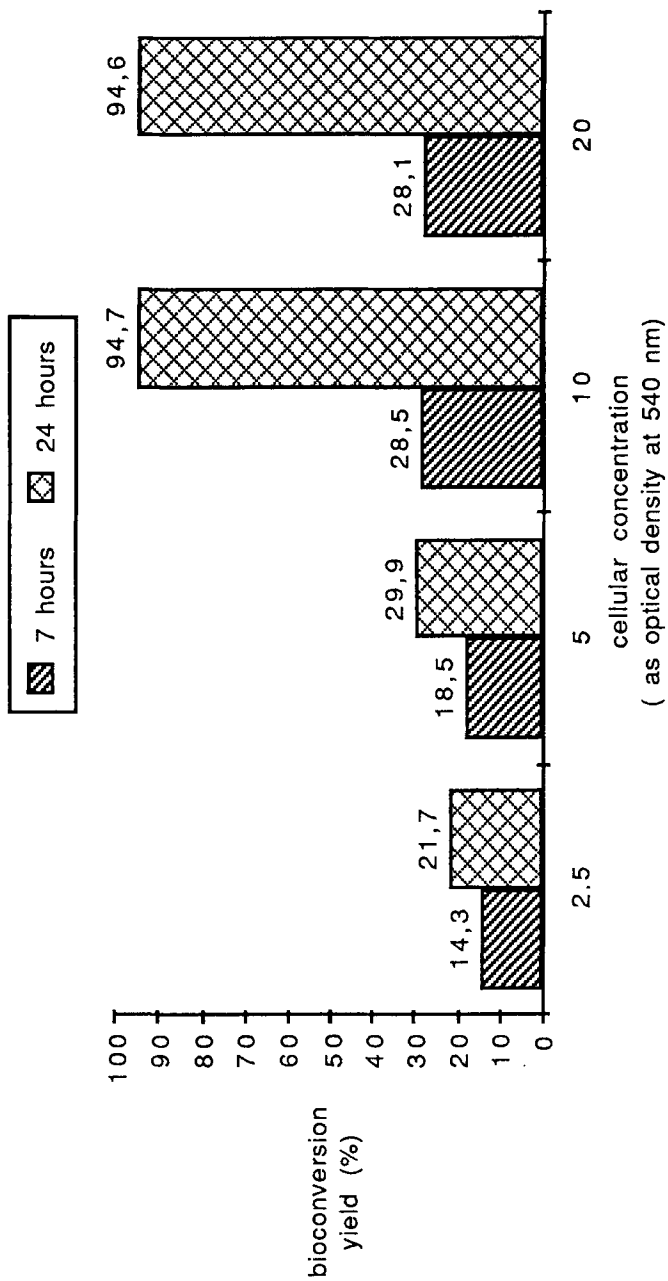


Fig. 3. Influence of cellular concentration in alginate beads on bioconversion yield (percent vanillin bioconverted) after 7 and 24 h of bioconversion in one-phase reactor (immobilized-cell reactor, 29°C, vanillin 2 g/L).

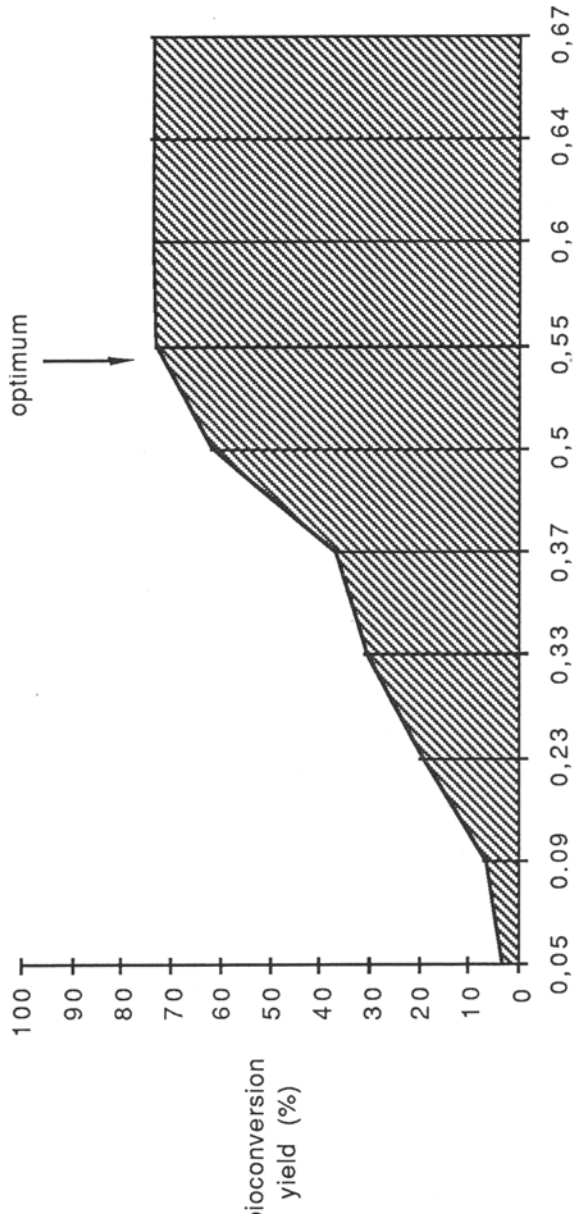


Fig. 4. Influence of the volume ratio of alginate beads over total volume on the bioconversion yield (percent vanillin bioconverted) in one-phase reactor (immobilized-cell reactor, 29°C, vanillin 2 g/L).

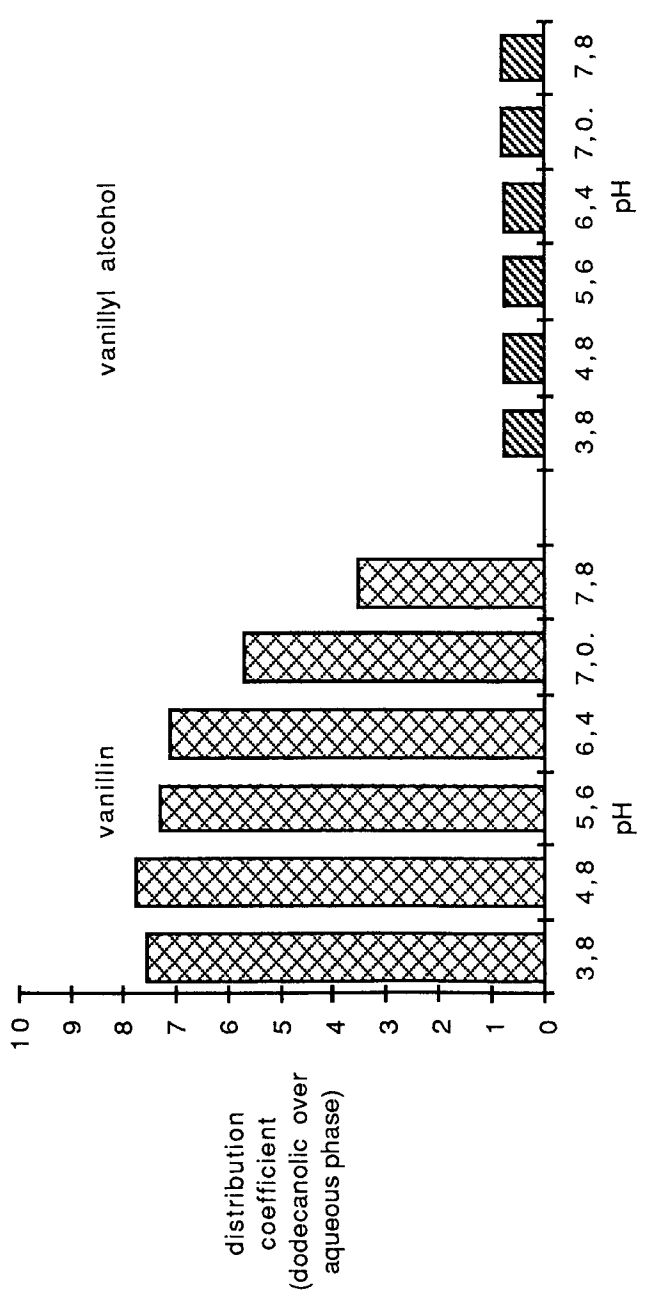


Fig. 5. Influence of pH, vanillin concentration, and volume ratio of aqueous over organic phase on the vanillyl alcohol productivity (g/L of reactor/d) in noncontinuous two-phase reactor (29°C). Productivities at optimal pH values are only reported.

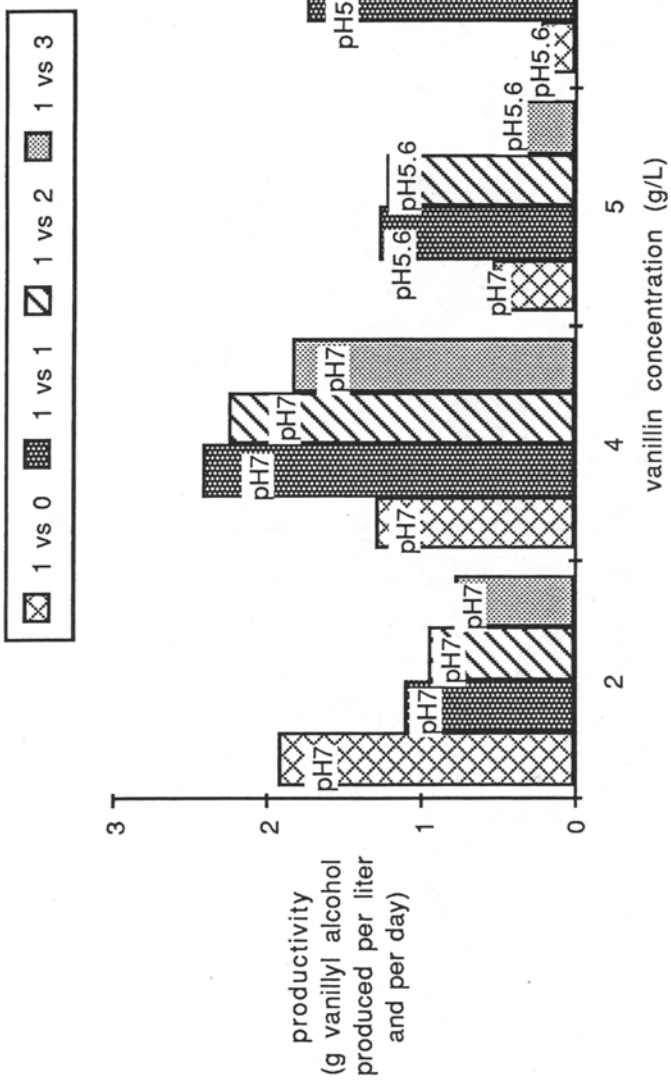


Fig. 6. Influence of pH on the distribution coefficient (ratio of aqueous phase concentration over organic phase concentration) of vanillin and vanillyl alcohol.

vanillin concentration in the reactor requires a lower diffusion of vanillin through the reactor and thus a lower pH value.

It can be observed in Fig. 6 that at a vanillin concentration of 5 or 6 g/L the pH optimum is pH 5.6. We can assume that for higher vanillin concentrations, this optimum would be 3.5. On the other hand, the distribution coefficient of vanillyl alcohol does not change significantly with pH (Fig. 5).

Optimized Reactors

Bearing in mind this parameter optimization, we have set up optimized two-phase (noncontinuous) and one-phase (continuous) reactors.

We report in Fig. 7 the productivities of two continuous one-phase reactors, respectively, with flow 8 and 45 mL/h. Although bioconversion yields (percent of vanillin bioconverted) did not change significantly, a flow increase from 8 to 45 mL/h raised the productivity dramatically (g vanillyl alcohol produced/L of reactor/d). Productivities attained in continuous one-phase reactors (vanillin concentration 3 g/L, flow 45 mL/h) *6.8 g vanillyl alcohol/L of reactor/d.*

On the other hand, we have set up noncontinuous two-phase reactors in optimized conditions (pH 7, vanillin concentration 4 g/L, volume ratio of aqueous over organic phase 1/1 and temperature 29°C. In these conditions, productivities varying from *3.7 to 4.1 g vanillyl alcohol produced/L of reactor/d* were reached.

DISCUSSION

To produce industrially specific chemicals by bioconversion, several conditions have to be met: the selection of a suitable microorganism (muted or not) for this bioconversion, the means to set up simple reactors for a long period of time, the ability to reduce the bioconversion inhibition by the substrate and/or the product, and relative ease in recovering the product.

The selection of a microorganism (mutant or not) is the first step in the optimization pattern. The selection of a hyperproducing mutant could increase the bioconversion yield 2–10-fold.

The other conditions are met in the setting up of two-phase reactors using immobilized cells. The immobilization, indeed, allows the separation of growth and bioconversion. Moreover, with this technique, the same cells (immobilized in optimal conditions) can be used for a long period of time without any change of the bioconversion yield (up to 250 h, in our case) (*21*).

The use of an organic solvent in which the substrate and the product are preferentially solubilized provides the opportunity to reduce the in-

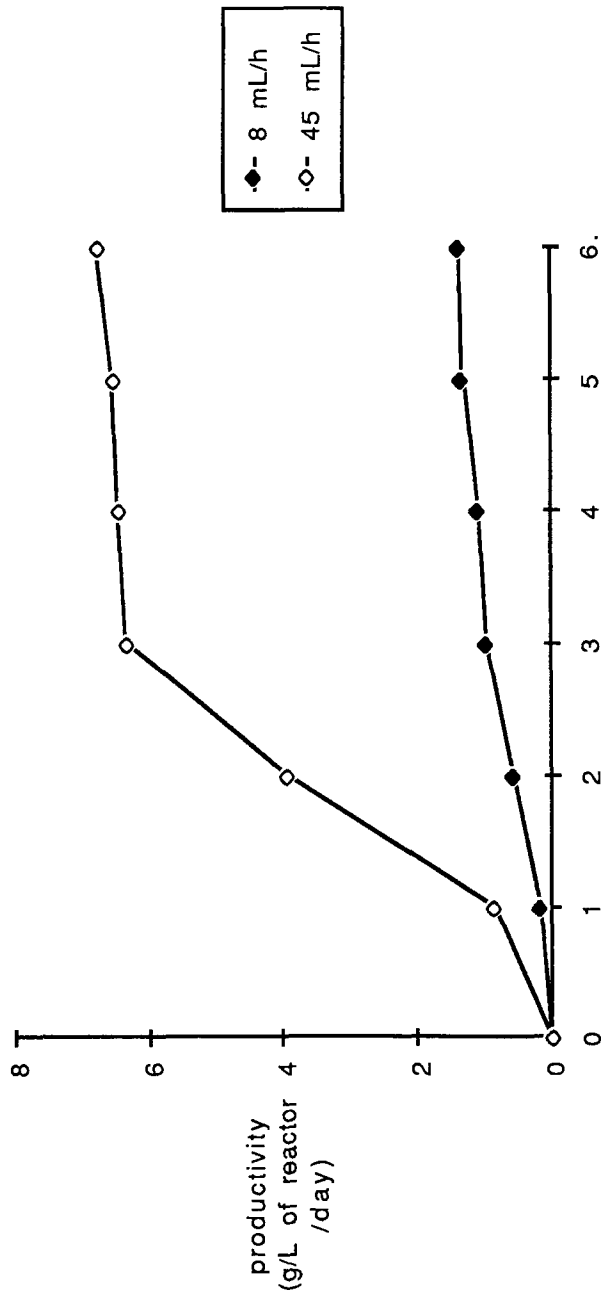


Fig. 7. Bioconversion of vanillin to vanillyl alcohol in continuous one-phase reactor. Influence of medium flow on the vanillyl alcohol productivity (g/L of reactor/d) (29°C, vanillin 2 g/L).

hibition of the bioconversion and to recover the product in the organic phase. Moreover, the product could be concentrated in this phase under specific conditions.

It is important to point out that the solvent choice must be repeated for every chemical that has to be bioconverted. The distribution coefficient could vary in a significant way from one solvent to another and from one chemical to another. However, the optimization pattern that we have followed is a good example for other bioconversions involved in the production of chemicals.

Next, we review the results of the optimization of the bioconversion of vanillin to vanillyl alcohol in two-phase reactors.

The mutant we have selected presents a bioconversion yield of vanillin twice as high as that of the wild type strain. Moreover, this mutant shows a lower bioconversion inhibition by the substrate and a faster enzyme induction. This behavior could be explained by a mutation affecting either the enzyme expression or the enzyme regulation. It is also possible that a mutation could affect cell permeability.

In the case of vanillin bioconversion, working in a two-phase reactor presents several advantages, such as, cutting down substrate inhibition and increasing productivity. It is very difficult to measure and to quantify the diffusion of the substrate from the organic phase to the cell, which is, with the substrate inhibition, the most important parameter. Indeed, the diffusion is the result of several interrelated parameters like vanillin concentration, pH (influence on the distribution coefficient), and volume ratio of aqueous over organic phase. PH influences the distribution coefficient of vanillin (Fig. 4); the volume ratio of aqueous over organic phase and the vanillin concentration influence the amount of vanillin that diffuses from the organic solvent to the cell. In this way, the diffusion optimum is a compromise of these parameters.

It has been observed that optimal productivity was attained for pH 7, vanillin 4 g/L, and a volume ratio of aqueous over organic phase of 1/1. It must be pointed out that the optimum pH in a two-phase reactor (pH 7) differs greatly from that in a one-phase reactor (pH 3.0–3.5), which is the bioconversion optimum at cellular level (*1*). Using a two-phase reactor technique, thus can completely change certain work conditions determined in a one-phase reactor, especially if the parameters studied greatly influence the substrate diffusion. However, other conditions, such as, cellular concentration in alginate beads or volume ratio of alginate beads over total volume, are always valid. These values are a cellular concentration of 3.9 mg dry matter per mL beads and a volume ratio of solid over solid and aqueous phases of 0.55.

In optimized conditions, the following productivities have been reached. In noncontinuous two-phase reactor, vanillyl alcohol productivity attained 4 g/L of reactor/d. In continuous one-phase reactor, productivity came to up to 6.8 g/L of reactor/d (Fig. 8).

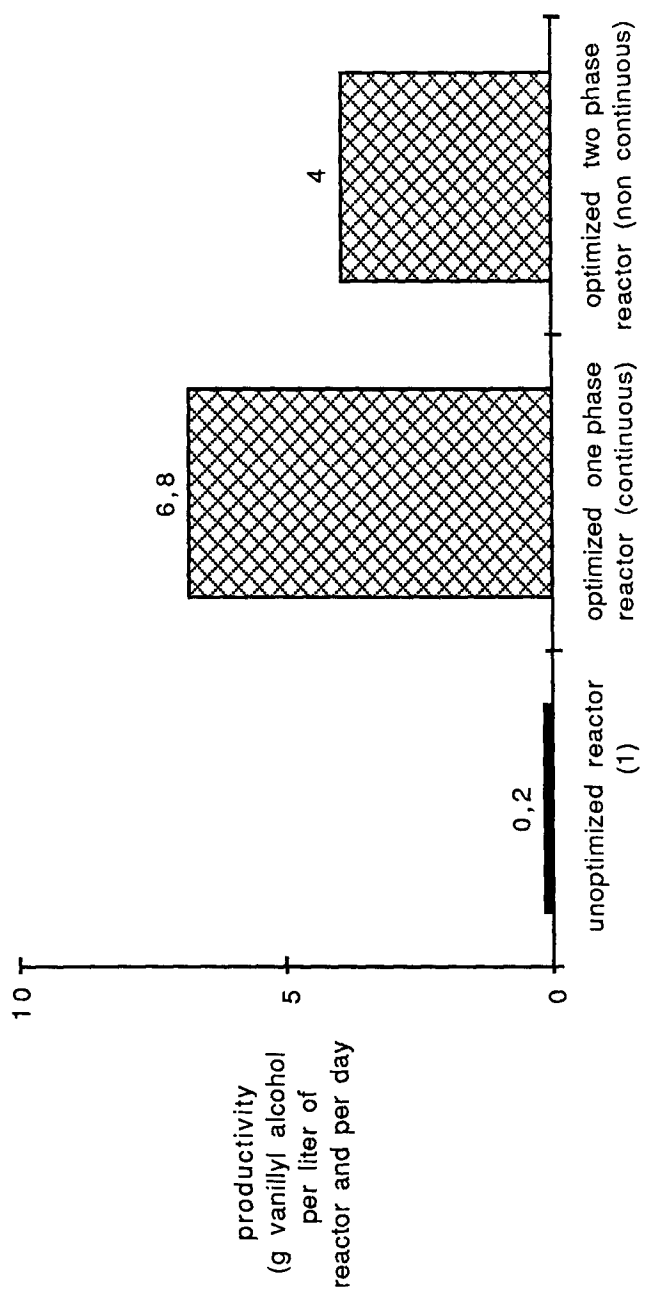


Fig. 8. Comparison of vanillyl alcohol productivities (g/L of reactor/d) of unoptimized reactor (*1*), optimized continuous one-phase reactor and optimized noncontinuous reactor.

Bearing this in mind, and remembering that our mutant strain can bioconvert twice as much efficiently as the wild type strain, we may expect in a continuous two-phase reactor to reach still higher productivities. Moreover, if we compare these results with the performance of our first nonoptimized reactor (*1*), we have already increased the productivity (from 1 to 35), and we expect to increase it by a factor of 50–100 in a continuous two-phase reactor.

This new kind of bioreactor makes it possible to work with chemicals that are either nearly insoluble in aqueous medium or strongly inhibitory. However, the use of living cells implies several limitations: The substrate must penetrate the cell to be bioconverted and the product must leave the cell without being metabolized. In these conditions, two-phase reactors, using living cells, provide the answer for bioconversion, implying cofactor regeneration.

REFERENCES

1. De Wulf, O., Thonart, Ph., Gaignage, Ph., Marlier, M., Paris, A., and Paquot, M. (1986), Biotechnol. Bioeng. Symp. No. 7, pp. 605–616.
2. De Wulf, O., Renaud, M., and Thonart, Ph. (1987), Poster presented at III seminar on enzymatic hydrolysis of biomass, Maringa, Brazil.
3. Chen, C., Zhou, B., Girdaukas, G., Shieh, W., Vanmiddleworth, F., Gopalan, A., and Sih, C. (1984), *Bioorg. Chem.* **12,** 98–117.
4. Thonart, Ph., Paquot, M., Baijot, B., and Dalemans, D. (1983), *Annales de Gembloux,* **89,** 221–237.
5. Fuganti, C. and Grasselli, P. (1982), *J. Chem. Soc., Chem. Commun.,* (4), 205–206.
6. Lanzilotta, R., Bradley, D., and Beard, C. (1975), *Appl. Microbiol.* **29** (2), 427–429.
7. Akita, H., Furuichi, A., Koshiji, H., Horikoshi, K., and Oishi, T. (1982), *Tetr. Lett.* **23** (39), 4051–4054.
8. Fuganti, C., Grasselli, P., and Marinoni, G. (1979–1980), *Tetra. Lett.* (13), 1161–1164.
9. Mori, K. (1981), *Tetr.* **37,** 1342.
10. Zhou, B., Gopalan, A., Vanmiddleworth, F., Sieh, W., and Sih, C. (1983), *J. Am. Chem. Soc.* **105,** 5925–5926.
11. Hirama, M., Shimizu, M., and Iwashita, M. (1983), *J. Chem. Soc., Chem. Commun.* 599–600.
12. Brooks, D., Kellogg, R., and Cooper, C. (1987), *J. Org. Chem.* **52,** 192–196.
13. Nakamura, K., Miyai, T., Nozaki, K., Ushio, K., Oka, S., and Ohno, A. (1986), *Tetr. Lett.* **27** (27), 3155–3156.
14. Sakai, T., Nakamura, T., Fukuda, K., Amano, E., Utaka, M., and Takeda, A. (1986), *Bull. Chem. Soc. Jpn.* **59,** 3185–3188.
15. Minier, M. and Goma, G. (1981), *Biotechnol. Bioeng.* **24,** 1565–1579.
16. Frank, G. and Sirkar, K. (1985), *Biotechnol. Bioeng. Symp.* **15,** 621–631.
17. Honda, H., Taya, M., and Kobayashi, T. (1986), *J. Chem. Eng. Jpn.* **19,** 268–273.

18. Bar, R. and Gainer, J. L. (1986), *Biotechnol. Bioeng. Symp.* N. 6, poster N.8.
19. Roffler, S., Blanch, H., and Wilke, C. (1984), *Trends Biotechnol.* **2** (5), 129–136.
20. Grenson, M., Mousset, M., Wiame, J., and Bechet, J. (1966), *Biophys. Biochem. Acta* **127,** 325–338.
21. Thonart, Ph. (1976), PhD thesis, Faculté des Sciences Agronomiques de l'Etat, 5800 Gembloux, Belgium.