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Bioconversion of a L-Carnitin Precursor in a One- or Two-Phase System

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ABSTRACT

The ability of the yeast *Saccharomyces cerevisiae* to bioconvert stereoselectively octyl-4-chloroacetoacetate (OCA) into the corresponding chiral alcohol, precursor of L-carnitin, an important physiological agent, was investigated.

In a monophasic system with free cells, more than 90% of OCA (0.018 *M*) bioconversion have been reached after 6 h (enantiomeric excess for the R form, ee_R:97%). Immobilized cells in alginate beads were less efficient in conversion of OCA than free cells.

In a two-phase system with free cells, the level of reduction of OCA (0.018 *M*) reached 85% after 48 h. With a medium containing a higher OCA concentration (0.270 *M*), 41% of this product were bioconverted after the same period. On the other hand, immobilized cells did not show any significant bioconversion of OCA in two-phase reactors.

The limiting factor of these reactors is the regeneration of the cofactors involved in the OCA reduction.

Index Entries: L-Carnitin precursor; *saccharomyces cerevisiae;* reduction; immobilized cell reactor; two-phase reactor.

INTRODUCTION

This last decade, there has been a surge of enthusiasm for carnitin, a molecule implicated in oxidation of fatty acids, especially in therapeutic applications, particularly in the treatment of heart diseases and muscle

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disorders (*1*). Because D-carnitin is a competitive inhibitor of L-carnitin acyltransferases (*2*) and can deplete the L-carnitin level of heart tissue, L-carnitin has been recommended for replacement therapy (*3*). The stereoselective step is, therefore, the key step in the production of L-carnitin.

If new "chemical" catalysts, especially organometallic compounds, are used in asymmetric synthesis, their use remains limited because of their cost and their greatly empirical action (*4,5*).

On the other hand, oxidoreductases are particularly suited for synthetic applications because they accept a broad range of compounds as substrates, and many of them are highly enantioselective (*6–8*).

In this investigation, we have exploited, in vivo, the unique chiral catalytic properties of *Saccharomyces cerevisiae* dehydrogenases. Using intact cells for this synthesis, a stumbling block that is encountered is the presence of several competitive enzymes generating products of opposite configuration. This difficulty can be overcome by choosing a substrate presenting a higher affinity for the enzyme generating the desired chiral precursor of carnitin, in order to favor this reaction as opposed to the other one (*9*). It is the reason why octyl ester of chloroacetoacetic acid (OCA) gives almost exclusively octyl-4-chloro-3-hydroxybutanoate (OCHB) of R configuration. This precursor can be transformed into L-carnitin with trimethylamine and HCl (*10,11*) (Fig. 1).

Moreover, in order to optimize the asymmetric production of the β-hydroxy ester, two nonexclusive alternatives of the traditional fermentations were investigated

1. Cell immobilization in alginate beads, which ensures better stability and easier recycling of the biocatalyst.
2. Extractive fermentation, especially a water–organic solvent two-phase system that allows a lower activity loss of the biocatalyst and *in situ* extraction of the bioconverted product.

In this paper, these techniques were investigated and compared with a bioconversion of the β-keto ester. The influence of glucose and OCA concentrations on the conversion and the cofactors regeneration were also studied.

MATERIALS AND METHODS

Strains

The dry baker's yeast commercialized by Bruggeman (Belgium) was used for the bioconversion and the strain *Saccharomyces cerevisiae* 1278b (*12*) for growth assays.

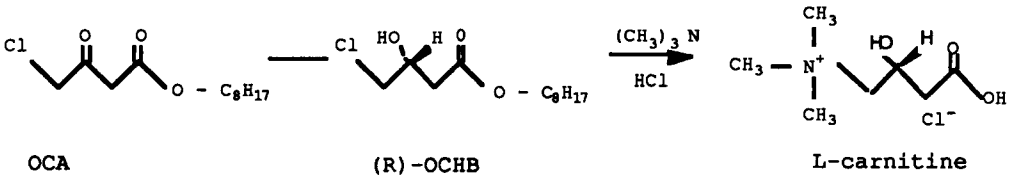

Fig. 1. Scheme of L-carnitin manufacturing, microbiological, and chemical steps.

Culture Media

The medium used for growth and bioconversion was a basic medium (SC medium) containing Yeast Nitrogen Base (Difco) 0.15%, ammonium sulphate 0.5%, and variable concentrations of glucose. Except for the free cell reactors, the media were always autoclaved. After cooling, OCA, with 0.125% chloramphenicol for the immobilized cell reactors, was added.

Growth and Viability Assays

Cell growth was measured by turbidimetry. 1 U of optical density (OD) at 540 nm was equal to 0.39 mg/mL of dry matter. Cell viability was estimated by methylene blue assay as described by Adams (*13*).

Free Cell Reactors

Each measurement of the bioconversion kinetics was carried out from a culture made by addition of 3.2 g "Bruggeman" yeast to 20 mL of SC medium containing 20 g/L of glucose. All the cultures were incubated at 30°C on an orbital shaker (Infors AG) after addition of OCA (0.018 *M* to 0.270 *M*) and dibutyl phtalate for the two-phase systems.

Immobilized Cell Reactors

Cells (160 mg/mL) in water were mixed with an equal volume of 4% sodium alginate. This mixture maintained in a cold water bath (in order to limit the fermentation during beads preparation) was dropped into 1% $CaCl_2$ solutions. The so-formed beads (about 24 g per flask) were left for 1 h in these solutions and suspended again for 10 min in 0.8% $BaCl_2$ solutions in order to make them harder. The beads were then filtered before use. In some experiments, the immobilized cells were conditioned at 30°C for 16 h in 20 mL of SC medium containing 2% glucose, 0.125% chloramphenicol and without OCA ("pretreatment"). The medium was replaced by a new SC medium with different concentrations of glucose (0% to 15%), chloramphenicol (0.125%), 0.054 *M* of OCA and, for the two-phase systems, dibutyl phtalate or dodecanol (20 mL). The immobilized cells were incubated again at 30°C in the orbital shaker (Infors AG).

Analyses of the Conversions Statement

For the monophase reactors, the reaction was stopped by extraction of the mixture with ethyl acetate (2×20 mL). The organic phase was dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness in vacuum. The residue was dissolved again in ethyl acetate for analysis. For the two-phase reactors, the bioconversion was stopped by separating, when it was necessary (centrifugation at 12,100*g* for 20 min) and by isolating the two phases. The statement of the reactions was monitored by gas chromatography (2 m, 5% FFAP on 80-100 mesh Chromosorb W-HP). Gas chromatography was performed with a Carlo Erba 452 instrument. The flow rate of nitrogen gas was 40 mL/min. The temperature programming was: 60°C for 5 min, 60–270°C (10°C/min) and 270°C for 15 min. The injector temperature was 220°C. The retention times for the ketone tautomer ($Cl\text{-}CH_3\text{-}CO\text{-}CH_2\text{-}COO\text{-}C_8H_{17}$) and the enol tautomer ($Cl\text{-}CH_2\text{-}COH=CH\text{-}COO\text{-}C_8H_{17}$) of OCA, and for the β-hydroxy ester (OCHB) were, respectively, 15.0, 18.6, and 26.2 min.

Purifying of the β-Hydroxy Ester

The residue obtained by evaporation of ethyl acetate after extraction was chromatographed over a silica gel column (110 g of MN-Kieselgel 60 (0.05–0.2 mm; 70–270 mesh ASTM) (2×70 cm), eluted with a solvent mixture consisting of hexane–ethyl acetate 10:1. Fractions of 20 mL were collected and analyzed by TLC (60–silica gel plates, 0.25 mm, Merck) with hexane–ethyl acetate 5:1. The spots of OCHB and enol were revealed by spraying the TLC plates with 0.1 *M* $K_2Cr_2O_7$ in 1*M* H_2SO_4 solution, and by heating the plates at 120°C for 30 min. Only the spots of enol became visible as revealed with 2% $FeCl_3$ as spray reagent.

Reduction of OCA by $NaBH_4$

Sodium borohydride (17 mmol) was dissolved at 0°C in 100 mL of tetrahydrofuranne with the β-keto ester (8.5 mmol). This mixture was left stirring for 3 h at room temperature. The reaction was quenched with saturated ammonium chloride solution (200 mL) and extracted with ethyl acetate (2×250 mL). The organic extracts were combined, dried ($MgSO_4$), filtered, and evaporated. The resultant OCHB was purified as described previously.

Enantiomeric Composition Determination

The optical purity of the β-hydroxy ester was determined by GC-MS analyses of its (R)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) esters. 50 mg of (R)-(+)-MTPA Cl was added to 50 mg of purified OCHB (*14*). After stirring for 1 min, 1 drop of anhydrous pyridine was added and the mixture was stirred for 72 h. The contents were diluted with 1 mL of water and extracted with ether (3×3 mL). The ethereal layer

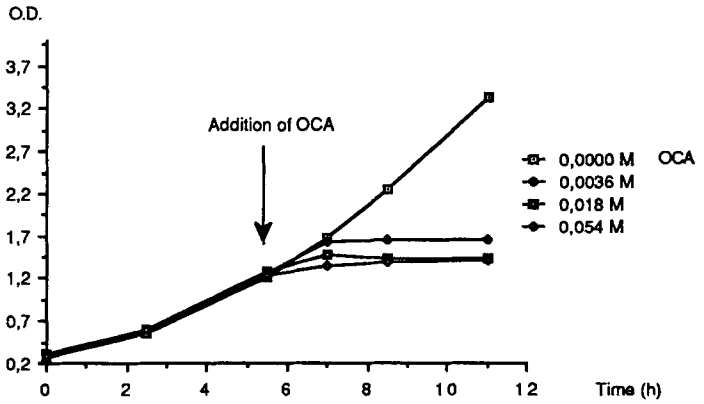

Fig. 2. Evolution of cell mass concentration as a function of time without and with addition of OCA at 3 C.

was washed with 10 mL of 1% HCl solution and then with water until neutral. After drying over $MgSO_4$, the ether layer was evaporated to dryness. The resultant (R)-(+)-MTPA esters were analyzed by gas chromatography (SPB5 capillar column, internal diameter, 0.25 mm, film thickness, 0.25 μm, length, 30 m) coupled with mass spectrometer (VG Analytical 7070 EQ; electron ionization).

RESULTS

Growth and Viability Assays

The effects of OCA on the growth and the viability of the bakers yeast have been investigated.

Cell growth is estimated by OD measurements at 540 nm for 11 h of culture. OCA is added to three cultures during their exponential phase (Fig. 2). With the addition of 0.0036 *M* OCA, growth is drastically reduced.

As any conversion of β-keto ester can't be detected by gas chromatography in our working conditions, only OCA is held responsible for reducing the growth rate. Cell viability is not affected by the OCA addition as estimated by methylene blue assays (data not shown).

Bioconversion of OCA by Free Cells

Saccharomyces cerevisiae is able to bioconvert OCA to OCHB (*10*). The OCA reduction by free cells has been studied in two types of reactors (one-phase and two-phase reactors).

One-Phase Reactors

Figure 3 shows that the reaction depends on the OCA concentration. Indeed, the balance of the reaction is almost completely in favor of formation of alcohol for the lowest OCA concentration (94% of OCHB after 6 h

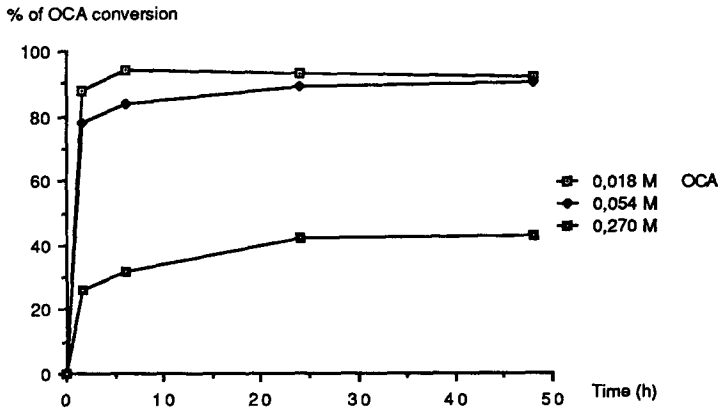

Fig. 3. Evolution of OCA bioconversion in free cell monophase reactors containing different concentrations of OCA.

for an initial concentration of 0.018 *M*) and barely reaches 40% of bioconversion for the highest OCA concentrations (0.270 *M*).

The enantiomeric composition has been determined (*see* Materials and Methods) for OCHB obtained by conversion of an initial OCA concentration of 0.018 *M* in a monophase reactor. This result has been compared with the one obtained with $NaBH_4$—reduced OCHB, also purified and treated with enantiomerically pure MTPA Cl ("control").

Figure 4 shows the computer pictures (mass chromatograms) obtained by adding the ion current recorded at regular intervals of an ion characteristic of the MTPA esters of chemically (A) and microbiologically (B) reduced OCHB (*see also* Material and Methods). Enantiomeric excess of microbiologically reduced OCHB is higher or equal to 97% (against about 25% for chemically reduced OCHB). R configuration of the enantiomer was established by polarimetry.

Two-Phase Reactors

Two-phase reactors were prepared by adding an organic phase (dibutyl phtalate) of the same volume to the monophase reactors. After 48 h, the conversion rates reach 85% for an initial OCA concentration of 0.018 *M* and 41% for an initial OCA concentration of 0.270 *M* (Fig. 5).

Bioconversion of OCA by Immobilized Cells

In order to increase stability and activity of the reactors and to improve their profitability by recycling the cells, immobilized cell reactors were set up. Cells were included in alginate beads.

Moreover, another factor may strongly influence the bioconversion. Indeed, OCA reduction is catalyzed by an alcohol dehydrogenase that requires NADPH as cofactor (*10*). The turnover of NADPH could be the limiting factor, and this turnover is in relation with the catabolism of saccharides. Thus, the influence of the glucose concentration in the medium on the bioconversion was investigated.

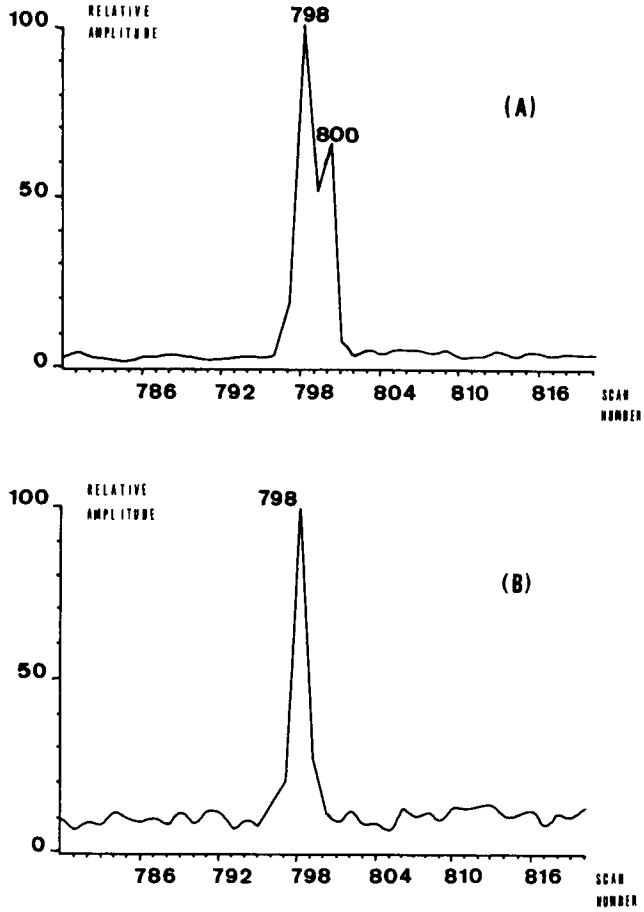

Fig. 4. Mass chromatograms of diastereoisomers of the MTPA esters of OCHB. A, chemical reduction of OCA; B, microbiological reduction of OCA.

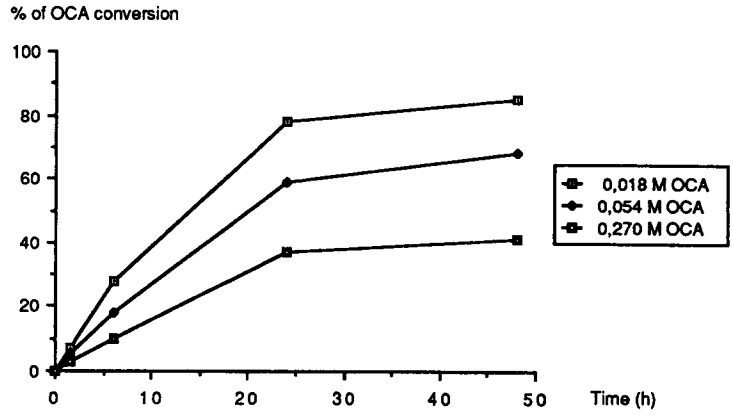

Fig. 5. Evolution of OCA bioconversion in free cell two-phase reactors containing different concentrations of OCA.

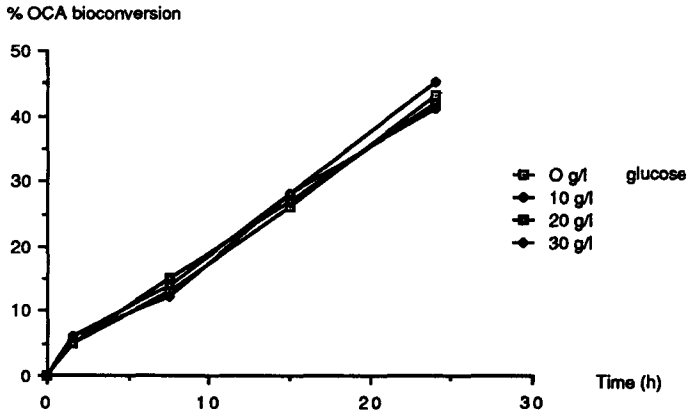

Fig. 6. OCA bioconversion by immobilized cells with various glucose concentrations.

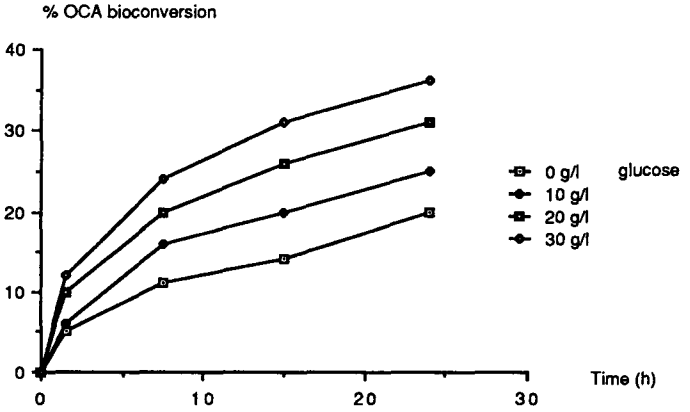

Fig. 7. OCA (initial concentration, 0,054 *M*) bioconversion by pretreated immobilized cells with various glucose concentrations.

One-Phase Reactors

A first set of experiments was performed in immobilized cells reactors with SC medium containing 0 to 30 g/L of glucose and 0.054 *M* of OCA. Measurements of these bioconversion kinetics are shown in Fig. 6.

Unlike the production curves observed with free cells, the reduction of the β-keto ester is linear with immobilized cells, from 0 to 24 h. Nevertheless, bioconversion is lower since, after 24 h, yield reaches about 43% only (for 89% with free cells).

A second set of experiments was carried out by conditioning the beads in SC medium (20 g/L of glucose) without OCA for 16 h before the bioconversion step. The pretreatment of the immobilized cells shows that OCA reduction is dependent on glucose concentration (from 20 to 36% of reduction after 24 h) (*see* Fig. 7). For the SC medium without glucose, the results could be explained by incomplete consumption of the saccharides during pretreatment. Nevertheless, the glucose concentration seems to

Table 1
Influence of Immobilized Cells Recycling on the Rates of OCA Bioconversion for 4 Different Concentrations of OCA

	Glucose concentrations			
4 days periods	20 g/l	50 g/l	100 g/l	150 g/l
1 st period	54 ± 2%	58 ± 2%	63 ± 2%	65 ± 2%
2 nd period	32 ± 5%	26 ± 5%	22 ± 5%	34 ± 5%
3 rd period	21 ± 5%	13 ± 5%	13 ± 5%	17 ± 5%

influence essentially the initial rate of the reduction, since the rate of the bioconversion as a function of time is independent of the glucose concentration after 7.5 h of growth.

In order to better estimate the reduction by immobilized cells, we have used SC media with a higher glucose concentration (20–150 g/L) and these media were successively replaced by new ones of identical compositions after 4 and 8 d of conversion (the beads were pretreated, and the initial OCA concentration for bioconversion was 0.054 *M*). The results are shown in Table 1.

The conversion percentages decrease at each new period of 4 d, whatever the glucose concentration may be.

Two-Phase Reactors

To prevent a possible effect of enzyme inhibition by substrate and/or by product, two-phase systems with immobilized cells were carried out with dibutyl phtalate or dodecanol as organic phases. In these cases, bioconversion efficiency is near zero, probably because of the bad transfer of the substrate (OCA) between the organic phase and the alginate beads. It is worth to notice that dibutyl phtalate and dodecanol are two polar solvents without toxicity against immobilized yeast (*15*).

Checking the Efficiency of the Cofactors Regeneration

In order to identify the limiting factor of this reaction, we modified the cell concentration for four β-keto ester and glucose concentrations (Fig. 8). This experiment was performed in monophase reactors with free cells, and OCA bioconversions were measured after 72-h period, after which no substrate reduction occurred any more. For these reactors, no significant cell growth was observed.

Figure 8 presents, for two substrate concentrations and two glucose concentrations, conversion yields obtained with various ratios "cell concentration (g/L)/OCA concentration (g/L)." In this experiment, bioconversion percentages increase with cell concentration, glucose concentration, and OCA concentration as well.

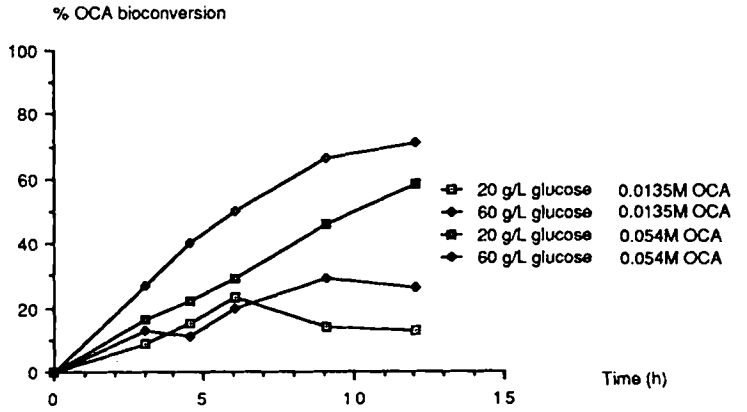

Fig. 8. Influence of cell, OCA, and glucose concentrations on OCA bioconversion yield (incubation time of the cells with OCA, 72 h).

DISCUSSION

The increasing use of only L-isomer of carnitin in medicine and in pharmacology is requiring the perfecting of an easy and efficient manufacturing process of this chiral chemical.

Whereas numerous researchers (*9,16,17*) have been looking into the making of high enantiomeric excess alcohols ("enantiomeric yield") by the means of yeast, optimal production of these alcohols ("chemical yield") was greatly neglected. This is why we have investigated the OCHB production, chiral precursor of L-carnitin, in various types of reactors.

Whereas cell viability is not affected by OCA, yeast growth is drastically reduced. Our investigations of OCA bioconversion with free cell monophase reactors has permitted us to reach conversion rates (90–95%) higher than the ones found in literature (70%) (*9*). Moreover, the enantiomeric purity of our product is near 100%.

On the other hand, productivity of the two-phase reactors (free cells) (Fig. 5) is always lower than the one obtained for the one-phase reactors (Fig. 3) after 48 h of bioconversion. Moreover, when the OCA concentration increases, the bioconversion yield decreases in the two-phase systems as in the monophase systems.

Otherwise, with two-phase reactors and with cells immobilized within alginate beads, no significant percentage of bioconversion is observed, probably because the transfer of the substrate from the organic to aqueous phase is very low.

In the immobilized cells reactor (monophase reactor), the yeast seems to use the intracellular saccharides (*18*) in preference to those of the medium in the first 24 h of bioconversion (Fig. 6). Indeed, bioconversion with immobilized cells pretreated for 16 h without OCA, is dependent on glucose concentration in the medium (Fig. 7). Nevertheless, the glucose concentration influences the bioconversion for the first 24 h of reaction, but is

not the only factor limiting the β-keto ester reduction since the conversion yield decreases as a function of time even with 150 g/L of glucose (Table 1). Summarizing, one can say that three of our results raised the question of the efficiency of the NADPH cofactors regeneration

1. The yeast growth quenching with OCA.
2. For the highest OCA concentrations, the reduction kinetics stabilize at lower conversion yields than for the other OCA concentrations (Figs. 3 and 5).
3. Glucose concentration influences the bioconversion for the first 24 h but is not the only limiting factor of the conversion.

The existence of a relationship (Fig. 8) between cell concentration and conversion yield after 72 h of bioconversion for a given OCA concentration supports the cofactor nonregeneration theory: if $NADP^+$ is not reduced, conversion stops through lack of one of two substrates necessary for the reaction.

Nevertheless, in Fig. 8, the increase of the reduction yield as a function of glucose and substrate concentration tends to prove cofactor regeneration at the beginning of bioconversion. Indeed, the greater the OCA and/or glucose concentration, the higher the $NADP^+$ recycling and the conversion. These comments are in accordance with our previous conversion kinetics.

Thus, the limiting factor of this reactor is the turnover of the cofactors involved in the OCA reduction; to overcome this limitation, physiological conditions that increase the turnover can be found.

Until now, all our experiments indicate conclusively that a free cell reactor is probably the most efficient system for OCA bioconversion.

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REFERENCES

1. Leibovitz, B. (1984), Carnitine, the Vitamine B_T Phenomenon, Dell, New York.
2. Fritz, I. B. and Schultz, S. K. (1965), *J. Biol. Chem.* **240**(5), 2188–2192.
3. Borum, P. R. (1981), *Nutrition Revs.* **39**(11), 385–390.
4. Kagan, H., Wilkinson, G., and F. G. A. (1982), Comprehensive Organometallic Chemistry, Abel, E. W., ed., Pergamon, Oxford, vol. 8, p. 463.
5. Bosnik, B. and Fryzuk, M. D. (1981), Topics in Inorganic and Organometallic Stereochemistry, Geoffroy, G. L., ed., Wiley, New York, p. 119.

6. Brooks, D. W., Kellog, R. P., and Cooper, C. S. (1986)), *J. Org. Chem.* **52,** 192–196.
7. Aragozzini, F., Maconi, E., and Craveri, R. (1986), *Appl. Microbiol. Biotechnol.* **24,** 175–177.
8. Manzocchi, A., Casati, R., Fiecchi, A., and Santaniello, E. (1987), *J. Chem. Soc. Perkin. Trans.* **1,** 2753–2757.
9. Sih, C. J., Shieh, W. R., Chen, C. S., Wu, S. H., and Girdaukas, G. (1986), *Ann. NY Acad. Sci.* **471,** 239–254.
10. Zhou, B. N., Gopalan, A. S., Vanmiddlesworth, F., Shieh, W. R., and Sih, C. J. (1983), *J. Am. Chem. Soc.* **105,** 5925–5926.
11. Sih, C. J., Zhou, B. N., Gopalan, A. S., Shieh, W. R., Vanmiddlesworth, F. (1983), Sih, Workshop conferences Hoechst-Sel. Goal. Synth. Effic. Proc. 14th, 251–261 (pub. 1984).
12. Grenson, M., Mousset, M., Wiame, J., and Bechet, J. (1966), *Biochem. Biophys. Acta* **127,** 325–338.
13. Adams, R. L. P., and Work, T. S. (1980), Cell Culture for Biochemists, Burdon, R. H., ed., Elsevier, Amsterdam, p. 261.
14. Dale, J. A., Dull, D. L., and Mosher, H. S. (1969), *J. Org. Chem.* **34**(9), 2543–2549.
15. De Wulf,O. and Thonart, Ph. (1987), *Appl. Biochem. Biotechnol.* **20/21,** 165–180.
16. Nakamura, K., Ushio, K., Oka, S., Ohno, A., and Yasui, S. (1984), *Tetrahedron. Lett.* **25**(36), 3979–3982.
17. Hirama, M., Shimizu, M., and Iwashita, M. (1983), *J. Chem. Soc. Chem. Commun.* 599, 600.
18. Nakamura, K., Inoue, K., Ushio, K., Oka, S., and Ohno, A. (1988), *J. Org. Chem.* **53,** 2589–2593.