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# Efficiency of bioconversion of steroids by *Fusarium oxysporum* into Δ1,4-androstadiene-3,17-dione using gas chromatography

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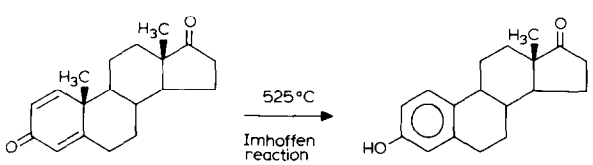
## Summary

Gas-liquid chromatography is suitable for the study of the bioconversion yield of dehydroisoandrosterone into Δ1,4-androstadiene-3,17-dione. *Fusarium oxysporum* (a wild isolated strain) exhibits the greatest activity for this bioreaction. The optimalization of the bioconversion efficiency has been studied in culture media or in buffered solutions using free and immobilized cells. Microbiological (cell weight, choice of strains and substrate, induction) and physicochemical (pH, temperature, ionic strength and choice of buffers, organic solvents, artificial cofactors) factors influencing the reaction yield are discussed. The same operating conditions can be used for free and immobilized cells. For both techniques, about 90% of Δ1,4-androstadiene-3,17-dione is formed in 48 h with non-induced cells and in 24 h with induced cells. A simple prototype of a laboratory bioreactor is employed and tested in order to describe the advantages and limitations.

## Introduction

The development and broad acceptance of oral contraceptives in the 1960s led to a great demand for 19-norsteroids, which are the chief components of the contraceptive compounds. Emphasis was placed on easily accessible steroids as precursors of these molecules, the problem being the removal of the angular methyl group at C-10. The original method for producing estrone on an industrial scale with yields over 70% consists of the pyrolysis of Δ1,4-androstadiene-3,17-dione as a mineral-oil solution at 525°C by the Imhoffen reaction (Krik-Othmer, 1983).




Δ1.4-androstadiene-3,17-dione — Estrone

Steroids have been produced by microbiological conversion for the first time in the 1950s (Perlman, 1952; Fried et al., 1953; Vischer and Wettstein, 1953; Zaffaroni et al., 1955; Shull and Kita, 1955; Hanc et al., 1957). However, with the advent of new analytical and separating methods (GC, NMR, HPLC, HPTLC,...), new fermentation processes and immobilized cell technologies,

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a recrudescence of the interest for steroids bioconversion appeared in laboratory researchs between 1975 and 1985 (Holland and Auret, 1975; Holland and Diakow, 1979; Holland and Taylor, 1980; Nambara et al., 1975, 1977; Jones et al., 1975; Zedan et al., 1976; Zedan and El-Tayeb, 1977; Kieslich, 1980; Lefebvre et al., 1980; Studebaker and Yang, 1981; Fauve and Kergomard, 1981; Broad et al., 1984; Williamson et al., 1985).

We therefore propose to develop an easier and faster method for screening those strains able to be used for bioconversion of steroids and to determine the yields. This method can be applied either to fungal suspensions in culture media or in buffered solutions as well as to immobilized cells in alginate microcarriers.

Vischer and Wettstein (1953) have shown that *Fusarium solani* can convert dehydroisoandrosterone, Δ4-androstene-3,17-dione, progesterone, 11-desoxycorticosterone and pregnenolone into Δ1,4-androstadiene-3,17-dione. Several wild strains of *Fusarium* have been tested in order to select the most active strain for Δ1,4-androstadiene-3,17-dione production. The influence of biomass, substrate, pH, temperature, organic solvent and buffer on the yield of the bioconversion has been studied. These microbiological and physicochemical factors are very similar to those affecting the biosensor response of microbial electrodes applied to analytical chemistry (Vincké et al., 1983a and b, 1984a, 1985a, b and c; Linders et al., 1985).

## Materials and Methods

*Apparatus*

An orbital incubator (100 rpm) is used for cells growth and conversion process.

A gas chromatograph (5710 A Hewlett-Packard) in conjunction with a Goërtz recorder, Model Servogor R.E. 512 with integrator is used for conversion measurements. Optimal separation conditions for the tested steroids on an 6 ft. × 1/4 in. × 4 mm glass column 3% OV-225 chrom. WHP 80-100 mesh are: detector 300°C; injector 300°C; column 240 → 260°C (0.4°C/min); carrier gas $N_2$ (70 ml/min); detector FID.

A circulating pump (L.K.B. 12,000 varioperpex) is used for continuous flow production of entrapped fungal cells in spheric alginate microbeads and for circulation of dehydroisoandrosterone (0.15 mg/ml) in buffered solution through the agar–agar gel column.

A Pharmacia (47 cm × 2.7 cm) thermostatic column is taken as a laboratory bioreactor prototype.

*Reagents*

All solutions were prepared with twice-distilled water.

All chemicals used were of analytical pure grade (Merck, Janssen, Difco).

TLC-plastic sheets silica gel 60 (layer thickness 0.2 mm, without fluorescent indicator) were used for thin-layer chromatography.

*Test organisms*

Three wild strains of *Fusarium* were chosen, namely *F. solani, F. oxysporum* and a *Fusarium sp.*

*Culture media*

(1) Stock cultures of the fungal strains were maintained at 4°C on Sabouraud Dextrose Agar (Difco) slants.

(2) Growth medium: Sabouraud Chloramphenicol Broth (SC broth) had the following composition (g/l):

| | |
|---|---|
| Neopeptone | 10 |
| Dextrose | 10 |
| Chloramphenicol | 0.2 |
| Distilled water | to 1 litre |

The medium was sterilized by heating at 121°C for 15 min.

*Cell immobilization techniques*

Two methods have been realized for *F. oxysporum* immobilization: a gel agar–agar column and alginate microcarriers.

*(1) Gel column.* 20 g of *F. oxysporum* are mixed under magnetic stirring with 250 ml of a sterile agar solution (1% w/v agar) at 45°C. After homogenization, the suspension is poured in the Pharmacia thermostatic column and cooled down.

*(2) Alginate microcarriers.* Cell entrapment in the alginate microcarriers is realized by precipita-

tion of the fungal suspension mixed with sodium alginate in a calcium chloride solution. A homogeneous suspension is made with 8 g of *F. oxysporum* in 100 ml of 2% w/v sodium alginate solution under stirring. This suspension is pumped in continuously with the L.K.B. pump (flow rate 1.5 $ml \cdot min^{-1}$) and dropped in a 4% w/v $CaCl_2$ solution. When the drop comes in contact with this solution, it forms directly an insoluble calcium alginate polymer which entraps the fungal cells in the material.

For the experiments, 22 g of those microbeads were tested in 100 ml of buffered solutions under stirring or 116 g are packed in the thermostatic column.

*Permeabilization procedure*

*F. oxysporum* cells were treated with dimethylsulfoxide after growth: washed cells were suspended at a concentration of 15 mg wet weight of cells per ml of 40% v/v $Me_2SO$. Cells were incubated at 30°C for 30 min, centrifuged and washed twice with cold distilled water.

*Methods*

The cells are grown in SC broth at 28°C under orbital agitation for 36–48 h. The cells are filtrated through a cellulose acetate membrane (Millipore HAWP 047S0, porosity 0.45 μm). The filter saturated with microorganisms is then washed with a physiological solution. A precise weight of dry cells is immobilized either in microcarriers or directly dipped in 100 ml SC broth or in buffered solution containing 15 mg of substrate in methanol at a concentration of 10 mg/ml alcohol. The conversion reactions occurred in 250 ml flasks. After 24 or 48 h the bioreaction is stopped by addition to the medium of an equal volume of methanol and subsequent filtration of the mixture.

The filtrate is then evaporated with a rotavapor, dipped again in 20 ml of bidistilled water and extracted 3 times with 50 ml of a mixture chloroform–dichloromethane (50% v/v) into a separatory funnel. The organic phase is evaporated and the residue is dissolved in 2 ml dichloromethane. 5 μl of this solution are injected into the chromatograph. The relative percentage of each steroidic compound is measured by integration.

## Results and Discussion

*Choice of the analytical method*

The problem was to choose an easy, fast, sensitive and quantitative method in bioconversion media. Furthermore compounds extracted simultaneously with the steroids such as fungal structures or metabolites must not interfere with their determination. For all those reasons, we have adopted gas-liquid chromatography, which is also the most commonly used chromatographic technique for steroids (Heftmann, 1976).

The described method allows a perfect correlation ($r = 0.9998$) between the peaks area and the steroids concentration for both the initial (dehydroisoandrosterone) and final (Δ1,4-androstadiene-3,17-dione) product in a linear range of 0.5–100%. The areas of the peaks are identical for both steroids as demonstrated by the same slope value.

As is shown in Fig. 1, the steroid peaks (1, 2, 4 and 4) are well defined and separated from the metabolites of *F. oxysporum* in SC broth, which have generally lower retention times. For each series of experiments, a corresponding blank solution is used for the identification of the peaks corresponding to the steroid compounds. These blank solutions are realized by extracting as previ-

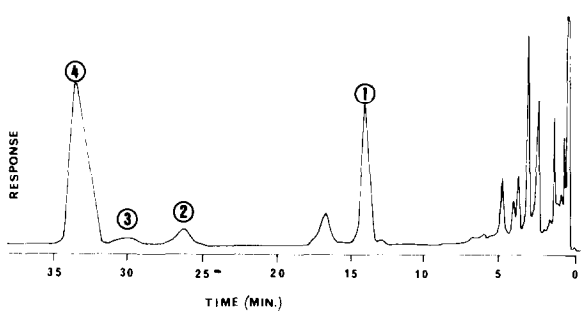


Fig. 1. Gas chromatograph of a Sabouraud Chloramphenicol Broth bioconversion medium collected after 24 h. 0.9 g % *Fusarium oxysporum* suspended, 0.15 mg/ml dehydroisoandrosterone, 28°C. 6 ft.×1/4 in.×4 mm glass column 3% OV-225 chrom. WHP 80-100 mesh: FID detector (128×10): 300°C, Injector 300°C, Column: 240 → 260°C (0.4°C/min), Carrier gas: $N_2$ (70 ml/min). 1 = dehydroisoandrosterone; 2 and 3 = steroids intermediary compounds; 4 = Δ1,4-androstadiene-3,17-dione.

TABLE 1

PERCENTAGE OF BIOCONVERSION AS A FUNCTION OF THE STRAINS CHOSEN AFTER 24 h (28°C)

| Strains | Untransformed dehydroisoandrosterone (%) | Intermediary products formed (%) | | Δ1,4-androstadiene-3,17-dione formed (%) |
|---|---|---|---|---|
| | | A * | B * | |
| *F. solani* | 61 | 8 | 5 | 27 |
| *F. oxysporum* | 34 | 4 | 4 | 58 |
| *Fusarium sp.* | 99 | 1 | 0 | 0 |

* A, B corresponding to retention times of 26.2 min (compound 2) and 29.7 min (compound 3), respectively, in Fig. 1.

ously described a filtrate of a fungal suspension without the steroids.

Another interest of gas-liquid chromatography is that two intermediates, which have not been observed by Vischer and Wettstein (1953) using thin-layer chromatography, were found to be present (peaks 2 and 3).

*Choice of strains*

Three strains of *Fusarium* have been studied in order to find the most active. *F. solani* proposed by Vischer and Wettstein (1953) is tested as well as two connected strains. The results are described in Table 1 for 0.8 g of each *Fusarium* in 100 ml of SC broth with 1.5 ml of dehydroisoandrosterone in methanol (10 mg/ml) during 24 h of bioreaction.

The total percentage for converted and unconverted products expressed in Table 1 is equal to 100% for a homogeneous description of the biotransformation process. Indeed, the described extraction procedure of four steroids (dehydroisoandrosterone, pregnenolone, testosterone and Δ1,4-androstadiene-3,17-dione) depends on the medium type: the yields of extraction are, respectively, 90–95% in SC broth and 95–98% in buffered solutions.

The reproducibility (CV%) of the yield values is about 3–5% for cell suspensions (in broth medium or in buffered solutions) and 7–10% for immobilized cell bioconversions.

Table 1 demonstrates that *F. oxysporum* is more active than *F. solani* and that *Fusarium sp.* is inactive. Therefore, *F. oxysporum* has been used in subsequent experiments.

*Choice of substrate*

Three main substrates have been studied for bioconversion, namely dehydroisoandrosterone, testosterone and pregnenolone. Experimental conditions are in this case: 0.5% w/v of *F. oxysporum* subcultured in SC broth for 24 and 48 h with 0.15 mg/ml of substrate. Results are recorded in Table 2.

TABLE 2

INFLUENCE OF SUBSTRATE ON *F. oxysporum* BIOCONVERSION YIELD (28°C)

The corresponding retention times (min) are: 13.9 for dehydroisoandrosterone; 19.0 for pregnenolone; 23.5 for testosterone; 26.2 for compound A; 29.7 for compound B and 33.4 for Δ1,4-androstadiene-3,17-dione.

| Substrate | Incubation time (h) | Dehydroisoandrosterone (%) | Pregnenolone (%) | Testosterone (%) | Intermediary compounds (%) | | Δ1,4-androstadiene-3,17-dione (%) |
|---|---|---|---|---|---|---|---|
| | | | | | A * | B * | |
| Dehydroisoandrosterone | 24 | 55 | – | – | 1 | 2 | 42 |
| | 48 | 4 | – | – | 0 | 3 | 93 |
| Pregnenolone | 24 | 4 | 93 | – | 1 | 0 | 2 |
| | 48 | 5 | 91 | – | 0 | 1 | 3 |
| Testosterone | 24 | – | – | 73 | 9 | 15 | 3 |
| | 48 | – | – | 45 | 15 | 26 | 14 |

* A, B corresponding respectively to compounds 2 and 3 in Fig. 1.

Progesterone cannot be chosen as substrate, because the OV-225 column does not allow the separation of progesterone and Δ1,4-androstadiene-3,17-dione. For this reason, the absence of progesterone in the bioconversion media had been tested by TLC (ethyl acetate/*n*-hexane/methanol 50 : 45 : 5) as migration solvents and $H_2SO_4$ 50% as revelator at 75°C).

The data of Table 2 indicates that the most appropriate substrate for yield measurements is dehydroisoandrosterone. For the three substrates the same intermediary compounds (A* and B*) are found. The Δ1,4-androstadiene-3,17-dione level is largely higher than those obtained by Zedan et al., 1976; Zedan and El-Tayeb, 1977) (< 50% in 72 h) using progesterone as substrate with *F. solani* and *Cylindrocarpon radicola* induced cells.

*Influence of cell weight*

The conversion efficiency for a constant substrate concentration is a function of cell weight, as it has been demonstrated in Fig. 2 for the SC broth.

The figure shows that the cell weights between 0.1 and 1.0% w/v give the greatest bioconversion yield differences. For higher weights than 1.0% w/v, the final product percentage increases slowly until it take a constant value.

*Influence of the bioreaction time*

The bioconversion process has been performed for identical substrate concentration in culture medium, buffered solution and with immobilized cells in microcarriers during 24 and 48 h. So, when the bioreaction time is doubled approximately a doubling percentage of the final product is obtained independently of the bioconversion mode. Indeed, the relation between the final product value after 48 h and after 24 h are, respectively, 2.2 and 2.5 for cell suspensions in SC broth and buffered solutions, and 1.9 for immobilized cells.

*Influence of physicochemical factors*

The physicochemical factors are tested in real bioconversion conditions: buffered solutions (Tris-HCl or phosphate 0.1 M) without any nutrients increasing the biomass. Indeed, the term "bioconversion" is valid when the microorganisms are used as a tool of transformation and then play the role of enzyme(s), independently from the cellular context.

*pH.* The effect of pH on the conversion has been realized for cell suspensions and immobilized cells. A wide pH scale can better be explored for cell suspensions with the use of phosphate and Tris-HCl buffers than for immobilized cells. Indeed, only Tris-HCl buffer can be used for alginate microbeads because phosphate buffer dissolves

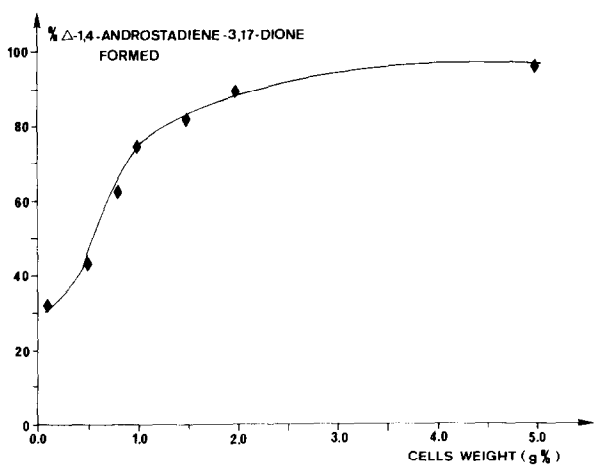


Fig. 2. Influence of cell weight on bioconversion. bioconversion realized in Sabouraud Chloramphenicol Broth during 24 h with 0.15 mg/ml dehydroisoandrosterone at 28°C.

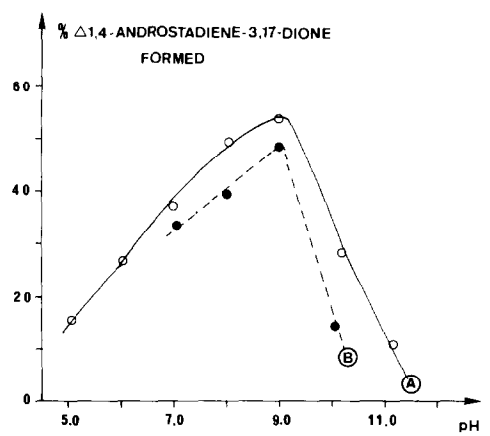


Fig. 3. Influence of pH on bioconversion yield. 0.15 mg/ml dehydroisoandrosterone, 28°C, 24 h. A = cells suspensions (0.8 g%, phosphate and Tris-HCl 0.1 M buffers). B = immobilized cells in microcarriers (22 g% of microbeads, Tris-HCl buffer).

the microcarriers by its complexing and precipitating actions on calcium.

As shown in Fig. 3, similar curves are obtained with immobilized cells and cell suspensions in the pH range 7.0–10.0. Thus, there is no influence of the microcarriers microenvironment on the pH dependency of the bioconversion yield. In both cases, an optimum is found at pH 9.0.

*Temperature.* Fig. 4 shows the action of temperature on the bioconversion yields. The same optimum of temperature range between 28 and 37°C is obtained for each condition. In the same way, the microenvironment of the immobilized cells has no influence on the bioconversion yield.

*Choice of buffer type and its ionic strength.* Biotransformations in Tris-HCl and phosphate buffers gives identical results at several pH values, but borate buffers inhibit the bioconversion. The maximum yield is obtained in Tris-HCl buffer between 0.1 and 0.01 M. For higher ionic strengths, the yield decrease is notable.

*Influence of the organic solvents.* Steroids are only soluble in organic solvents. So, the substrate must be added dissolved in an organic water-miscible solvent. However, this solvent must also have inhibiting action on the biotransformation. This inhibition depends on the solvent type and its concentration. Optimum yield is obtained using 1.5% v/v methanol but N,N′-dimethylformamide, acetone or higher methanol concentrations produce a strong decrease of the bioconversion.

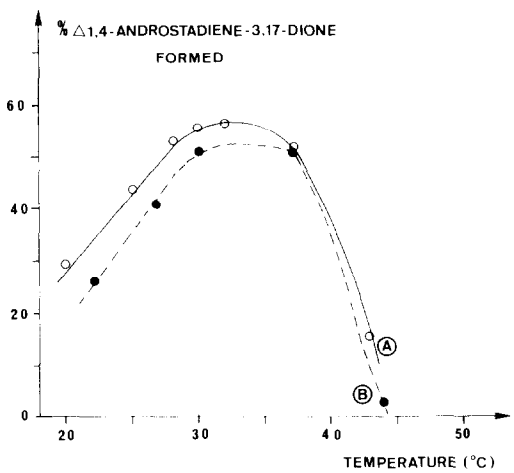


Fig. 4. Influence of temperature on bioconversion yield. 0.15 mg/ml dehydroisoandrosterone, Tris-HCl 0.1 M pH 9.00, 24 h. A = cell suspensions. B = immobilized cells in alginate microcarriers.

*Influence of cell pretreatments*

Pretreatment of fungal cells before their bioconversion study shows that only the method of induction has an influence on the yield (Table 3). The induction method is realized by a subculture of the growing cells in SC broth with 0.15 mg/ml of substrate during 24 h. The induced cells are then tested for their bioconversion. This induction provides an increase of the bioreaction yield which is nearly the same for cell suspensions or for microcarriers. This increase is equal to 1.6 (89/56) for cell suspensions and 1.5 (76/50) for immobilized cells in microbeads.

The absence of yield increases in the case of permeabilized cells with 40% v/v dimethylsulfoxide and with the addition of an artificial cofactor for oxidative enzymatic process ($FeCN_6^{3-}$) (Vincké et al., 1984b, 1985a) shows the very low enzymatic kinetics of this bioconversion. This fact has been confirmed by the sluggish and weak response obtained by potentiometry with *F. oxysporum* suspensions under similar conditions to those described recently for L-lactate determinations (Vincké et al., 1985a).

*Bioconversions with immobilized cells in a bioreactor prototype*

Immobilized microorganisms have attracted increasing interest as catalysts in the past few years (Larsson et al., 1976; Ohlson et al., 1978; Mattiasson and Ramstorp, 1981; Birnbaum et al., 1981; Maddox et al., 1981; Kim et al., 1982; Bihari et al., 1984). They have the same operational advantages as those inherent in immobilized enzymes (Trevan, 1980); they are reemployable, well suited for continuous operation in controlled conditions and furthermore, there is no stringent demand for asepsis during the operation. Immobilized microcarriers offer additional advantages such as the use of enzymes in their natural environment, without costly and tedious isolation steps and without the need of a cofactor.

As described, the prototype of a laboratory bioreactor is composed by a simple thermostatic

TABLE 3

EFFECT OF CELL PRETREATMENTS ON THE CONVERSION EFFICIENCY: 0.8 G % OF CELL SUSPENSIONS IN Tris-HCl 0.1 M BUFFER pH 9.00 AT 30°C DURING 24 h

| Pretreatment of *F. oxysporum* | Untransformed dehydroisoandrosterone (%) | Intermediary compounds formed (%) | | Δ1,4-androstadiene-3, 17-dione formed (%) |
|---|---|---|---|---|
| | | A * | B * | |
| Induced (0.15 mg/ml of inducer) | 3 | 4 | 4 | 89 |
| Non-induced | 26 | 3 | 15 | 56 |
| Non-induced but permeabilized | 34 | 6 | 2 | 58 |
| Induced and permeabilized | 9 | 4 | 1 | 86 |
| Non-induced, but with an artificial cofactor ($8 \times 10^{-3}$ M hexacyanoferrate III) | 12 | 21 | 10 | 57 |

* A and B corresponding to retention times of 26.2 min (compound 2) and 29.7 min (compound 3), respectively, in Fig. 1.

column filled with either a gel agar–agar or microbeads entrapping induced cells. The difference in constitution of both systems due to their fluid outflow resistance necessitates a continuous flow system for the agar (flow rate = 5 ml/h) and a steady mode for alginate microbeads.

A total soluble substrate must be used for getting through the gel column (15 mg dehydroisoandrosterone are dissolved either into 100 ml of a dimethylformamide/Tris-HCl 0.1 ml (10/100 v/v) or into a methanol/Tris-HCl 0.1 M (15/100 v/v solution). The results are, respectively, 2% and 5% Δ1,4-androstadiene-3,17-dione formed during 24 h.

For the alginate beads, 1.5% v/v methanol in Tris-HCl 0.1 M is used as in previous experiments. This buffer containing the bioconversion products is collected after 24 h. Only 27% of Δ1,4-androstadiene-3,17-dione is obtained after this time. This value is much lower than that expected from the earlier results (over 80%). This fact implicates the necessity of stirring conditions and needs the future elaboration of a new stirred bioreactor like those described by Trevan (1980) for enzyme reactors and by Larsson et al. (1976) for immobilized cells.

However, the immobilized cells in alginate beads are reusable daily during 5 days without important loss of the bioconversion activity.

## Conclusions

In the present work, we have shown that gas-liquid chromatography is well suited for an easier and faster technique for screening strains able to be used for steroid bioconversion and to determine the yield of this biotransformation. The judicious choice of the strain, the substrate and the improvement by cell induction allows an increase of the bioconversion above previous works (Zedan et al., 1976; Zedan and El-Tayeb, 1977). About 90% of Δ1,4-androstadiene-3,17-dione can be formed in 48 h either with uninduced cell suspensions in SC broth or in buffered solutions as well as uninduced immobilized cells in microbeads of calcium alginate. When induced *F. oxysporum* are used those yields can be obtained in only 24 h. We have demonstrated that similar results can be obtained with free and immobilized cells in buffered solutions. The bioreactor prototype filled with alginate microcarriers is more suitable than the gel agar column and is reemployable. However, this fix packed bed reactor shows a lower bioconversion than the stirred solutions. Thus, some improvements are needed in order to realize a more suitable bioreactor for industrial processes.

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