

## Influence of microbial cultivation during bio-reduction with in-situ product crystallization (ISPC)

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### ABSTRACT

An in-situ product crystallization process (ISPC) was developed for a crystalline product formed during bioreduction coupled with biocatalyst cultivation. The model reaction was the asymmetric reduction of 4-oxoisophorone (OIP) by baker's yeast (*Saccharomyces cerevisiae*). Yeast cells were cultivated fed-batch in the reactor with an initial cell concentration ( $C_{Xi}$ ) of 1 gdw.L<sup>-1</sup> to reach a maximum concentration of 30 gdw.L<sup>-1</sup>. The desired product, 6*R*-dihydro-oxoisophorone (DOIP), is also degraded by baker's yeast mainly to an unwanted by-product (4*S*,6*R*-actinol); thus, it was removed immediately from the fermentor via an external crystallization unit in the integrated process. The OIP reduction rate was five times higher ( $\cong 0.33$  mmol.gdw<sup>-1</sup>.h<sup>-1</sup>) with growing cells as compared to the reduction rate with resting cells. During the integrated process, OIP reduction was started when the optimum cell concentration was already reached in the reactor as the substrate (OIP) was found to inhibit cell growth at a constantly high concentration ( $C_{OIP} \geq 55$  mM) in the reactor. Final DOIP yield and selectivity were 85% and 99%, respectively. The product crystals were readily recovered, rod-like in shape, and tend to form aggregates. Typical DOIP crystals have an average diameter of 12  $\mu$ m and length of 20  $\mu$ m.

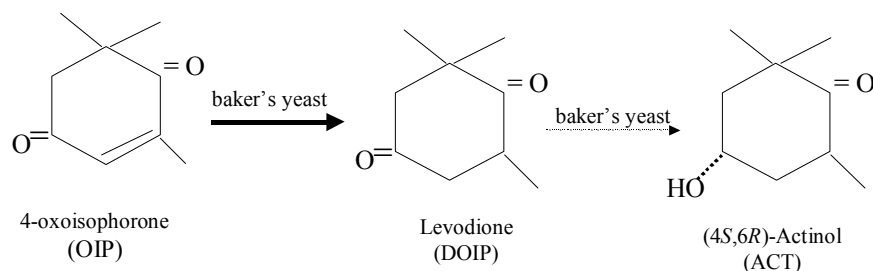
## INTRODUCTION

Nowadays, the range of products that can be produced by fermentation (biotransformation) is increasing as productivity improves dramatically using protein and metabolic engineering. In addition, maximizing the biocatalyst concentration in the reactor and/or immediately removing inhibiting or degrading products during the process can also raise bioreactor productivity. The former requires cell cultivation during the process especially when the biocatalyst is not commercially or readily available and the latter involves in-situ product removal [Lye and Woodley, 1999; Stark *et al.*, 2003; Buque-Taboada *et al.*, 2004a].

This work aims to demonstrate experimentally the feasibility of implementing cell cultivation concomitant with bio-reduction and in-situ product crystal formation in an integrated fermentation-crystallization process. In-situ product crystallization is considered in this work as it can directly provide the desired product (in already pure form) without the need for an auxiliary phase [van der Wielen and Luyben, 1992; Buque-Taboada *et al.*, 2004a]. This approach can be generally applicable in processes, especially those involving cell cultivation prior to or simultaneously with the biocatalytic formation of the product and its subsequent crystallization. This process strategy is also widely suitable for processes where it is required to control and reduce the product concentration in the reactor in order to prevent product toxicity, inhibition and/or degradation, and in addition, simplify the product separation and recovery steps.

The chosen model reaction was the asymmetric reduction of 4-oxoisophorone (OIP) using baker's yeast (*Saccharomyces cerevisiae*) as biocatalyst (Figure 1). The desired product is known as 6*R*-dihydro-oxoisophorone (DOIP), which is a key intermediate in carotenoid synthesis [Leuenberger, 1985] and in the production of saffron and tobacco flavours [Sode *et al.*, 1987]. As baker's yeast is known to also degrade DOIP mainly to 4*S*,6*R*-actinol, an unwanted by-product [Leuenberger *et al.*, 1976], this must be removed from the fermentor as soon as it is formed to prevent low product yield and selectivity. In this case, in-situ product crystallization (ISPC) is appropriately applied. In the previous work [Buque-Taboada *et al.*, 2004a], it was shown that with resting cells of *S. cerevisiae*, the integrated process was the most efficient compared to batch and fed-batch alternative configurations. Fed-batch processing might also be favourable for process systems where the biocatalyst needs to be cultivated in the reactor prior to or concomitant with bio-reduction and product recovery.

Although baker's yeast is not an exceptional organism as it is cheap, readily available, and thus, does not need to be cultivated in the reactor, it is an interesting case to perform reductions with pre-cultivated yeast cells. In many instances, using whole cells as biocatalyst would prove economically favourable as this allows for an easy and *in-vivo* cofactor regeneration in the cell, which sustains catalytic activity for redox reactions. Aeration and nutrient feeding can, however, be the most important constraints to consider, and can potentially complicate the whole biocatalytic process.



**Figure 1.** Reduction of 4-oxoisophorone to DOIP by baker's yeast.

## MATERIALS AND METHODS

### **Micro-organism and chemicals**

Active dry baker's yeast (Fermix®; 97.5 % dry weight) was kindly provided by DSM-Gist (Delft, The Netherlands). OIP (>98%) was supplied by Fluka Biochemika (Buchs, Switzerland). DOIP and actinol (ACT) standard samples were kindly provided by F. Hoffmann-La Roche-VFCD (Basel, Switzerland). The purity of all other chemicals used was at least laboratory grade.

### **Cell cultivation and batch OIP reduction**

Cell cultivations were carried out aerobically in a 2-L bioreactor (Applikon, Schiedam, The Netherlands). The fermentor was equipped with two six-blade Rushton-type impellers (diameter 4.5 cm; impellers 0.5 cm above each other) and with an air outlet condenser. The total working volume was 1 L, the airflow was 0.25 vvm and the stirrer speed was 800 rpm. The temperature of the fermentor and its attached condenser was set at 30°C and 2-4°C, respectively. The control of pH at 5.5 with 1M H<sub>2</sub>SO<sub>4</sub> and 2M KOH was done via a biocontroller (Applikon, ADI 1010). BIOEXPERT software (Applikon, NL) was used as data-acquisition program. The parameters measured on-line during the experiment were the dissolved oxygen concentration, pH, temperature, and stirrer speed.

The complete medium for baker's yeast cultivation was used. The standard procedure was to add 0.7 L medium solution to the fermentor followed by a known amount of baker's yeast (1 g dw yeast cells) suspended in 0.30 L medium solution to make up the 1 L total working volume. The mixture was stirred and aerated for 30 min to acclimatize (pre-incubate) the yeast. Then, glucose as electron donor was supplied in medium solution at a rate of 3 mL.h<sup>-1</sup>. Different glucose feed concentrations (*as specified*) were also used in various experiments. The reactor system was allowed to attain stable/stationary oxygen consumption for 2 h at 30°C. In the course of the experiment, liquid samples were taken from the reactor and analysed for biomass dry weight and glucose concentration. Ethanol and acetate concentrations in the samples were also checked occasionally.

For batch OIP reductions, the reaction was started by addition of a known amount in the range of 5-10 g of pure OIP, after the 2 h acclimatization period when oxygen consumption in the reactor was stabilised. For these experiments, the liquid samples were analysed for OIP, DOIP and actinol in addition to biomass dry weight and concentrations of glucose, ethanol, and acetate.

### **Integrated fermentation-crystallization experiment for OIP reduction**

In these experiments, the same set-up and protocols were employed as in cell cultivation and batch OIP reductions with the following modifications. The initial cell concentration employed was about 1 gdw.L<sup>-1</sup>. Glucose as electron donor was supplied in medium solution at a rate of 3 mL.h<sup>-1</sup>; but its feed concentration ( $C_{Gluc,feed}$ ) was varied (*as specified*) at different stages of the experiment. After 48 h, OIP reduction was started by addition of about 8 g pure OIP and at the same time, fed at 1.5 mL.h<sup>-1</sup> (10.2 mmol.h<sup>-1</sup>) to maintain a concentration level in the reactor that avoids substrate inhibition [Leuenberger *et al.*, 1976; Buque-Taboada *et al.*, 2004b]. The rest of the integrated fermentation-crystallization procedures described previously were followed [Buque-Taboada *et al.*, 2004a].

### **Analytical Methods**

OIP, DOIP, and actinol in the supernatant of the reaction mixture were analyzed as described previously [Buque-Taboada *et al.*, 2004a]. Glucose, ethanol, acetate and the biomass dry weights in the reaction mixture were analyzed as described elsewhere [Buque *et al.*, 2002; Chin-Joe *et al.*, 2001]. The measured amounts of OIP, DOIP, ACT, glucose and biomass were corrected for the actual volume in the reactor as well as the amounts taken out during sampling.

The product crystal morphology and the crystal size distribution (CSD) were determined using an Image Analyzer (IA) consisting of a Sony CCD video camera module (XC-77CE), an Olympus Stereo zoom microscope (SZH) and a PC with IA software LEICA Qwin version 3 (Olympus). Crystals produced during the experiments were compared with the pure (standard) sample.

## RESULTS AND DISCUSSION

### ***Cell cultivation and batch OIP reduction***

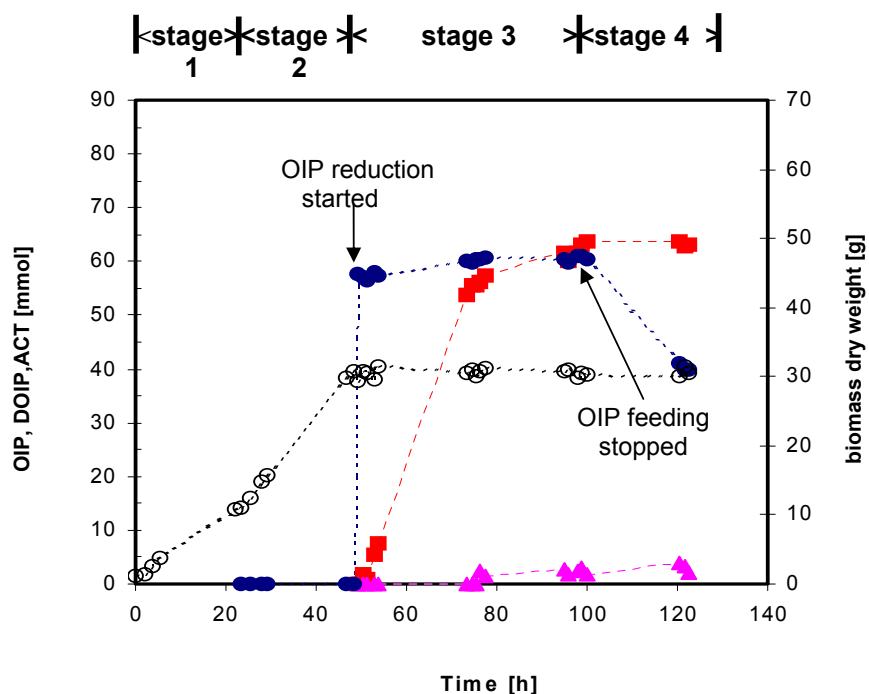
The biomass concentration in the reactor was measured during the course of cell cultivation. The growth patterns of baker's yeast cells during fed-batch cultivation varied at different glucose feeding rates. From an initial cell concentration  $C_{X_i} = 1.3 \text{ gdw.L}^{-1}$ , biomass accumulation in the reactor reached  $\approx 10 \text{ gdw.L}^{-1}$  after 24 h with glucose feed rate of  $0.32 \text{ mmol.h}^{-1}$  at a glucose concentration  $C_{Gluc,feed} = 108.2 \text{ mM}$ . Increasing the glucose feed concentration to over 5 times higher ( $1.68 \text{ mmol.h}^{-1}$  at  $C_{Gluc,feed} = 560.9 \text{ mM}$ ) would favour an increase in biomass from  $C_{X_i} = 10.6 \text{ gdw.L}^{-1}$  to a maximum amount of  $\approx 30 \text{ gdw.L}^{-1}$  after another 24 h.

When cell cultivation was coupled with batch OIP reduction, the growth patterns of baker's yeast cells changed. This was obviously due to additional processes taking place in the cell requiring carbon and energy, namely: cell growth and maintenance, and product formation. At a glucose feed rate of  $1.68 \text{ mmol.h}^{-1}$  in the medium, cell growth as well as bioreduction happened simultaneously. Cell accumulation in the reactor reached  $\approx 30 \text{ gdw.L}^{-1}$  from an initial amount of  $10.2 \text{ gdw.L}^{-1}$  concomitant with batch OIP reduction at an average biomass-specific rate of  $\approx 0.33 \text{ mmol.gdw}^{-1}.\text{h}^{-1}$ . However, when OIP was fed such that its concentration in the reactor was  $C_{OIP} \geq 55 \text{ mM}$ , cell growth was inhibited. Thus, OIP reduction should be started when the maximum cell concentration in the reactor is already reached to maximize productivity.

### ***Integrated fermentation-crystallization process for OIP reduction***

This experiment was performed in several stages in combining cell cultivation, bioreduction and in-situ product crystallization. Microbial cultivation was done in stages 1 and 2. OIP reduction was started in stage 3 when the cell concentration in the reactor had already reached  $30 \text{ gdw.L}^{-1}$  to avoid growth inhibition and maximize bioreactor productivity (see Figure 2). OIP was added in the reactor at  $56.5 \text{ mmol}$  ( $8.6 \text{ g}$ ) and at the same time, fed at  $1.5 \text{ mL.h}^{-1}$  ( $10.2 \text{ mmol.h}^{-1}$ ) to maintain a constant OIP concentration in the reactor that favours maximum reduction rate and avoid substrate inhibition. The glucose feed rate was also raised to  $6.04 \text{ mmol.h}^{-1}$  at  $C_{Gluc,feed} = 2.01 \text{ M}$  to further sustain product formation and cell maintenance. Results (Figure 2) showed that biomass concentration in the reactor was indeed maintained during OIP reduction in stage 3 as the average biomass dry weight was  $30.55 \text{ g}$ . OIP concentration in the reactor was constant at  $C_{OIP} \approx 60 \text{ mM}$ , which indicates that the OIP fed was continuously converted to the product (DOIP) and the OIP feed rate ( $1.5 \text{ mL.h}^{-1} = 10.2 \text{ mmol.h}^{-1}$ ) was equal to the observed OIP reduction rate ( $\approx 0.33 \text{ mmol.gdw}^{-1}.\text{h}^{-1}$ ) at the given constantly high biomass dry weight. This was a favourable situation for an integrated process where OIP reduction rate was maximal, keeping the substrate concentration in the reactor at a constant level avoiding substrate inhibition and cell lysis. At different stages in the process, the glucose feed rate was raised appropriately to sustain the required metabolic reactions in the cell. Dissolved oxygen during the experiment was above 50% of air saturation. Metabolic by-products such as ethanol and acetate were negligible as their concentrations in the sample were below detection limits.

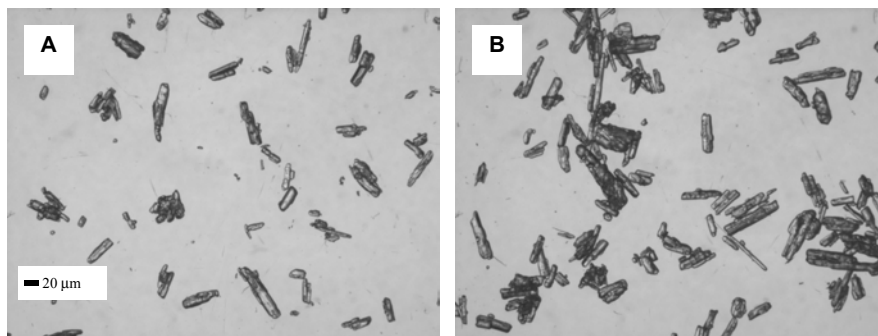
In this process scheme, the final yield and selectivity of the product DOIP were 85% and 99%, respectively, the latter indicates a negligible degradation of the product, rendering ISPC an effective method for in-situ product removal during the integrated process. DOIP crystals ( $60.50 \text{ g}$ ) were accumulated in the crystallizer and were readily recovered. The enantiomeric excess (e.e.) of 6R-DOIP remained very high at  $\text{e.e.} \geq 98\%$ , making the biocatalyst an attractive choice for enantioselective OIP reduction. The biocatalyst consumption rate was  $0.42 \text{ kg}$  per  $\text{kg}$  of product obtained; this can further be reduced when the integrated reduction process is continued up to several days more before the close-down phase (stage 4). This can be a potential benefit for other processes with expensive biocatalyst. The volumetric productivity was  $0.55 \text{ g.L}^{-1}.\text{h}^{-1}$  and can certainly increase when the bioreduction process is optimally lengthened and/or the biocatalyst cultivation period reduced.



**Figure 2.** Amounts of OIP (●), DOIP (■), ACT (▲) and biomass (○) in the reactor. OIP reduction was started after 48 h (stage 3), when  $C_X = 30 \text{ gdw.L}^{-1}$ . OIP feeding was done at a rate of  $1.5 \text{ mL.h}^{-1}$ , implementing a reduction rate of about  $0.33 \text{ mmol.gdw}^{-1}.\text{h}^{-1}$  as OIP concentration level was constant in the reactor. During the close-down phase (stage 4), OIP feeding was stopped but reduction was continued for another 24h.

### ***Morphology and DOIP crystal size distribution***

The product (DOIP) crystals are white in color and rod-like in shape (Figure 3A). They are quite stable and tend to form aggregates (Figure 3B). Crystal size data were obtained by measuring the crystal size distribution using the Image Analyser set-up described in the Materials and Methods section. Most of the crystals have a length range of 1-30  $\mu\text{m}$  and a diameter range of 1-20  $\mu\text{m}$ . Considering a crystal population of 1500, the average length and diameter were 20 and 12  $\mu\text{m}$ , respectively. The crystal morphology and size distribution were comparable with the standard sample.



**Figure 3.** (A) Typical DOIP crystals, which are rod-like in shape, and tend to form aggregates (B).

## CONCLUSIONS

The integrated reduction-crystallization process strategy is applicable to any biocatalytic (i.e. reduction) systems where crystalline products are obtained; however, when microbial cultivation is necessary, substrate/product inhibition, toxicity and degradation as well as the sufficient supply of energy and carbon sources must be taken into account during the process design, amongst others, in order to obtain an economically promising biocatalytic process.

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