PROPOSITIONS

accompanying the PhD thesis:

_In situ_ removal of solid products during whole-cell biocatalysis
by Evelyn M. Buque

1. *In situ* product removal is not the _mere_ removal of the product formed.

2. The influence of certain impurities during crystal formation can only be truly known by experimental investigation.

3. *In situ* product removal results in process integration while the reverse is not necessarily true.

4. Process intensification is not always process integration.

5. Motherhood is the essence of being a woman.

6. A masterpiece invokes great beauty and value but entails an equally great pain and sacrifice.

7. The parents’ love for their children can best be manifested by their love for each other.

8. No man ever steps in the same river twice. For with the flow and the tide, an opportunity lost is lost forever.

   _Rephrased quote from Heraclitus_

   “You can never step into the same river, for new waters are always flowing on to you.”
   _Heraclitus, Greek philosopher. 544-483 BC_

9. Social integration while encouraged in many cultures has limitations.

10. The best indicator of corruption as a severe social illness is the uneven proportion between the wealthy minority and the poor majority.

11. The use of renewable resources will one day be the only option.

12. Being happy is simply learning to see life beyond the imperfections.

_These propositions are considered opposable and defensible and as such have been approved by the supervisors,
Prof. dr.ir. L.A.M. van der Wielen and Prof.dr.ir. J.J. Heijnen._
STELLINGEN

behorende bij het proefschrift:

In situ verwijdering van vaste producten tijdens biokatalyse met hele cellen
door Evelyn M. Buque

1. In situ productverwijdering is niet slechts de verwijdering van het gevormde product.
2. De invloed van bepaalde onzuiverheden tijdens kristalvorming kan slechts door experimenteel onderzoek echt worden gekend.
3. In situ productverwijdering resulteert in procesintegratie terwijl het omgekeerde niet noodzakelijk het geval is.
4. Procesintensivering is niet altijd procesintegratie.
5. Moederschap is de essentie van een vrouw te zijn.
6. Een meesterwerk leidt tot grote schoonheid en waarde maar draagt een even grote pijn en opofferingsleed met zich mee.
7. De liefde van de ouders voor hun kinderen kan het best door hun liefde voor elkaar worden geuit.
8. Geen mens stapt ooit tweemaal in dezelfde rivier. Want door de stroom en het getijde is een gemiste kans voor altijd verloren.

Geherformuleerd citaat van Heraclitus

"U kunt nooit in dezelfde rivier stappen, voor nieuwe wateren stromen altijd op u."
Heraclitus, Griekse filosoof, 544-483 V.CHR.

10. De beste indicator van corruptie als ernstige sociale ziekte is de ongelijke proporties tussen de rijke minderheid en de arme meerderheid.
11. Het gebruik van hernieuwbare grondstoffen zal ooit de enige optie zijn.
12. Gelukkig zijn is simpelweg te leren het leven te zien voorbij de onvolmaaktheden.

Deze stellingen worden opponeerbaar en verdedigbaar geacht en zijn als zodanig gedgekeurd door de promotoren,
Prof. dr.ir. L.A.M. van der Wielen en Prof.dr.ir. J.J. Heijnen.
In Situ Removal of Solid Products during Whole-cell Biocatalysis

Evelyn M. Buque
In Situ Removal of Solid Products during Whole-cell Biocatalysis

PROEFSCHRIFT

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For Jay, Venise, and Nico
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Chapter 1

INTRODUCTION

E.M. Buque-Taboada, A.J.J. Straathof, J.J. Heijnen, and L.A.M. van der Wielen

Submitted.
INTRODUCTION

Producing high-value materials by exploiting biotechnology is one of the latest challenges of the chemical industry. The increasing need to use renewable feedstocks and to reduce CO₂ emission due to fossil fuel consumption already cause a gradual shift from chemical to biochemical production processes [1]. While this undertaking may take decades to implement and may be impossible to some extent, the existing trend is to look for an alternative biochemical route for an already existing chemical process. In many cases, a combination of biochemical and chemical methods are developed and implemented. These efforts lead to an increase in the range of compounds that can be produced by fermentation, most of which are desired in their solid or crystal form.

The growing interest in biochemical processes can be attributed to their potential capability to significantly increase productivity, purity, and yield using novel protein and metabolic engineering coupled with innovative process engineering. For the latter strategy, a promising long-time target for chemicals production is fermentation or biotransformation with \textit{in situ} recovery of the products formed. These processes should be cost-efficient, robust, clean, based on renewable materials, and widely applicable.

\textbf{IN SITU PRODUCT RECOVERY}

Biocatalytic processes usually have limited productivity, which is commonly due to product inhibition that causes a decrease in biocatalytic activity, or product degradation that results in lower yield [2]. Thus, attempts to increase volumetric productivity and yield by optimizing physiological and technological parameters are essential in the further development of biocatalytic processes. Typical trends of product inhibition and degradation are schematically shown in Figure 1.1 where it is clearly seen that increasing the product concentration in the reactor can lead to pronounced effects of inhibition or degradation. Keeping the dissolved product concentration low in the reactor can obviously circumvent these limitations. An approach that can accomplish this task is the implementation of an \textit{in situ} product recovery (ISPR) technique [2-7].
Figure 1.1. **Typical trends of product inhibition and degradation in a biocatalytic process.**

*In situ* product recovery (ISPR) techniques are proven to solve these issues [2-8]. These lead to the processes where the product is removed as soon as it is formed in the reactor. In doing so, the waste streams as well as the number of downstream processing steps can be reduced. ISPR is part of the general concept of process integration or intensification [2-7], which represents the improved coordination of upstream, reaction, and downstream technologies. The selection of a proper ISPR strategy, appropriate process configuration and best mode of operation must be considered *a priori* in the implementation of this technique in biocatalytic processes.

The techniques employed for *in situ* product removal depend on the properties of the target product, on the biocatalyst involved, as well as on the expected benefit that ISPR needs to achieve. Some examples are already known and established, and are leading to useful applications in industry [2-8]. By far, extraction, adsorption and evaporation have been the most commonly employed techniques for ISPR [2,4,7,8]. In some cases, electrodialysis, precipitation, complexation, and membrane-assisted separation techniques have been employed [2,4,7,9]. Product categories involved are organic solvents, acids, aromas and flavors, secondary metabolites and proteins.
The basic configurations for *in situ* product removal as applied to the established techniques are shown in Figure 1.2. Separation of the cells and product can be achieved by employing an [6-8]:

a) *External configuration*, where the target product is removed in an external unit and the liquid broth is returned to the fermenter; or

b) *Internal configuration*, where the product in the fermenter is separated from the fermentation broth.

In either case, the cells can be in *direct* or *indirect* contact with the product-containing phase. The choice of configuration is also dependent on the properties of the target product and biocatalyst, the number of relevant phases involved as well as the type of recovery process employed. In most approaches, the product is removed by using an auxiliary phase, which complicates the process design and potentially increase production costs.

![Figure 1.2. Different configurations for ISPR [6-8].](image)

A process configuration with indirect contact of the cells is well-established and straightforward. This can be done for example by ultrafiltration (for external ISPR) or by cell immobilization (for internal ISPR). Thus, fermentation can be run close to optimum operating conditions. However, it is necessary to achieve controlled product removal at a
reasonable rate without making large changes in pH or temperature between fermenter and separator as this could potentially complicate liquid recycling to the fermenter. Elsewhere comparable approaches have been successfully applied in enzymatic processes employing ISPR with external configuration [8-14].

However, in situ product recovery by crystallization during a whole-cell catalyzed reaction has hardly been considered. When in situ product crystallization is employed, it might directly provide the desired product (in solid or crystal form) without the need for an auxiliary phase.

Crystallization is one of the oldest yet the most effective processes for the preparation of solid products. High purities and stabilities can be obtained with distinct morphologies, and functional properties. Crystallization is important in different areas of biotechnology. For instance, crystallization of proteins, viruses, nucleic acids, and macromolecular complexes has been done for a variety of reasons such as for X-ray diffraction analysis, as part of the formulation of pharmaceutical or fine-chemical products, and as a means of purification in bioseparation processes [15].

MICROBIAL FORMATION OF CRYSSTALLINE PRODUCTS

When a certain substrate S enters a microbial cell, it is converted to product P as schematically shown in Figure 1.3. The cell then has at least two options on how to deal with the product P. In some cases, the cell does not secrete the product and tends to store it. When this happens, the concentration of the product in the cell ($C_{Pin}$) increases and will be greater than the concentration outside the cell assuming a continuous import of substrate into the cell; then, $C_{Pout}$ is low or zero as $C_{Pin}$ is maximized in the cell (Figure 1.3A). This leads to the accumulation and potential crystallization of the product inside the cell, which sometimes result in cell damage. However, when the cell needs to maintain a low concentration of the product inside the cell (e.g. due to its toxicity), it has to secrete the product; then, $C_{Pout}$ is greater than $C_{Pin}$ as $C_{Pin}$ remains low in this case (Figure 1.3B). When this occurs, product crystals may start to form outside the cell when the solubility limit is reached. In this sense, the crystal formation of certain molecules may be intracellular (in the cell) or extracellular.
(outside the cell). If secretion is slow as compared to product formation, both intracellular and extracellular crystals might be formed.

![Diagram showing conversion of substrate S to product P in the cell.]

**Figure 1.3.** Conversion of substrate S to product P in the cell.

Most often, this situation leads to the formation of crystals of organic, inorganic or biological nature. However, it is important to be aware that the types of compounds for which crystals may be formed vary extremely widely. For a wide range of organic compounds, crystallization may occur during an industrial fermentation process. However, also under less controlled conditions such organic crystals may form. For example, calcium lactate formation may occur on Cheddar cheeses as a result of microbial action [16]. Crystallization may be also due to completely natural processes. Storage polymers such as cellulose may have crystalline regions [17]. A more exotic case is the accumulation of heme crystals by *Plasmodium* parasites [18]. Also, crystalline proteins may accumulate, for example for the toxins of *Bacillus thuringiensis* used as insecticide [19]. *Paramecium* species even seem to discharge protein-crystal containing trichosyst organelles, as a defense against other microorganisms [20]. An antibiotic (of the nifimycin A₁-scopafungin group) can also spontaneously crystallize during submerged fermentation of *Actinomyces hygroscopeicus* [21]. More importantly, microorganisms play a role in the deposition of many minerals, for example for polyphosphates, calcium carbonates, silicas, metal sulfides, metal oxides, and gold [22].
Industrially-relevant examples include: amino acids (phenylalanine, glutamine), carboxylic acids, calcium salts of carboxylic acids (lactate, malate), steroids (prednisolone, etc), antibiotics (tetracycline, oxytetracycline), carotenoids, riboflavins, chiral compounds formed by reducing precursors (DOIP, hydroxysulphone, nitroalcohol, diltiazem precursor), monoglycerides, bacterial cellulose (showing crystalline regions), polyhydroxyalkanoates (e.g. PHB), inorganic mineral compounds (e.g. polyphosphates), special biological compounds such as protein inclusion bodies, core and virus-like particles, and macromolecular complexes.

Thus, it is not surprising that microbial catalysis could be exploited for the production of crystalline products of organic, inorganic, or biological nature. When crystals are formed and stored intracellularly, this requires cell disruption to release and recover the crystal. In such cases, in situ product removal is not applicable.

On the other hand, when the product is secreted, crystals are formed extracellularly. In this situation, in situ product removal by crystallization may be helpful in increasing productivity and yield. In chemical technology, these types of processes are often referred to as reactive crystallization if an internal configuration is used as shown in Figure 1.2 [23-24].

**IN SITU PRODUCT REMOVAL BY CRISTALLIZATION**

In many fermentations and bioconversions [25-39], crystalline product formation has hardly been seen as a potentially beneficial phenomenon for an efficient product recovery. However, for many processes where isolated, crude, or pure enzymes are used, a crystallizer unit is employed in combination with the bioreactor [10-14, 40-42]. For whole-cell catalyzed reactions, the separation of product crystals from the cells is usually carried out by addition of organic solvent, hence precipitating the cell debris and re-dissolving the product. Then, the product is crystallized again [36-38]. Such procedures lead to high amount of waste streams and high production costs.

In situ separation of intracellular crystals from cells without affecting the viability of the cells is an unexplored area and seems complicated. Such a separation may be based on size,
density and/or surface tension. Complexation can also be exploited to give a distinct property of the target product, which can lead to easier separation [2]. Centrifugation, flotation and interfacial partitioning may be considered. These techniques recently also have been considered for separation of biological particles [43-44].

In some cases, the presence of extracellular crystals may cause stress and transport limitations, thus, is not favorable at all to the cells. However, in a large number of whole-cell catalysed processes, product inhibition or degradation occurs at a concentration below the solubility limit and product crystals are hardly or not formed at all in the fermenter. In these situations, ISPR by external crystallization with indirect contact between cell and product-containing phase will be an attractive solution. The product is removed from the fermenter as soon as it is formed, thereby, lowering intracellular and extracellular product concentrations and maximizing the fermentation process performance. The subsequent discussion will focus then on ISPR by external crystallization with indirect contact.

**Cell retention options**

Figure 1.4 shows some options for cell retention in ISPR by crystallization with external configuration. In these schemes, product crystallization is performed outside the bioreactor. In Figure 1.4A, a membrane filter separates the product-containing supernatant (liquid) from the cells. While the retentate is recycled to the reactor, the filtrate is directly fed to the crystallizer where the target product is crystallized. The product-depleted mother liquor in the crystallizer is also recycled to the reactor. In this case, it is also important to consider the residence time of the fermentation broth in the membrane filter as the cells may suffer from nutrient or oxygen limitation. In scheme B, a spin filter or an acoustic perfusion system is installed in the bioreactor for cell retention. This strategy, which is usually applied for processes employing mammalian cells [45], allows the direct and continuous withdrawal of the cell-free liquid broth from the bioreactor while the cells are retained, avoiding cell stress and potential nutrient or oxygen limitation. However, complete retention is difficult and would require a second-stage filtration unit, making this option less attractive in combination with a crystallization process. Immobilized cells can also be used (scheme C) where cells are retained in the bioreactor. This leads to a relatively simple configuration, but requires an immobilization step. Of course, Figure 1.4 is by no means exhaustive.
Figure 1.4. External configurations for ISPR with cooling crystallization. Cell retention is done by (A) external filter, (B) spin filter inside the bioreactor, (C) cell immobilization. In all cases, the filtrate is directly fed to the external crystallization loop to crystallize the product. The product-depleted mother liquor is recycled to the bioreactor.
Chapter 1

Criteria for process implementation
ISPR by external crystallization is logically applied if the target commercial product is solid or crystalline at storage conditions. The following criteria must be considered in deciding whether it is applicable or beneficial for a certain whole-cell biocatalyzed process. Some are inter-related and are readily known. It must be noted that the goal is to remove the product as soon as it is formed in the reactor to avoid product toxicity or degradation, thereby, increasing fermenter productivity and yield.

Product toxicity, degradation, and solubility
When the product is toxic to the microorganism, its dissolved concentration level must be kept low as the concentration that triggers inhibition in the reactor is usually below its solubility. When the product degrades, the dissolved product concentration in the reactor must be kept low also to avoid a high degradation rate. However, product (super)saturation must be achieved in the crystallizer unit. Thus, it is imperative to determine the allowable dissolved product concentration in the reactor in relation to the solubilities of the product and also of other compounds involved in the process at the conditions in the reactor as well as at the conditions in the crystallizer. In any case, the crystallization rate must be greater than the net production rate to implement ISPR by external crystallization in the process. Otherwise, in situ product removal by crystallization is less efficient, if not illogical. Thus, a typically reasonable crystallization rate must be assumed, if not yet determined.

Production rates and volumetric productivity
The production rate must be fast enough to achieve the product solubility limit (saturation) in the reactor or in the crystallizer rather quickly. For economically attractive processes, the final product concentrations are in the range of 50-100 g.L⁻¹, with 100 g.L⁻¹ as a typical value [46]. For biocatalytic processes involving oxygen transfer, a typical volumetric productivity of 1 g.L⁻¹.h⁻¹ is a good start. This implies that the residence time is about 100 h (4 days) for a fermentative process. Furthermore, crystal growth rates of well-soluble compounds are typically in the range of $10^{-8} - 10^{-7}$ m.s⁻¹. Simple separation units are capable of handling particles ranging in size of 0.2 - 1 mm. To reach these sizes, generally a residence time of crystals in the crystallizer of 0.5 to 5 h is needed. This implies that the product formation rate
will be rate-limiting and integrated fermentation-crystallization processes will operate at low supersaturation ratios such that seeding may be required in the crystallizer in some cases.

**Biocatalyst**

When whole-cells are employed as biocatalyst, the best physiological state of the cells must be considered, more so, when the reaction involves process integration via ISPR. The start of the reaction, which is often dictated by the time of precursor addition, has a profound influence on productivity. In case where enzyme induction is needed, it is necessary to add small amounts of precursor at the beginning. Addition of precursor in the late exponential (state) growth phase usually avoids toxicity effects. Whether to employ growing cells or resting cells, the choice must be clear beforehand; in some cases, both conditions should be considered as the metabolic activities (thus, also biocatalytic activities) of each are not the same.

**Aseptic or non-aseptic conditions**

When growing or susceptible cells are employed as biocatalyst, it is usually imperative to keep the process under aseptic conditions. It may be difficult or expensive to implement this situation when ISPR by crystallization is involved. However, it is possible that the cell growth stage is done in a sterile condition while the bioconversion phase is implemented under non-aseptic conditions. In this case, cell growth and cultivation can be carried out in one large fermenter and the harvested cells are stored properly for subsequent bioconversion.

**Mode of operation**

When the biocatalytic process is a fed-batch or a continuous process operating at a constant volumetric productivity, the process might reach a steady state with respect to precursor and product concentrations in which the production rate in the bioreactor is equal to the product crystallization rate. This results in a constant dissolved product concentration in the reactor and an accumulation of product crystals in the crystallizer. The continuation of the process is then dependent on sustained biocatalytic activity. ISPR may promote sustained biocatalytic activity because product toxicity and by-product formation are minimized. However, if the
by-product keeps accumulating, it must be purged, otherwise the process should eventually be discontinued.

**Product crystal morphology and purity**

When ISPR by external crystallization is implemented, product crystals are formed in the presence of reaction medium containing other solutes and impurities. These can have varying effects to the crystallization of the product [15,47-48], but so far, this is still difficult to predict. In some cases, co-solutes can accelerate or inhibit crystal formation and growth; sometimes, they have no effect at all. In other cases, the solubility of the crystal in the reaction medium may be lowered by the presence of these co-solutes, thus, rendering crystallization as a favorable recovery technique.

Crystallization involves nucleation and crystal growth. These two sub-processes must be considered in order to describe the crystallization behavior of the product crystals and the influence of impurities. While it is important to determine crystallization kinetics of a certain product, this should not hinder the implementation of ISPR in the process. Supposing that the desired crystal morphology and purity are affected by the presence of co-solutes or impurities in the reaction medium, recrystallization is the common approach to solve the problem [47-48].

**Other factors**

Other factors may or may not be important in deciding whether ISPR by crystallization is a viable option. In some cases, it is easy to imagine that energy consumption (i.e. due to cooling or evaporation) may be a significant issue to consider in the process. Heat integration of the process may be required. The cold, product-depleted, mother liquor is recycled to the fermenter, which can help in keeping the fermenter temperature constant, thus, cooling may not be necessary, resulting in less energy costs and less investment. Over all, the choices to be made should be driven by and should simply result to more efficient and cost-effective processes.
MODEL BIOCATALYST

For redox reactions, employing whole-cells as biocatalyst instead of isolated enzymes allows the regeneration of cofactors in the cell, which sustains the catalytic activity. However, this benefit should not be outweighed by intricate downstream processing steps. ISPR by crystallization might be a favorable option for downstream processing. In this study, *Saccharomyces cerevisiae*, commonly known as baker's yeast, is used as the model biocatalyst. While it is known to catalyze a wide range of redox reactions with suitable reaction rates [49-53], it is also readily available, easy to handle, cheap, non-toxic and non-pathogenic. The reaction process can also be done in a non-aseptic condition, which eliminates the problems associated with aseptic processes.

MODEL REACTION

The model reaction chosen with *S. cerevisiae* as catalyst is the reduction of 4-oxoisophorone (OIP) to 6R-dihydro-oxoisophorone (DOIP), also known as levodione (Figure 1.5). DOIP is a white crystalline solid with a typical solubility of about 10 g.L\(^{-1}\) at the fermentation temperature of 30°C. It is used as a key intermediate in the production of some carotenoids [36-37] and saffron flavors [54]. This reaction suffers from substrate inhibition and product degradation as baker's yeast also catalyzes the reduction of DOIP to an unwanted by-product 4S,6R-actinol (ACT). Thus, feeding the substrate to maintain a concentration level which is non-toxic to the biocatalyst is required. The target product DOIP must also be removed from the reactor as soon as it is formed in order to minimize, if not eliminate, its degradation.

![Chemical structure](image)

**Figure 1.5.** Synthesis of 6R-dihydro-oxoisophorone (DOIP) via reduction of 4-oxoisophorone (OIP) by baker's yeast.
AIM OF THESIS

This study aims to start the systematic development of integrated fermentation-crystallization processes employing the in situ product removal by external crystallization strategy whereby fully metabolizing whole-cells are used as biocatalyst. Such a strategy can lead to truly sustainable processes in producing high-value materials from renewable feedstocks. Inherent with this aim is to experimentally demonstrate the concept, delineate the advantages and disadvantages of the process, and to distinguish a wider scope of application.

STRATEGY AND SCOPE OF THESIS

The most common fermentation process is an aerobic fed-batch operation. This pre-defines, though not strictly, the integrated fermentation-crystallization process in this study to be fed-batch and aerobic. Instead of directly using a growing-cell system that has all the complexities of aseptic experimentation due to the dynamic nature of the process, initially resting cells of baker’s yeast (S. cerevisiae) are employed in a fed-batch system with aeration and with nutrient feeding. An established protocol [55] of nutrient feeding is employed to eliminate complications caused by the formation of metabolic by-products. Once the working concept has been established with resting cells, cultivation of the cells will be included in the process.

Cell immobilization is one of the main options for cell retention. Its influence and applicability during the bioreduction of a 3-oxo ester is studied in Chapter 2. Although, this does not involve the model reaction, it demonstrates that cell immobilization has large effects on the reaction rate and enantioselectivity. Therefore, ultrafiltration is used instead as a cell retention technique in the rest of the thesis. In Chapter 3, the working concept of in situ removal of a solid product using an external crystallization loop is established for bioreduction of 4-oxoisophorone. The kinetics of substrate inhibition and product degradation during OIP reduction by baker’s yeast are investigated in Chapter 4 in order to allow process optimization. For many microbial transformations, the biocatalyst is not readily available and the reaction is typically preceded by the cultivation of cells. During this situation, the metabolism of the growing cells is not the same as in the resting mode such that
in some cases biotransformation has been done during the growth phase to attain higher productivity. Thus, the integrated fermentation-crystallization process coupled with cell growth is explored in Chapter 5. After demonstrating the feasibility of integrated fermentation-crystallization process at this point, the influence of co-solutes present in the reaction medium on the nucleation and growth of product crystals is investigated in Chapter 6. This shows that crystal growth is an important sub-process during crystallization as ≥ 99% of the mass of crystals is usually accounted to (the growth of) large crystals. To combine the results and insights gathered so far, and to reveal other relevant factors to be considered for the integrated fermentation-crystallization process, Chapter 7 describes a conceptual design of this process as applied to DOIP production and compares it with the design of a known conventional process equivalent. Finally, a general outlook on the different aspects and possibilities of integrated fermentation-crystallization processes employing ISPR by crystallization is given in Chapter 8.
REFERENCES


Chapter 2

Immobilization affects the rate and enantioselectivity of 3-oxo ester reduction by baker’s yeast

This work was done in collaboration with Ifoeng Chin-Joe.

E.M. Buque, I.Chin-Joe, A.J.J. Straathof, J. A. Jongejan, and J.J. Heijnen

*Enzyme and Microbial Technology* 2002, 31, 656-664.
ABSTRACT

The asymmetric reduction of ethyl 3-oxobutanoate to ethyl (S)-3-hydroxybutanoate by baker’s yeast (Saccharomyces cerevisiae) immobilized in calcium alginate was studied. The reaction was carried out under aerobic conditions with glucose fed as electron donor. Using immobilized cells at a concentration of 6 g dw biomass per liter of reaction volume and with mean particle diameter $d_{p,\text{mean}} = 1.2$ mm resulted in the same specific reduction rate as in the reduction using freely suspended cells. The enantiomeric excess of ethyl (S)-3-hydroxybutanoate was $\geq 98\%$. At larger particle sizes, the specific reduction rate decreased while the enantiomeric excess of ethyl (S)-3-hydroxybutanoate remained high at $\geq 98\%$. At $d_{p,\text{mean}} = 1.2$ mm but using higher cell concentrations (22-37 g dw.L$^{-1}$), the average specific reduction rate was reduced by 22% compared to the specific reduction rate using low cell concentrations or using free cells. Under these conditions, the enantiomeric excess of ethyl (S)-3-hydroxybutanoate decreased to 83-90%. Calculation of the concentration profiles in alginate beads showed that among the reacting species, severe diffusion limitation is expected only for oxygen. This may have led to the lower reduction rates of the immobilized cells at larger particle sizes and at high cell concentrations and in addition, to the change in enantioselectivity.
INTRODUCTION

The production of enantiomerically pure compounds is of increasing importance in the fine chemicals industry. Many of these compounds can be obtained by stereoselective reduction of prochiral ketones like 3-oxo esters. In biocatalytic conversions [1-6], whole cells are used more often than isolated enzymes because cofactor regeneration is required for sustained catalytic activity. Baker’s yeast (Saccharomyces cerevisiae) is an economically attractive biocatalyst due to its availability and low costs, ease of handling and disposal, safety for food and pharmaceutical applications [2] as well as its capacity to catalyze a wide range of stereoselective reductions [1-6].

A frequently occurring problem in biocatalytic processes is the production of dilute and complex product streams, requiring concentration and purification for the recovery of the desired product. Retention of cells by immobilization can facilitate product recovery, in particular when in-situ product removal is performed. Immobilized cells may be re-used, reducing cell wastes and minimizing loss of product in cases where it is absorbed by the cells. Entrapment of cells in calcium alginate is the most widely used immobilization technique in the biocatalytic production of chemicals [7-11]. Alginate is cheap and readily available, has a high affinity for water, and has the ability to form gels under mild conditions, which are suitable for most cells. It is non-toxic and non-pathogenic which makes it attractive for applications in food and pharmaceutical industry [10].

Several 3-oxo ester reductions using immobilized cells have been reported in the literature. In many cases, baker’s yeast immobilized in calcium alginate [12-15] was used. Other microorganisms [16] as well as other immobilization matrices such as polyurethane [12], celite [13], carrageenan [15], and chrysotile fibers [17] have also been described. The 3-oxo ester reductions with immobilized cells were preferably performed in water for easy handling and separation of products and to avoid solvent toxicity; however, reductions were also carried out in organic solvents like hexane [12,14,15] and isooctane [18]. Studies on the batch-wise reduction of 3-oxo esters using immobilized baker’s yeast showed a significant change in the enantiomeric excess values of the desired product [12-15] as compared to reductions with free cells. In addition, the specific reduction rates using immobilized cells were found to be slower than the reduction rates using free cells, unless cells were
immobilized in small diameter beads (i.e. $d_{p_{\text{mean}}} \leq 1.5 \text{ mm}$) [15]. These observations were usually speculated to be due to changes of the reaction conditions at the surface of the yeast cells or a difference in the concentration of substrates inside and outside the beads. The actual origin of the effects of immobilization cannot be traced back from these studies. We supposed that concentration gradients in the beads and diffusion limitation of 3-oxo ester, electron donor (i.e. glucose) or oxygen might have caused these effects.

In this work, the batchwise enantioselective reduction of ethyl 3-oxobutanoate (EOB) to ethyl (S)-3-hydroxybutanoate ((S)-EHB) in water using immobilized baker's yeast under aerobic conditions is chosen as the model reduction reaction (Figure 2.1). To elucidate the influence of cell immobilization to the over-all reduction performance, the effects of varying particle sizes and cell concentrations in the alginate beads will be studied, using the established protocol [6] to identify possible transport limitations. The concentration profiles of all reacting compounds in the beads will be calculated. By controlling the electron donor supply such that neither biomass increase nor production of metabolic by-products occurred, the reduction process is simplified.

![Diagram](image)

**Figure 2.1.** Simplified reaction scheme for the reduction of ethyl 3-oxobutanoate to ethyl (S)-3-hydroxybutanoate by baker's yeast. The regeneration of cofactors involves more reaction steps than displayed.
MATERIALS AND METHODS

Micro-organism
Fresh pressed baker’s yeast (Koningsgist, 28-33% dry weight) was kindly provided by DSM-Gist (Delft, The Netherlands). The yeast was stored at 4°C and used within three weeks.

Chemicals
Ethyl 3-oxobutanoate (>99%) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Struktol® solution J-673 was obtained from Schill and Seilacher (Struktol Co., Stow, OH, USA) and sodium polyphosphate was from Sigma-Aldrich (St. Louis, MO, USA). Sodium alginate (>98%) from brown algae (for immobilization of micro-organisms) was obtained from Fluka Biochemika (Buchs, Switzerland). The purity of all other chemicals used was at least laboratory grade.

Preparation of alginate gel beads
Calcium alginate beads were prepared at ambient temperature (≈ 18-22°C) as described [7]. The immobilization equipment has been described elsewhere [11]. A 1 L aqueous 2% w/w sodium alginate solution, which was stirred for 24 h, was prepared for cell immobilization. Beads were left to harden in 5 L aqueous 0.2 M CaCl₂ solution for 2 h, filtered, dried with paper towel and immediately used for the reduction process, or stored in 0.2 M calcium chloride solution at 4°C overnight.

Three different particle sizes were prepared using syringe nozzles with inner diameters of 0.1 mm, 0.2 mm, and 0.5 mm. Bead diameters were measured using a micrometer ocular (Peak Scale Lupe, 10x magnification) and further checked using an Image Analyser (IA). The IA set-up consists of a Sony CCD video camera module (XC-77CE), an Olympus stereo zoom microscope (SZH), and a PC with IA software CUE II version 4.7 (Olympus). The mean particle diameters ($d_{p,\text{mean}}$) determined at ambient temperature from a sample of 100 beads were 1.2 mm, 1.7 mm, and 2.4 mm with standard deviations of 11%, 14%, and 17%, respectively.
**Bead shrinkage test**

A known amount of alginate beads (0.1, 0.2, and 0.3 L; each in duplicate), was placed in a vessel containing buffer solution (composition and pH as used in bioreduction experiments). The mixture (with total volume ranging from 0.3–1 L) was placed in a water bath at \( T = 30^\circ\text{C} \) for 24 h. The initial and final mass and volume of the beads were determined. Samples of the supernatant liquid were analyzed for biomass content.

**Partitioning of EOB and EHB in alginate beads**

The partitioning experiments were performed using the standard conditions of airflow rate, stirring speed, reactor temperature, and pressure, and the standard procedure for bioreduction described before [6] with minor modifications. A 0.5 L buffer solution (composition and pH as used in bioreduction) was added to the reactor followed by 0.3 L alginate beads without cells and 0.2 L buffer solution to make up the working volume of 1 L. The mixture was stirred and aerated for 30 min prior to the addition of 10-20 mmol of EOB and EHB; after which it was continuously stirred and aerated for 24 h. In the course of the experiment, liquid samples were taken and analyzed for EOB and EHB. The partition coefficients were calculated from the difference between the amount of EOB and EHB originally added and the amount measured in the liquid.

**Ester reduction**

Because alginate beads require calcium ions to maintain their mechanical properties and high phosphate concentrations tend to dissolve them, the buffer solution used in the experiments slightly differed from the standard protocol described earlier [6]. Minor modifications of the reduction set-up and protocol [6] were required. Ester reductions were performed with a buffer composed of 5 mM potassium phosphate (pH adjusted to 5.5 with KOH solution), 5 mM MgSO₄, 50 mM calcium chloride and 0.2 mL L⁻¹ Struktol® solution J-673 as antifoam agent. The standard procedure was to add 0.5 L buffer solution to the reactor followed by 0.3 L immobilized cells and 0.2 L buffer solution to make up the 1 L 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 solution at a rate of 5 mL h⁻¹. The specific glucose supply rate was constant at \( F_{Gl} = 0.06 \) mmol.g dw⁻¹.h⁻¹ by varying the concentration in the feed solution from 69 to 447 mM depending on the initial biomass concentration in the reactor. The system was allowed to attain stationary oxygen consumption for 2 h.
Subsequently, the reduction was started by addition of 1.3 g EOB (10 mmol). In the course of the reduction, liquid samples were taken and analyzed for EOB, EHB and its enantiomeric excess, residual glucose, ethanol, and acetate.

**Analytical Methods**

EOB, EHB, ethanol and acetate in the supernatant of the reaction mixture were measured by gas chromatography as described before [19]. Determination of the enantiomeric excess of S-EHB was performed by gas chromatography with a chiral diAc-tBuSi-β-cyclodextrin column (MEGA, Italy) as described [19]. Glucose in the supernatant of the reaction mixture was analyzed by a commercial glucose oxidase and peroxidase kit (BIOTROL®, Biotrol Diagnostic, France); a UV-Vis spectrophotometer (Ultrspec 2000, Pharmacia Biotech, Cambridge, England) was used for the absorbance measurements at 550 nm. Measurements of oxygen consumption and carbon dioxide production were done as described elsewhere [6].

To estimate cell dry weight, about 2 g of beads was dissolved in 50 mL of 10% w/w sodium polyphosphate solution. The suspension was filtered through a pre-dried and pre-weighed filter (Gelman Sciences membrane filters: \( \phi = 47 \text{ mm, } 0.2 \mu \text{m; Gelman Sciences Inc., Ann Arbor, MI, USA} \) and washed with demineralized water. The biomass collected was dried at 105°C to constant weight. To check cell leakage during the immobilization process, samples of the 0.2 M calcium chloride gelling solution were also analyzed for biomass content.

**Quantification of reductions**

The reduction process was treated as a two-phase system. Only the concentrations of EOB, EHB, residual glucose, ethanol and acetate, in the liquid were measured. In the calculation of initial biomass specific reduction rates \((q_r)\), these measurements were corrected for the actual volume in the reactor, the amounts taken out during sampling and the amounts that partitioned to the beads (in the case of EOB and EHB). The pH and dissolved-oxygen (DO) concentrations in the reactor as well as the oxygen and carbon dioxide concentrations in the off-gas were monitored and measured on-line [6]. The latter were corrected for the ambient pressure and temperature and were used to calculate the biomass specific oxygen consumption rates \((q_{\text{O}_2})\) and carbon dioxide production rates \((q_{\text{CO}_2})\). Values of initial cell dry weight were used in the calculation of specific rates \((q)\).
RESULTS AND DISCUSSION

Cell dry weight measurement in beads
Cell dry weight measurement by re-dissolving the alginate beads in 10% w/w sodium polyphosphate solution was found to be reliable and reproducible. Batches of immobilized baker's yeast with varying cell concentrations were analyzed for cell dry weight. Results showed that the measured biomass dry weight in the beads was proportional to the original amount added. In the 0.2 M CaCl₂ gelling solution, where freely suspended cells may be found, the measured cell dry weights were negligible. This showed that immobilization of baker's yeast by entrapment in calcium alginate was 99-100% efficient.

Bead shrinkage
Calcium alginate beads have swelling and shrinkage characteristics that are dependent on various factors such as pH, temperature, mechanical stress, CaCl₂ concentration, and sodium alginate concentration [20-21]. In the present study, it was observed that fresh alginate beads coming from the gelling solution at ambient temperature (≈ 18-22°C), shrunk by 15% (by mass and by volume) after subsequent exposure to the reduction temperature of 30°C for 24 h. As cell leakage was absent, it was concluded that exposure of the beads to the reduction temperature of 30°C caused a considerable amount of water to escape into the aqueous solution. In the bioreduction experiments, it was observed that beads also shrunk by about 15% (by mass and by volume), which could be attributed to water loss upon exposure to the reduction temperature of 30°C. Thus, bead shrinkage due to mechanical stress (i.e. stirring) and other factors could be neglected.

Partitioning of EOB and EHB in alginate beads
Initial concentrations of 10-20 mM each of EOB and EHB were used in the determination of the partition coefficients. At equilibrium, both compounds were observed to be in the supernatant liquid as well as in the beads in nearly equal molar concentrations. The partition coefficients of EOB and EHB between the gel beads and liquid were 1.0±0.1. Because of this value, bead shrinkage was not expected to have any adverse effect to the over-all reduction performance.
**Kinetic model of ester reduction with free cells**

A model based on the mass balance equations for the batch-wise reduction of ethyl 3-oxobutanoate under aerobic conditions was set-up using the reaction rates of ester reduction with free cells.

EOB balance:

\[
\frac{dC_{EOB}}{dt} = -r_{EOB} = -q_{EOB} \cdot C_X = -(r_{(S)-EHB} + r_{(R)-EHB}) \tag{2.1}
\]

(S)-EHB balance:

\[
\frac{dC_{(S)-EHB}}{dt} = r_{(S)-EHB} = q_{(S)-EHB} \cdot C_X = q_{(S)-EHB}^{\text{max}} \cdot \frac{C_{EOB}}{K_{M,EOB}^S + C_{EOB}} \cdot C_X \tag{2.2}
\]

(R)-EHB balance:

\[
\frac{dC_{(R)-EHB}}{dt} = r_{(R)-EHB} = q_{(R)-EHB} \cdot C_X = q_{(R)-EHB}^{\text{max}} \cdot \frac{C_{EOB}}{K_{M,EOB}^R + C_{EOB}} \cdot C_X \tag{2.3}
\]

In addition, the enantiomeric excess (ee) of ethyl (S)-3-hydroxybutanoate was expressed as:

\[
\text{ee} = \frac{(C_{(S)-EHB} - C_{(R)-EHB})}{(C_{(S)-EHB} + C_{(R)-EHB})} \cdot 100\% \tag{2.4}
\]

The simulation of equations 2.1-2.4 and fitting these results with experimental data [6] were performed for EOB reduction with a glucose feed rate of 0.06 mmol.g$^{-1}$h$^{-1}$ using free cells (Figure 2.2). The following kinetic parameters of the reduction reaction were found: $q_{(S)-EHB}^{\text{max}} = 0.25$ mmol.g$^{-1}$h$^{-1}$, $q_{(R)-EHB}^{\text{max}} = 0.03$ mmol.g$^{-1}$h$^{-1}$, $K_{M,EOB}^S = 0.2$ mM, and $K_{M,EOB}^R = 45$ mM.

**Ester reduction with immobilized cells**

*General Observations*

The biomass concentration in the alginate beads was measured after each reduction experiment. In no case biomass increase was observed. Negligible amounts of cells had leaked out to the supernatant liquid. The measured residual glucose concentrations in the bulk liquid at any time during the course of the reduction were in the same order of magnitude at
\( C_{\text{Glc}} \leq 1 \text{ mM} \) for all experiments performed with immobilized cells. Metabolic by-products were negligible during the reduction process as residual ethanol and acetate in the liquid samples were below detection limits. In the reduction experiments using freely suspended cells where neither biomass increase nor production of metabolic by-products occurred, the specific glucose feed rate was at 0.06 mmol.g \( \text{dw}^{-1}.\text{h}^{-1} \) [6]. Therefore, during the reductions with immobilized cells, glucose was supplied at a feed rate of 0.06 mmol.g \( \text{dw}^{-1}.\text{h}^{-1} \).

![Figure 2.2](image_url)  

**Figure 2.2.** *Time course of ethyl 3-oxobutanoate reduction using free cells (\( C_X = 5.94 \text{ g dw.L}^{-1} \)) with glucose (electron donor) feed rate of \( q_G = 0.057 \text{ mmol.g dw}^{-1}.\text{h}^{-1} \). Markers are experimental data for EOB (○), (S)-EHB (▲), (R)-EHB (●), and enantiomeric excess of (S)-EHB (□). Lines are simulation results.*

The dissolved-oxygen (DO) concentrations in the reactor were in the range of 93-97% of air saturation at low cell concentrations regardless of the particle size used and were 67-80% at high cell concentrations. At low cell concentrations, the measured values of \( q_{O2} \) and \( q_{CO2} \) were 0.25 and 0.34 mmol.g \( \text{dw}^{-1}.\text{h}^{-1} \), respectively, the same as in the reduction using free cells [6]. At high cell concentrations, negligible changes in specific oxygen consumption and carbon dioxide production rates were observed.

The ester balance showed slight errors as \( q_{(S)}-\text{EHB} \) and \( q_{(R)}-\text{EHB} \) do not sum up to \( q_{\text{EOB}} \) (Tables 2.1 and 2.2). This could be attributed to experimental inaccuracy. However, the established
trends are clear. During the reduction of EOB using immobilized cells with $d_{p,mean} = 1.2 \text{ mm}$ at a biomass concentration, $C_X = 6.2 \text{ g dw.L}^{-1}$, complete conversion was achieved after 7 h (Figure 2.3). Using larger particle sizes, complete conversion was achieved a few hours later as reduction rates were lower. Figure 2.4 shows the time course of the reduction of EOB at high cell concentration, $C_X = 28.8 \text{ g dw.L}^{-1}$, where EOB conversion was completed within 3 h. In other cases of reductions at high cell concentrations, complete conversion was also achieved within 3 h. The reduction process was reproducible as can be seen by the duplicate results (Table 2.1, $d_{p,mean} = 1.2 \text{ mm}$).

**Figure 2.3.** Time course during ethyl 3-oxobutanoate reduction with immobilized baker’s yeast at $d_{p,mean} = 1.2 \text{ mm}$ and $C_X = 6.25 \text{ g dw.L}^{-1}$. Markers are experimental data for EOB (○), (S)-EHB (▲), (R)-EHB (●), and enantiomeric excess of (S)-EHB (□). Lines are simulation results using the free cell model.

**Influence of different particle sizes at low cell concentrations**

Results of ester reduction at low cell concentrations using immobilized beads of different particle sizes are presented in Table 2.1. The specific production rates of S-EHB ($q_{(S)-EHB}$) as well as R-EHB ($q_{(R)-EHB}$) at these conditions decreased with increasing particle size. However, the ratio between the two remained largely unchanged since the enantiomeric excess of S-EHB (Table 2.1) at any particle size was consistently a bit higher than the
enantiomeric excess during the reduction using free cells. The time course of the reduction of EOB using free cells and immobilized cells at \( d_{pmean} = 1.2 \) mm were similar (Figures 2.2 and 2.3). The specific reduction rates (\( q_{EHB} \)) using free cells and using immobilized cells at \( d_{pmean} = 1.2 \) mm were comparable as simulation results fitted well; however, this is not the case anymore at larger particle sizes. In previous work [15], almost the same reduction rates were reported using free cells and also immobilized cells having small sizes (i.e. \( d_{pmean} = 1.5 \) mm). Although the reduction conditions employed were slightly different from those in this study, it is clear that immobilized beads with small particle sizes facilitate ester reduction.

**Table 2.1. Influence of particle size on specific rates of ethyl 3-oxobutanoate, ethyl (S)-3-hydroxybutanoate and ethyl (R)-3-hydroxybutanoate during ethyl 3-oxobutanoate reduction using immobilized baker’s yeast at low cell concentrations.**

<table>
<thead>
<tr>
<th>( d_{pmean} ) (mm)</th>
<th>( C_X ) (g dw.L(^{-1}))</th>
<th>( F_{DC} ) (mmol.g dw(^{-1}.h(^{-1}))</th>
<th>( q_{EOB} ) (mmol.g dw(^{-1}.h(^{-1}))</th>
<th>( q_{(S)-EHB} ) (mmol.g dw(^{-1}.h(^{-1}))</th>
<th>( q_{(R)-EHB} ) (mmol.g dw(^{-1}.h(^{-1}))</th>
<th>ee(^{a}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>free cells</td>
<td>5.9</td>
<td>0.057</td>
<td>0.231</td>
<td>0.236</td>
<td>0.004</td>
<td>97.5</td>
</tr>
<tr>
<td>1.2</td>
<td>6.1</td>
<td>0.057</td>
<td>0.227</td>
<td>0.235</td>
<td>0.003</td>
<td>98.9</td>
</tr>
<tr>
<td>1.2</td>
<td>6.2</td>
<td>0.059</td>
<td>0.221</td>
<td>0.227</td>
<td>0.002</td>
<td>98.5</td>
</tr>
<tr>
<td>1.7</td>
<td>5.8</td>
<td>0.060</td>
<td>0.173</td>
<td>0.182</td>
<td>0.002</td>
<td>98.0</td>
</tr>
<tr>
<td>2.4</td>
<td>6.3</td>
<td>0.061</td>
<td>0.136</td>
<td>0.127</td>
<td>0.001</td>
<td>98.6</td>
</tr>
</tbody>
</table>

\(^{a}\) Enantiomeric excess (ee) at the end of the reduction process.

**Influence of high cell densities at the same particle size**

Reductions using immobilized baker’s yeast with mean particle diameter \( d_{pmean} = 1.2 \) mm at high cell concentrations of 22-37 g dw.L\(^{-1}\) proceeded as summarized in Table 2.2. The average specific reduction rate at these cell concentrations was about 22% lower than the specific reduction rate at low cell concentration of 6.2 g dw.L\(^{-1}\). In addition, simulation results using the kinetic model of freely suspended cells did not agree well with experimental data (Figure 2.4 is shown as an example). The enantiomeric excess of S-EHB significantly decreased to about 83-90% (Table 2.2) whereas it was ≥ 98% at low cell concentrations. This was not only caused by a decrease in the specific production rate of the desired S-enantiomer (\( q_{(S)-EHB} \)) but also an unexpected increase in the specific production rate of the undesired R-enantiomer (\( q_{(R)-EHB} \)).
Figure 2.4. Time course during ethyl 3-oxobutanoate reduction with immobilized baker's yeast at $d_p = 1.2$ mm and $C_X = 28.85$ g dw.L$^{-1}$. Markers are experimental data for EOB (○), (S)-EHB (▲), (R)-EHB (●), and enantiomeric excess of (S)-EHB (□). Lines are simulation results using the free cell model, showing that this model does not fit.

Table 2.2. Influence of different cell concentrations during ethyl 3-oxobutanoate reduction using immobilized baker's yeast with mean diameter ($d_p$) of 1.2 mm.

<table>
<thead>
<tr>
<th>$C_X$ (g dw.L$^{-1}$)</th>
<th>$q_{GLC}$ (mmol.g dw$^{-1}$.h$^{-1}$)</th>
<th>$q_{EOB}$ (mmol.g dw$^{-1}$.h$^{-1}$)</th>
<th>$q_{(S)-EHB}$ (mmol.g dw$^{-1}$.h$^{-1}$)</th>
<th>$q_{(R)-EHB}$ (mmol.g dw$^{-1}$.h$^{-1}$)</th>
<th>ee$^a$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td>0.059</td>
<td>0.221</td>
<td>0.227</td>
<td>0.002</td>
<td>98.5</td>
</tr>
<tr>
<td>21.66</td>
<td>0.059</td>
<td>0.192</td>
<td>0.182</td>
<td>0.006</td>
<td>89.1</td>
</tr>
<tr>
<td>24.56</td>
<td>0.061</td>
<td>0.230</td>
<td>0.179</td>
<td>0.013</td>
<td>83.4</td>
</tr>
<tr>
<td>28.85</td>
<td>0.066</td>
<td>0.168</td>
<td>0.149</td>
<td>0.009</td>
<td>90.6</td>
</tr>
<tr>
<td>37.15</td>
<td>0.060</td>
<td>0.181</td>
<td>0.159</td>
<td>0.012</td>
<td>83.6</td>
</tr>
</tbody>
</table>

$^a$ Enantiomeric excess (ee) at the end of the reduction process.

Concentration profiles in alginate beads

When using free cells during the reduction of EOB, the local concentration levels of EOB, glucose and oxygen surrounding the cells are nearly the same as in the bulk liquid. During the
reduction with immobilized cells, however, lower concentration levels of these compounds may be expected in the solid phase (alginate beads), where the reduction reaction actually takes place, due to slow diffusive transport (see Figure 2.5) [22]. A concentration gradient of one of not all of these reacting compounds in the alginate beads might affect the over-all reduction performance. Thus, concentration profiles of the reacting species in the beads were calculated.

A mass balance over an infinitely thin layer of the biocatalyst bead was used [22]:

$$\frac{D_{i,\text{eff}}}{R^2} \frac{d}{dR} \left( R^2 \frac{dC_i}{dR} \right) - r_i = 0$$  \hspace{1cm} (2.5)

To solve this expression for the concentration of $i$ ($i = \text{EOB, glucose, oxygen}$) as a function of radial distance ($R$) in the biocatalyst bead, the following assumptions were made:

1. The external diffusion limitation of $i$ is negligible. Calculations (not included) showed that this assumption was acceptable.

2. The effect of inter-molecular friction forces inside the particle is negligible.

3. The reaction rates of glucose can be described by zero- or first-order kinetics while oxygen can be assumed to have zero-order kinetics. The reaction rates of EOB, S-EHB and R-EHB can be described by equations (2.1-2.3).

4. The diffusion coefficients of glucose and oxygen in calcium alginate ($D_{\text{Glc-eff}}, D_{\text{O2-eff}}$) were taken as 91% and 81% of their diffusivities in water, respectively [8,23]. No literature data on diffusion coefficients of EOB, (S)-EHB and (R)-EHB in water and in calcium alginate were found; those of ethanol were taken instead. The diffusivity coefficients of EOB, (S)-EHB and (R)-EHB in calcium alginate ($D_{\text{EOB-eff}}, D_{(S)-\text{EHB-eff}}, D_{(R)-\text{EHB-eff}}$) were taken as 91% of the diffusivity of ethanol in water [8].

5. Cells are homogeneously distributed in alginate beads and concentration gradients in the beads are established instantaneously (compare [24]). For the reactants (i.e. EOB, glucose and oxygen), the boundary conditions $C_i(R) = C_{i,\text{bulk}}$ at $R = R_{\text{particle}}$, $C_i(R) = 0$ and $dC_i(R)/dR = 0$ at $R = R_o$ were applied [22]. The actual experimental values of the initial concentrations of the reacting species in the bulk liquid were used as initial conditions.
For EOB as well as (S)-EHB and (R)-EHB, the calculated profiles in the beads showed negligible gradients. The internal effectiveness factor ($\eta_e$) was 1 in all cases, indicating the absence of diffusion limitation even if diffusivity coefficients of these compounds were taken twice as low as that of ethanol. As the reduction rate is only dependent on EOB concentration according to equations (2.1-2.3), simulation results of the time course of EOB, (S)-EHB and (R)-EHB concentration in the reactor using immobilized cells were expected to be the same as with free cells.

![Schematic diagram](image)

**Figure 2.5.** Schematic diagram of the concentration profile of substrates in a spherical immobilized catalyst.

The glucose consumption rate may either be described by zero-order kinetics where $q_{Glc} = q_{Glc}^{\text{max}}$ or by first-order kinetics using $q_{Glc} = k \cdot C_{Glc}$ where $k = q_{Glc}^{\text{max}} / K_{M,Glc}$. Assuming first-order kinetics with $q_{Glc}^{\text{max}} = 1 \text{ mmol.g dw}^{-1} \cdot \text{h}^{-1}$ [6] and with a varying $K_{M,Glc}$ value of 0.1-1.0 mM [25], the glucose concentration profile inside the immobilized beads showed a negligible gradient in any case at the specific glucose feed rate $F_{Glc} = 0.06 \text{ mmol.g dw}^{-1} \cdot \text{h}^{-1}$. Assuming zero-order kinetics at the same specific glucose feed rate, the glucose concentration profile also showed a negligible gradient in the beads regardless of the mean.
particle diameter and cell concentration. These results indicated that no glucose transport limitation occurred in the beads during reductions using immobilized cells at particle diameters ranging from 1.2-2.4 mm and at any cell concentration employed.

In the prediction of oxygen concentration profiles in the immobilized beads, the oxygen consumption rate was described by zero-order kinetics where \( q_{O_2} = q_{O_2}^{\text{max}} \). For \( C_X = 6 \text{ dw.L}^{-1} \), significant oxygen concentration gradients in gel beads were calculated at \( d_{p_{\text{mean}}} = 1.7 \text{ mm} \) and 2.4 mm (Figure 2.6). About 85% of the oxygen concentration in the bulk liquid \( (C_{O_2}) \) was available for the cells in the center of the beads with a \( d_{p_{\text{mean}}} \) of 1.2 mm. The latter situation may still suffice for the immobilized cells, as the reduction rate at this condition was comparable to the reduction rate using free cells. However, the oxygen concentration in the center of the beads was reduced by one-third and two-thirds for immobilized cells having \( d_{p_{\text{mean}}} = 1.7 \text{ mm} \) and 2.4 mm, respectively (Figure 2.6). At high cell concentrations in the beads with \( d_{p_{\text{mean}}} = 1.2 \text{ mm} \), strong gradients in the oxygen profiles were calculated (Figure 2.7). In figure 2.7, practically no oxygen was available in the center of the beads for a cell concentration of \( C_X = 28.8 \text{ g dw.L}^{-1} \). The penetration of oxygen in the beads was only about halfway across the particle radius at a cell concentration of \( C_X = 37.2 \text{ g dw.L}^{-1} \). The occurrence of this severe oxygen limitation might explain the slower reduction rates observed. It is known that during ester reduction, the regeneration of the cofactor NADPH in the cell is necessary to sustain catalytic activity. This regeneration process is coupled to the oxidation of NADH, which is dependent on the oxygen concentration \([5]\). At severe oxygen gradients in the beads, the rate and course of the metabolism in the cells might have changed, not only slowing down the reduction capacity of the cells but also changing their enantioselectivity. The latter phenomenon had also been reported in previous studies with reductions using immobilized yeast \([12-15]\).

If anaerobic conditions occurred in the center of the beads, glucose might be fermented to ethanol, which may be further converted to carbon dioxide in the outer part of the beads. It is known that the reduction of EOB proceeds differently at aerobic and anaerobic conditions, and with glucose or ethanol as electron donor \([6,26-31]\). We did not quantitatively model these effects because this would require detailed knowledge of the influence of oxygen on the intrinsic reaction kinetics during the reduction of 3-oxo esters.
Figure 2.6. Simulation results for oxygen concentration profiles in gel beads during reduction of ethyl 3-oxobutanoate using immobilized baker’s yeast at different mean particle diameters: $d_{p_{\text{mean}}} = 1.2$ mm (---), $d_{p_{\text{mean}}} = 1.7$ mm (-----) and $d_{p_{\text{mean}}} = 2.4$ mm (-----).

Figure 2.7. Simulation results for oxygen concentration profiles in gel beads during reduction of ethyl 3-oxobutanoate using immobilized baker’s yeast with mean particle diameter ($d_{p_{\text{mean}}} = 1.2$ mm at high cell concentrations: $C_{X} = 21.66$ g dw.L$^{-1}$ (-----), $C_{X} = 24.56$ g dw.L$^{-1}$ (-----), $C_{X} = 28.85$ g dw.L$^{-1}$ (-----) and $C_{X} = 37.15$ g dw.L$^{-1}$ (-----).
CONCLUSIONS

A suitable protocol was developed for quantitatively studying the batch reduction of EOB in water under aerobic conditions using baker's yeast immobilized in calcium alginate. The specific reduction rate using immobilized cells at low cell concentration with particle size $d_{p,mean} = 1.2$ mm corresponded very well with the reduction rate using freely suspended cells at the same cell concentration. The enantiomeric excess of S-EHB reached $\geq 98\%$, which was slightly higher than with freely suspended cells. For larger particle sizes and higher cell concentrations, the reduction rate decreased, and in the latter case, the enantiomeric excess of (S)-EHB also decreased, which could possibly be attributed to the occurrence of oxygen diffusion limitation.

ACKNOWLEDGEMENTS

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**LIST OF SYMBOLS**

- $C_i$: concentration of species $i$ in the liquid, mM
- $C_X$: biomass concentration, dry weight per unit reactor volume, g dw.L$^{-1}$
- $d_{p,mean}$: mean particle diameter, mm
- $D_{i,eff}$: effective diffusivity coefficient of species $i$, m$^2$.h$^{-1}$
- $DO$: dissolved oxygen concentration in the liquid, % of air saturation
- $ee$: enantiomeric excess, %
- $k$: first-order kinetic constant, L.g dw$^{-1}$.h$^{-1}$
- $K_{M,EOB}^S$: Michaelis-Menten constant for EOB with $S$-specific enzyme, mM
- $K_{M,EOB}^R$: Michaelis-Menten constant for EOB with $R$-specific enzyme, mM
- $K_{M,Glc}$: Monod constant for glucose consumption of $S. ~cerevisiae$, mM
- $q_i$: biomass specific reaction rate of species $i$, mmol.g dw$^{-1}$.h$^{-1}$
- $q_i^{max}$: maximum biomass specific reaction rate of species $i$, mmol.g dw$^{-1}$.h$^{-1}$
- $R$, $R_{particle}$: radial distance of particle, mm
- $R_o$: radial distance when concentration of species $i$ is zero, mm
- $r_i$: rate of reaction of species $i$, mmol.L$^{-1}$.h$^{-1}$
REFERENCES


Chapter 3

*In situ* product removal using a crystallization loop in the asymmetric reduction of 4-oxoisophorone by *Saccharomyces cerevisiae*

E.M. Buque-Taboada, A.J.J. Straathof, J.J. Heijnen, and L.A.M. van der Wielen

Chapter 3

ABSTRACT

In situ product crystallization was investigated for solid product crystals that were obtained during fermentation. The model reaction was the asymmetric reduction of 4-oxoisophorone (OIP) using baker's yeast (S. cerevisiae) as a biocatalyst. The target product was 6R-dihydro-oxoisophorone (DOIP), also known as levodione, a key intermediate in carotenoid synthesis. DOIP was degraded by baker's yeast mainly to (4S,6R)-actinol, an unwanted by-product in the process. Actinol formation reached up to 12.5% of the initial amount of OIP in the reactor during a batch process. However, better results were obtained when the dissolved DOIP concentration was controlled using an integrated fermentation-crystallization process since (a) actinol formation was reduced to 4% and (b) DOIP crystal formation in the reactor was avoided. DOIP productivity was improved by 50% and its selectivity was raised from 87 to 96% relative to the batch process. In the integrated process, most of the product was recovered as pure crystals; this may already minimize if not eliminate the use of organic solvents in the final purification steps. An almost 6-fold reduction in biocatalyst consumption per kg product was achieved, which also can contribute to the minimization of waste streams.
INTRODUCTION

Product inhibition and/or product degradation become the limiting factors when a fermentation process is developed to the level that leads to high product concentrations [1]. Often, low yields and productivities are encountered [2-3]. In order to develop economically promising biochemical processes, these limitations must be overcome. In situ product removal (ISPR) is usually applied to improve bioreactor performance [2,4]. Many studies have focused on high-volume low-value products such as organic acids and solvents in their removal from fermentation broth [2-3,5]. Advantages of ISPR have been mentioned in most cases, for example, increases in the productivity or yield of a biocatalytic reaction by either overcoming inhibitory or toxic effects, shifting unfavorable reaction equilibria, or minimizing product losses owing to degradation or uncontrolled release [2-3,5]. In these processes, the consumption of auxiliary chemicals such as organic solvents can be reduced if not eliminated, and waste production can be minimized. More importantly, ISPR could reduce the total number of steps in downstream processing, which is in many cases the cost-limiting factor [2-3,5]. ISPR can be varied in its configuration and application, depending usually on the properties of the target product, the guiding principle exploited during product removal, and the desired potential benefit to be implemented in the process [2,5].

Product crystallization as an ISPR method has hardly been explored in bioprocesses using whole cells, although most chemicals produced by fermentation or biotransformation are solids at room temperature with a production process involving crystallization or precipitation steps. Occasionally, crystallization already occurs in the reactor when the product concentration exceeds the solubility during the reaction. There are numerous examples of this involving a single enzymatic reaction, either by a purified enzyme or crude cell, but in this paper we focus on reactions catalyzed by metabolizing whole cells. Industrially relevant examples are the production of riboflavin [6], L-tryptophan [7], steroids [8-9], tetracycline and oxytetracycline [10], diltiazem precursor [11], a chiral hydroxysulfone [12], a chiral nitro-alcohol [13], 6R-dihydro-oxoisophorone [14-15], calcium lactate [16] and 6-hydroxy nicotinic acid [17]. These in situ product crystallization processes are not using in situ product recovery in a strict sense, because neither the final product concentration in the reactor was reduced nor the product recovery process was simplified. In these cases, the
crystalline product usually had to be dissolved again in order to be recovered and purified. Only for riboflavin [6] and 6R-dihydro-oxoisophorone [14-15], it was mentioned that the product crystals could be easily separated from the cells by filtration.

Easy product recovery combined with reduced product concentration in the reactor might be achieved by using an external crystallization loop rather than internal crystallization. So far, bioreactor-crystallizer set-ups have been reported only for enzymatic conversions in immobilized systems: L-malic acid production by L-malease in immobilized Brevibacterium flavum cells [18-19], L-alanine production by L-aspartate β-decarboxylase in immobilized Pseudomonas dacunhae cells [20-21] and calcium gluconate by immobilized glucose oxidase [22]. The results of these studies clearly show that a bioreactor-crystallizer system may have large advantages as compared to non-integrated systems.

The aim of this work is to show experimentally that also for metabolizing cells it is possible to achieve such advantages. Product inhibition and degradation can be minimized by controlling the product concentration in the fermenter via its removal by crystallization in an external loop. When the cells are retained in the fermenter, this should also lead to a simplification of the product recovery process.

As compared to the aforementioned enzymatic systems, the most important additional constraint with metabolizing cells is that they usually require aeration. In aerobic systems, the commercial potential of cell retention by immobilization in particles is limited because oxygen diffusion limitation in the particles may easily lead to loss of productivity [23] or selectivity [24]. In the present study, cell retention by membrane confinement will be used instead.

Our model reaction is the reduction of 4-oxoisophorone (OIP) to 6R-dihydro-oxoisophorone (DOIP) using S. cerevisiae as the biocatalyst (Figure 3.1). DOIP is an important key intermediate in carotenoid synthesis [14-15] and in the production of tobacco and saffron flavors [25]. It is degraded to an unwanted by-product known as (4S,6R)-actinol (ACT) by the same biocatalyst. The reaction also suffers from substrate inhibition [14-15].
It is very well established that baker's yeast (Saccharomyces cerevisiae) catalyzes a wide range of redox reactions [26]. Because it is readily available, cheap and easy to handle, it has been one of the most widely used biocatalysts. It is very attractive for food and pharmaceutical applications as it is non-toxic, non-pathogenic and generally regarded as safe (GRAS). Above all, using whole cells as such allows regeneration of cofactors in the cell, providing sustained catalytic activity for redox reactions [27]. In S. cerevisiae, two enone reductases have been found to catalyze the reduction of OIP to DOIP [28]. The cofactors were NADPH and NADH, respectively, which can easily be regenerated in the cell. Recently, the old yellow enzyme 2 (OYE2) of S. cerevisiae, expressed in E.coli was also used as biocatalyst for this reaction [29]. For the conversion of DOIP to ACT, various enzymes may be involved such as the highly selective levodione reductases recently cloned, characterized and employed in the reaction [29-31]. For reduction of ethyl 3-oxobutanoate by S. cerevisiae, glucose feeding at aerobic conditions has been shown to be the preferred option to avoid production of metabolic by-products [32], and this option is also used in the present study for OIP reduction. We will experimentally compare for the latter reaction the performance of an integrated bioreaction-crystallization process to the batch and fed-batch alternatives for OIP supply to the reactor, with respect to yield, selectivity, final product concentration, biocatalyst consumption per kg product, and volumetric productivity.
MATERIALS AND METHODS

Micro-organism
Active dry baker’s yeast (Fermix®; 97.5 % dry weight) was kindly provided by DSM-Gist (Delft, The Netherlands).

Chemicals
OIP (>98%) was supplied by Fluka Biochemika (Buchs, Switzerland). DOIP (98%) and actinol (98%) were kindly provided by F. Hoffmann-La Roche (Basel, Switzerland), with enantiomeric excesses (e.e.’s) of 99% for 6R-DOIP and 4R,6R-ACT as measured, respectively. The purity of all other chemicals used was at least laboratory grade.

Solubility measurement
The solubility measurements of OIP, DOIP, and ACT in water at the reduction and crystallization temperatures of 30°C and 5°C, respectively, were performed in 30 mL glass tubes with screw caps. The solute was added in the tubes in excess to ensure solubility determination. The tubes were immersed in water bath at the temperature setting and shaken vigorously for more than 48 h to ensure equilibration. The mixtures were allowed to settle before sampling. Samples were withdrawn using syringes with attached 0.2-μm filters to avoid entrainment of the solids and were analyzed by gas chromatography.

OIP reduction
The bioreduction set-up and protocols described elsewhere [33] were employed with some minor modifications. The standard procedure was to add 0.7 L buffer solution to the fermenter followed by 30 g dw yeast cells suspended in 0.3 L phosphate buffer solution to make up the 1 L 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 solution at a rate of 3 mL.h⁻¹ and a specific glucose feeding rate of 0.20 mmol.g dw⁻¹.h⁻¹. The reactor system was allowed to attain stationary oxygen consumption for 2 h at 30°C. Subsequently, the batch reduction was started by addition of weighed amount in the range of 5-10 g pure OIP. In the case of fed-batch and integrated reductions, OIP was supplied at 0.066 mmol.g dw⁻¹.h⁻¹ to allow a concentration level in the reactor that avoids substrate inhibition [14].
Feeding of glucose and OIP was done using a precision-type syringe pump (Harvard Apparatus pump model 11, Holliston, MA, USA) equipped with a gastight glass syringe (Hamilton Company, Nevada, USA). For the integrated reduction experiment (coupled conversion and crystallization), the reactor content was continuously pumped through an ultrafiltration membrane module (UFP-500-C-3MA, 500kDa NMWC, φ fibre 0.5 mm, A = 140 cm²; A/G Technologies, USA), which was inserted in an external loop (dead volume of membrane loop ca.100mL, flow over module 62.5 mL.h⁻¹, module retentate flow ≈ 42.5 mL.h⁻¹). The same feeding rates of glucose and OIP in the reactor were used and permeate flow rate to the crystallizer was 20 mL.h⁻¹. The permeate was collected into a cooling crystallizer unit (2-L jacketed-vessel, equipped with two six-blade Rushton-type impellers, diameter = 4.5 cm; impellers 0.5 cm above each other, 100 rpm; Applikon, Schiedam, The Netherlands), which was cooled to 2-5°C through a cryostat. The recirculation rate of the liquid solution from the crystallizer to the reactor was such that the net permeate collected in the crystallizer was 5 mL.h⁻¹. This was done with the help of 0.2-μm filters attached to the recirculation tubing to avoid entrainment of the solid crystals. In the course of the reduction, liquid samples were taken from the reactor and from the crystallizer (for the integrated reduction set-up) and analyzed for OIP, DOIP, and actinol. Concentrations of glucose, ethanol and acetate in the reactor were also checked occasionally. The pH and dissolved oxygen (DO) levels in the reactor were monitored and measured on-line.

**Analytical Methods**

OIP, DOIP, and actinol in the supernatant of the reaction mixture were extracted with ethyl acetate (1:1 v/v) and measured by gas chromatography using a high-performance capillary column HP-Innowax (cross-linked polyethylene glycol) with length = 15 m, i.d. = 0.53 mm, and film thickness = 1 μm; oven and injection temperatures were 140°C and 180°C, respectively. At this condition, the retention time was 4.4 min for OIP, 5.8 min for 6R-DOIP, 6.0 min for 6S-DOIP, 15.7 min for 4R,6R-actinol, and 15.9 min for 4S,6R-actinol. The enantiomeric excess (e.e.) of 6R-DOIP was measured from the peak areas of the two enantiomers observed between the retention times of 5.8-6.0 min. Enantiomeric excess (e.e.) was expressed as [R-S]/[R+S] in %. Glucose in the reaction mixture was analyzed as described elsewhere [24]. Analysis of ethanol and acetate as well as estimation of cell dry weight in the bioreactor was done as described previously [33].
Quantification of experimental results
In the calculation of OIP, DOIP, and ACT concentrations as well as the initial specific OIP reduction rates, the measurements were corrected for the actual volume in the reactor and the amounts taken out during sampling. Values of the initial cell dry weight in the reactor were used in the calculation of biomass-specific rates, because the cell mass did not change significantly during the experiments. The final yield was expressed in % as the ratio of the total DOIP produced to the total OIP fed while the final DOIP selectivity was expressed as the ratio of the total DOIP obtained to the total DOIP and ACT produced. Volumetric productivity was expressed in terms of total grams DOIP produced per liter per hour.

RESULTS AND DISCUSSION

Solubility data
OIP is soluble in water up to 98.7 mM (≈ 15 g.L⁻¹) at 30°C. DOIP is soluble up to 64.9 mM (≈ 10 g.L⁻¹) at the same temperature and its solubility decreased by 50% at 5°C. At this temperature, the aqueous solubility of OIP decreased to 65.8 mM (≈ 10 g.L⁻¹) so that maintaining an OIP concentration below this value in the reactor (i.e. by feeding) would allow the OIP to remain in solution at 5°C while only DOIP would crystallize.

General observations
No variation in biomass concentration in the reactor was observed during the course of the reduction experiment. Metabolic by-products were negligible during the reduction process as the measured ethanol and acetate concentrations in the liquid samples were below detection limits. However, at low glucose feeding rate (i.e. at 0.06 mmol.g dw⁻¹.h⁻¹), biomass concentration in the reactor dropped, suggesting that microbial storage compounds such as glycogen and trehalose may have been consumed during the process [34]. Increasing the glucose-feeding rate to 0.2 mmol.g dw⁻¹.h⁻¹ kept the biomass concentration constant throughout the experiment, and the metabolic by-products below detection limits. This rate was employed in the succeeding experiments. It is much lower than the maximal uptake capacity of the cells, causing immediate and complete consumption of the fed glucose.

50
Batch OIP reduction

A stable and consistent initial OIP reduction rate of 10 mg.g dw⁻¹.h⁻¹ was obtained during batch reductions. The total ketone balance in the reactor was 95-98% (Figure 3.2). Substrate (OIP) concentration in the reactor approached zero in 48 h. About 85% of OIP was converted to DOIP and the rest to actinol (by-product). Volumetric productivity was about 0.20 g.L⁻¹.h⁻¹, which is comparable to literature values [14-15]. The final DOIP concentration is rather low at about 58 mM (8.9 g.L⁻¹); this is limited by the amount of substrate that can be added because OIP is known to inhibit the reaction rate at concentrations greater than 78.9 mM (12 g.L⁻¹) in the reactor [14]. However, this situation can be avoided if the substrate is fed to the reactor such that its concentration will not reach inhibitory levels (see section Fed-batch OIP reduction). During OIP reduction at an aeration rate of 0.25vvm, the dissolved oxygen level in the reactor remained above 60%, which shows that the oxygen supply was not limiting.

Figure 3.2. Course of batch OIP reduction at a biomass concentration of 30 g dw:L⁻¹ and initial OIP concentration of 68.4 mM (10.4 g.L⁻¹). Markers are experimental data for OIP (●), DOIP (♦), ACT (▲), and the total mass balance (□).
Fed-batch OIP reduction

In this configuration, OIP was fed to the reactor, like glucose, to avoid substrate inhibition which occurs above 78.9 mM (≈ 12 g.L⁻¹) OIP concentration. During the experiment, the working volume increased to 1.65 L. The total amount of OIP in the reactor remained low at about 30 mmol (≈ 5 g) during the first 80 h of reaction time (Figure 3.3). DOIP crystals were first observed in the reactor after 30 h when the DOIP concentration reached 100 mmol (≈ 15.4 g). Gradually the suspension became very dense. After 80 h, the amount of OIP in the reactor increased, suggesting a decrease in biomass reduction capacity at this time, which could be due to accumulation of DOIP crystals or actinol causing product inhibition or biomass lysis, for example. As a result, the final DOIP yield was only at 68%, equivalent to a total production of almost 240 mmol. Actinol (by-product) accumulated in the reactor up to 20 mmol, accounting for 5.7% of the total OIP fed and 8.4% of the total DOIP produced.

![Graph](image)

**Figure 3.3.** Course of fed-batch OIP reduction at a biomass concentration of 30 g dw.L⁻¹ and OIP feed rate of 10 mg gw⁻¹.h⁻¹. Markers are experimental data for OIP (●), DOIP (♦), ACT (▲), total mmoles of OIP, DOIP and ACT accounted in the reactor (★) and total OIP fed to the reactor (□). The reactor volume increased linearly from 1 to 1.65 L.

A significant gap in the total mass balance was observed, notably after 80 h (Figure 3.3), which was attributed to evaporation of OIP and DOIP as this experiment was conducted at a
higher aeration rate of 0.75 vvm. The fermentation broth resulting from fed-batch reduction contains, amongst others, dissolved as well as crystallized DOIP, thus requiring a considerable train of downstream processing steps to remove biomass debris and to obtain the pure product crystals.

**Integrated continuous OIP reduction**

In an integrated reduction experiment, the fermenter was attached to the crystallizer, and cells were retained in the reactor by inserting an ultrafiltration membrane in an external loop in between the two units. The mother liquor (without the crystals) from the crystallizer was recirculated through a filter (Figure 3.4). The start-up phase was determined by the residence time ($\tau \equiv 16$ h), and the system reached its hydrodynamic steady state in about 80 h. The working volume in the reactor was constant at 1.0±0.04 L throughout the experiment. In this reduction configuration, an OIP level of about 35-40 mmol ($\equiv 5.3-6.1$ g) in the reactor was maintained at steady-state, which suggests a constant biomass specific reduction capacity which lasted 170 h (Figure 3.5). DOIP was maintained at a level of about 60 mmol ($\equiv 9.2$ g), to avoid product crystallization in the reactor. In the crystallizer, an accumulation of DOIP crystals occurred and during the 7-day operation, a total of 256.5 mmol ($\equiv 39.5$ g) was produced. The working volume in the crystallizer increased to about 850 mL in 170 h. Product degradation occurred as indicated by the presence of actinol in the reactor, but it only reached a maximum of about 8 mmol ($\equiv 1.25$ g), being only 4.0% of the total OIP fed, and 4.7% of the total DOIP produced, a considerable improvement over the batch and the fed-batch reduction processes. The final DOIP yield is 85%, which is comparable to the batch process, however, its selectivity is 96%, much better than both the batch and fed-batch processes (Table 3.1). Volumetric productivity increased by 50% relative to the batch process and 100% compared to the fed-batch process. The biocatalyst consumption per kg of product is about 6-fold lower compared to the batch process. For other types of reactions with expensive biocatalyst instead of baker’s yeast, this would be a potential benefit. In any case, however, the waste stream is minimized. Above all, this configuration already allows for direct product recovery without the need for an auxiliary phase. This process still can be optimized further in particular when the reaction and crystallization kinetics are known in detail.
Figure 3.4. Schematic diagram of the integrated fermentation-crystallization process for OIP reduction.

Figure 3.5. Course of OIP reduction employing the integrated fermentation-crystallization process. Markers are experimental data for OIP (■), DOIP (●) and ACT (▲).
It is important to note that in the different process configurations employed, the enantiomeric excess (e.e.) of 6R-DOIP was consistently ≥ 98%. This is in line with the observation that for batch, repeated batch (90 h) and fed-batch (18 days) operations, enantioselectivity of 6R-DOIP remained very high and was not affected at all by the implemented reduction modes [14-15].

<table>
<thead>
<tr>
<th>Parameters, units</th>
<th>Batch reduction</th>
<th>Fed-batch reduction</th>
<th>Integrated reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final yield (DOIP/OIP fed), %</td>
<td>85</td>
<td>68</td>
<td>85</td>
</tr>
<tr>
<td>Final selectivity (DOIP/(DOIP+ACT)), %</td>
<td>87</td>
<td>92</td>
<td>96</td>
</tr>
<tr>
<td>Final product concentration, mM</td>
<td>57.9</td>
<td>145.1</td>
<td>57.7^a/301.8^b</td>
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<tr>
<td>Biocatalyst consumption, kg biocat/kg prod</td>
<td>3.52</td>
<td>0.89</td>
<td>0.67</td>
</tr>
<tr>
<td>Volumetric productivity, g.L^{-1}.h^{-1}</td>
<td>0.20</td>
<td>0.15</td>
<td>0.30</td>
</tr>
<tr>
<td>Direct recovery of product crystals</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
</tr>
</tbody>
</table>

^a In fermenter; ^b In crystallizer.

**CONCLUSION**

A novel example of an aerobic biotransformation with free cells is shown where the product is immediately recovered in an external crystallization loop, avoiding its unfavorable degradation. This results in an integrated process where the desired product is directly obtained as pure crystals. Relative to the conventional batch and fed-batch processes, the integrated process configuration has a potentially better performance with respect to the most important process parameters such as yield, selectivity and productivity.

**ACKNOWLEDGEMENTS**

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Chapter 4

Substrate inhibition and product degradation during the reduction of 4-oxoisophorone by *Saccharomyces cerevisiae*

E.M. Buque-Taboada, A.J.J. Straathof, J.J. Heijnen, and L.A.M. van der Wielen

ABSTRACT

The aromatic diketone 6R-dihydro-oxoisophorone (DOIP) is an important intermediate in the synthesis of some naturally-occurring carotenoids. Its preparation via the reduction of 4-oxoisophorone (OIP) by baker’s yeast has previously been developed to a pilot-scale process. In this work, the kinetics of substrate inhibition and of product degradation during the reduction of OIP using resting baker’s yeast cells as catalyst is studied. Substrate inhibition during the reduction can be described by a non-competitive type of inhibition. Product is degraded to an unwanted by-product 4S,6R-actinol by baker’s yeast. This reaction can very well be described by a second-order rate equation with respect to DOIP concentration, which is an exceptional case for a whole-cell-catalyzed reaction system.
INTRODUCTION

The biocatalytic production of fine chemicals used for the synthesis of naturally occurring carotenoids, vitamins, pigments, flavors and fragrances, is becoming the preferred route for commercial-scale production. Biocatalytic processes, occurring at mild conditions using renewable resources, are usually slow but with competitive yields and desirable enantioselectivities. Levodione, also known as 6R-dihydro-oxoisophorone (DOIP), a key intermediate in the synthesis of optically active xanthophylls, xanthoxin, and zeaxanthins [1-2] as well as aroma constituent of tobacco and saffron [3-4] can be produced by the reduction of 4-oxoisophorone (OIP) with high yield and enantioselectivity. This conversion has been carried out via hydrogenation of the substrate (OIP) over alumina-supported Pt and Pd catalysts [5] or over a Pd/Al2O3 catalyst [6] at a pressure of up to 50 bars. The yield was about 80%, which has been improved further by employing organic solvents in the reaction medium and by varying the pressure and temperature; but the enantioselectivity towards 6R-DOIP was very low in any case. Using baker’s yeast (Saccharomyces cerevisiae) as catalyst gave a comparable yield of 80-85% DOIP and a very attractive enantiomeric excess of e.e. > 98% [1,7]. Although baker’s yeast also degrades the product to an unwanted by-product, known as 4S,6R-actinol, also referred to as S-ACT (Figure 4.1), this process has been regarded as the best option for industrial application [8]. Other yeasts such as Saccharomyces rouxii, S. delbrueckii, S. williamus, Zygosaccharomyces bailii and Candida tropicalis have also been found to catalyze OIP reduction [9].

Aspergillus niger converted OIP to only 55% DOIP and to six by-products [10]. The thermophilic bacteria Thermomonospora curvata cultured at 50-51°C yielded four times higher reduction rates than baker’s yeast [3-4]. Using immobilized Thermomonospora curvata yielded lower rates and the process experienced diffusion and other transport limitations [11]. Various types of reactors like hollow-fiber, fluidized and packed bed reactors [4,12] and different reduction modes such as batch, fed-batch, repeated-batch [1] and integrated fermentation-crystallization processes [7] have also been studied to improve productivity of 6R-DOIP. In a few cases, the sequential two-step reduction of OIP to 4R,6R-actinol (R-ACT) was also explored using two different species of bacteria, namely: Thermomonospora curvata and Bacillus stearothermophilus [13-14]. In none of these studies, the mechanism and kinetics of the reaction were given much attention, despite its
significance in understanding better the behaviour of the biocatalysts involved in the reaction. Such information can be relevant in gaining insights as to how such processes, in particular the integrated fermentation-crystallization process [7] can be optimized and improved further to attain maximum productivity at minimum costs.

![Chemical Diagram]

**Figure 4.1. OIP reduction to DOIP and DOIP's subsequent reduction to actinol.**

It is for this inherent purpose that this work aims to describe the kinetics of (a) the reduction of OIP to DOIP and (b) the subsequent degradation of DOIP to ACT using resting cells of baker's yeast as catalyst. Baker's yeast is used as biocatalyst as it is cheap, readily available, non-toxic, non-pathogenic and more importantly, cofactor regeneration is possible which is essential in sustaining catalytic activity in the cell. NADPH-dependent and NADH-linked oxido-reductases are involved in the reduction of OIP and DOIP in baker's yeast. Some of them have been isolated from baker's yeast, purified and characterized. Two enone reductases [15] and the so-called old yellow enzyme [16] have been found to be responsible for the reduction of OIP. Levodione reductases catalyze the second subsequent reaction from DOIP to actinol [17-20].

**MATERIALS AND METHODS**

**Micro-organism**
Active dry baker's yeast (Fermix®; 97.5 % dry weight) and pressed Koningsgist (33% dry weight) were kindly provided by DSM (Delft, The Netherlands). The other baker's yeast strains used were commercially available and obtained locally.
Chemicals

OIP (>98%) was supplied by Fluka Biochemika (Buchs, Switzerland). 6R-DOIP (98%; e.e. ≥ 99%) and 4R,6R-actinol (R-ACT, 98%, e.e. ≥ 99%) were kindly provided by F. Hoffmann-La Roche, presently DSM (Basel, Switzerland). 4S,6R-actinol (S-ACT) was prepared employing the procedure for DOIP degradation by baker’s yeast (see section on Reduction experiments in fermenters). The purity of all other chemicals used was at least laboratory grade. The buffer solution used was 50 mM potassium phosphate (pH 5.5) containing 5 mM MgSO4·7H2O.

Chemical degradation and stability experiments

These experiments were done using the bioreactor set-up and protocols employed in batch reductions (see section on Reduction experiments in fermenters). Known aliquots of pure OIP, DOIP and ACT were added to the buffer solution. The working volume was 1 L and the operating temperature was maintained at 30°C via a recirculating waterbath. Stirrer speed was set at 800 rpm with no aeration. Baker’s yeast and glucose were not added to the reactor. The mixture was stirred for 8 days and liquid samples were taken regularly and analyzed for OIP, DOIP and ACT concentrations.

Shake-flask experiments

This method was used for the selection of the best baker’s yeast strain for reducing OIP as well as for determining the effect of initial OIP concentration on OIP reduction. The general procedures for all of these experiments were the same. About 100 mL of buffer solution was placed in a 1-L flask followed by about 6 g dry weight yeast suspended in 50 mL buffer solution. In a separate operation, 5 g glucose was dissolved in 50 mL buffer solution. The yeast suspension was shaken (250 rpm) for 30 min to acclimatize (pre-incubate) the yeast, after which the glucose solution was added (initial glucose concentration in the medium was 25 g.L⁻¹), making up the total working volume of about 200 mL. This was pre-incubated for 2 h before the reaction was started by addition of known amounts of pure OIP. Several 200-mL reaction mixtures in 1-L flasks were prepared. The bioreductions were carried out at 30°C and 250 rpm for 24-48 h. Samples were taken periodically and analyzed for OIP, DOIP, ACT, and biomass dry weight.
Reduction experiments in fermenters

The bioreduction set-up and protocols described previously [7] were employed with a few minor modifications. The general procedure was to add 0.7 L of the buffer solution to the fermenter followed by 30 g dw yeast cells suspended in 0.3 L buffer solution to make up the 1 L working volume. The mixture was stirred and aerated for 30 min to acclimatize (pre-incubate) the yeast. Then, glucose solution was supplied at a rate of 3 mL h⁻¹ and a specific glucose feeding rate of 0.20 mmol.g dw⁻¹.h⁻¹. The reactor system was allowed to attain stationary oxygen consumption for 2 h at 30°C. Subsequently, the batch reduction was started by addition of known amounts of pure OIP. Liquid samples were withdrawn from the reactor during the course of the reduction experiment and were analyzed for OIP, DOIP, ACT and biomass dry weight. Occasionally, the glucose, ethanol and acetate contents were also checked. In experiments where there was DOIP or actinol initially present in the reactor, this had been dissolved in 0.025 L buffer solution and added to the reactor immediately before the addition of OIP.

For DOIP degradation experiments, a known amount of pure DOIP dissolved in 0.050 L buffer solution was added to the reactor. Therefore, the 30 g dw baker’s yeast was dissolved in 0.250 L (instead of 0.30 L) buffer solution. In experiments using very high initial DOIP concentrations (> 65 mM), 0.050-L slurry containing DOIP crystals was added. The mixture in the reactor was stirred for about 1-2 minutes to dissolve DOIP before taking the first sample (set at reaction time = 0). No OIP and actinol were added in these experiments. In the production of S-ACT, a known amount of pure DOIP was used as substrate and the reaction was run to complete conversion. The fermentation broth was collected and centrifuged at 10,000 rpm and 4°C for 20 mins to separate the cell debris. The clarified broth was then collected and stored at 4°C ready for use. The concentrations of DOIP and ACT (S-ACT and R-ACT) were measured by gas chromatography. In all experiments, measures were taken to prevent infection and/or contamination.

To check for the reversibility of substrate inhibition, OIP reduction was carried out with an initial OIP concentration of about 113 mM and a total working volume of 0.75 L. After 20 h of reduction, another 0.75 L buffer solution was added to the reactor to make up a total working volume of 1.5 L. Thereby the OIP concentration decreased below the inhibitory
level. OIP reduction was continued at this condition. Samples were withdrawn regularly and the concentrations of OIP, DOIP, and ACT and the biomass dry weight were analyzed.

**Analytical Methods**

The samples were centrifuged at 12,000 rpm, 4°C for 5 minutes and the supernatant liquid was decanted. The samples with DOIP crystals were appropriately diluted with known amounts of demineralized water to dissolve the crystals before centrifugation. OIP, DOIP, and ACT in the supernatant of the samples were extracted with ethyl acetate (1:1 v/v for initial concentrations of up to 15 mM and 1:10 v/v for higher initial concentrations) and measured using a GC-17A gas chromatograph (Shimadzu Co., Ltd., Japan) equipped with a flame ionization detector and a high-performance capillary column HP Innowax (cross-linked polyethylene glycol) with length = 15 m, i.d. = 0.53 mm, and film thickness = 1 μm. The oven and injection temperatures were 140°C and 180°C, respectively. The carrier gas was helium at 1.0 mL.min⁻¹. Glucose, ethanol and acetate in the reaction mixture were analyzed as described previously [21].

To determine cell dry weight, the samples were centrifuged at 12,000 rpm, 4°C for 5 minutes and filtered through pre-dried and pre-weighed filters (Gelman Sciences membrane filters: Ø 47 mm, 0.2 μm; Gelman Sciences, Inc., Ann Arbor, MI, USA). The collected biomass was washed with demineralized water and dried at 105°C to constant weight.

**Quantification of experimental results**

The experimental data gathered during the course of the experiments were corrected for the actual liquid volume in the reactor and the amounts taken out during sampling before they were used in the calculation of initial specific OIP reduction and DOIP production/degradation rates. Since the biomass dry weight concentrations in the reactor did not change significantly (at least, during the first 6-7 h of the reduction experiments), the values of the initial cell dry weight in the reactor were used in the calculation of biomass-specific rates.
MODELING

The batch reduction of OIP to DOIP and DOIP's subsequent degradation to ACT (S-ACT + R-ACT) is described by using equations derived from mass balances at constant reactor volume as follows:

OIP balance: \[ \frac{dC_{OIP}}{dt} = -r_{DOIP} \] (4.1)

DOIP balance: \[ \frac{dC_{DOIP}}{dt} = r_{DOIP} - r_{S-ACT} - r_{R-ACT} \] (4.2)

S-ACT balance: \[ \frac{dC_{S-ACT}}{dt} = r_{S-ACT} \] (4.3)

R-ACT balance: \[ \frac{dC_{R-ACT}}{dt} = r_{R-ACT} \] (4.4)

where \( r_i \) is the rate of the reaction leading to the formation of species \( i \) (mmol.L\(^{-1}.h\(^{-1}\)).

A kinetic model is proposed which may describe substrate inhibition during OIP reduction using baker's yeast. The mechanism of inhibition will be evaluated using hyperbolic kinetics extended with an inhibition term [22]:

\[ r_{DOIP} = q_{DOIP}^{max} \cdot \frac{C_{OIP}}{C_{OIP} + K_{M,OIP}} \cdot \left(1 - \frac{C_{OIP}}{C_{OIP}^{*}}\right)^n \cdot C_X \] (4.5)

where \( q_{DOIP}^{max} \) is the maximum specific rate constant (mmol.gdw\(^{-1}.h\(^{-1}\)), \( C_{OIP} \) is the concentration of OIP (mM), \( C_{OIP}^{*} \) is the critical concentration of OIP above which the reaction stops (mM), \( K_{M,OIP} \) is the apparent half-velocity constant for the substrate (mM), \( C_X \) is the concentration of biomass (gdw.L\(^{-1}\)), and \( n \) is the constant for the degree of inhibition. The rate equations of actinol formation will be discussed later.
RESULTS AND DISCUSSION

General Observations
In shake-flask and bioreactor experiments, the biomass dry weight in the course of the experiment was generally constant except for the experiments (as indicated below) with very high initial OIP concentrations where the biomass significantly decreased and cell lysis occurred. No ethanol or acetate (metabolic by-products) was detected in the liquid samples, which were occasionally analyzed. The dissolved oxygen levels in the bioreactor were monitored and were generally above 60% of air saturation. Glucose concentrations in the bioreactor were measured to be \( \leq 1 \) mM during the course of the experiments, which indicates that the resting cells are fully-metabolizing and utilizing the fed carbon source for maintenance, reduction, and other cellular activities.

Experiments were carried out to check for the chemical degradability and stability of the important reactants and products in the reduction experiments. OIP, DOIP, and ACT concentrations in the reactor did not change significantly during the 8-day experiment at the conditions employed, which indicates that these compounds are chemically stable in the bioreduction experiments.

Selection of baker’s yeast strain for OIP reduction
Baker’s yeast is available from various commercial sources. Table 4.1 shows that baker’s yeast’s catalyzing power for OIP reduction depends on the source. Although several entries gave comparatively high reduction capacities, Fermix® was chosen for the subsequent experiments as it gave the most consistent (reproducible) OIP reduction rates, especially at cell concentrations of about 30 gdw.L\(^{-1}\).

Effect of aeration
Aeration, if not properly monitored and controlled, becomes an important factor that affects mass balances in aerated biochemical reaction systems as such. Bioreduction experiments at different aeration rates (0.25-0.75 vvm) were performed. Significant gaps in the over-all mass balances (up to 25%) were detected at aeration rates of 0.50 and 0.75 vvm (Figures 4.2A-4.2B). These gaps were attributed to evaporation of OIP and DOIP involved in the reaction and would lead to large errors in kinetic studies. However, at an aeration rate of 0.25
vvm, the mass balance closed to 95-99%. Thus, this aeration rate was employed in the subsequent bioreduction experiments.

Table 4.1. Selection of baker’s yeast strains for OIP reduction.

<table>
<thead>
<tr>
<th>Baker’s yeast (commercial name)</th>
<th>Specific OIP reduction rates (mg/gdw⁻¹.h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gist Levure® (active-dry, 97% dw)</td>
<td>10.0</td>
</tr>
<tr>
<td>Fermix® (active-dry, 97% dw)</td>
<td>10.1</td>
</tr>
<tr>
<td>Instant Gist® (active-dry, 97% dw)</td>
<td>10.4</td>
</tr>
<tr>
<td>Algist Bruggeman® (pressed, 33% dw)</td>
<td>5.8</td>
</tr>
<tr>
<td>Koningsgist® (pressed, 33% dw)</td>
<td>5.2</td>
</tr>
<tr>
<td>Bakers Choice® (active-dry, 96% dw)</td>
<td>5.5</td>
</tr>
<tr>
<td>Diamond® (active-dry, 93% dw)</td>
<td>6.4</td>
</tr>
<tr>
<td>Fermipan® (active-dry, 95% dw)</td>
<td>6.8</td>
</tr>
<tr>
<td>Fleischmann® (active-dry, 96% dw)</td>
<td>7.9</td>
</tr>
<tr>
<td>Mauripan® (active-dry, 96% dw)</td>
<td>5.4</td>
</tr>
<tr>
<td>Red Star® (active-dry, 92% dw)</td>
<td>5.6</td>
</tr>
<tr>
<td>Saf Instant® (active-dry, 95% dw)</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Effect of initial OIP concentration during reduction

The substrate (OIP) is known to be toxic to many micro-organisms [10] and inevitably inhibits the reduction process under certain conditions [1]. To determine the extent of this effect, bioreductions at different initial OIP concentrations were conducted and results are summarized and presented in Figures 4.3-4.4. The biomass concentration profiles did not change significantly during the reduction at initial OIP concentrations of 80 mM and below (see example trend in Figure 4.3). However, a large biomass decrease was noted at initial OIP concentrations greater than 80 mM. About 18% and 27% decrease in biomass dry weight were observed after 48 h with 95.7 mM and 123.5 mM initial OIP concentrations, respectively. At this condition, yellowish biomass aggregates were seen adhering to the walls of the reactor, indicating biomass disintegration and lysis. Severe foaming also occurred especially on the second day of the reduction experiment.
Figure 4.2A. Total amount of OIP (broken lines) and DOIP (solid lines) during the course of OIP reduction at different aeration rates of 0.75 vvm (○,●), 0.50 vvm (□,■), and 0.25 vvm (△,▲). Open and closed markers are at initial OIP concentrations of about 68 mM and 35 mM, respectively.

Figure 4.2B. Total amount (OIP+DOIP+ACT) during the course of OIP reduction at different aeration rates of 0.75 vvm (○,●), 0.50 vvm (□,■), and 0.25 vvm (△,▲). Open and closed markers are at initial OIP concentrations of about 68 mM and 35 mM, respectively.
There is an increase in the reduction rates of OIP with increasing initial OIP concentration, but above 80 mM OIP, the reduction rates started to slow down remarkably (Figure 4.4). In the OIP concentration range of 50-80 mM, the OIP reduction rates were relatively high, at about 0.066 mmol.gdw^{-1}.h^{-1} (≈ 10 mg.gdw^{-1}.h^{-1}). To describe this trend, equation (4.5) was employed. \( C_{OIP}^{c} \) was determined by trial and error and evaluated together with the constants \( q_{DOIP}^{\text{max}}, K_{M,OIP} \) and \( n \) of equation (4.5) using the experimental data. Simulation and data-fitting (using MathCad software) yielded values of the kinetic parameters as follows: \( q_{DOIP}^{\text{max}} = 1.95 \pm 0.22 \) mmol.gdw^{-1}.h^{-1} and \( K_{M,OIP} = 855.99 \pm 102.90 \) mM with fixed \( n = 1 \); and \( C_{OIP}^{c} = 130 \) mM. As \( n > 0 \), equation (4.5) represents a noncompetitive type of substrate inhibition [22]. Duplicate experiments confirmed the reproducibility of the reduction experiments. The initial rates of OIP and DOIP indicated a slight difference of up to 6%, which can be attributed to experimental errors. Mass balances closed at 95-99%.

**Figure 4.3.** Biomass concentration profiles at initial OIP concentrations below 80 mM (◇ 31.6 mM OIP; △ 68.4 mM OIP) and above 80 mM (□ 95.7 mM OIP; ○ 123.5 mM OIP). Cell lysis occurred during reductions at initial OIP concentrations above 80 mM after the 24-h period.
To prove that the low rates at high OIP concentrations were not due to (irreversible) inactivation of the cells but to (reversible) substrate inhibition, an experiment was performed. The initial OIP concentration was about 113 mM, which was at the inhibitory level (Figure 4.5). At this condition, the observed initial reduction rate of OIP was 1.19 mmol.L\(^{-1}\).h\(^{-1}\), which agreed with the predicted value using equation (4.5). Mass balances closed at >99% as the specific DOIP production rate was the same. However, when the reaction liquid was diluted by adding buffer solution, thereby decreasing the OIP concentration to a non-inhibitory level (44.5 mM), the subsequent initial reduction rate of OIP increased dramatically to 2.02 mmol.L\(^{-1}\).h\(^{-1}\) (Figure 4.5), which actually was even higher than predicted by equation (4.5) but indicates that substrate inhibition had occurred at high OIP concentrations without biocatalyst inactivation. However, prolonged exposure (>24 h) of the biocatalyst to high substrate concentrations (> 80 mM) can be damaging to the cells as observed in Figure 4.3.
Figure 4.5. Check for the reversibility of OIP inhibition during the course of OIP reduction employing different initial OIP concentrations. Open and closed markers are experimental data at initial OIP concentrations of 44.5 mM and 113 mM, respectively. Experimental data are shown for: OIP (○,●), DOIP (◇,◆), and ACT (△,▲).

Effect of actinol and DOIP

Bioreduction experiments were conducted to determine the effect of the presence of ACT (R-actinol and S-actinol) during OIP reduction. Results showed that these compounds did not have any effect at all on OIP reduction in the concentration range studied. Furthermore, the specific reduction rates as well as the DOIP final yields and selectivity were essentially the same at different R-ACT and S-ACT concentrations (see Table 4.2). However, DOIP yields and selectivity generally showed a decreasing trend when measured after 24 h and 48 h (comparing results with S-ACT and R-ACT), as more of the DOIP was degraded to actinol. This degradation process can be minimized, if not avoided at all, when DOIP is withdrawn from the reactor immediately after it is produced (i.e. by in situ product removal technique [7]).

Figure 4.6 shows that also the presence of DOIP does not influence the course of OIP reduction, as the initial reduction rates of OIP did not change significantly at this condition. The specific reaction rates of OIP and DOIP without the initial presence of DOIP were the
same at 0.071 mmol.gdw⁻¹.h⁻¹; while these were 0.080 and 0.067 mmol.gdw⁻¹.h⁻¹ for OIP and DOIP, respectively, with the initial presence of DOIP at a concentration of 35.3 mM. The slight difference in the specific rates of OIP and DOIP in the latter case was due to the fact that the subsequent degradation of the readily available DOIP to actinol was observed. However, DOIP when produced in the reactor as crystals during a fed-batch operation can potentially contribute to transport limitations during the reduction process with baker’s yeast as catalyst [7].

Table 4.2. Specific rates, yields and selectivity during OIP reduction at different S-ACT and R-ACT concentrations.

<table>
<thead>
<tr>
<th>C_{OIP, init} (mM)</th>
<th>C_{ACT, init} (mM)</th>
<th>Initial rates (OIP) (mg.gdw⁻¹.h⁻¹)</th>
<th>Initial rates (DOIP) (mg.gdw⁻¹.h⁻¹)</th>
<th>DOIP Final Yield* (%)</th>
<th>DOIP Final Selectivity** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>51.6</td>
<td>2.3</td>
<td>11.5</td>
<td>11.8</td>
<td>83.6</td>
<td>83.3</td>
</tr>
<tr>
<td>54.9</td>
<td>5.5</td>
<td>10.7</td>
<td>11.1</td>
<td>86.8</td>
<td>87.4</td>
</tr>
<tr>
<td>53.9</td>
<td>11.4</td>
<td>10.3</td>
<td>10.2</td>
<td>87.1</td>
<td>87.5</td>
</tr>
<tr>
<td>56.0</td>
<td>23.9</td>
<td>9.5</td>
<td>9.0</td>
<td>87.6</td>
<td>88.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C_{OIP, init} (mM)</th>
<th>C_{ACT, init} (mM)</th>
<th>Initial rates (OIP) (mg.gdw⁻¹.h⁻¹)</th>
<th>Initial rates (DOIP) (mg.gdw⁻¹.h⁻¹)</th>
<th>DOIP Final Yield* (%)</th>
<th>DOIP Final Selectivity** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.8</td>
<td>0</td>
<td>10.7</td>
<td>10.4</td>
<td>84.8</td>
<td>86.4</td>
</tr>
<tr>
<td>56.1</td>
<td>6.1</td>
<td>9.7</td>
<td>9.6</td>
<td>82.4</td>
<td>84.1</td>
</tr>
<tr>
<td>54.6</td>
<td>17.8</td>
<td>10.2</td>
<td>10.3</td>
<td>79.5</td>
<td>80.9</td>
</tr>
<tr>
<td>53.2</td>
<td>31.8</td>
<td>11.2</td>
<td>11.0</td>
<td>83.7</td>
<td>84.6</td>
</tr>
<tr>
<td>54.5</td>
<td>51.1</td>
<td>10.6</td>
<td>10.5</td>
<td>82.6</td>
<td>83.2</td>
</tr>
</tbody>
</table>

* Yield is the amount of DOIP produced per amount of OIP fed.
** Selectivity is the amount of DOIP produced per total amount of DOIP and ACT produced.
* Measured after the 24-h and 48-h experimental process for S-ACT and R-ACT, respectively.

Batch DOIP degradation

To describe the kinetics of DOIP degradation to actinol, independent bioreduction experiments were carried out at different initial DOIP concentrations from 7.9 mM to 92.4 mM. Figure 4.7 showed an increase in specific reduction rates with increasing initial DOIP concentration in the reactor. At low DOIP concentrations, only S-ACT was produced whereas at very high DOIP concentrations (above the solubility limit of about 65 mM), R-ACT also starts to be produced, although, at a comparatively lower rate than S-ACT. The trend indicates a second-order rate of reaction with respect to DOIP concentration. As such, equations 4.6-4.7 can be used to describe the rates considering a constant volume batch reaction:
S-ACT rate: \[ r_{S-\text{ACT}} = k_{S-\text{ACT}} \cdot (C_{\text{DOIP}})^2 \cdot C_X \] (4.6)

R-ACT rate: \[ r_{R-\text{ACT}} = k_{R-\text{ACT}} \cdot (C_{\text{DOIP}})^2 \cdot C_X \] (4.7)

Upon data-fitting using these equations, the following kinetic parameters were estimated: \( k_{S-\text{ACT}} = 2.4 \times 10^{-6} \text{ L}^2\text{mmol}^{-1}\text{gdw}^{-1}\text{h}^{-1} \) and \( k_{R-\text{ACT}} = 9 \times 10^{-7} \text{ L}^2\text{mmol}^{-1}\text{gdw}^{-1}\text{h}^{-1} \) with \( R^2 = 99.8\% \). Figure 4.8 shows the DOIP, S-ACT and R-ACT concentration profiles with the simulation results.

Thus the rate of DOIP reduction to ACT is second-order with respect to DOIP concentration, which is an exceptional trend for a whole-cell-catalyzed reaction system. This indicates that the rate of DOIP degradation is very highly dependent on its concentration in the reactor. Amongst the hypotheses that were tested, only one would be able to explain this trend. This hypothesis assumes that during this reaction, DOIP is simultaneously required for ACT formation as well as for a reversible reaction of DOIP to OIP (see Figure 4.9). It is supposed that ACT formation requires NADPH, and NADPH can only be regenerated readily by the oxidation of DOIP to OIP, a fast but an unfavourable equilibrium reaction. If regeneration of NADPH via glucose metabolism is virtually absent or extremely low compared to NADH regeneration via glucose metabolism, OIP may be reduced readily to DOIP using NADH-linked enzymes only. Computer simulations (see Appendix) of the proposed reaction model indeed gave a second-order dependence of the rate on DOIP concentration for certain combinations of kinetic parameters. Studies have shown that with isolated enzymes, NADH is suitable for the preparative production of DOIP as a coenzyme as it yielded very high productivity or specific activity than with NADPH [15,18]. While this phenomenon is in itself interesting and requires more experimental proof, it is supposed to sufficiently explain in this work the peculiar second-order dependency of the rate of DOIP degradation by baker’s yeast with respect to DOIP concentration.
Figure 4.6. Course of OIP reduction with and without the initial presence of DOIP. Initial concentrations of OIP and DOIP were 61.5 mM and 35.3 mM (open markers) and 64.5 mM and 0 mM (closed markers), respectively. Experimental data are shown for: OIP (○, ●), DOIP (◇, ◆), and ACT (▲, ▲).

Figure 4.7. Biomass-specific rates of DOIP reduction (●) and ACT production (S-ACT, ■; R-ACT, ▲) at different initial DOIP concentrations. Lines are fits of the second-order rate equations.
Figure 4.8. Concentration profiles of (A) DOIP, (B) S-ACT and (C) R-ACT at different initial DOIP concentrations. Data points are experimental results and lines are simulation results using the model equations. Experimental data were: 92.4 mM DOIP (●); 62.4 mM DOIP (■); 49.7 mM DOIP (○); 32.7 mM DOIP (▲); 18.4 mM DOIP (●); and 7.9 mM DOIP (∗).
Figure 4.9. Hypothetical reaction scheme in the cell during DOIP degradation to ACT involving cofactor regeneration, which would lead to a second-order dependence of DOIP conversion rate on DOIP concentration.

With this model reaction, it is obviously a better strategy to feed the substrate OIP in the reactor such that its concentration is constantly below the inhibitory level during the reduction process. In addition, the desired DOIP must be removed from the reactor as soon as it is formed in order to minimize, if not avoid, product degradation and product crystal formation in the reactor. In this manner, maximum reduction rates can be attained and DOIP yield and selectivity optimized.

The model equations developed in this work were applied to simulate OIP reductions performed previously [7]. The model could reasonably describe the reduction process, thereby rendering these results useful in optimizing and improving the reactor performance with OIP reductions using resting cells.
CONCLUSIONS

In the production of 6R-dihydro-oxoisophorone, which is an important chiral intermediate in the synthesis of naturally-occurring carotenoids and flavors, via the reduction of 4-oxoisophorone (OIP) by baker’s yeast, OIP inhibits the reaction and can cause cell disintegration at a concentration above 80 mM. Baker’s yeast also degrades the product mainly to an unwanted by-product 4S,6R-actinol. A noncompetitive type of inhibition can describe the kinetics of substrate inhibition during OIP reduction. Product degradation is simply the further reduction of DOIP to actinol by the same biocatalyst. This reaction, although has a slower rate compared to OIP reduction, can be described by a second-order rate equation with respect to DOIP concentration, which is an exceptional behaviour so far for a bio-catalyzed reaction system. This is supposed to be due to the complex interaction of the reactants and products with NADH-linked and NADPH-dependent enzymes during the process of cofactor regeneration in the cell.

ACKNOWLEDGEMENTS

This research is funded by the MHO-USC-DUT Project in Chemical Engineering. We kindly thank DSM (Delft, The Netherlands) for providing baker’s yeast strains and F. Hoffman-La Roche-VFCD, now DSM (Basel, Switzerland) for DOIP and actinol. EMBT would like to thank Max Zomerdijsk (TU Delft), Victoria F. Napsa and her laboratory staff (USC), and Florie Gil Y. Pino (research assistant, USC) for all the support and assistance in conducting this work. The following students at USC are also gratefully acknowledged for their valuable contribution to this project: Lester R. Gimenez, Christine C. Rubia, Florie Gil Y. Pino, Henry Castro, Niño Cugias and Helen Salvame.
LIST OF SYMBOLS

\( C_i \)  \hspace{1cm} \text{concentration of species} \ i, \ M \\
\( C_{\text{OIP}} \)  \hspace{1cm} \text{critical concentration of OIP above which the reaction stops,} \ M \\
\( C_X \)  \hspace{1cm} \text{biomass concentration,} \ \text{gdw.L}^{-1} \\
\( K_{M,i} \)  \hspace{1cm} \text{apparent half-velocity constant for the substrate} \ i, \ M \\
\( k_{S-ACT}, k_{R-ACT} \)  \hspace{1cm} \text{rate constant for} \ S-\text{ACT and} \ R-\text{ACT, respectively,} \ L^2.\text{mol}^{-1}.\text{gdw}^{-1}.h^{-1} \\
\( n \)  \hspace{1cm} \text{constant for the degree of inhibition} \\
\( q_{i}^{\text{max}} \)  \hspace{1cm} \text{maximum biomass-specific reaction rate of species} \ i, \ \text{mol.gdw}^{-1}.h^{-1} \\
\( r_i \)  \hspace{1cm} \text{rate of reaction leading to the formation of species} \ i, \ \text{M.h}^{-1}
APPENDIX: Simulation details for batch DOIP degradation to actinol

Rates:
\[
\begin{align*}
    r_1 &= k_1 \cdot \left( C_{DOIP} \cdot C_{NADP} - \frac{C_{OIP} \cdot C_{NADPH}}{K_{eq}} \right) \\
    r_2 &= k_2 \cdot C_{DOIP} \cdot C_{NADPH} \\
    r_3 &= k_3 \cdot \left( C_{DOIP} \cdot C_{NAD} - \frac{C_{OIP} \cdot C_{NADH}}{K_{eq}} \right) \\
    r_4 &= k_4 \cdot C_{NAD}
\end{align*}
\]

Macroscopic balances:
\[
\begin{align*}
    \frac{dC_{ACT}}{dt} &= r_2 \\
    \frac{dC_{NADPH}}{dt} &= r_1 - r_2 \\
    \frac{dC_{OIP}}{dt} &= r_1 + r_3 \\
    \frac{dC_{NADH}}{dt} &= r_3 + r_4
\end{align*}
\]

Stoichiometric balances:
\[
\begin{align*}
    C_{DOIP} &= C_{DOIP,0} + C_{OIP,0} + C_{ACT,0} - C_{OIP} - C_{ACT} \\
    C_{NADP} &= C_{NADP,0} + C_{NADPH,0} - C_{NADPH} \\
    C_{NAD} &= C_{NAD,0} + C_{NADH,0} - C_{NADH}
\end{align*}
\]

Parameters and rate constants:
\[
\begin{align*}
    k_1 &= 1 \\
    k_2 &= 1000 \\
    k_3 &= 100 \\
    k_4 &= 10000 \\
    K_{eq} &= 0.001
\end{align*}
\]

Initial concentrations:
\[
\begin{align*}
    C_{OIP,0} &= 0 \\
    C_{NAD,0} &= 0.001 \\
    C_{NADPH,0} &= 0.001 \\
    C_{DOIP,0} &= 10 \\
    C_{NADH,0} &= 0.001 \\
    C_{ACT,0} &= 0 \\
    C_{NADP,0} &= 0.001
\end{align*}
\]

Computer simulation of this combination of kinetic parameters would show second-order dependence of the rate of DOIP degradation on DOIP concentration.
REFERENCES


Chapter 5

Microbial reduction and *in situ* product crystallization coupled with biocatalyst cultivation during the synthesis of 6\(R\)-dihydro-oxoisophorone

E.M. Buque-Taboada, A.J.J. Straathof, J.J. Heijnen, and L.A.M. van der Wielen

Chapter 5

ABSTRACT

An in situ product crystallization procedure was developed for a crystalline product formed during microbial reduction coupled with cell cultivation. The model reaction was the asymmetric reduction of 4-oxoisophorone (OIP) by baker’s yeast (Saccharomyces cerevisiae). Yeast cells were cultivated fed-batch to reach a maximum concentration of 30 gdw.L⁻¹. The desired product, 6R-dihydro-oxoisophorone (DOIP), may be further reduced by baker’s yeast to an unwanted by-product; thus, DOIP was removed immediately from the fermenter via an external crystallization loop in this procedure. The OIP reduction rate was five times higher (≪ 0.33 mmol.gdw⁻¹.h⁻¹) as compared to the reduction rate with resting cells. OIP reduction was started when the optimum cell concentration had already been reached in the reactor because the substrate (OIP) at 55 mM concentration inhibited cell growth. An appropriate supply of glucose as carbon and energy source was necessary to support the coupled reactions involving cell growth and maintenance and product formation while avoiding formation of metabolic by-products. Final DOIP yield and selectivity were 85% and 99%, respectively, while over 100 g.L⁻¹ of product was obtained in the crystallizer. The product crystals with favorable properties were readily recovered from the crystallizer. These results indicate that product crystallization is not impaired by the solutes present in the fermentation medium.
INTRODUCTION

The range of products that can be produced by microbial biotransformation is increasing nowadays as productivity improves dramatically by metabolic and process engineering. In the latter approach, maximizing the biocatalyst concentration in the reactor and/or immediately removing inhibiting or degrading products during the process can raise bioreactor productivity. This requires cell cultivation during the process especially when the biocatalyst is not commercially or readily available and/or involves in situ product removal [1-5]. However, simplifying the product separation and purification steps still remains a challenge as it is the cost-limiting factor in most cases [1-4]. When the desired product crystallizes in the reactor, it is often re-dissolved using organic solvents, separated and re-crystallized. If the product crystallizes during fermentation and can be separated from the cells directly, production costs might be reduced dramatically [5-11].

In previous work [5], it was shown that with resting cells of Saccharomyces cerevisiae, an in situ product crystallization procedure by an integrated process (Figure 5.1) was more efficient than the non-integrated batch and fed-batch configurations. Still, such a process can be improved further by implementing a close-down phase when substrate feeding is stopped and bioreduction is continued to achieve a zero- or low-level substrate concentration in the reactor. In various cases, the biocatalyst (micro-organism) needs to be cultivated prior to or concomitant with biotransformation and product crystallization. It would be interesting to incorporate cell growth in this in-situ product crystallization procedure. Such an integration can possibly contribute to differences in the biotransformation and crystallization kinetics as growing cells may follow different metabolic routes compared to resting cells.

Thus, this work aims to demonstrate experimentally the impact of implementing cell cultivation concomitant with bio-reduction and in-situ product crystal formation in this integrated fermentation-crystallization process. This integration strategy should be generally applicable to processes that involve cell cultivation, biocatalytic formation of a product, and subsequent product crystallization. This approach might also be 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, to simplify the product separation and recovery steps.
The chosen model reaction was the asymmetric reduction of 4-oxoisophorone (OIP) using baker’s yeast (S. cerevisiae) as biocatalyst (Figure 5.2). The desired product is known as 6R-dihydro-oxoisophorone (DOIP), which is a key intermediate in carotenoid synthesis [12-13] and in the production of saffron and tobacco flavors [14-15]. As baker’s yeast is known to also degrade DOIP mainly to 4S,6R-actinol, an unwanted by-product, DOIP must be removed from the fermenter as soon as it is formed, to prevent low product yield and selectivity. In this case, in situ product crystallization (ISPC) is appropriately applied.

Baker’s yeast is cheap, readily available, and thus is an exceptional organism as it does not need to be cultivated in the reactor. Still, it is an interesting case to perform reductions with pre-cultivated yeast cells. Using whole cells as biocatalyst would prove economically favorable as this allows for an easy and in-vivo cofactor regeneration in the cell, which sustains catalytic activity for redox reactions [16-17]. Aeration and nutrient feeding can however be the most important constraints to consider, which can potentially complicate the whole biocatalytic process.
In this work, fed-batch cell cultivation was employed where nutrient (glucose) feed rates were varied to maximize the utilization of carbon source and to minimize formation of metabolic by-products. Fed-batch cell cultivation was also performed concomitantly with batch reduction where initial OIP concentrations were varied. Results were used to evaluate which conditions would better suit the subsequent integrated experiment involving in-situ product crystallization when a close-down phase is implemented towards the end of the process.

**MATERIALS AND METHODS**

**Chemicals**
OIP (>98%) was supplied by Fluka Biochemika (Buchs, Switzerland). DOIP and actinol (ACT) standard samples were kindly provided by DSM (Basel, Switzerland). The purity of all other chemicals used was at least laboratory grade.

**Cell cultivation and batch OIP reduction**
Cells were cultivated aerobically in a 2-L bioreactor (Applikon, Schiedam, The Netherlands). The fermenter 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 fermenter and its attached condenser was set at 30°C and 2-4°C,
respectively. The control of pH at 5.5 with 1M H₂SO₄ 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.

A mineral growth medium for baker's yeast cultivation [18] was prepared. The standard procedure was to add 0.7 L medium solution to the fermenter followed by a known amount of baker's yeast (1 g active dry baker's yeast, Fermix®; 97.5 % dry weight, DSM-Gist, Delft, The Netherlands) 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. Subsequently, glucose was supplied at a rate of 0.33 mmol.h⁻¹ with C_Gluc,feed = 110 mM in medium solution. Other glucose feed rates (as specified) were also used in various experiments. The reactor system was allowed for 2 h to attain stationary oxygen consumption at 30°C. In the course of the experiment, liquid samples were taken from the reactor and analyzed 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 stabilized. For these experiments, the liquid samples were analyzed for OIP, DOIP and actinol in addition to biomass dry weight and concentrations of glucose, ethanol, and acetate.

**Synthesis of DOIP**

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⁻¹. Glucose feed rate was varied (as specified) at different stages of the experiment. After 24 h, OIP reduction was started by addition of about 8 g pure OIP and at the same time, fed at 0.50 mL.h⁻¹ (3.4 mmol.h⁻¹) to maintain a concentration level in the reactor that avoids substrate inhibition [13,19]. The OIP feed rate was increased to 1.5 mL.h⁻¹ (10.2 mmol.h⁻¹) after 48 h as the cell concentration was expected to increase threefold at this time. The rest of the integrated fermentation-crystallization procedures described previously [5] were followed. In the course of the experiment, liquid samples were taken
from the reactor and the crystallizer and analyzed for OIP, DOIP, and actinol. Concentrations of glucose, ethanol and acetate as well as biomass dry weight in the liquid samples from the reactor were also determined. The crystallizer volume was over 0.6 L.

Analytical Methods

OIP, DOIP, and actinol in the supernatant of the reaction mixture were analyzed as described previously [5]. The enantiomeric excess (e.e.) of DOIP was checked using a gas chromatograph (Shimadzu GC 17A) equipped with an FID and a chiral diAc-tBuSi-β-cyclodextrin column (MEGA, Legnano, Italy) 25 m x 0.25 mm i.d., film thickness 0.25 μm, carrier gas helium, flow rate 0.58 mL min⁻¹, split ratio 1:50, temperature of injector and detector 250°C and 280°C, respectively. Retention times for 4-oxoisophorone (OIP), 6R-dihydro-oxoisophorone (6R-DOIP), 6S-dihydro-oxoisophorone (6S-DOIP), 4R,6R-actinol, and 4S,6R-actinol were 2.7, 5.0, 5.2, 11.1 and 11.3 min, respectively. Glucose, ethanol, acetate as well as the biomass dry weight in the reaction mixture were analyzed as described elsewhere [20-21]. 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).

RESULTS AND DISCUSSION

Cell cultivation and batch OIP reduction

The fed-batch cultivation of baker’s yeast cells is already well-established in literature [22-24]. In this work, baker’s yeast cultivation varied at different glucose feeding rates as expected (see Table 5.1A). From an initial cell concentration $C_{Xt} = 1.3$ gdw.L⁻¹, biomass accumulation in the reactor reached 10.5 gdw.L⁻¹ after 24 h with a glucose feed rate of 0.32 mmol.h⁻¹ ($C_{m0,a}$). Increasing the glucose feed concentration to over 5 times higher at 1.68 mmol.h⁻¹ ($C_{m5,a}$) would favor further increase in biomass from $C_{Xt} = 10.6$ gdw.L⁻¹ to an
amount of >30 gdw.L⁻¹ after 24 h. This glucose feed rate can apparently support cell growth and maintenance better than the other feed rates used (i.e. Cₘ₁ₐ and Cₘ₃ₐ in Table 5.1A). Combining strategies involved in experiment Cₘ₀ₐ and Cₘ₅ₐ would lead to cell cultivation up to 30 gdw.L⁻¹ in 48 h by employing the appropriate nutrient feed rates.

**Table 5.1A. Biomass concentration during cell cultivation at different glucose feed rates for 24 h.**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Biomass concentration (gdw.L⁻¹)</th>
<th>Nutrient (glucose) feed rate (mmol.h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial, Cₓ₁</td>
<td>Final, Cₓ₁</td>
</tr>
<tr>
<td>Cₘ₀ₐ</td>
<td>1.3</td>
<td>10.5</td>
</tr>
<tr>
<td>Cₘ₁ₐ</td>
<td>10.6</td>
<td>12.5</td>
</tr>
<tr>
<td>Cₘ₃ₐ</td>
<td>10.2</td>
<td>18.3</td>
</tr>
<tr>
<td>Cₘ₅ₐ</td>
<td>10.6</td>
<td>37.4</td>
</tr>
</tbody>
</table>

**Table 5.1B. Biomass concentration during cell cultivation and batch OIP reduction at different glucose feed rates for 24 h.**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Biomass concentration (gdw.L⁻¹)</th>
<th>Nutrient (glucose) feed rate (mmol.h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial, Cₓ₁</td>
<td>Final, Cₓ₁</td>
</tr>
<tr>
<td>Cₘ₀₆</td>
<td>0.8</td>
<td>5.3</td>
</tr>
<tr>
<td>Cₘ₁₆</td>
<td>10.2</td>
<td>10.0</td>
</tr>
<tr>
<td>Cₘ₃₆</td>
<td>9.8</td>
<td>10.2</td>
</tr>
<tr>
<td>Cₘ₅₆</td>
<td>10.2</td>
<td>31.6</td>
</tr>
</tbody>
</table>

When fed-batch cell cultivation was coupled with batch OIP reduction, then biomass concentration in the reactor changed (Table 5.1B). This was obviously due to product formation, competing in the cell with cell growth and maintenance for the carbon and energy source. Results in Table 5.1B showed that the coupled reactions could be sustained at an approximately five-fold increase in medium nutrient feed rate (Cₘ₅₆ at 1.68 mmol.h⁻¹) when cell growth as well as bioreduction occurred simultaneously. In this case, cell accumulation in the reactor reached ≥ 30 gdw.L⁻¹ from an initial amount of 10.2 gdw.L⁻¹ (Table 5.1B) concomitant with batch OIP reduction at an average biomass-specific rate of ≥ 0.33 mmol.gdw⁻¹.h⁻¹ (Figure 5.3). This condition was therefore applied subsequently in the integrated fermentation-crystallization process.
The observed initial biomass-specific OIP reduction rate of 0.33 mmol.gdw⁻¹.h⁻¹ was essentially the same at different medium nutrient feed rates (Figure 5.3) and was about five times higher than in reductions using resting cells [5]. This suggests that metabolic reactions with growing cells can be very different as compared with resting cells, which in this case favors higher reduction capacity. Glucose as the carbon/energy source was utilized preferably for product formation rather than for cell growth as no further increase in biomass was observed when the nutrient (glucose) feed rate was < 1.68 mmol.h⁻¹ (Table 5.1B; Cₘ₅ₐₖₙ and Cₘ₃ₐₖₙ). Furthermore, the final conversion rates varied; complete (100%) conversion was attained within 24 h with Cₘ₅ₐₖₙ (Figure 5.3), as more glucose was available for product formation and cell growth than with Cₘ₅ₐₖₙ and Cₘ₃ₐₖₙ, where degrees of conversion were 85.5% and 92.2%, respectively.

At different initial OIP concentrations, the observed initial biomass-specific reduction rates varied (Figure 5.4). A maximum OIP reduction rate of 0.33 mmol.gdw⁻¹.h⁻¹ was achieved at an initial OIP concentration (Cₒᵢₕᵢ) of 53.8 mM (≈ 8.2 g.L⁻¹), but this rate decreased to 0.25 mmol.gdw⁻¹.h⁻¹ and 0.23 mmol.gdw⁻¹.h⁻¹ at Cₒᵢₕᵢ = 67.5 mM (≈ 10.3 g.L⁻¹) and 37.2 mM (≈ 5.7 g.L⁻¹), respectively. This indicates that whilst OIP reduction rates were much faster with growing cells than with resting cells, the maximum rate with the former was observed at a slightly lower substrate concentration (Cₒᵢₕᵢ = 55 mM) than the latter which was at Cₒᵢₕᵢ = 79 mM [5,13]. Consequently, the OIP concentration in the reactor must be maintained (i.e. by feeding) at Cₒᵢₕ ≥ 55 mM to implement the observed maximum reduction rate with growing cells. Towards the end of the batch reduction (see Figure 5.4), OIP conversion was not complete (only 83-89%) for all experiments at the same nutrient feed rate (0.33 mmol.h⁻¹) and initial biomass concentration (Cᵨᵢ = 10 gdw.L⁻¹). No biomass growth was observed in these cases; rather a slight decrease in biomass dry weight was noted at the end of the 48-h experiment. Obviously, the glucose supplied in these experiments was not sufficient to sustain all the required metabolic reactions in the cell.
Figure 5.3. Total amounts of (A) OIP and (B) DOIP and ACT at different nutrient feed rates during cell cultivation with simultaneous batch reduction of OIP: ○ ○ +, $C_{m1}$ (0.31 mmol h⁻¹, $C_{\text{gluc,feed}} = 102.6$ mM, $C_{Xf} = 10.2$ g dw L⁻¹); □ □ ●, $C_{m3}$ (1.06 mmol h⁻¹, $C_{\text{gluc,feed}} = 333.1$ mM, $C_{Xf} = 9.8$ g dw L⁻¹); △ △ *, $C_{m5}$ (1.68 mmol h⁻¹, $C_{\text{gluc,feed}} = 560.9$ mM, $C_{Xf} = 10.2$ g dw L⁻¹).
Figure 5.4. Total amounts of (A) OIP and (B) DOIP and ACT at different initial concentrations of OIP during cell cultivation with simultaneous batch reduction:

- ○○+, $C_{OIP,i} = 67.5$ mM;
- □□●, $C_{OIP,i} = 53.8$ mM;
- △△●, $C_{OIP,i} = 37.2$ mM.
Integrated fermentation-crystallization procedure for OIP reduction

This process was performed as shown in the previous schematic diagram (Figure 5.1) where the fermenter was attached to the crystallizer and in between the two units an ultrafiltration membrane was placed in an external loop to recycle the biomass. From the crystallizer, the mother liquor (excluding the crystals) was recirculated back to the fermenter through a 0.2-μm filter [5]. Two process schemes were designed. Process scheme A was an integrated fermentation-crystallization process where the OIP reduction stage was carried out concomitant with further biomass cultivation after 24 h from the start of the experiment. Process scheme B was a modification of process scheme A where the OIP reduction stage was implemented when the biomass concentration in the reactor had already reached an optimum level. Further elaborations of these processes are given subsequently.

Process scheme A

This experiment was done more efficiently with the previous favorable results implemented. It was performed in several stages, combining cell cultivation, bioreduction and in situ product crystallization. As shown in Figure 5.5, the first stage of 24 h consisted of yeast cell cultivation from an initial concentration (C_{X0}) of 1.23 gdw.L⁻¹ to 9.84 gdw.L⁻¹ when the medium nutrient (glucose) feed rate was 0.32 mmol.h⁻¹. In the second stage (from 24 to 48 h), OIP reduction was started by OIP addition of 55.5 mmol (8.4 g) and feeding at 0.5 mL.h⁻¹ (3.4 mmol.h⁻¹). The glucose feed rate was increased to over five-fold (1.67 mmol.h⁻¹) to further support cell growth and product formation. In the third stage, OIP feeding was increased to 1.5 mL.h⁻¹ (10.2 mmol.h⁻¹) and the glucose feed rate was raised to 6.01 mmol.h⁻¹ as biomass was expected to increase to a maximum amount of about 30 gdw.L⁻¹. However, results in Figure 5.5 showed that biomass dry weight did not increase in stage 2 when OIP reduction was implemented. Rather, it remained constant during this period (contrary with batch OIP reduction when cell dry weight simultaneously increased as shown earlier). Eventually, it decreased in stage 3 when the OIP concentration in the reactor started to increase due to its accumulation. Biomass disintegration and lysis occurred at this stage, resulting to the formation of biomass (debris) aggregates, which caused severe clogging in the ultrafiltration membrane. After 72 h, the biomass concentration (C_{X}) was 6.28 gdw.L⁻¹.
When OIP was fed such that its concentration was constant in the reactor (i.e. $C_{OIP} \geq 55$ mM), cell growth was inhibited as illustrated in Figure 5.5. The expected maximum amount of cells in the reactor was not attained. Nevertheless, OIP reduction occurred as DOIP increased in time. This indicates that the cells favored the utilization of glucose for product formation (OIP reduction) rather than cell growth. Hence, OIP reduction should be started at the moment that the targeted maximum biomass concentration has been reached in order to maximize bioreactor productivity.

![Graph](image)

**Figure 5.5.** Process scheme A: Total amounts of OIP (●), DOIP (■), ACT (▲) and biomass (○) in the reactor. OIP reduction was started after 24 h (stage 2) and no further increase in biomass was observed after this time.

**Process scheme B**

In this experiment, the observations and results obtained in process scheme A were considered. Thus, process scheme B was performed with further modifications of scheme A. As shown in Figure 5.6, the OIP reduction was started in stage 3 when the cell concentration
in the reactor had already reached 30 gdw.L\(^{-1}\) to avoid growth inhibition and maximize bioreactor productivity. OIP was added in the reactor at 56.5 mmol (8.6 g) and at the same time, fed at 1.5 mL.h\(^{-1}\) (10.2 mmol.h\(^{-1}\)) to maintain the OIP concentration in the reactor that allows the maximum reduction rate. The glucose feed rate was also raised, to 6.04 mmol.h\(^{-1}\), to further sustain product formation and cell maintenance. Results (Figure 5.6) showed that the biomass concentration in the reactor was indeed maintained during OIP reduction in stage 3 as the average biomass dry weight was 30.5 g. The OIP concentration in the reactor was constant at \(C_{OIP} \approx 60\) mM, which indicates that the OIP feed rate (1.5 mL.h\(^{-1}\)= 10.2 mmol.h\(^{-1}\)) was equal to the observed OIP reduction rate (\(\approx 0.33\) mmol.gdw\(^{-1}\).h\(^{-1}\)). This was a favorable situation for an integrated process because the OIP reduction rate was maximal by keeping the substrate concentration in the reactor at a constant level while avoiding substrate inhibition and cell lysis.

At different stages during the experiment, the glucose feed rate was raised appropriately to sustain the required metabolic reactions in the cell, and support biocatalytic activity. Dissolved oxygen during the experiment was constantly above 50% of air saturation. Metabolic by-products in the samples such as ethanol and acetate were negligible.

Table 5.2 shows the important process parameters usually used to assess the efficiency of a certain biocatalytic process [25]. 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 \textit{in situ} product removal during the integrated process. In addition to the dissolved DOIP, such as shown in Figure 5.6 for the reactor, DOIP crystals (60.50 g) accumulated in the crystallizer and were readily recovered. Final washing with ice-cold water, filtration and drying gave product crystals of 99.5% purity. The enantiomeric excess (e.e.) of 6R-DOIP was \(\geq 98\%\), making the aforementioned procedure an attractive option for enantioselective OIP reduction. The biocatalyst consumption rate was 0.42 kg per kg of product obtained; this can further be reduced when the ketone reduction process is continued up to several days more before the close-down phase. This is a potential benefit in particular for other processes with more expensive microbial biocatalysts. The volumetric productivity was 0.55 g.L\(^{-1}\).h\(^{-1}\) and would certainly increase when the bioreduction process would be optimally lengthened and/or the biocatalyst cultivation period reduced.

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Figure 5.6. Process scheme B: 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 mL.h\(^{-1}\), implementing a 
reduction rate of about 0.33 mmol.gdw\(^{-1}.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 24 h.

Table 5.2. Process parameters for the integrated fermentation-crystallization experiment (scheme B).

<table>
<thead>
<tr>
<th>Parameter [units]</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final yield (DOIP/OIP fed), [%]</td>
<td>85</td>
</tr>
<tr>
<td>Final selectivity (DOIP/(DOIP+ACT)), [%]</td>
<td>98.7</td>
</tr>
<tr>
<td>Final product concentration [g.L(^{-1})]</td>
<td>8.98(^a) / 101.45(^b)</td>
</tr>
<tr>
<td>Biocatalyst consumption [kg/kg prod]</td>
<td>0.42</td>
</tr>
<tr>
<td>Volumetric productivity [g.L(^{-1}.h(^{-1})]</td>
<td>0.55(^c) / 0.92(^d)</td>
</tr>
</tbody>
</table>

\(^a\) In fermenter; 
\(^b\) In crystallizer; 
\(^c\) During cultivation and reduction stages; 
\(^d\) During reduction stage only.
Morphology and DOIP crystal size distribution

It is important to assess the morphology and crystal size distribution of the resulting product as its properties (i.e. dissolution rates, viscosity, color, shape, impurity content) have a large impact on its applications and handling [26-28]. The product (DOIP) crystals obtained were white in color and rod-like in shape (Figure 5.7A). They were mechanically quite stable and tended to form large complex aggregates (Figure 5.7B). Crystal size data were obtained by measuring the crystal size distribution using the Image Analyser set-up described in the experimental section. Results in Figure 5.8 showed that most of the crystals have a length range of 1-30 μm and a diameter range of 1-20 μm. Considering a crystal population of 1500, the average length and diameter were 20 and 12 μm, respectively. However, due to its tendency to form aggregates, the crystal size distribution may vary in favor of the larger particles especially when these crystals have undergone storage. When the produced crystals underwent final washing and drying to increase the purity up to 99.5% (the same purity as that of the standard sample), the crystal morphology and size distribution were comparable. This indicates that re-crystallization is not required, despite the presence of many solutes in the fermentation medium used for cell growth.

Figure 5.7. (A) Typical DOIP crystals, which are rod-like in shape, and tend to form large complex aggregates (B).
Microbial reduction and ISPC coupled with biocatalyst cultivation during the synthesis of DOIP

![Graph showing crystal size distribution](image)

**Figure 5.8.** DOIP crystal size distribution.

This process strategy might be applicable to many biocatalytic (e.g. reduction) systems where crystalline products are obtained. Many examples of reactions where ISPR via crystallization might be employed are known [5,26,29,30]. Products from this process can include specialty biochemicals, pharmaceuticals, and fine chemicals such as amino acids, steroids, proteins, esters and ketones. The biocatalyst involved may range from wild-type baker’s yeast (S. cerevisiae) or bacteria (Escherichia coli) to genetically-engineered micro-organisms. However, when microbial cultivation is necessary, substrate/product inhibition, toxicity and degradation, sufficient supply of energy and carbon sources, as well as aeration and agitation, must be taken into account during the process design, amongst others, in order to obtain an economically promising biocatalytic process.
CONCLUSIONS

An efficient procedure was developed for DOIP synthesis involving cell cultivation coupled with OIP reduction and subsequent in-situ product crystal formation. Process implementation is feasible, however, for OIP reduction, biocatalyst cultivation must be done prior to bio-reduction and in-situ product crystallization as OIP inhibits cell growth when its concentration in the reactor is high (≥ 55 mM). An appropriate supply of the carbon and energy source such as glucose is necessary as it is preferably utilized for product formation rather than cell growth. Furthermore, the rate of OIP reduction using growing cells was five times higher than the rate with resting cells, which was favorable. A product concentration of over 100 g.L\(^{-1}\) was obtained in the crystallizer at the end of the integrated experiment. The product crystals were readily recovered and did not require re-crystallization.

ACKNOWLEDGEMENTS

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REFERENCES


Chapter 6

Influence of fermentation co-solutes on the nucleation and growth of 6R-dihydro-oxoisophorone crystals

E.M. Buque-Taboada, A.J.J. Straathof, J.J. Heijnen, and L.A.M. van der Wielen

Submitted.
ABSTRACT

The influence of co-solutes present in the medium to the crystallization kinetics of 6R-dihydro-oxoisophorone (DOIP) is studied as it provides some insights on the process design of in situ removal of DOIP via direct crystallization during its biocatalytic synthesis. The solubility of DOIP slightly decreases in phosphate buffer and in culture medium as compared to water. However, there are no significant differences in the kinetic parameters during crystallization in these three media. The nucleation and growth rates are dependent on the supersaturation ratio S and can be described by the following relations: \( J = 2.0 \times 10^5 \exp \left[ -0.023/(\ln S_0)^2 \right] \text{m}^{-3}\text{s}^{-1} \) and \( G = 1 \times 10^{-8} (S-1)^{0.86} \text{m.s}^{-1} \), respectively. Thus, the co-solutes in the medium increase the crystallization rate of DOIP as a particular DOIP concentration leads to a higher supersaturation ratio in the presence of co-solutes, due to lower DOIP solubilities. The interfacial tension (\( \gamma \)) was 0.0092 J.m\(^{-2}\), indicating a primary heterogeneous nucleation mechanism. From the process engineering point of view, crystal growth is the most significant sub-process to consider during crystallization as \( \geq 99\% \) of the mass of crystals is accounted to (the growth of) large crystals.
INTRODUCTION

Biocatalysis, using isolated enzymes or living cells, has become a viable alternative in the production of high-value specialty compounds in the pharmaceutical and fine-chemical industries [1,2]. Coupled with the demand to increase product purity and productivity, the challenge is now to explore new concepts for either the development of new processes or the improvement of old ones.

A biocatalytic reaction leads to a multicomponent system, usually containing dissolved nutrients, salts, unreacted substrate, and by-products, in addition to the desired product. Direct product crystallization from the reaction medium may be employed as a recovery and purification technique, but the presence of these co-solutes will influence formation of crystal nuclei and growth of crystals [3,4]. Co-solute adsorption at the surface of product crystals can either accelerate or inhibit the crystallization, which consequently leads to the modification of the crystal shape, size, morphology and structure [5-11]. In some cases, however, co-solutes did not influence crystallization [4,11-13].

Integrated reaction-crystallization systems are already applied and tested in biocatalytic processes [14-25]. In most of these processes, enzymes which are either crude, purified or partly purified are employed, yet the crystallization kinetics of the target product has not been studied, except in the production of calcium malate [24], aspartame precursor [25] and calcium gluconate crystals [26]. Still, the influence of co-solutes present in the medium was neither considered nor clearly elucidated. In our previous studies [14-15], whole-cells have been employed as catalyst and the vital addition of nutrients and salts in the reaction medium caused an increase in the bulk of dissolved co-solutes, which included the unreacted substrate and the dissolved by-product. Thus, it is crucial to obtain a clear understanding of the mechanisms on how co-solutes (impurities) can influence the crystallization kinetics in these integrated processes and which factors affect product crystal formation.

The crystallization of 6R-dihydro-oxoisophorone (DOIP) is considered in this work. It is a key intermediate in the synthesis of some carotenoids and flavors, and synthesized via the enantioselective reduction of 4-oxoisophorone by *Saccharomyces cerevisiae* (baker’s yeast), where *in situ* removal of the product by direct crystallization was employed [14,15]. Figure
6.1 shows the synthesis reaction involved. As this is a promising process option, it calls for the investigation of the effects of fermentation co-solutes during in situ crystallization of the target product.

Figure 6.1. Synthesis of DOIP from 4-oxoisophorone (OIP) by baker's yeast. By-product formed is actinol (ACT). The desired product (DOIP) must be recovered from the reactor by i.e. in situ crystallization to avoid degradation [14-15].

Thus, the influence of co-solutes in the medium on the crystallization kinetics of DOIP will be described. Nucleation and crystal growth kinetic parameters are simultaneously determined using the experimental data on (a) the desupersaturation curve at different initial supersaturation ratios $S_0$, where the DOIP concentration in the aqueous phase is monitored in time at the crystallization temperature $T = 278 \, \text{K}$, and (b) the crystal size distribution (CSD) using image analysis. Low values of initial supersaturation ratios $S_0$ are used as these are typical for in situ crystallization processes. Model validation is performed for a seeded batch crystallization experiment.

**THEORY AND MODEL**

During crystallization, two main processes take place: nucleation and crystal growth. The evolution of these processes can be followed by experimental measurements of the course of the desupersaturation, and of the crystal size distribution (CSD), also known as population density. Various mathematical approaches are known [4,27-33] and the integral approach
[29] is adapted to derive the kinetic parameters of nucleation and crystal growth as this allows the use of these two types of measurements from a single experiment. This method minimizes error in calculation and yields better estimation of parameters.

**Nucleation kinetics**

The supersaturation ratio \( S \) of a solute with a concentration \( C_{eq} \) in an aqueous solution is expressed as:

\[
S = \frac{C}{C_{eq}} \tag{6.1}
\]

The nucleation rate \( J \) as a function of \( S \) is given by the classical expression [27]:

\[
J = k_n \cdot \exp\left(\frac{-B}{(\ln S)^2}\right) \tag{6.2}
\]

which can be simplified to the following power-law expression [27]:

\[
J = k_n \cdot (S - 1)^{h} \tag{6.3}
\]

In a batch reactor, the particle number concentration is related to the nucleation rate [29] by

\[
n = \int_{0}^{t} J \cdot dt \tag{6.4}
\]

The particle number concentration can be determined experimentally using the integral equation [29]:

\[
n = \int_{0}^{\infty} N \cdot dL' \tag{6.5}
\]

where \( N \) is the crystal size distribution density function (#/m^4) at time \( t \) and where size is expressed in crystal length \( L' \).

Combining equations (6.2) and (6.4) gives equation (6.6), which is less sensitive to experimental errors [29]:

\[
n - n_0 = \int_{0}^{t} k_n \cdot \exp\left(\frac{-B}{(\ln S)^2}\right) dt \tag{6.6}
\]
Alternatively, equation (6.6) can be expressed as:

\[ n - n_0 = \int_0^t k_n \cdot (S - 1)^b \cdot dt \]  

(6.7)

From the particle number concentration \( n \) measured at time \( t \) while monitoring \( S \), the nucleation kinetic parameters \( k_n, B, \) or \( b \) can be estimated using equation (6.6) or equation (6.7). As \( S \) changes in time, equation (6.6) or (6.7) can be integrated with respect to time and \( S \) for each experiment conducted in various media.

**Crystal growth kinetics**

The over-all linear growth rate \( G \) of a crystal can be described by the simplified equation [27]:

\[ G = \frac{dL^*}{dt} = k_g \cdot (S - 1)^g \]  

(6.8)

Using the aqueous concentration profile with respect to time (from the desupersaturation curve), the linear growth rate can be described with the given estimates of the kinetic parameters \( k_g \) and \( g \). From the mass balance, assuming the mass due to nucleation of crystals is negligible compared to the mass due to crystal growth, the \( C^* \) profile can be fitted by simulation of the following equation (see Appendix for more details):

\[ \frac{dC^S}{dt} = -\frac{dC^{eq}}{dt} = \frac{3 \cdot M_o^S}{L_o^c \cdot M_o} \cdot k_g \left( \frac{C^{eq}}{C^{eq}_{eq}} - 1 \right)^g \]  

(6.9)

**Population balance**

The classical population balance of crystals without aggregation or breakage during a batch crystallization process is [27]:

\[ \frac{\partial N}{\partial t} + \frac{\partial (N \cdot G)}{\partial L^*} = 0 \]  

(6.10)

Multiplying equation (6.10) by the mole amount per crystal \( c^o = \rho V^o / M_o \) and differentiating the expression with respect to the length gives the following expression [29]:

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\[
\frac{dC^S}{dt} = \frac{3\rho^\text{cr} \cdot L^\text{cr}}{M_w} \int_0^{t^\text{cr}} \phi_s \cdot (L^\text{cr})^2 \cdot G \cdot N \cdot dL^\text{cr}
\] (6.11)

Equation (6.11) assumes that the mass due to nucleation is negligible. Integration gives [29]:

\[
C^S - C_0^S = \frac{3\phi_s \rho^\text{cr} \cdot t^\text{cr}}{M_w} \int_0^{t^\text{cr}} \left( \int_0^{L^\text{cr}} \right) G \cdot N \cdot dL^\text{cr} \cdot dt
\] (6.12)

From the nucleation kinetic expressions (equations 6.4 and 6.5) where:

\[
n = \int_0^{t^\text{cr}} J \cdot dt_n = \int_0^{t^\text{cr}} N \cdot dL^\text{cr}
\]

the change in molar concentration (equation 6.12) becomes:

\[
C^S - C_0^S = \frac{3 \cdot \rho^s \cdot \phi_e \cdot t^s \cdot G \cdot dt_g \cdot J \cdot dt_n}{M_w}
\] (6.13)

where \( t_g \) = time for crystal growth and \\
\( t_n \) = time for crystal nucleation.

Equation (6.13) is an expression directly showing the nucleation (\( J \)) and growth (\( G \)) rate relations.

The population density \( N \) can be expressed in terms of the concentration \( C^S \) as:

\[
N = \frac{C^S \cdot M_w \cdot w}{\rho^s \cdot \phi_e \cdot (L^\text{cr})^3}
\] (6.14)

where \( w \) = weight fraction of crystals per size class.

The simplified population balance relations (equations 6.12-6.14) can be solved and simulated using values and kinetic parameters of nucleation and crystal growth determined from the experimental data of the desupersaturation curve and crystal size distribution.
Chapter 6

EXPERIMENTAL SECTION

Chemicals
Levodione, also known as 6\(R\)-dihydro-oxoisophorone (DOIP), was kindly provided by DSM (Basel, Switzerland). The purity of all other chemicals used was at least laboratory grade.

Solubility measurements
The procedure for solubility measurement of DOIP is described elsewhere [14]. These measurements were reproduced using the crystallization vessel described in Crystallization experiments section. The measured values were averaged with previous results, for each medium and temperature.

Crystallization experiments
Freshly prepared solutions were saturated with DOIP at a fixed temperature in a 2-L jacketed vessel containing three baffles and two six-blade Rushton-type impellers (diameter 4.5 cm; impellers 0.5 cm above each other). The crystallization experiments were done using three different solvents or media, namely: pure water, 50 mM potassium phosphate buffer (pH 5.5) with 5 mM MgSO\(_4\).7H\(_2\)O, 60 mM 4-oxoisophorone (OIP) and 0.2 mL\(^{-1}\) Struktol\textsuperscript{®} J673 as antifoam agent; and culture medium with dissolved 60 mM OIP, having a composition (per L solution) as follows [34]: 20 g glucose, 3 g KH\(_2\)PO\(_4\), 5 g (NH\(_4\))\(_2\)SO\(_4\), 0.5 g MgSO\(_4\).7H\(_2\)O, 2.67 mL trace element solution, 2.67 mL vitamin solution, and 0.2 mL Struktol\textsuperscript{®} J673 as antifoam agent. The trace element solution has a composition (per L solution) of: 15 mg EDTA, 4.5 mg ZnSO\(_4\).7H\(_2\)O, 1 mg MnCl\(_2\).4H\(_2\)O, 0.3 mg CuSO\(_4\).5H\(_2\)O, 0.3 mg CoCl\(_2\).6H\(_2\)O, 1 mg H\(_3\)BO\(_3\), 0.4 mg Na\(_2\)MoO\(_4\).2H\(_2\)O, 3 mg FeSO\(_4\).7H\(_2\)O, 4.5 mg CaCl\(_2\).2H\(_2\)O, and 0.1 mg KI. The vitamin solution has a composition (per L solution) of: 0.05 mg biotin, 1 mg calcium pantothenate, 1 mg nicotinic acid, 25 mg myo-inositol, 1 mg pyridoxine.HCl, 0.2 mg para-aminobenzoic acid, and 1 mg thiamine.HCl. The latter two media (phosphate buffer and culture medium) are used in the actual integrated bioreactor-crystallization processes described in previous studies [14-15] where the substrate OIP was fed and kept constant at a concentration level avoiding microbial toxicity.

Batch crystallization experiments were conducted in a 2-L jacketed vessel containing three baffles and two inter-MIG II impellers (0.5 cm above each other), with a stirring speed of 100
Influence of fermentation co-solutes on the nucleation and growth of DOIP crystals

rpm. In some experiments, a stirring speed of 300 rpm was employed to determine its influence on the crystallization behavior of DOIP. The crystallization temperature was set at \( T_c = 5^\circ C \) and the working volume was 1 L. The temperature profile in the crystallizer was monitored during the experiment via a biocontroller (Applikon, ADI 1010).

After 5-20 minutes, the temperature in the crystallizer reached the setpoint \( T_c = 5^\circ C \) and at this time (time \( t \) is set to zero), about 0.5 g DOIP seed crystals were added and sampling was carefully done at various time intervals. Aliquots of samples were carefully filtered using 0.2 μm non-pyrogenic sterile cellulose acetate filters (Gelman Sciences). The filtrates were appropriately diluted and prepared for subsequent DOIP analysis by gas chromatography as described previously [14]. In the calculation of DOIP amounts, the measurements were corrected for the actual volume in the crystallizer and the amounts taken out during sampling.

After each experiment, the collected final product crystals were carefully kept in their final aqueous environment from the crystallizer and directly analysed for crystal size distribution, to avoid alteration of the crystal shape and size. The values of particle number concentration \( n (L) \) were determined from the image analysis of about 1500 particles observed via light microscopy (Image Analyzer (IA) consisting of a Sony CCD video camera module (XC-77CE), an Olympus stereozoom microscope (SZH) and a PC with IA software Leica Qwin version 3 (Olympus)). The particle number concentrations \( n (L) \) obtained were converted to weight fraction of crystals per size class \( w (L) \).

**Calculation of the objective function**

The objective function [29], a statistical tool calculated according to equations (6.15-6.16), indicating the difference between the experimental and calculated values, are determined and minimized, where \( x \) is the number of experiments and \( y \) the number of measurements, as follows: For nucleation:

\[
\Delta_{\text{nucleation}} = \frac{1}{x \cdot y} \sum_{j=1}^{x} \sum_{i=1}^{y} \left( \frac{N_{\text{exp}}(t_{i,j}) - N_{\text{calc}}(t_{i,j})}{N_{\text{exp}}(t_{i,j})} \right)^2
\tag{6.15}
\]

For growth:

\[
\Delta_{\text{growth}} = \frac{1}{x \cdot y} \sum_{j=1}^{x} \sum_{i=1}^{y} \left( \frac{C_{\text{exp}}^{eq}(t_{i,j}) - C_{\text{calc}}^{eq}(t_{i,j})}{C_{\text{exp}}^{eq}(t_{i,j})} \right)^2
\tag{6.16}
\]
RESULTS AND DISCUSSION

Solubility
The solubility of DOIP in various media (pure water, phosphate buffer, and culture medium) as a function of temperature $T$ is shown in Figure 6.2. A slight difference in the solubility of DOIP in these media is observed, affecting the equilibrium concentration and consequently the supersaturation ratio $S$ during crystallization. From Figure 6.2, DOIP is most soluble in pure water and least soluble in the culture medium at the crystallization temperature employed. This difference can be attributed to the presence of salts in the phosphate buffer and in particular in the culture medium. The presence of these salts could have made the solution more polar, thereby reducing its capacity to dissolve the rather nonpolar DOIP. The solubility ($C_{eq}$) of DOIP with respect to temperature can be described by equation (6.17) with the corresponding constants ($k$ and $\Delta H$) in various media presented in Table 6.1:

$$C_{eq} = k \cdot \exp \left( \frac{-\Delta H}{RT} \right)$$  \hspace{1cm} (6.17)

![Figure 6.2. Solubility of DOIP as function of temperature in various solvents or media: Pure water (O), Phosphate buffer (Δ), and culture medium (□). Lines are the best fit.](image)

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These relations are applied in the determination of the nucleation and crystal growth kinetics during DOIP crystallization in various media.

**Table 6.1. Constants and standard enthalpy of DOIP in various media**

<table>
<thead>
<tr>
<th>Medium (Solvent)</th>
<th>$k$ (mol.m$^{-2}$)</th>
<th>$\Delta H$ (J.mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure water</td>
<td>$0.53 \times 10^6$</td>
<td>-22,970</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>$2.93 \times 10^6$</td>
<td>-27,500</td>
</tr>
<tr>
<td>Culture medium</td>
<td>$3.62 \times 10^6$</td>
<td>-28,270</td>
</tr>
</tbody>
</table>

**Morphology and crystal size distribution**

The DOIP crystals obtained were white and rod-like in shape. During image analysis, a length range of 10-500 $\mu$m was considered for the crystal size distribution. Figure 6.3 shows typical population densities as well as the corresponding sample images of the seed and product crystals. The average aspect ratio ($A_R$) of the seed crystals is 1.85. The product crystals have a morphology similar to the seed crystals, except that occasionally needle-like shapes were obtained (as shown in Figure 6.3); but the average aspect ratio remains relatively constant, considering a sample of 1500 particles during the image analysis.

The population density of the product crystals show a peak at the crystal mean length $L_i = 15$ $\mu$m (size class between 10 and 20 $\mu$m) in all media at the initial supersaturation ratio $S_o \leq 1.3$, except in culture medium where this still holds at $S_o = 1.65$. At higher initial supersaturation ratios ($S_o > 1.3$), the population density peak is observed at the crystal mean length $L_i = 25$ $\mu$m (size class between 20 and 30 $\mu$m) but in the culture medium this is the case at $S_o > 1.65$. These suggest that nucleation of new crystals at the lowest size class occurred at a fast rate during crystallization, rendering product crystals of very fine sizes, like in some other cases [35-36]. In all our experiments, crystal growth is evident as seen by the increase in population density per size class from the mean length $L_i = 15$ $\mu$m to at least $L_i = 55$ $\mu$m.

Thus, a crystallization model, which includes both nucleation and crystal growth processes, was used to describe the mechanism of crystal formation and growth during DOIP crystallization. The crystal size distribution, which shows the particle number concentration per size class, can then be predicted during model validation employing the population balance.
Figure 6.3. Microscopic images of DOIP crystals at different magnifications and its corresponding population density. Product crystals are occasionally needle-like as shown, although, the rod-like shape is dominant.

Nucleation

In some experiments, nucleation seemed to occur before \( t = 0 \) according to mass balances, but this was negligible compared to the seed crystals. Estimation of the nucleation kinetic parameters was done by taking all the data points in various media. Figure 6.4 shows the plot of \( 1/(\ln S_0)^2 \) vs. \( \ln (n-n_0) \), which gives \( k_n = 2 \times 10^5 \ #.m^{-3}.s^{-1} \) and \( B = 0.023 \) using equation (6.6). The \( k_n \) and \( B \) values were also fitted for the experiments in each individual medium, which are in the same order of magnitude (see Table 6.2), but due to the scatter in the data no
dependence on the media was revealed. In all cases, equation (6.6) gives a more accurate fit of the data given than equation (6.7) (results not shown). Thus, the nucleation rate of crystals in various media can be described more accurately by the classical equation (6.2) with the aforementioned $k_n$ and $B$ values.

![Graph](image)

**Figure 6.4.** Nucleation kinetic parameter determination using plot of $1/(\ln S_o)^2$ versus $\ln (n-n_0)$ for each experiment done in pure water (O), phosphate buffer (△), and culture medium (□). Line is the best fit.

The nucleation kinetic parameter $B$ is related to the interfacial tension or surface energy ($\gamma$) as shown in equation (6.18) [29] below. With the determined value of $B = 0.023$, the interfacial tension ($\gamma$) = 0.0092 J.m$^{-2}$, which corresponds to a primary heterogeneous mechanism of nucleation [36].

$$B = \frac{4 \cdot \gamma^3 \cdot M_n^2 \cdot \phi_s^2}{27 \cdot (\rho^{\sigma r})^2 \cdot \phi_v^2 \cdot R^2 \cdot k_B \cdot T^3} \quad (6.18)$$

**Table 6.2.** Nucleation kinetic parameters in various media

<table>
<thead>
<tr>
<th>Medium (Solvent)</th>
<th>Nucleation rate coefficient $k_n$ ($\text{m}^3\text{s}^{-1}$)</th>
<th>Nucleation kinetic parameter $B$ (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure water</td>
<td>$3.74 \times 10^5$</td>
<td>0.052</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>$2.07 \times 10^5$</td>
<td>0.038</td>
</tr>
<tr>
<td>Culture medium</td>
<td>$1.88 \times 10^5$</td>
<td>0.034</td>
</tr>
</tbody>
</table>
Desupersaturation curves

The desupersaturation curves at different initial supersaturation ratios $S_o$ in various media are shown in Figures 6.5A-6.5C. Reproducibility is shown in Figure 6.5A for two experiments at $S_o = 1.27$ and in Figure 6.5B for the concentration profiles at $S_o = 1.21$ and $S_o = 1.22$. According to the results in Figure 6.5A for $S_o = 1.26$ at 300 rpm and for $S_o = 1.27$ at 100 rpm, the stirring speed has in this range no significant influence on the change in aqueous concentration with respect to time. The same trend is observed in Figure 6.5C with the concentration profiles at $S_o = 1.91$ and $S_o = 1.93$, where the stirring speed was 300 and 100 rpm, respectively. This indicates that mass-transfer limitations are absent and that a surface integration mechanism is dominant during crystallization at the conditions indicated.

In terms of crystal mass deposition, the total mass of crystals in a size class increased with increasing length class up to at least 280 $\mu$m (see example in Figure 6.6A). A very high percentage ($\geq 99\%$) of the total mass in the solid-phase is due to the large crystals (having a length of 50-300 $\mu$m), although these correspond to a lower particle number concentration compared to small crystals (i.e. with length of 10-40 $\mu$m) as shown in Figure 6.6B. This observation was consistent for all experiments. Since nucleation of new crystals occurred at the lowest size class, the corresponding total mass of new crystals is negligible compared to the mass due to crystal growth. Thus, in performing mass balances, only the total mass due to (crystal growth of) the large crystals (i.e. $L_f = 50-500\, \mu m$) was taken into account.

Crystal growth

The linear crystal growth rate as described by equation (6.8) was determined by fitting equation (6.9) to the experimental aqueous concentration ($C^{aq}$) profiles. Figures 6.5A-6.5C include the simulation results using equation (6.9). The fitted crystal growth kinetic parameters in various media are of the same order of magnitude (see Table 6.3). Combining all data in various media and fitting these results (see Figure 6.7) yields overall values of the crystal growth kinetic parameters where $k_e = 1 \times 10^{-8} \, \text{m.s}^{-1}$ and $g = 0.86$. 

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Figure 6.5A. Desupersaturation curves for DOIP crystallization in pure water at different initial supersaturation ratios $S_0$: 1.26 ($\blacklozenge$), 1.27 ($\bigtriangleup$), 1.31 ($\bigcirc$), and 1.37 ($\square$). Closed markers are at 300 rpm and open markers are at 100 rpm. Lines are simulation results.

Figure 6.5B. Desupersaturation curves for DOIP crystallization in phosphate buffer at different initial supersaturation ratios $S_0$: 1.21 ($\bigtriangleup$), 1.22 ($\square$), 1.51 ($\bigtriangleup$), 1.86 ($\bigcirc$). All experiments were done at 100 rpm. Lines are simulation results.
Figure 6.5C. Desupersaturation curves for DOIP crystallization in culture medium at different initial supersaturation ratios $S_o$: 1.29 (○), 1.65 (□), 1.91 (○) and 1.93 (○). Closed markers are at 300 rpm and open markers are at 100 rpm. Lines are simulation results.

Figure 6.6. (A) Mass concentration and (B) population density of DOIP crystals with respect to crystal size length. Example shown was for experiment in phosphate buffer at $S_o = 1.86$. Gray bars (in B) indicate the population density of the seed crystals used in the experiment.
In many cases, the value of the power number \( g \) in the growth rate equation indicates the growth mechanism during crystallization. This corresponds to the birth and spread mechanism (B+S model) of crystal growth where \( g = 5/6 \) [4,36]. Although other mechanisms might apply as well, this mechanism describes the growth development from surface nucleation that occur at the edges, corners and on the faces of the crystal and further development of surface nuclei on the monolayer as they spread across the crystal face [4,36].

Figure 6.7 indicates that although the growth rate parameters remain constant, slight increases in supersaturation as caused by the presence of co-solutes, lead to significantly higher crystal growth rates. In this regard, it is favorable to have more co-solutes (i.e. salts) in the medium as this leads to more favorable in situ crystallization conditions for the integrated fermentation-crystallization processes [14-15].

![Graph showing ln(G) vs ln(S-1)](image)

**Figure 6.7.** Crystal growth kinetic parameter determination using equation (6.8) for each experiment done in pure water (○), phosphate buffer (△), and culture medium (□). Line is the best fit.

**Table 6.3.** Crystal growth kinetic parameters in various media

<table>
<thead>
<tr>
<th>Medium (Solvent)</th>
<th>Growth rate coefficient ( k_c (m.s^{-1}) )</th>
<th>Order of crystal growth, ( g (-) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure water</td>
<td>( 1.24 \times 10^4 )</td>
<td>0.99</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>( 1.02 \times 10^4 )</td>
<td>1.00</td>
</tr>
<tr>
<td>Culture medium</td>
<td>( 0.94 \times 10^3 )</td>
<td>0.97</td>
</tr>
</tbody>
</table>
To illustrate further the effect of solubility on the crystallization rate, Figure 6.8 is shown as an example where equation (6.9) is simulated using an initial aqueous concentration of the solute $C_{sw}^{ini} = 30$ mM for DOIP crystallization in water, phosphate buffer, and culture medium at the same crystallization conditions. The aqueous concentration profiles (Figure 6.8) indicate that the rate of DOIP decrease is fastest when the culture medium is employed. This is due to the lower equilibrium concentration (solubility) in the culture medium at the crystallization temperature, resulting in higher supersaturation ratios and faster crystallization rates.

![Figure 6.8](image)

**Figure 6.8.** Simulation of DOIP concentration profile during crystallization in pure water (-- -- -- --), phosphate buffer (-- -- -- --), and culture medium (---) at the same crystallization conditions.

Model consistency

Model validation is not performed to show that independent results can be predicted, but to show that a model with over-all parameters can describe all data. The population density is calculated according to the relations (equations 6.12-6.14) derived from the population balance equation (6.10). With the known kinetic parameters of nucleation and crystal growth, the population densities in various media are simulated well. It was observed that the
population density was high at the lowest size classes, although growth prevailed with crystals at higher size classes, as mentioned earlier.

Parity plots of the experimental and calculated values of the population densities and aqueous DOIP concentrations are shown in Figure 6.9. This means that the crystallization kinetic parameters in various media can be described by one overall set of kinetic parameters, which are derived from all experimental data. The model can adequately describe the population density during DOIP crystallization in pure water, phosphate buffer or culture medium, with the determined kinetics of nucleation and crystal growth. In addition, the mathematical approach chosen minimized the errors in the calculation.

With respect to crystal purity, the DOIP product crystals obtained from crystallization are 99.5% pure after filtration, washing with ice-cold demineralised water and drying. The same crystal purity was obtained in integrated fermentation-crystallization experiments described elsewhere [14-15]. This suggests that re-crystallization may not be necessary as these crystals are used as starting material for the synthesis of higher-value specialty products. From the production process-engineering point of view, the mass of crystals formed during crystallization is an important parameter in determining yield, productivity and purity. In this view, crystal growth is the only significant sub-process to consider during crystallization. However, when the morphology and physical characteristics of the product play important role, both the nucleation and crystal growth sub-processes must be considered in optimizing the crystallization process.
Figure 6.9. Parity plots of experimental and calculated (A) population density and (B) aqueous DOIP concentration (examples) for the experiments done in pure water (○), phosphate buffer (△), and culture medium (□).
CONCLUSIONS

The crystallization kinetics of DOIP have been determined using the experimental data on desupersaturation curves and crystal size distribution. The nucleation and crystal growth kinetic parameters in various media show no significant differences; but the solubility of DOIP decreases slightly in the presence of co-solutes, leading to faster crystal growth. This means that crystallization in culture medium is favorable in this case. Considering all the data points in various media, overall kinetic parameters can be used which leads to a more straightforward description of the experimental results. When crystallization is employed with the aim of implementing an integrated process as in the case of in situ removal of DOIP crystals, the mass balance can be completed with the known kinetics of crystal growth. Nucleation can be neglected as a very high percentage of the mass of crystals is accounted for by the (growth of) large crystals. However, when the particle number concentration per size class is an important parameter in determining morphology and characteristics of the product crystal, the nucleation process should also be taken into account.

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS

$A_R$ average aspect ratio

$b$ order of nucleation

$B$ nucleation kinetic parameter

$c$ mole amount

$C$ molar concentration

$d$ diameter

$g$ order of crystal growth

$G$ crystal growth rate

$\Delta H$ standard enthalpy

$k$ constant for the solubility equation

$k_B$ Boltzmann constant

$k_g$ growth rate coefficient

$k_n$ nucleation rate coefficient

$J$ nucleation rate

$L$ crystal length

$m$ mass amount

$M$ mass concentration

$M_w$ molecular weight

$n$ particle number concentration of crystals

$N$ crystal size distribution density function

$r$ radius

$R$ gas constant

$S$ supersaturation ratio

$T$ temperature

$t$ time

$V$ volume

$V_{cr}$ volume per crystal

$w$ weight fraction per size class

$x$ number of experiments

$y$ number of measurements in an experiment

Greek letters

$\phi_s$ surface shape factor

$\phi_v$ volume shape factor

$\rho$ density

$\gamma$ interfacial tension or surface energy

Superscript

$aq$ aqueous phase

$cr$ of a crystal

$s$ solid phase

Subscript

$eq$ equilibrium saturation (solubility)

$i$ size class

$o$ initial
Appendix. Detailed derivations

Concentration profile in the aqueous phase

To measure crystal growth rate, the time course of the dissolved DOIP concentration during crystallization with seeding is determined at different initial supersaturation ratios $S_p$. This method is based on the assumption that the change in dissolved DOIP concentration is due to the lattice formation of the DOIP molecules on the surface of the seed crystals [4,.26-28]. The cylindrical (rod-like) geometry of the crystals is assumed with the mean length $L^c$ and radius $r^c$ based on the image analysis data.

The volume $V^c$ of a single seed crystal is:

$$V^c = \pi \cdot r^c \cdot \frac{L^c}{2}$$  \hspace{1cm} (A.1)

Considering the average crystal aspect ratio $A_R$:

$$A_R = \frac{\text{length}}{\text{diameter}} = \frac{L^c}{2} = \frac{L^c}{2r^c}$$  \hspace{1cm} (A.2)

This means that the length of the crystal ($L^c$) is $A_R$ times its diameter ($d^c$), which indicates that the growth rate of seed crystals in the longitudinal direction can be assumed to be $A_R$ times higher than in the radial direction. If the increase in the length of a rod-like crystal in a short period $dt$ is $dL^c$, then, the proportional increase in its radius in the same period should be:

$$dr^c = \frac{dL^c}{2A_R}$$  \hspace{1cm} (A.3)

Defining the molar growth rate of DOIP per unit crystallization volume as $dC^S/dt$, the mass balance can be developed as shown below based on the population density $n$ of the crystals:

$$\frac{dC^S}{dt} = \frac{d(n \cdot c^c)}{dt} = n \frac{dc^c}{dt} + c^c \frac{dn}{dt}$$  \hspace{1cm} (A.4)
Assuming negligible nucleation, the particle number concentration is \( n = n_o \) and equation (A.4) becomes

\[
\frac{dC^s}{dt} = n_o \frac{dc^{cr}}{dt} \tag{A.5}
\]

Considering the initial population density \( n_o \) of the crystals and the amount of moles \( c^{cr} \) per single crystal to be:

\[
n_o = \frac{M^S_o}{V^{cr}_o \rho^s} \tag{A.6}
\]

\[
c^{cr} = \frac{\rho^s \cdot V^{cr}_o}{M_w} = \frac{\rho^s}{M_w} \left( \pi \cdot r^{cr^2} L^{cr} \right) \tag{A.7}
\]

The over-all mass balance becomes:

\[
\frac{dC^s}{dt} = \frac{M^S_o}{V^{cr}_o \rho^s} \cdot \frac{d}{dt} \left[ \pi \cdot r^{cr^2} L^{cr} \right]
\]

\[
\frac{dC^s}{dt} = \frac{M^S_o \cdot \pi}{V^{cr}_o M_w} \left( \frac{L^{cr} \cdot d(r^{cr^2}) + r^{cr^2} \cdot dL^{cr}}{dt} \right)
\]

\[
\frac{dC^s}{dt} = \frac{3 \cdot M^S_o}{L^{cr^2} \cdot M_w} \cdot \frac{dL^{cr}}{dt} \tag{A.8}
\]

The term \( dC^s/dt \) is also equivalent to the molar rate of change per unit volume of dissolved DOIP in the crystallizer as:

\[
\frac{dC^s}{dt} = -\frac{dC^{\text{DOIP}}}{dt} = \frac{3 \cdot M^S_o}{L^{cr^2} \cdot M_w} \cdot \frac{dL^{cr}}{dt} \tag{A.9}
\]

Combining the crystal growth rate (equation (6.8) in the text) and supersaturation ratio (equation (6.1) in the text), the simplified equation becomes:

\[
\frac{dC^s}{dt} = -\frac{dC^{\text{DOIP}}}{dt} = \frac{3 \cdot M^S_o}{L^{cr^2} \cdot M_w} \left[ k_s \left( \frac{C^{\text{DOIP}}}{C^{\text{DOIP,eq}}} - 1 \right)^s \right] \tag{A.10}
\]
REFERENCES


Chapter 7

Design of an *in situ* product crystallization process for the production of 6R-dihydro-oxoisophorone

E.M. Buque-Taboada, A.J.J. Straathof, J.J. Heijnen, and L.A.M. van der Wielen

*Submitted.*
ABSTRACT

In this work, a case study is presented where a conceptual process design of *in situ* product crystallization (ISPC) with external configuration is compared with the *base case*, which is a known conventional process equivalent, for the production of 6R-dihydro-oxoisophorone (DOIP). DOIP is a key intermediate in the synthesis of some carotenoids and saffron flavors. The comparison indicates that employing ISPC has potential advantages over the conventional process (base case) in terms of increased productivity and yield with a corresponding decrease in the number of downstream processing steps as well as in the quantity of waste streams. This leads to an economically more interesting process alternative.
INTRODUCTION

In biochemical industry, downstream processing is often the cost-limiting factor during the production of pharmaceutical and fine-chemical products because low product concentrations are usually obtained from the reactor, requiring a large train of separation and purification steps. On the other hand, when fermentation or biotransformation is run to reach high product concentrations, volumetric productivity is often limited by either product inhibition or degradation, such that in situ product removal (ISPR) is useful in these cases [1].

This case study involves the synthesis of 6R-dihydro-oxoisophorone (DOIP), a white crystalline solid, with a solubility of about 10 g.L⁻¹ in water [2,3]. The synthesis reaction (Figure 7.1) involves the asymmetric reduction of 4-oxoisophorone (OIP) using baker's yeast (Saccharomyces cerevisiae) [2-4] or Saccharomyces rouxii [4] as biocatalyst. The main product is 6R-dihydro-oxoisophorone (DOIP), which is further degraded by the yeast mainly to (4S,6R)-actinol, an unwanted by-product in the process [2-4]. These yeasts are capable of producing DOIP with an enantiomeric excess of ≥ 98% [2-4].

![Diagram of chemical reactions](image)

**Figure 7.1.** Synthesis of 6R-dihydro-oxoisophorone via reduction of 4-oxoisophorone.

As product degradation occurs in the fermenter, it is imperative to keep the product concentration low; this can be done by employing an ISPR technique where the product is removed as soon as it is formed in the reactor. We are exploring the potential of ISPR by external crystallization and aim to demonstrate its pros and cons by designing a conceptual process and comparing its performance with a known conventional process.
As DOIP is a key intermediate in the synthesis of high-value products such as zeaxanthin and saffron flavors in which a relatively expensive substrate (OIP) is employed, stringent requirements on purity, (enantio)selectivity, productivity, and yield must be complied. Nowadays, DOIP is not available in the open market and is thus expected to be produced in-house or in a rented multipurpose plant (MPP) by the producers of carotenoids and flavors (see Table 7.1).

Little is known about the current industrial production process for DOIP and its current production levels, as these are usually classified industrial information. However, a recent patent literature by F. Hoffmann-La Roche AG [4], one of the producers of zeaxanthin and some flavors, describes a process for the production of DOIP (also known as levodione) with immobilized yeast biomass; this is assumed to be the process used in industrial practice. For the sake of comparison, this process is regarded as the base case and the process description is changed slightly to fit the specifications set in the case study, as will be discussed later on.

**Table 7.1. Known DOIP and carotenoid manufacturers in the world [5].**

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOIP</td>
<td>F. Hoffmann-La Roche AG (VFCD)*</td>
<td>Basel, Switzerland</td>
</tr>
<tr>
<td></td>
<td>Fluka Chemie A.G.</td>
<td>Buchs, Switzerland</td>
</tr>
<tr>
<td></td>
<td>Interchim S.A.</td>
<td>Montlucon, France</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>Apin Chemical Ltd.</td>
<td>Oxon, United Kingdom</td>
</tr>
<tr>
<td></td>
<td>Extrasyntheses S.A.</td>
<td>Genay Cedex, France</td>
</tr>
<tr>
<td></td>
<td>F. Hoffmann-La Roche AG (VFCD)*</td>
<td>Basel, Switzerland</td>
</tr>
<tr>
<td></td>
<td>Indofine Chemical Company Inc.</td>
<td>Sommerville NJ, United States</td>
</tr>
<tr>
<td></td>
<td>Carl Roth GmbH &amp; Co. Chemische Fabrik</td>
<td>Karlsruhe, Germany</td>
</tr>
<tr>
<td></td>
<td>Sigma Chemical Company</td>
<td>St. Louis MO, United States</td>
</tr>
<tr>
<td></td>
<td>Spectrum Chemical MFG. Corp.</td>
<td>Gardence CA, United States</td>
</tr>
<tr>
<td></td>
<td>DSM</td>
<td>Heerlen, The Netherlands</td>
</tr>
</tbody>
</table>

* Now owned by DSM.

Considering its application, DOIP has expectedly a rather small production volume (estimated herein at 2-6 tons per year), such that the process is designed to attain production targets in a multipurpose plant setting where other fine-chemical products are produced, thus, taking advantage of economy of scale. The designed processes are intended to replace the existing process; therefore, there is no impact on the world market.
The design challenge is to produce 6 tons per year of DOIP crystals in the size range of 0.01 - 0.1 mm [6-7] with a purity of 99.5 %w/w and an enantiomeric excess (e.e.) of ≥98%. Inherent to this challenge is to compare the production costs per kg of DOIP of the process employing ISPR by crystallization and the base case on the same basis.

A conceptual process design (CPD) approach is adapted in this case study as described in detail elsewhere [8-13]. The procedures indicated therein have been carefully followed and this work is the condensed and simplified version of the detailed CPD.

**PROCESS DESIGN**

A process for producing 6R-dihydro-oxoisophorone (DOIP) from 4-oxoisophorone (OIP) catalysed by whole-cells (yeasts) is conceptually designed. To overcome the problem of product degradation, *in situ* product crystallization (ISPC) with external configuration is applied. To evaluate the ISPC process performance, it is compared to a base case DOIP production process based on a recent industrial patent literature [4]. For the ISPC case, the biocatalyst used is a typical baker's yeast (*S. cerevisiae*) with reaction kinetics and production rates described previously [3,14]. For the base case, immobilized *Saccharomyces rouxii* is employed where the reaction rates and productivities are described previously [4]. The enantiomeric excess of DOIP produced by *S. cerevisiae* and *S. rouxii* is ≥ 98% [2-4], which meets the specifications. Economic evaluation is done on the same basis considering current costs of raw materials, equipment and utilities [15-16]. An operating time of 6000 hours a year is chosen for the production of 6 tons DOIP in a multipurpose plant (MPP) that has flexibility for use in the production of other compounds.

**Process description**

The process flow diagram of the base case is shown in Figure 7.2. The base case employs a bubble column fermenter with immobilized cells as biocatalyst as this gave good volumetric productivity and low estimated costs [4]. For downstream processing, extraction or adsorption may be done prior to crystallization in organic solvent medium to produce the DOIP crystals. For this process design, extraction with ethyl acetate as described elsewhere [2] is chosen to avoid too much speculation in the design of adsorption columns where the
required parameters are not available for the base case process. Crystallization occurs from the ethyl acetate extract upon cooling and solvent evaporation [4]. A single multipurpose vessel is used for extraction and crystallization, as fermentation is the process rate-limiting step according to the batch cycle diagram.

The process flow diagram for the ISPC case is shown in Figure 7.2. For this process, a stirred-tank fermenter coupled with an external crystallizer is considered where an ultrafiltration unit is placed in between these units to separate the liquid from the cells and to recycle the cells to the fermenter as described previously [3]. Crystallization is done by cooling at \( T_c = 5^\circ C \) and for process simulation, the crystallization kinetics described previously [6] is adapted. The final filtration step of the product crystals is done using a Nutsche filter and nitrogen gas is employed for crystal drying to prevent explosions.

**Basic Assumptions**

*Location and plant life*

It is desired to perform the production in a multipurpose plant (MPP). At such a plant there is a wide variety of process equipment available for processing fine chemicals. The equipment is used for different production processes over the year.

At the MPP, infrastructure for communication and transportation of raw materials and products are available. The facility is able to supply heat transfer oil at a wide temperature range, approximately between −10 to 150\(^\circ\)C and electricity, among others. A wastewater treatment plant (WWTP) and waste handling facility is also installed in the MPP. It is assumed that the MPP facility will be partly used for DOIP production when a smaller production capacity is desired, but for the sake of economic analysis, a full-time usage of the MPP is adapted.

The economical plant life for large production plants for bulk products is in general about 20 years. In contrast, fine chemicals are produced with much smaller capacities, as its market is quite flexible with demands changing in a couple of years. Thus the economical plant life for the design cases is assumed to be only 15 years.
Figure 7.2. Process flow diagram for (A and B) base case and (C) ISPC case.
Conventional equipment, which is usable in the production of other fine-chemical products, has an equipment life of 15 years while unconventional (specialized) equipment has an assumed life of 10 years.

**Feedstock**
A feedstock of OIP is required for the synthesis of DOIP and is expected to be available locally. The biomass feedstock is assumed to be produced in-house. Other fermentation chemicals required such as: glucose (the C-source), oxygen, buffer, acid, base and anti-foaming agent are also available locally. The fermentation liquid is sterilised process water.

**Battery limit**
The biomass used as catalyst is cultivated outside the battery limit for both cases. However, regeneration of the immobilized biomass is implemented within the battery limit for the base case.

Further, the bioconversion and downstream processing equipment are implemented within the battery limit. For the base case, this includes units for reaction, filtration, extraction, evaporation, crystallization and solid handling. For the ISPC case, devices are limited to reactors, filters, crystallizers and the solid handling units. For both cases auxiliary equipment is desired such as storage vessels, pumps, piping, valves, and process control devices.

The fermentation (feedstock) solution is composed of many different components usually needed in small quantities. For simplicity, this is condensed to three types of solution: media solution, reactivation solution (for biomass regeneration in the base case), and the biomass suspension (used in the ISPC case). The feedstock for these solutions are acquired from outside the battery limit and subsequently mixed within the battery limit.

Wastewater treatment is done outside the battery limit. Spent biomass goes with the spent process water to a wastewater treatment plant (WWTP). The immobilized biomass cannot be processed in a WWTP and needs to be discarded in another way at the end of the process. In the base case, the spent solvent needs to be discarded in an organic waste treatment facility. Utilities such as electricity, heating and cooling are in the battery limit. Table 7.2 summarizes the ins and outs of the battery.
Table 7.2. Definition of units/operations in- and outside the battery limit.

<table>
<thead>
<tr>
<th>Base case</th>
<th>ISPC case</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inside battery limits</strong></td>
<td><strong>Outside battery limits</strong></td>
</tr>
<tr>
<td>Fermenter</td>
<td>Acquisition of feedstock</td>
</tr>
<tr>
<td>Filters</td>
<td>Waste treatment</td>
</tr>
<tr>
<td>Extractor</td>
<td>Cooling duties</td>
</tr>
<tr>
<td>Evaporator</td>
<td>Sterilisation of process water</td>
</tr>
<tr>
<td>Crystallizer</td>
<td></td>
</tr>
<tr>
<td>Solid Handling</td>
<td></td>
</tr>
<tr>
<td>Auxiliary equipment</td>
<td></td>
</tr>
<tr>
<td>Regeneration of biomass</td>
<td></td>
</tr>
</tbody>
</table>

**Process block schemes**

A simplified process flowsheet for the base case is shown in Figure 7.3 where the mass streams, pressures and temperatures are indicated. The thicker lines show the flow of main reactant (OIP) and product (DOIP). The values indicated (in ton per ton of product) in each process stream have been used in the economic analysis. In this case, the overall process yield of DOIP on OIP was 79 %w/w during the production of 6.2 tons of DOIP in approximately 6000 hours. Details of the major equipment employed in the process are summarized in Table 7.3 and described in the Appendix. Fermenter profile is also shown in the Appendix.

![Figure 7.3. Simplified block scheme diagram of DOIP production process (base case).](image-url)
Table 7.3. Specifications of major equipment used in the base case.

<table>
<thead>
<tr>
<th>Specification</th>
<th>Fermenter R101</th>
<th>Extractor S201</th>
<th>Crystallizer S201</th>
<th>Filters S101</th>
<th>Filters S202</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>Bubble Column</td>
<td>MPV(^a)</td>
<td>MPV(^a)</td>
<td>Microfilter</td>
<td>Nutsche</td>
</tr>
<tr>
<td>Volume [m(^3)]</td>
<td>1.65</td>
<td>4.5</td>
<td>4.5</td>
<td>0.07</td>
<td>0.8</td>
</tr>
<tr>
<td>Area [m(^2)]</td>
<td>0.44</td>
<td>1.33</td>
<td>1.33</td>
<td>49.2</td>
<td>1</td>
</tr>
<tr>
<td>Flux [m(^3).m(^-2).h(^-1)]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Number</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>- series</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>- parallel</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Multipurpose vessel with agitation accessories.

A simplified flowsheet of the ISPC case is shown in Figure 7.4 where the yield per process step is indicated. The overall process yield of DOIP on OIP is 91% w/w. A process cycle of one batch is about 400 hours, which results in a total of 15 batches a year to produce the desired amount. The ISPC process has characteristics of both a continuous and a batch process; however, the streams for a continuous process indicated are employed in the economic analysis. Details of the major equipment required in the process are summarized in Table 7.4 and described in the Appendix. The process profile is also shown in the Appendix.

---

**Figure 7.4.** Simplified block scheme diagram of DOIP production process (ISPC case).
Table 7.4. Specifications of major equipment used in the ISPC case.

<table>
<thead>
<tr>
<th>Specification</th>
<th>Fermenter</th>
<th>Crystallizer&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Filters</th>
<th>Filters</th>
<th>Filters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;sup&gt;R101&lt;/sup&gt;</td>
<td>&lt;sup&gt;S102&lt;/sup&gt;</td>
<td>&lt;sup&gt;S101&lt;/sup&gt;</td>
<td>&lt;sup&gt;S102&lt;/sup&gt;</td>
<td>&lt;sup&gt;S103&lt;/sup&gt;</td>
</tr>
<tr>
<td>Type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume [m³]</td>
<td>6 (4.1&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>3.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Area [m²]</td>
<td>2.7</td>
<td>1.5</td>
<td>6</td>
<td>0.07</td>
<td>1.8</td>
</tr>
<tr>
<td>Flux [m³.m⁻².h⁻¹]</td>
<td>-</td>
<td>-</td>
<td>0.07</td>
<td>0.61</td>
<td>0.22</td>
</tr>
<tr>
<td>Number</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>- series</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>- parallel</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Fermenter working volume.
<sup>b</sup> With internal microfilter.
<sup>c</sup> Installed inside the crystallizer.

**PROCESS ANALYSIS**

**Base case**

The base case design makes use of proven technology. There is sufficient know-how of the sub-processes, which are widely applied in large-scale industrial operation. This makes the base case process a reliable one with respect to the individual operations of the equipment. The process is flexible but the overall operation is not straightforward, which can cause potential setbacks (i.e. disruption of equipment scheduling) on the over-all production process.

Using immobilized biomass as the biocatalyst is well-considered. Volumetric productivity is high and the short batch process time in a bubble-column fermenter is an attractive feature of the process, as it reduces the required equipment size.

The base case has an over-all yield of 79% w/w, which is not attractive when a relatively expensive substrate is employed, as is the case for OIP. Furthermore, a large amount of process water (202 tons per ton product) is required, resulting in a dilute product stream and large waste streams. The large amount of organic solvent required (18.4 tons per ton of product) in the process leads to a considerable cost in the operation, more so when a good quantity of spent organic solvent makes its way to the WWTP for disposal.
**ISPC case**

A large-scale production system of the ISPC case is designed, which is technically and economically feasible. However, the technology is new and needs to be enhanced (e.g. by performing pilot-scale studies) to improve process reliability. The ISPC case employs less equipment (thus, less manpower) but its semi-continuous and integrated nature can be disastrous to the production process itself. Appropriate troubleshooting schemes should be implemented. The process requires a relatively large fermenter with stirring equipment, which is more costly to install, operate, and maintain than a bubble-column fermenter employed in the base case.

The ISPC case has an over-all yield of 91% w/w, which is a substantial improvement of the base case having a yield of 79% w/w. This is due to the fact that product degradation is minimized, stream recycles are implemented, a long process time is also implemented, and water build-up in the system is prevented. Process water required is 14.4 tons per ton product, which is 14 times smaller than in the base case. In addition, the ISPC case does not use organic solvent, which leads to an even smaller and easy-to-treat waste stream.

Most equipment choices for the ISPC case are attractive options. The ultrafilter does not lead to high costs, the stirred-tank fermenter has higher yield (although costly) and cooling crystallization is efficient. The stream recycles are an important feature of the design, however, careful implementation is needed to avoid potential process disturbances and failures.

**ECONOMIC ANALYSIS**

The cost of the main substrate OIP is estimated at 100 Euro per kg and DOIP is four times more expensive (400 Euro/kg) [16]. All other costs were estimated according to available sources [15-16]. The total cost as well as the production cost per kg of DOIP produced in each case is shown in table 7.5. The ISPC case has a lower annual production costs and thus a higher net cash flow (NCF). It has a higher economic potential, scoring better on all economic criteria (table 7.6). This is attributed to the higher yield and the absence of solvent usage. In the base case, the lower yield causes a lower net cash flow and the use of organic solvent resulted in higher variable costs, which includes waste costs. Although the equipment
costs (PEC) of the ISPC case are higher, the final total investment required is the same for both processes. The higher PEC for the ISPC case is due mostly to the high costs of the fermenter. The variable costs due to cooling in the crystallization loop in the ISPC case turn out to be modest.

### Table 7.5. Final economic figures in both cases

<table>
<thead>
<tr>
<th>Cost Items</th>
<th>Base case</th>
<th>ISPC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Purchased Equipment Costs (PEC) [k€]</strong></td>
<td>661</td>
<td>907</td>
</tr>
<tr>
<td><strong>Investment Costs [k€]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct Costs (DC)</td>
<td>1,740</td>
<td>1,620</td>
</tr>
<tr>
<td>Indirect Costs (IC)</td>
<td>1,040</td>
<td>970</td>
</tr>
<tr>
<td>Direct Fixed Capital (DFC)</td>
<td>3,160</td>
<td>2,970</td>
</tr>
<tr>
<td>Total Investment costs (IT)</td>
<td>4,000</td>
<td>3,720</td>
</tr>
<tr>
<td><strong>Operating Costs [k€/a]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fixed Costs (FC)</td>
<td>860</td>
<td>580</td>
</tr>
<tr>
<td>Variable Costs (VC)</td>
<td>1,080</td>
<td>761</td>
</tr>
<tr>
<td>Raw materials</td>
<td>798</td>
<td>709</td>
</tr>
<tr>
<td>Consumables</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>Utilities</td>
<td>146</td>
<td>28</td>
</tr>
<tr>
<td>Waste treatment and disposal</td>
<td>104</td>
<td>1</td>
</tr>
<tr>
<td>Miscellaneous expenses</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Other costs</td>
<td>129</td>
<td>87</td>
</tr>
<tr>
<td><strong>Annual Production Costs (APC) [k€/a]</strong></td>
<td>2,069</td>
<td>1,428</td>
</tr>
<tr>
<td><strong>Income [k€/a]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Revenues</td>
<td>2,490</td>
<td>2,560</td>
</tr>
<tr>
<td>Net Cash Flow (NCF)</td>
<td>420</td>
<td>1,130</td>
</tr>
<tr>
<td><strong>DOIP produced per year [kg/a]</strong></td>
<td>6222</td>
<td>6393</td>
</tr>
<tr>
<td><strong>Production Costs [€/kg]</strong></td>
<td>333</td>
<td>223</td>
</tr>
<tr>
<td><strong>Margin (NCF/kg DOIP) [€/kg]</strong></td>
<td>68</td>
<td>177</td>
</tr>
</tbody>
</table>

### Table 7.6. Economic criteria in both cases.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Base case</th>
<th>ISPC case</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of Return [%]</td>
<td>11</td>
<td>31</td>
</tr>
<tr>
<td>Pay-back time [years]</td>
<td>10.5</td>
<td>4.3</td>
</tr>
<tr>
<td>DCFRR* [%]</td>
<td>6.4</td>
<td>26.1</td>
</tr>
<tr>
<td>Return on Investment [%]</td>
<td>2</td>
<td>17</td>
</tr>
</tbody>
</table>

*Discounted cash flow rate of return*
Possible economic improvements
Since substrate cost is a major cost factor as it is relatively expensive, improving the yield of DOIP on OIP is recommended to increase the net cash flow especially in the base case. A cheaper, more efficient, easy-to-dispose-of kind of organic solvent must be employed if it cannot be avoided in the base case, so that the utilities costs are decreased, as will be the waste costs. Reducing the amount of used process water and the amount of solvent loss will save on utilities and waste treatment/disposal costs. The ISPC case is in many ways already an improvement of the base case, but can still be enhanced. The yield and productivity can be improved further by optimising flow and recycle streams and by employing a biocatalyst with better biocatalytic activity, amongst others. Table 7.7 shows the DOIP productivity employing yeasts at different conditions. Free cells of *S. rouxii* shows a productivity which is almost 3 times better than *S. cerevisiae*. Growing cells of *S. cerevisiae* shows the highest productivity but this might be economically compensated by the cost of nutrients required in growing cells. Alternative fermenters should be explored as the current stirred-tank fermenter (including its operation) is a major cost factor in the process. Upscaling would significantly reduce investments for both options.

Although the acquisition of biomass feedstock is outside of the battery limit, it is economically attractive to consider acquiring or producing it on a yearly basis by cultivating the biomass in a large rented fermentation facility and storing it properly to maintain its viability throughout the year.

Table 7.7. DOIP productivity by two important yeasts [2-4,17]

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Productivity [mg (g cells)⁻¹ h⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resting cells⁺</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> [3,17]</td>
<td>10</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> [2]</td>
<td>Free cells</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> [4]</td>
<td>9</td>
</tr>
<tr>
<td><em>Saccharomyces rouxii</em> [4]</td>
<td>26</td>
</tr>
</tbody>
</table>

⁺The productivity is in terms of gram dry weight of cells.
CONCLUSIONS

A conceptual process design of an in situ product removal technique by external crystallization for the production of DOIP has been developed and the production performance has been evaluated and compared with a conventional process equivalent (base case). It is established that the ISPC process is the technically and economically more attractive option in the production of solid products employing whole-cell biocatalysts as it has higher over-all yield and productivity and does not require an auxiliary phase. It has smaller waste streams and requires less downstream processing steps, resulting in lower production costs. However, further research must be done to improve process reliability as it is a novel technology.

ACKNOWLEDGEMENTS

This study has been funded by the MHO-USC-DUT Project in Chemical Engineering. The following persons are gratefully acknowledged for their important contribution to this work: Sjoerd Blokker, Marcel Dabkowski, Willem Groendijk, Dirk Renckens, Jeroen de Rond, and Prof.dr.ir. Johan Grievink of Delft University of Technology.
APPENDIX. DETAILS OF THE PROCESSES

Base case

A bubble column is used as fermenter in the base case as this has good mass and heat transfer properties in the fluid phase and oxygen transfer between gas and liquid phase. Fluidization of the immobilized biomass via the rising liquid and gas bubbles and thereby the lack of a need for a stirrer has a positive effect on the lifespan of the immobilized biomass. A bubble column is less expensive than a stirred-tank fermenter and it has no moving parts, which can break down. It can be operated with atmospheric top pressure and a temperature of 30°C. To ensure that no solids or particulates (cell debris) are present in the broth from the fermenter, a 1 µm microfilter is placed on-line.

The fermenter concentration profile is shown in Figure A.7.1. The kinetic model for the base case is derived from the patent literature [4]. The parameters in Table A.7.1 are the input variables of the model to simulate the fermentation process. In Figure A.7.1, a fermentation time of 8 hours gives almost 100% conversion of OIP. After this time, fermentation is stopped and the fermenter is drained and subsequently filled with washing water to wash the immobilized biomass and the fermenter. Reactivation of the immobilized cells is done after every 20 batch fermentation cycles as described [4].

<table>
<thead>
<tr>
<th>Input variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction time [h]</td>
<td>8</td>
</tr>
<tr>
<td>Glucose concentration [kg.m⁻³]</td>
<td>30</td>
</tr>
<tr>
<td>OIP concentration [kg.m⁻³]</td>
<td>10</td>
</tr>
<tr>
<td>Immobilized biomass concentration [kg.m⁻³]</td>
<td>230</td>
</tr>
</tbody>
</table>

A mixer-settler set-up has been chosen for the extraction unit for its simplicity and high efficiency. A normal stirred vessel can be used, where the solvent (ethyl acetate) is added to the filtered fermentation broth. The extraction takes place at atmospheric pressure and temperature of 20°C as the distribution coefficients of the compounds are measured at these conditions. The number of stages is calculated according to a 98% extraction yield. In practice 3 stages indicates that the aqueous solution coming from the fermenter goes through 3 extraction stages, where the solvent used is recycled. Fresh solvent is needed to compensate for solvent loss in the raffinate and the solvent recycle purge. After 3 extraction stages, the raffinate (aqueous solution) is discharged to the wastewater treatment facility.
An evaporative crystallization is employed since the solubility of DOIP in the solvent is high at a crystallization temperature of 5°C (84.2 g. L⁻¹). By evaporating the solvent the product concentration increases and crystallization is more effective. After approximately 6 hours the evaporation is stopped and the crystallization is continued, leading to a total time of 12.6 hours [4]. For crystallization some seed crystals are added, but this is not accounted for in the mass balances, as the amount involved is negligible. The same vessel for extraction and evaporative crystallization can be used. With all the typical process times known for each step, this option is possible, but some extra buffer vessels are necessary. These multipurpose vessels (MPV) should have special features to be able to be used for both unit operations. The cycle time of the complete process is determined by the reactivation step, which occurs every 176 hours [4]. For biocatalyst activation, the bubble column is filled in 30 minutes with reactivation solution as described elsewhere [4]. The biomass is reactivated in 23.4 hours. The spent reactivation solution is transferred to the WWTP. The indicative operation times of the processes are shown in Table A.7.2.

**Table A.7.2. Operation times of processes in the base case.**

<table>
<thead>
<tr>
<th>Process</th>
<th>Time [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Re-)activation biomass / start up</td>
<td>24.0</td>
</tr>
<tr>
<td>Fermentation</td>
<td>9.5</td>
</tr>
<tr>
<td>Extraction</td>
<td>5.7</td>
</tr>
<tr>
<td>Evaporation</td>
<td>6.3</td>
</tr>
<tr>
<td>Crystallization</td>
<td>6.3</td>
</tr>
<tr>
<td>Solid Handling</td>
<td>10.5</td>
</tr>
</tbody>
</table>

153
ISPC case

In the fermenter there is a possibility of evaporation of OIP and DOIP resulting in yield loss. This however only occurs at high aeration rates [3]; therefore the fermenter is designed in such a way that there is high oxygenation efficiency at lower aeration rates. A measure that could help achieve this is by employing a high stirrer speed or by enriching the air with oxygen.

In using an ultrafilter for cell separation and recycling, clogging or fouling can occur, which is due to the presence of aggregated cell debris and the excreted proteins of disrupted cells, for example. Thus, two ultrafilters are installed in parallel so that if one fails, another one is readily online. This set-up also allows regular cleaning without disrupting the process flow.

In the crystallizer, the cooling process might cause some problems. If the crystallizer wall is too cold, crystals would be formed at the wall. This decreases the cooling efficiency and DOIP cannot be crystallized at a sufficient rate. When the incoming stream is pre-cooled, this problem can be avoided. The crystallizer is installed with a plastic scraping mechanism. This “scraper” scrapes the formed crystals off the wall and stirs the crystallizer at the same time. The crystallizer is installed with a microfilter to prevent the crystals from going back to the fermenter during the recirculation of the mother liquor. Table A.7.3 shows the operation times of processes in ISPC case.

Process profile

For the ISPC case, the kinetic model and the input variables described elsewhere [3,14] are adapted. Figure A.7.2 shows the process profiles with the following output:

Concentrations at the end of each reduction process (t = 370h):

<table>
<thead>
<tr>
<th></th>
<th>OIP</th>
<th>DOIP</th>
<th>ACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>EndFerm</td>
<td>4.6</td>
<td>28.8</td>
<td>6.1</td>
</tr>
<tr>
<td>EndCrys</td>
<td>5.9</td>
<td>25.6</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vcr</td>
<td>2.8</td>
<td>m³</td>
</tr>
<tr>
<td>DOIP Production</td>
<td>426.2</td>
<td>kg</td>
</tr>
<tr>
<td>Yield DOIP</td>
<td>97.5</td>
<td>%</td>
</tr>
<tr>
<td>Yield ACT</td>
<td>1.4</td>
<td>%</td>
</tr>
<tr>
<td>Cryst percentage</td>
<td>93.6</td>
<td>%</td>
</tr>
<tr>
<td>Yield cryst</td>
<td>91.2</td>
<td>%</td>
</tr>
</tbody>
</table>

Volume crystallizer
Production of DOIP crystals
Yield total DOIP on OIP
Yield ACT on DOIP
Percentage crystals on total DOIP
Yield DOIP crystals on OIP
Table A.7.3. Operation times of processes in ISPC case.

<table>
<thead>
<tr>
<th>Process</th>
<th>Time [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting up</td>
<td>5</td>
</tr>
<tr>
<td>OIP feeding</td>
<td>300</td>
</tr>
<tr>
<td>Closedown phase</td>
<td>70</td>
</tr>
<tr>
<td>Filtration</td>
<td>6</td>
</tr>
<tr>
<td>Washing</td>
<td>6</td>
</tr>
<tr>
<td>Drying</td>
<td>30</td>
</tr>
<tr>
<td>Solid Handling/Cleaning</td>
<td>28</td>
</tr>
</tbody>
</table>

Figure A.7.2. Process profiles of the ISPC case.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT</td>
<td>Actinol</td>
</tr>
<tr>
<td>APC</td>
<td>Annual production cost</td>
</tr>
<tr>
<td>CPD</td>
<td>Conceptual process design</td>
</tr>
<tr>
<td>DC</td>
<td>Direct costs</td>
</tr>
<tr>
<td>DCFRR</td>
<td>Discounted cash-flow rate of return</td>
</tr>
<tr>
<td>DFC</td>
<td>Direct fixed capital</td>
</tr>
<tr>
<td>DOIP</td>
<td>(6R)-dihydro-oxoisophorone</td>
</tr>
<tr>
<td>e.e.</td>
<td>Enantiomeric excess</td>
</tr>
<tr>
<td>FC</td>
<td>Fixed costs</td>
</tr>
<tr>
<td>IC</td>
<td>Indirect costs</td>
</tr>
<tr>
<td>ISPR/ISPC</td>
<td><em>in situ</em> product removal/<em>in situ</em> product crystallization</td>
</tr>
<tr>
<td>IT</td>
<td>Total investment costs</td>
</tr>
<tr>
<td>MPP</td>
<td>Multi purpose plant</td>
</tr>
<tr>
<td>MPV</td>
<td>Multi purpose vessel</td>
</tr>
<tr>
<td>NCF</td>
<td>Net cash flow</td>
</tr>
<tr>
<td>NPV</td>
<td>Net present value</td>
</tr>
<tr>
<td>OIP</td>
<td>4-oxoisophorone</td>
</tr>
<tr>
<td>PEC</td>
<td>Purchased equipment costs</td>
</tr>
<tr>
<td>VC</td>
<td>Variable costs</td>
</tr>
<tr>
<td>WWTP</td>
<td>Wastewater treatment plant</td>
</tr>
</tbody>
</table>
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Chapter 8

OUTLOOK

E.M. Buque-Taboada, A.J.J. Straathof, J.J. Heijnen, and L.A.M. van der Wielen

Submitted.
OUTLOOK

Crystallization as an in situ product recovery technique is proven to be an attractive option in the production of solid compounds employing whole cells as biocatalyst. A case study, presented as an example, shows that employing in situ product crystallization in a typical whole-cell catalysed reaction can lead to a more economical production process where increased productivity and yield with minimal waste streams is possible at lower production costs. The process does not require the use of auxiliary phases such as organic solvents. This process strategy may be applicable to many biocatalytic (e.g. reduction) systems where crystalline products are obtained, which include specialty biochemicals such as amino acids, steroids, antibiotics, proteins, esters and ketones. Furthermore, this is an attractive option for processes employing relatively expensive substrates.

However, the design approaches and choices for such a process are crucial for its successful implementation. As this study is the start of a systematic development for ISPC processes, further research is required to unravel the different factors and issues concerning this process. In the end, in situ product recovery by crystallization should be a common practice in the design and implementation of robust integrated processes involving fermentation or biotransformation.

More specifically, the following issues of the process must be addressed:

- Extensive application in biocatalyzed processes, leading to a wider scope of product and biocatalyst categories,
- Different cases of implementing in situ product crystallization (ISPC),
- Strategies for ISPC implementation in aseptic conditions,
- Approaches in particle-particle separation,
- Crystallization aspects (e.g. generalized correlation on the influence of co-solutes on the product crystal, improved (re-)crystallization techniques),
- Improvement of process reliability and consistency (by pilot-scale studies),
- Process optimisation

At this stage, many aspects of ISPC are yet to be established and generalized to make it broadly applicable. It is imperative to investigate different cases of implementing ISPC and
explore a wider range of applications, which encompass a larger set of product and biocatalyst categories. As this is a promising strategy for process integration, the challenge now is how this strategy can be developed further systematically and implemented quickly for each specific case in order to shorten product or process development period in industry.

More specifically, the various configurations for ISPR, which can be applied to ISPC, should be tested. In this work, the external configuration with indirect contact was presented. Other possible external configurations are possible as well; it is interesting how this can be implemented in a number of relevant cases. On the other hand, it is challenging to see how an internal configuration of ISPC, which is potentially a more simple design, for instance should work for a given case. Thus, methods to select the best configuration for ISPC for a particular case must be established to speed-up the development period of the process. Inherent to this endeavor is the investigation of the various separation techniques for particle-particle systems (i.e. cell-crystal or solid-solid systems) having particles of almost the same properties or nature.

The implementation of ISPR with external crystallization depends critically on appropriate cell recycling or cell retention techniques. Many options are already proven to be reliable in large-scale operations; however, improved and more efficient techniques must also be explored and tested further for their potential in ISPC processes.

As microorganisms catalyse different reactions resulting to a wide range of solid products, wide applications of ISPC are expected, having demonstrated in this study increased productivity and yield at competitive production costs. The fermenter or reactor is the critical part of this process as it is where the product is formed. When improved productivities are desired at lower operating costs, alternative reactors and reactor configurations must be investigated in the implementation of ISPC.

In this study, the ISPC process is not tested at aseptic conditions. Depending on the susceptibility of the biocatalyst involved and the sterilization process required or chosen, maintaining an aseptic condition during this process can be a demanding and costly task due to the integrated nature of the process where recycle streams are implemented. Thus, research endeavors dealing with this challenge must be encouraged.
Chapter 8

Crystallization is obviously the main downstream processing step for the ISPC process, where the crystals are formed in the reaction medium. It is important to determine how co-solutes or impurities present in the reaction medium can affect crystal morphology, purity, and crystallization rates. For a large number of cases, varying effects are found; thus, it is still difficult so far to predict how certain co-solutes or impurities can affect the formation of crystals. For biocatalytic processes involving crystalline products, only a few cases investigated the crystallization behavior of the target crystals as well as the effect of impurities. Thus, it is important to develop further the crystallization approaches that can be applied for a variety of products formed in integrated biocatalytic reaction systems.

Normally, a study on crystallization behavior is in itself time-consuming. It would be helpful if these crystallization approaches are developed in combination with other state-of-the-art techniques (i.e. for *in situ* monitoring of crystal size distribution profiles) so that time of implementation in industry is shortened.

In fine-chemical industry, purity and (enantio)selectivity is of utmost importance. When the co-solutes or impurities in the reaction medium affect the purity of the product crystals, re-crystallization is the common practice to address the problem. In this regard, developing further the re-crystallization techniques can contribute to improving product quality.

Although, the ISPC process consists of known and proven sub-processes (i.e. crystallization, fermentation), it is a novel technology in the sense that it addresses fermentation limitations and leads to robust integrated processes where whole-cells are employed as biocatalyst. The main interesting features include the logical implementation of recycle streams and the elimination of auxiliary phases in the system. There is a need to enhance the process reliability and consistency, which can be dealt with by conducting pilot-scale studies for promising cases.

All these future endeavors must include cost and benefit analysis as this always provides the logic for process implementation. Evaluation of the various aspects and issues concerning this process must also be carried out in combination with other suitable, advanced, emerging or state-of-the-art techniques in order to speed up the development time.


Summary

In situ removal of solid products during whole-cell biocatalysis

PhD Thesis by Evelyn M. Buque

Innovative process engineering coupled with novel protein and metabolic engineering pave the way for biochemical processes to become attractive and feasible alternatives in the chemical industry. From the process engineering's perspective, chemicals production via fermentation or biotransformation with in situ recovery of the products formed, would be an efficient, straightforward, and cost-effective technology.

A crucial bottleneck in the biochemical industry is the tedious task of product separation and purification, which is mainly caused by the low product concentrations obtained during fermentation. Of course, high product concentrations are desired but this is often limited as the process suffers from product inhibition or degradation. Employing in situ product removal (ISPR) techniques is useful in these cases.

In this study, in situ product removal by direct crystallization during a whole-cell catalyzed reaction is considered as this removal can directly provide the desired product (in solid or crystal form) without the need for an auxiliary phase. In doing so, product concentrations are kept low in the reactor, thereby, maximizing biocatalyst performance, which results in high yields and volumetric productivities.

In the implementation of ISPR, various options for cell recycling or cell retention must be explored to efficiently obtain a cell-free liquid broth from the fermenter. In this regard, the effect of using baker's yeast immobilized in calcium alginate during the batch reduction of ethyl 3-oxobutanoate (EOB) was studied. The specific reduction rates as well as the enantiomeric excess of the desired product (S-EHB) using free and immobilized cells (with particle size \(d_{pmean} = 1.2 \text{ mm}\)) are the same at low cell concentrations. However, for larger particle sizes and higher cell concentrations, the reduction rates decrease, and in the latter case, the enantiomeric excess of (S)-EHB also decreases which is attributed to the occurrence of oxygen diffusion limitation. In general, employing immobilized cells for cell retention is
efficient when small particles and low cell concentrations are employed, thus, minimizing mass transfer limitations.

Implementing ISPR by direct crystallization is done during the synthesis of 6R-dihydrooxoisophorone (DOIP), a white crystalline product, with a solubility of about 10 g.L\(^{-1}\) in water at the fermentation temperature of 30°C. DOIP is an important chiral intermediate in the synthesis of naturally-occurring carotenoids and flavors. The synthesis reaction involves the asymmetric reduction of 4-oxoisophorone (OIP) using baker’s yeast (*Saccharomyces cerevisiae*) as biocatalyst. The main product DOIP is further degraded by the yeast mainly to (4S,6R)-actinol, an unwanted by-product in the process. An enantiomeric excess (e.e.) ≥ 98% for DOIP is obtained in this process.

During the reduction process, the substrate (OIP) inhibits the reaction and can cause cell disintegration at a concentration above 80 mM. Thus, substrate feeding is implemented to maintain a low concentration level (about 50-60 mM) in the reactor. A non-competitive type of inhibition can describe the kinetics of substrate inhibition during OIP reduction. The product degrades by the consecutive reduction of DOIP to actinol by baker’s yeast. The kinetics of product degradation can be described by a second-order rate equation with respect to DOIP concentration, which is an exceptional behavior so far for a bio-catalyzed reaction system. This implies that product concentration in the reactor must be kept low to avoid massive degradation.

*In situ* product crystallization (ISPC) using an external configuration is successfully implemented during DOIP production employing ultrafiltration as a cell recycling technique. During the aerobic biotransformation with free cells, the product is immediately recovered in the external crystallization loop, avoiding its unfavorable degradation. In the crystallizer, accumulation of crystals occurs during the entire process, as the product-depleted mother liquor is recycled to the fermenter. This results in an integrated process where the desired product is directly obtained as pure crystals. Relative to the conventional batch and fed-batch processes, this integrated process configuration has a better performance with a final yield of 85%, final selectivity of 96%, and productivity of 0.30 g.L\(^{-1}\).h\(^{-1}\). A biomass-specific reduction rate of 10 mg.gdw\(^{-1}\).h\(^{-1}\) is reproducible.
Having successfully demonstrated ISPC using resting cells, an efficient procedure is
developed for DOIP synthesis involving cell cultivation. In order to implement the process,
cell cultivation must be done prior to bio-reduction and in situ product crystallization as the
substrate OIP inhibits cell growth when its concentration level in the reactor is kept high (≥
55 mM). In this case, it is necessary to appropriately supply glucose (as the carbon and
energy source) because the cells preferably utilize the nutrient for product formation rather
than cell growth. The rate of OIP reduction using growing cells is five times higher than the
rate with resting cells, which leads to a final product concentration of over 100 g.L⁻¹ in the
crystallizer at the end of the integrated experiment. The process performance is improved
further as the final selectivity is 98.7% (indicating minimum product degradation) and
volumetric productivity is 0.92 g.L⁻¹.h⁻¹ (6 mmol.L⁻¹.h⁻¹), which is an industrially attractive
output for a biocatalytic process.

Inherent to the ISPC process is the crystallization of the product in the reaction medium
where the presence of co-solutes and impurities can potentially affect the product crystal
purity, morphology, and structure. Thus, the crystallization kinetics of DOIP is determined
using the experimental data on desupersaturation curves and crystal size distribution profiles.
The crystallization (nucleation and crystal growth) kinetic parameters in water, phosphate
buffer, and culture medium show no significant differences. However, the solubility of DOIP
decreases slightly in the presence of co-solutes, leading to faster crystal growth in the culture
medium. Thus, crystallization in the culture medium is a favorable situation in this case.
From the process-engineering point of view, crystal growth is the important sub-process
during crystallization as the mass of crystals due to nucleation is negligible compared to the
mass of crystals accounted for by the (growth of) large crystals. However, when the crystal
morphology, structure, and size distribution are important parameters, the nucleation process
must be considered.

A conceptual process design of an in situ product crystallization process as applied to DOIP
production is performed implementing the external configuration with indirect contact of the
cells. In this work, it is demonstrated that the process is technically and economically
feasible, performing better in terms of yield and production costs when compared with a
known conventional process.
The ISPC process is an attractive approach for process integration where recycle streams are implemented (minimizing waste streams) and the process is intensified by involving only a few steps. It does not require the use of auxiliary phases, which reduces costs and simplifies the entire procedure. This process strategy may be applicable to many biocatalytic (e.g. reduction) systems where crystalline products are obtained, which include specialty biochemicals such as amino acids, steroids, antibiotics, proteins, esters and ketones.

As this study is the start of a systematic development of ISPC processes, further research is required to address the various issues concerning the process. For instance, it is imperative to explore a wide range of applications for this process by investigating cases where different ISPC configurations are implemented. The major criteria for process implementation have been set and are expected to help ease-up and speed-up this task. It is required to generalize this approach as much as possible for an extensive application in biotechnology. As ISPC is a novel technology, it is important to fast-track the process development time for industry by evaluating bottlenecks in combination with other advanced and emerging technologies. In the end, in situ product recovery by crystallization should be a common practice in the design and implementation of robust integrated processes involving fermentation or biotransformation. For all these endeavors, cost and benefit analysis must be done to emphasize the logic of ISPC implementation.
Samenvatting

_In situ verwijdering van vaste producten tijdens biokatalyse met hele cellen_

Proefschrift door Evelyn M. Buque

Door de koppeling tussen innovatieve procestecnologie, protein engineering en metabolic engineering worden biochemische processen een steeds aantrekkelijker alternatief voor chemische processen. Vanuit het oogpunt van een procestecnoloog is de productie van chemische verbindingen met behulp van fermentatie of biotransformatie in combinatie met _in situ_ productverwijdering (ISPV) een directe, efficiënte en rendabele technologie.

Een belangrijke bottleneck in de biochemische industrie is productopwerking en -zuivering, vanwege de lage product concentraties die over het algemeen worden behaald in fermentatieve processen. In veel gevallen wordt de productiviteit beperkt door productinhibietie en productafbraak. _In situ_ productverwijdering kan in deze gevallen een uitkomst bieden.

Dit proefschrift beschrijft de _in situ_ verwijdering van biokatalytische producten door middel van kristallisatie uit een reactiemengsel dat hele cellen bevat. Deze opwerkingsroute levert het gewenste product direct in vaste vorm zonder dat daarbij een hulfpase nodig is. Op deze wijze blijft de productconcentratie in de reactor laag, zodanig dat de biokatalytische activiteit gemaximaliseerd wordt, waardoor een hoge opbrengst en volumetrische productiviteit kunnen worden behaald.

Voor de implementatie van I PV moeten verschillende methoden van celrecirculatie en celretrentie voor het verkrijgen van een cel-vrij reactiemengsel worden overwogen. Vanuit dit oogpunt is het effect van celimmobilisatie in calciumalginaat op de batch-reductie van ethyl-3-oxobutanoaat (EOB) door bakkersgist onderzocht. Het is aangetoond dat de specifieke reductiesnelheid en de enantiomere overmaat van het gewenste product (S-EHB) met en zonder celimmobilisatie (deeltjesdiameter van 1.2 mm) gelijk blijven bij lage celconcentraties. Echter, bij grotere deeltjes en hogere celconcentraties is de reductiesnelheid lager. Tevens is de enantiomere overmaat van (S)-EHB lager bij hogere celconcentraties. Dit
wordt toegeschreven aan diffusielimitatie van opgeloste zuurstof in de calciumalginaat bolletjes. In het algemeen is celretentie door middel van immobilisatie efficiënt wanneer transportlimitaties worden beperkt door het gebruik van kleine deeltjes en lage celconcentraties.

De implementatie van ISPV door middel van direkte kristallisatie is getest voor de synthese van 6R-dihydroxyoxisophoron (DOIP). Dit is een wit kristallijn product met een oplosbaarheid in water van ongeveer 10 g.L\(^{-1}\) bij 30 °C. Het is een belangrijk chiraal intermediair in de synthese van natuurlijke carotenoïden en smaakstoffen. Voor de productie van deze stof wordt gebruik gemaakt van de asymmetrische reductie van 4-oxoisophoron (OIP) door bakkersgist (Saccharomyces cerevisiae). Het voornaamste product, DOIP, wordt door de gistcellen omgezet tot (4S,6R)-actinol, wat een ongewenst bijproduct van de reactie is. In het proces werd een enantiomere overmaat van DIOP van ≥ 98% behaald.

Het katalytische reductieproces wordt geremd door substraatinhibitie (OIP). Tevens kan celdesintegratie plaatsvinden bij substraatconcentraties boven 80 mM. Vandaar dat het substraat geleidelijk aan wordt toegediend om de concentratie ervan in de reactor te beperken. Het mechanisme achter de substraatinhibitie is noncompetitief. Het product, DIOP, wordt door bakkersgist in meerdere stappen gereduceerd tot actinol. Dit proces kan worden beschreven met een tweede-orde afhankelijkheid van de DOIP-concentratie, wat ongebruikelijk is voor biokatalytische reactiesystemen. Deze conclusie impliceert dat de productconcentratie in de reactor laag moet worden gehouden om overmatige afbraak ervan te voorkomen.

*In situ* product kristallisatie (ISPK) van DOIP in een externe recyclestroom is succesvol gebleken in een proces waarbij de cellen met behulp van ultrafiltratie terug naar de reactor werden gevoerd. Gedurende de aerobe bioconversie met gesuspendeerde cellen kon het product direct worden teruggewonnen in de externe kristallisatiestap met als gevolg dat productdegradatie kon worden gereduceerd. Gedurende het gehele proces vond ophoping van kristallen plaats in de kristalliser en werd de vloeistof teruggevoerd naar de fermentor. Dit resulteerde in een geïntegreerd proces waarin het gewenste product direct in de vorm van pure kristallen werd gewonnen. Het proces had een opbrengst van 85%, een selectiviteit van 96%, en een productiviteit van 0.30 g.L\(^{-1}\).h\(^{-1}\), wat beduidend beter is dan voor conventionele
batch en fed-batch processen. De biomassa-specifieke reductiesnelheid van 10 mg.gdw\(^{-1}\).h\(^{-1}\) die werd behaald in dit proces was reproduceerbaar.

De succesvolle demonstratie van ISPK met behulp van niet-groeiende cellen heeft geleid tot de ontwikkeling van een efficiënte procedure voor DIOP synthese met behulp van microbiële cellen. De implementatie van dit proces vereist dat de cellen worden gecultiveerd alvorens ze gebruikt worden in de bioreductie en in ISPK vanwege de substraat-inhibitie (OIP) die plaatsvindt bij concentraties boven 55 mM. De glucose die door de cellen als koolstof- en energiebron wordt gebruikt moet met beperkte maar voldoende snelheid worden toegediend zodat de cellen dit substraat voornamelijk gebruiken voor productvorming en niet voor groei. De reductiesnelheid van OIP door de groeiende cellen is vijf keer zo hoog dan door niet-groeiende cellen. Dit leidt tot een productconcentratie in de krisallisator boven de 100 g.L\(^{-1}\) aan het eind van het geïntegreerde experiment. Op deze manier werd de selectiviteit van het proces verhoogd tot 98.7%, wat impliceert dat productafbraak minimaal was. Verder was de volumetrische productiviteit 0.92 g.L\(^{-1}\).h\(^{-1}\) (6 mmol.L\(^{-1}\).h\(^{-1}\)). Dit alles maakt het biokatalytische proces aantrekkelijk voor industriële toepassing.

Inherent aan het ISPK proces is kristallisatie van het product in het reactiemengsel waarin andere opgeloste componenten de zuiverheid, morfologie en structuur van de productkristallen kunnen beïnvloeden. Vandaar dat de kristallisatiekinetiek van DOIP is bepaald aan de hand van experimenteel bepaalde kristalgrootteverdeling en afnames van oververzadigingsconcentraties. De parameters voor de kristallisatiekinetiek (nucleatie en kristalgroei) in water, fosfaatbuffer en fermentatiemedium zijn gelijk gebleken. Wel neemt de oplosbaarheid van DOIP lichtelijk af in aanwezigheid van andere opgeloste stoffen, wat leidt tot snellere kristalgroei in het fermentatiemedium. Kristallisatie in het fermentatiemedium is in dit geval dus gunstiger. Vanuit het oogpunt van de procestechnoloog is kristalgroei het belangrijkste sub-proces in kristallisatie aangezien de kristalmassa als gevolg van nucleatie verwaarloosbaar klein is ten opzichte van de kristalmassa die door kristalgroei wordt gevormd. Niettemin, het nucleatieproces blijft belangrijk voor het controleren van de morfologie, structuur en grootteverdeling van de kristallen.

Een conceptueel procesontwerp is gemaakt van het ISPK proces voor de productie van DOIP. Dit bevat een externe kristalisatiestap waarbij de cellen niet in contact komen met de
Samenvatting

kristallen. Dit procesontwerp toont aan dat het proces zowel technisch als economisch haalbaar is en beter is dan conventionele processen met betrekking tot opbrengst en productiekosten.

Het ISPK proces is een aantrekkelijke manier om procesintegratie uit te voeren. De recyclestomen minimaliseren afvalstromen, en procesintensificatie vindt plaats omdat het proces slechts uit een paar stappen bestaat. Het proces behoeft geen hulpfase waardoor de kosten worden gereduceerd en de gehele procedure wordt vergemakkelijkt. De gepresenteerde strategie is mogelijk toepasbaar op vele biokatalytische reattiesystemen (bijvoorbeeld reducties) waarin de producten in kristalvorm worden verkregen. Binnen deze systemen vallen onder andere specialties zoals aminozuren, steroïden, antibiotica, eiwitten, esters en ketonen.

De studie die in dit proefschrift beschreven staat geeft een aanzet tot de systematische ontwikkeling van ISPK processen. De verschillende aspecten van de procesvoering dienen dan ook in vervolgonderzoek in meer detail te worden bekeken. Het is bijvoorbeeld verstandig om een groot aantal mogelijke toepassingen van het proces te bestuderen met behulp van cases waarin verschillende configuraties van het proces worden geïmplementeerd. De belangrijkste criteria voor de implementatie van het proces zijn in dit proefschrift geïdentificeerd, zodat procesontwikkeling vergemakkelijkt en versneld kan worden. Een algemene aanpak voor de implementatie van het proces is gewenst zodat het in veel biotechnologische processen kan worden toegepast. Omdat ISPK een nieuwe technologie is, is het belangrijk de benodigde tijd voor industriële procesontwikkeling te reduceren door evaluatie van de bottlenecks. Waar mogelijk kunnen geavanceerde opkomende technieken worden ingezet om de bottlenecks aan te pakken. In de toekomst moet in situ product verwijdering door middel van kristallisatie algemeen toepasbaar worden voor het ontwerpen van en de implementatie in robuuste fermentatie- en biotransformatie-processen. Het nut van ISPK moet voor alle gevallen apart worden beschouwd door een kosten-baten analyse.

Thanks to Pim van Hee for the translation.
Curriculum Vitae

Evelyn Montajes Buque was born in General Santos City, Philippines on 1 August, 1969. She graduated high school as class valedictorian from the University of Cebu, Philippines in 1986 and was a recipient of gold medal awards for leadership and academic excellence. She took up B. S. in Chemical Engineering at the University of San Carlos (USC), Cebu, Philippines and graduated *cum laude* in 1991.

She taught high school chemistry for a year at USC and in 1992 joined the Department of Chemical Engineering as staff member doing teaching, research and management work. She received a six-month fellowship for research and course development at the Department of Biotechnology, Delft University of Technology (DUT) in the Netherlands in November 1996 funded by the MHO-USC-DUT Project in Chemical Engineering. Prior to this, she was awarded a scholarship grant from the Philippine Department of Science and Technology for graduate studies at the University of the Philippines (Diliman, Quezon City), finishing M. S. Chemical Engineering in 1998. Her master’s thesis was on the purification methods of lipase from *Rhizopus delemar*.

In 1999, she received joint fellowship grants from the Institute of Biotechnological Sciences Delft Leiden (BSDL) and MHO-USC-DUT Project in Chem Eng to pursue postgraduate studies at DUT, finishing the advanced degree of Master in Biotechnology with specialization in Bioprocess Engineering in 2001. Her graduation project was on the asymmetric reduction of 3-oxo esters by immobilized baker’s yeast, supervised by Dr.ir. A.J.J. Straathof and Prof.dr.ir. J.J. Heijnen. Expansion of this work led to the study of *in situ* product removal by crystallization during whole-cell biocatalysis, the results of which are written in this thesis. Part of this research was presented in September 2004 during ESBES-5 in Stuttgart, Germany and was runner-up for the Malcolm Lilly Award for best young scientist’s work.

She is married to J.B. Taboada and in the course of this project gave birth to two lovely and charming children. Currently, she is working as staff member of the Department of Chemical Engineering, USC, Philippines.
Publications and Presentations

Journal Articles


Oral Presentations


**Poster Presentations**


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Sincerely,

Evelyn