BIOPROCESS DEVELOPMENT FOR ASYMMETRIC REDUCTIONS BY *Sachharomyces cerevisiae* ENZYMES

By

PARAG ANIL PAREKH

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2006

This document is dedicated to my Family.

ACKNOWLEDGMENTS

I would like to thank my parents for their continuous encouragement for my graduate education. I would also like to thank my brother, Nitin. Thanks go to all the Stewart group members.

TABLE OF CONTENTS

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

BIOPROCESS DEVELOPMENT FOR ASYMMETRIC REDUCTIONS BY
Saccharomyces cerevisiae ENZYMES

By

Parag Anil Parekh

May 2006

Chair: Jon D. Stewart
Major Department: Chemistry

Biotechnological methods are becoming increasingly important in industrial production of fine chemicals. But, the total number of compounds made from biocatalysis on an industrial scale is still limited due to disadvantages like long reaction times and low productivity associated with bioprocesses. We used *Saccharomyces cerevisiae* enzymes to asymmetrically reduce β keto esters to chiral hydroxyl esters, which are important building blocks as versatile chiral synthons for asymmetric synthesis of pharmaceuticals and agrochemicals.

Different levels of consideration for development of bioprocess were applied to achieve the maximum bioprocess efficiency.

Biocatalyst	Enzyme	vs.	Whole cells
Form	Free	vs.	Immobilized
Medium	Water	vs.	Organic

We focused on reduction of three different substrates, ethyl butyryl acetate, ethyl 4-chloro acetoacetate and ethyl acetoacetate. Different problems varying from product inhibition, substrate toxicity and poor substrate solubility were tackled using techniques like *in situ* product removal, aqueous two-phase system, reactions with cell-free extracts, etc. Reduction in the operating costs of the process was achieved by using immobilized cells and application of metabolic engineering tools. Studies of these bioprocesses in non-conventional media like ionic liquids or other immobilization techniques like cross-linked enzyme aggregates will further improve the bioprocess efficiency of the system.

CHAPTER 1
BACKGROUND

Biocatalysis is an attractive alternative to chemical conversions.[1-4] The reaction conditions are mild, the solvent is generally water and the biocatalysts are easily decomposed after use. Biocatalysis is therefore environmentally friendly green chemistry and a sustainable technology. Additionally, biocatalysis often circumvents protection and deprotection steps in the synthesis of products with chiral centers. These properties give biocatalysis an edge over chemical conversions.

One of the main disadvantages encountered in biocatalysis is the application of biocatalysis at large scale, i.e. the scale-up of reactions and low space-time yield. [5]

The biochemical engineering required to develop a mature process from early observations in the lab is demanding and therefore the number of compounds made from biocatalysis is relatively limited. [6] The aim of this study is to apply various techniques to overcome these limitations and make a model bioprocess more efficient.

Oxidoreductases

Oxidoreducatases comprise approximately 25% of presently known enzymes. [7] Almost 30% of all industrial biocatalytic processes involve oxidoreductases. [6] They perform interesting reactions such as reduction, epoxidation, hydroxylation, dihydroxylation etc. There are two major categories of oxidoreductases: dehydrogenases, also known as reductases, and oxygenases.

Reductases are enzymes that reduce carbonyl groups yielding chiral products like alcohols, acids or their esters or amino acids respectively. [8] Examples of these reactions are shown in Figure1-1.

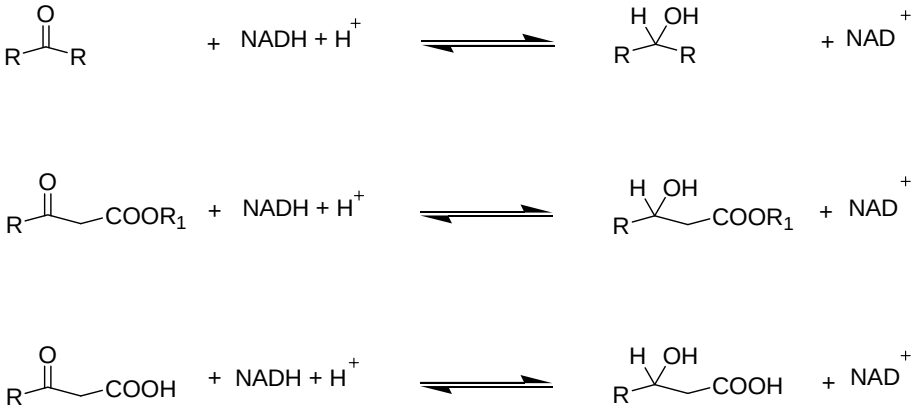


Figure 1-1. Enzyme catalyzed reductions

Oxygenases use molecular oxygen as co-substrate and are used for oxidation of non-functionalised C-H or C═C bonds yielding hydroxyl or epoxy products. [9] Examples of these reactions are shown in Figure1-2.

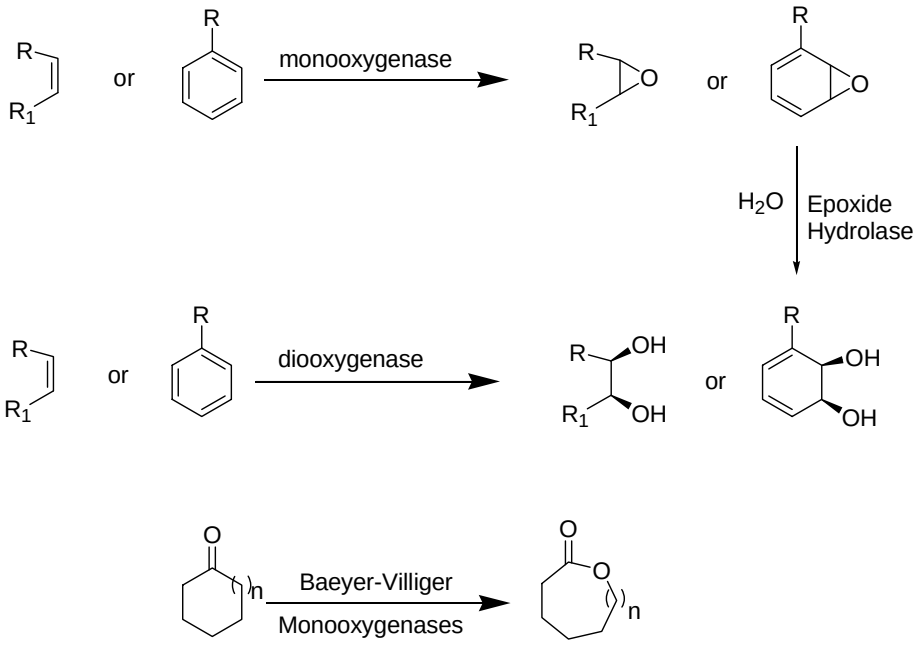
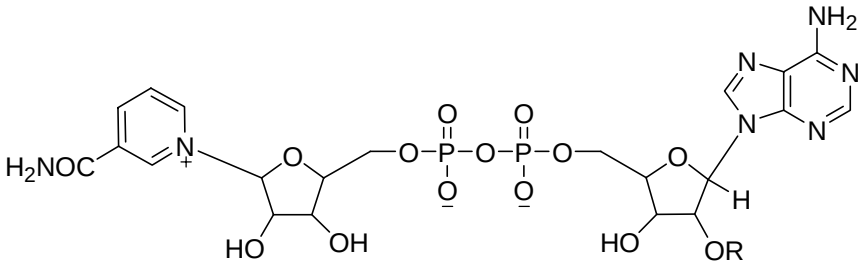


Figure 1-2. Enzyme catalyzed oxidations

Cofactors

Oxidoreductases require redox cofactors, or coenzymes, which accept or donate chemical equivalents for reduction or oxidation. Most oxidoreductases require Nicotinamide Adenine Dinucleotide (NAD^+) or Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$) as the cofactor; some require Flavin Adenine Dinucleotide (FAD^+) or Flavin Mononucleotide (FMN). These cofactors are very expensive and are relatively unstable.

In the enzymatic reduction of a substrate, these cofactors participate in the redox reaction via the direct transfer of hydride **(**H^-) ions either to or from the cofactor and a substrate. Oxidoreductases acting as oxygenases abstract a hydride ion from the donor and transfer it to the nicotinamide moiety. Oxidoreductases acting as reductases abstract a hydride ion from the reduced nicotinamide and transfer it to the carbonyl to reduce the substrate.



NAD^+ R = H
$NADP^+$ R = PO_3^{-2}

Figure 1-3. Nicotinamide cofactors NAD^+ and $NADP^+$

Mechanism of Hydride Delivery

Hydride transfer is highly stereoselective. The delivery of the hydride from the *si* or *re* face of the carbonyl results in its reduction to yield either *(R)* or *(S)* alcohol. There are four possible stereochemical patterns that enable transfer of hydride from the cofactor to

the substrate. This hydride transfer is well studied and results from the specific binding of the substrates as well as coenzymes on the chiral enzyme surface in close proximity and defined geometry. [10, 11] The four different hydride transfers, two from the *re* face and two from the *si*-face, result in either *(R)* or *(S)* alcohols corresponding to transfer of pro-R and pro-S hydrides from the cofactors.

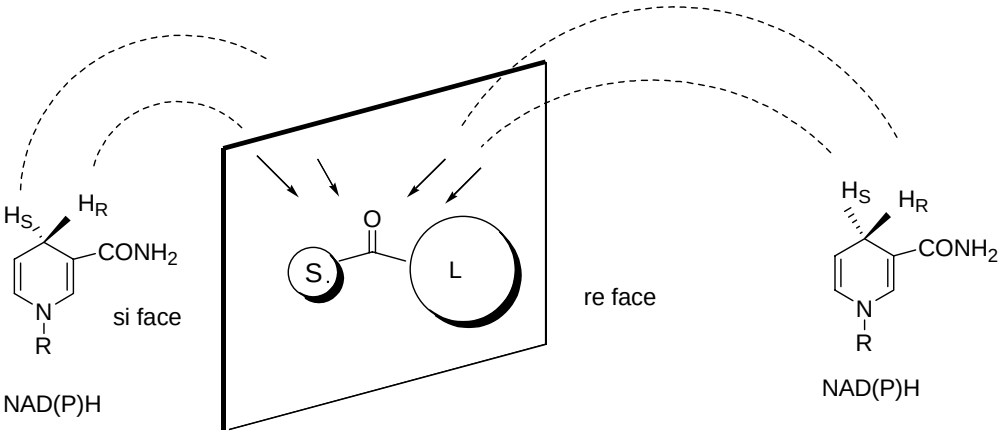


Figure 1-4. Stereochemistry of hydride transfer from NAD(P)H to the carbonyl carbon on the substrate (S = small and L = large group) [12]

Asymmetric Reductions Using Bakers' Yeast Enzymes

Baker's Yeast (BY), *Saccharomyces cerevisiae,* is cheap, readily available and easy to use. Thus it is a very popular catalyst for asymmetric reductions of carbonyl compounds. [13] Baker's Yeast has more than 40 different carbonyl reductases in its genome which may have overlapping substrate specificities, but with different stereoselectivites, yielding a mixture of stereosiomeric alcohols. [14] To overcome this, Iwona Kaluzna in the Stewart lab made a library of twenty β-keto ester reductases from Bakers' Yeast, *S.cerevisae,* over expressed in *E.coli* cells with a Glutathione S-transferase tag. [15] These reductases are used for the synthesis of hydroxyl esters which hold a very important place in organic chemistry as they can be transformed into various functionalities, without racemization, to synthesize industrially important chemicals. [16]

Applications of Baker's Yeast Enzymes

Isolated Enzyme Catalysis

Isolated dehydrogenases, when used as biocatalysts, give cleaner reactions, less side-products and less purification problems than the whole cell reactions. [17] The main disadvantage of using isolated dehydrogenases is the cofactor requirement, which in most of the Baker's yeast carbonyl reductases is NADPH. [18] NADPH is relatively unstable and expensive and thus is impractical to use in stochiometric amounts. Therefore an *in-situ* regeneration of NADPH is a prerequisite for large-scale applications to be economically feasible. Recycling of cofactors can be done chemically, electrochemically, enzymatically, etc. [19-21] The efficiency of recycling is measured by the number of cycles that can be achieved before a cofactor molecule is destroyed. It is expressed as total turnover number (TTN), which is the total number of moles of product formed per mole of cofactor during the complete course of the reaction. The most successful application of cofactor regeneration is by enzymatic methods, either by coupled-substrate or coupled-enzyme processes. [22]

Substrate-coupled regeneration

In substrate-coupled regeneration, a single enzyme, a dehydrogenase, accepts an additional substrate that regenerates the cofactor for further use. This additional substrate is generally an aliphatic alcohol which is oxidized to a ketone to regenerate the cofactor. [23] Though this approach is elegant it has several disadvantages such as enzyme deactivation and co-substrate inhibition due to presence of large amounts of auxiliary substrate. [17]

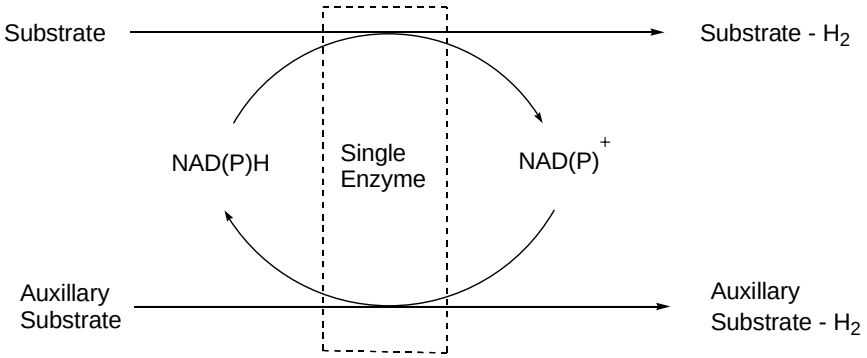


Figure 1-5. Cofactor recycling by the coupled-substrate method [17]

Enzyme-coupled regeneration

In enzyme-coupled regeneration, two enzymes are employed. In this case, two parallel redox reactions are catalyzed by two different enzymes, i.e. conversion of main substrate and cofactor recycling. [24] For best results, both enzymes should have different specificities for their respective substrates so that the substrates do not have to compete for the active site of a single enzyme, but are efficiently converted independently by the two biocatalysts.

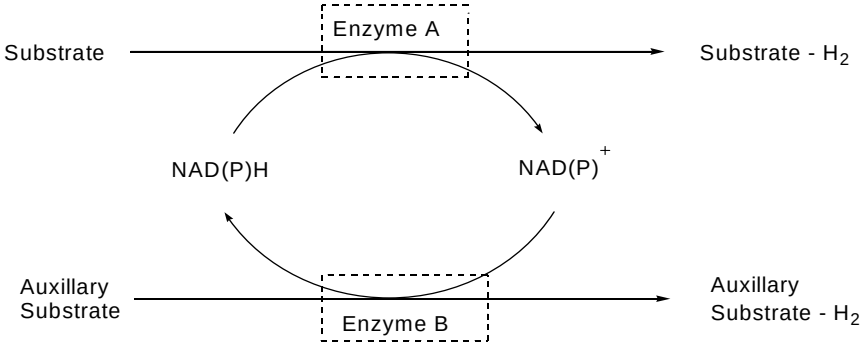


Figure 1-6. Co-factor recycling by coupled – substrate method [17]

Formate dehydrogenase, which oxidizes formate to carbon dioxide, is the enzyme of choice for NADH regeneration. Carbon dioxide is easily removed and not toxic to protein with concomitant reduction of NAD^+ to NADH. [25] Recycling of NADPH with glucose dehydrogenase or glucose 6-phosphate dehydrogenase is widely employed. [26, 27]

Glucose is oxidized to gluconolactone that spontaneously hydrolyzes to gluconic acid. These regeneration techniques require pure proteins. Special equipment and training is required to purify proteins further increasing costs of the process.

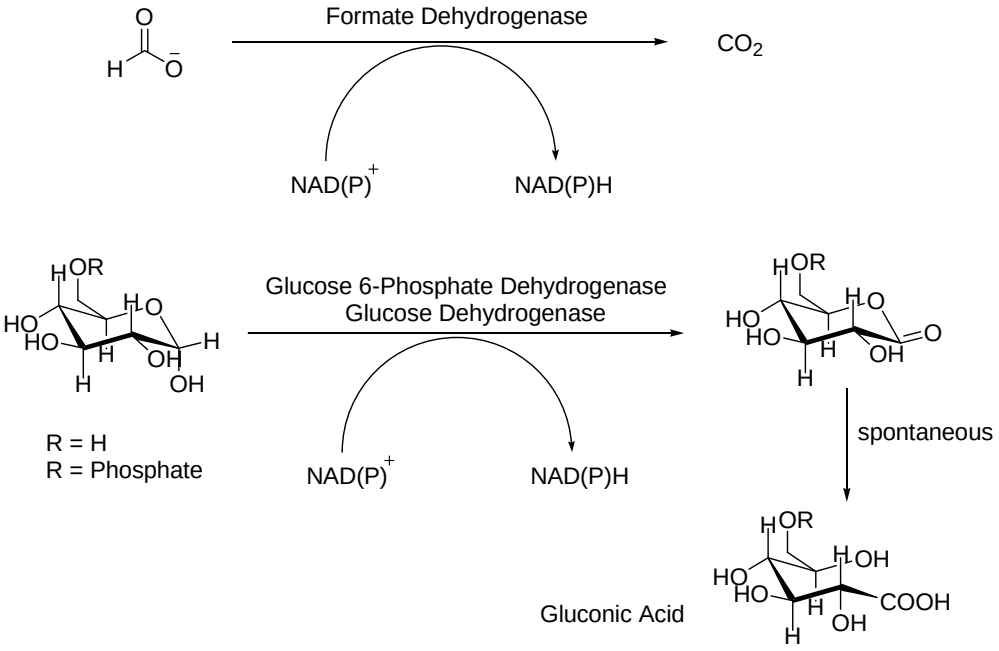


Figure 1-7. Cofactor recycling by coupled – enzyme method

Whole Cell Biocatalysis

The cofactor regeneration problems can be circumvented by *in vivo* application of dehydrogenases since the host cell regenerates cofactors as a part of its normal metabolism. [28] The whole cell biocatalysts function as miniaturized reaction vessels, which produce functional enzyme, regenerate cofactor, take up the substrate and convert substrate to product that diffuses out of the cell. Cofactor recycling *in vivo* requires metabolically active cells; when reducing equivalents are no longer generated, the desired reaction also stops. Therefore the cells must be maintained in a state that permits high co-factor regeneration. A minimum requirement for this is that the cell membrane remains intact during the biocatalysis process.

Major drawbacks of whole cell biocatalysis are formation of by-products and purification of the product. Essentially no special training is required to run whole cell mediated reactions.

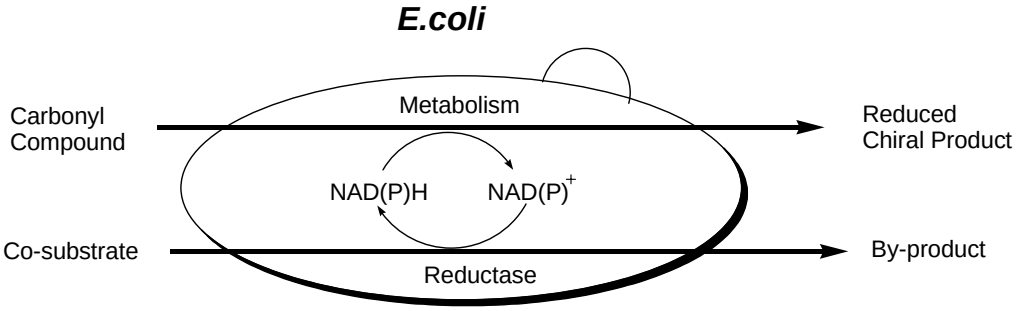


Figure 1-8. Principle of whole-cell reduction of carbonyl compounds [29]

Long Term Objectives

We want to study reductions of β-keto esters and increase the bioprocess efficiency of the system. The bioprocess efficiency is defined as the main parameters influencing the cost of the process. It is addressed in terms of yield, final product concentration (g/L of product), volumetric productivity (i.e. the space time yield g/L/hr) and catalyst consumption (product/catalyst ratio gm/gm or biocatalyst/substrate ratio g/g).

The goal of this work is to standardize protocols and develop a more efficient bioprocess using genetic techniques and optimization of reaction conditions and apply them to make a pool of chiral products within an infrastructure /technology platform.

CHAPTER 2
RESULTS

We have classified our work in four different sections for reductions of three different β-keto esters *viz.* ethyl butyrylacetate (EBA), 4-chloro ethyl acetoacetate (ECAA) and ethyl acetoacetate (EAA) and solved different problems encountered in the bioprocess development.

Biotransformations

Section I: Bioprocess Development for Reduction of Ethyl butyrylacetate

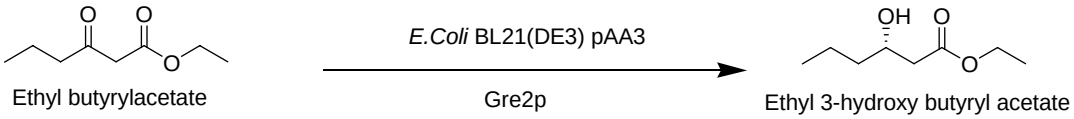


Figure 2-1. Reduction of Ethyl butyrylacetate

Enzymatic reduction of ethyl butyryl acetate to ethyl 3-hydroxy butyryl acetate (EHBA) was carried out in our lab yielding an enantiomeric excess of >98% using several of the yeast reductases found in Bakers Yeast. Of the enzymes present in our library, Gre2p was selected and over expressed in *E.coli* strain BL21 (DE3) containing the plasmid pAA3 to give the S alcohol with >98% enantiomeric excess. [16] The plasmid contained genes for kanamycin resistance and Gre2p, whose expression is under the control of phage T-7 RNA polymerase.

When induced by isopropy-β-thiogalactopyranoside (IPTG), the efficient T-7 promoter over expresses Gre2p that equals about 20% of the cell dry weight.

Biotransformations with Non-growing Cells

The whole cell reductions were carried out with non-growing cells using the general procedure of Walton and Stewart. [30] The cells were grown in Luria-Bertani medium (LB) with 4g/L glucose and were harvested just before the cells reached stationary phase. The cells were resuspended in 1 L of minimal composed of M-9 medium without NH_4Cl to prevent cell growth.

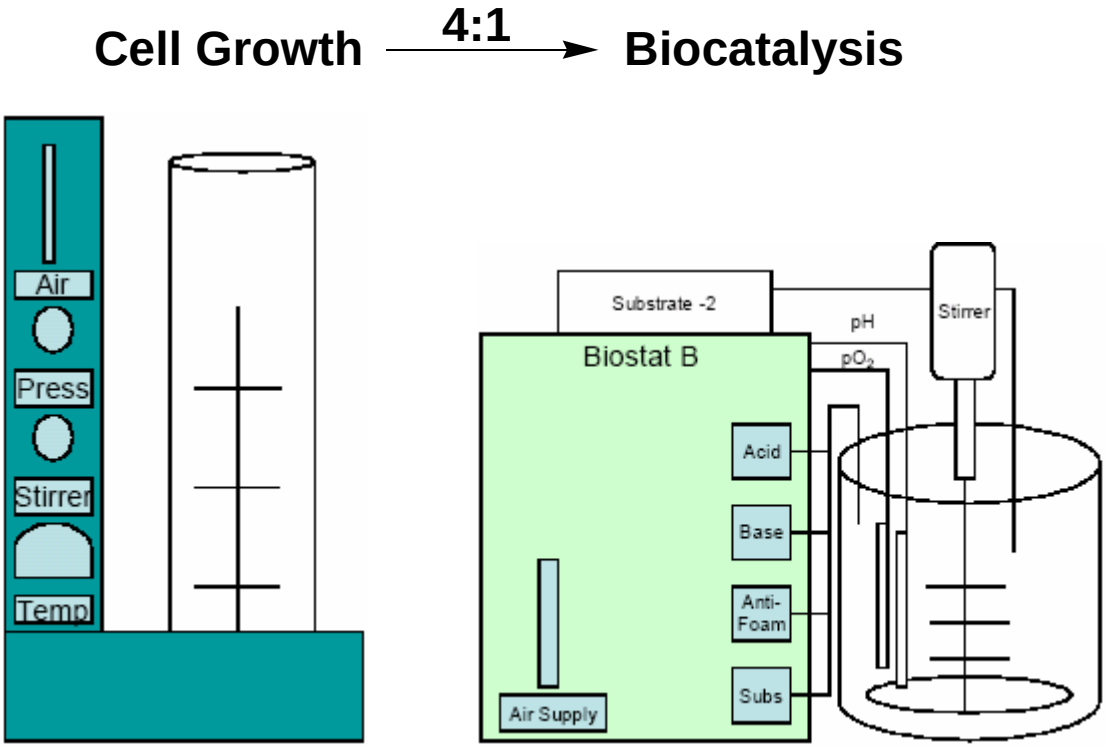


Figure 2-2. Cell growth in a 4L New Brunswick fermentor and bioconversion carried out in 1L Braun Bioststat B Fermentor

Parameters such as temperature, pH, and dissolved oxygen tension were optimized to give the best results and then were kept constant. The reductions were carried out at 30 ^{o}C, pH = 7.0, and the dissolved oxygen tension at 75% saturation. The bioconversions were conducted at one-liter scale with biomass loading at OD_{600} 18. Different substrate feed rates to the reactor with a pump was used to find the maximum rate of bioconversion as shown in Figure 2-3.

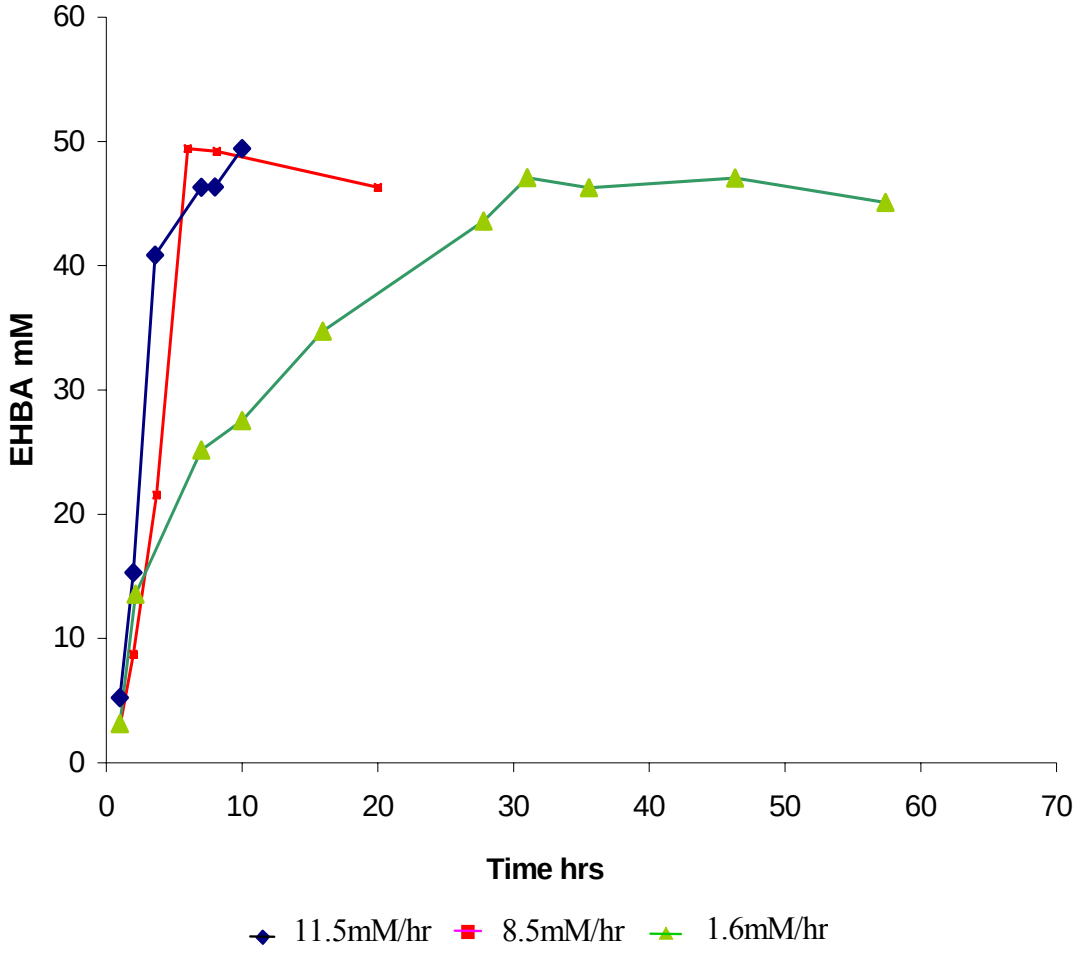


Figure 2-3. Increasing substrate (EBA) feed rate

The product formation stopped after the product reached a concentration of about 50 mM. Even after increasing the cell loading in the system, there was no observable increase in the product formation. This led us to believe that the product was inhibiting the biocatalyst, i.e. the product is toxic to the catalyst. Product inhibition was confirmed by performing an experiment wherein along with a substrate addition of 15 mM/hr we did bolus addition of 50 mM at 1.25hrs. No further product formation was observed confirming the product inhibition of the biocatalyst (Figure2-4).

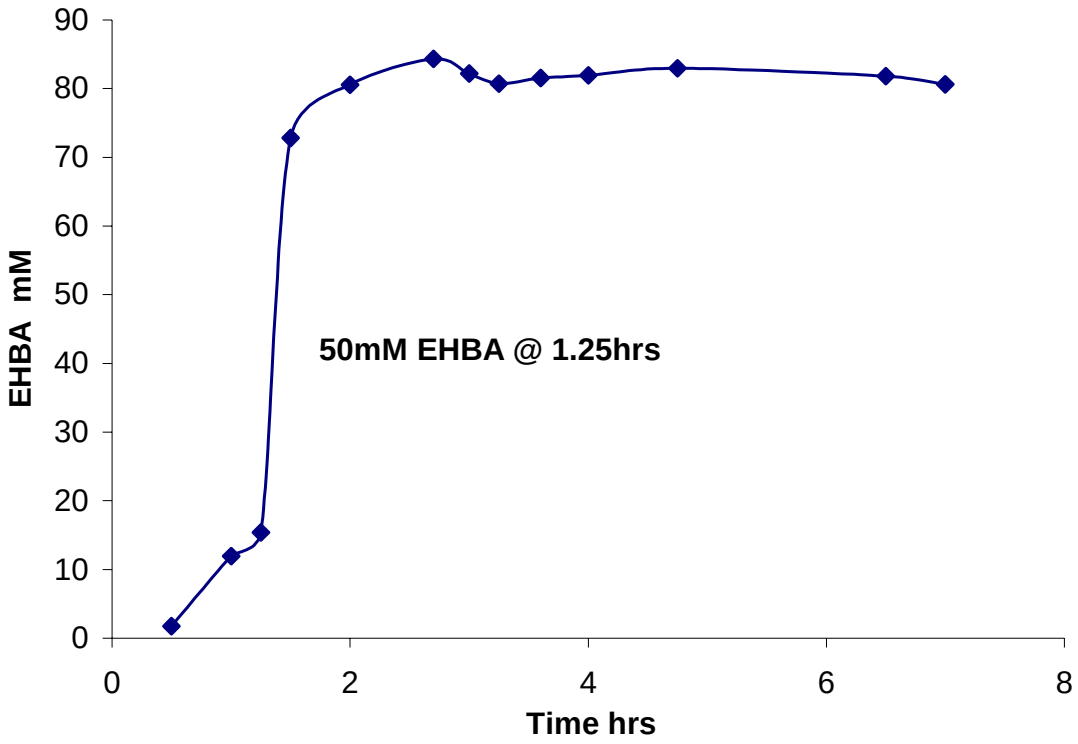


Figure 2-4. No additional product formed after bolus addition of ethyl 3-hydroxy butyrylacetate at 1.25 hrs.

When substrates or products are inhibitors of biocatalysts, the eventual product concentrations are very low, making the product recovery laborious and expensive. Therefore, strategies were developed to maintain sub-toxic product concentration in the reactor to attain high process yields.

Strategy I: Centrifugation

A very basic strategy was used where the biocatalyst was resuspended in fresh aqueous buffer after the product reached the critical concentration. The reaction media was centrifuged at 6000 rpm and the biocatalyst then resuspended in a new aqueous medium for a new round of bioconversion. Substrate was added at rate of 15 mM/hr.

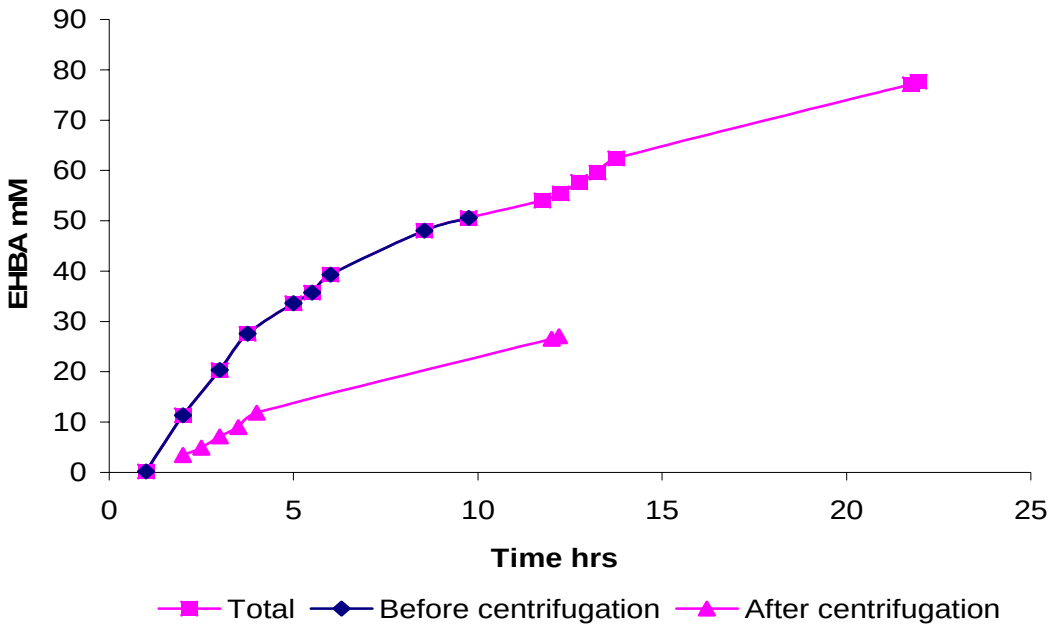


Figure 2-5. Product removal by centrifugation. Whole cells resuspended in fresh aqueous media.

While the final product concentration achieved was much higher, this strategy proved to be only of limited use and could not be applied due to the inconsistent behavior of the whole cells after centrifugation.

During the whole cell catalysis, the cell membrane is affected due to long exposure to the substrate and the product. These cells cannot withstand the high forces exerted on it during centrifugation. This causes many of the cells to become metabolically inactive, which in turn causes the biotransformation to be less productive.

Strategy II: *In situ* product removal (ISPR)

As a result of the product inhibition, the adverse effect on the biocatalyst can be minimized by the removal of the product as soon as it is formed. This also helps to increase the productivity of the system. *In situ* product removal techniques address this

problem by removing product (or maintaining low concentrations of substrate) formed from the vicinity of the biocatalyst. [31]

The separation of product from the reactor can be carried out in different modes. Different techniques are available for batch, fed-batch or continuous process. [32,33] The separation step can occur within the bioreactor or outside it. Different physical and chemical properties like volatility, molecular size, solubility, charge, hydrophobicity, etc are exploited as the driving force for separation of the product (or substrate). Different techniques like distillation, gas stripping, membranes (micro filtration and ultra filtration techniques are available), pervaporation or perstraction, extraction, supercritical carbon-dioxide, precipitation, crystallization, ion-exchange, electrodialysis, hydrophobic-interaction chromatography, adsorption and different affinity methods can be employed for the separation of product. [34] The most commonly used separation technique for *in situ* product removal relies on adsorption of product (or substrate) to a hydrophobic resin. The hydrophobic Amberlite XAD resins have been reported to be used most widely in industrial context. [35, 36] We selected XAD 4 from XAD 4, 16 and 18, because it had the highest capacity to adsorb ethyl butyrylacetate.

The easiest way to perform an ISPR is to preadsorb substrate onto a resin and add the resin to the reactor where the resin would act as a second phase. The resin would adsorb the product formed, and during the reaction, the concentration of the starting material or the product would not increase beyond a certain point due to adsorption and desorption of the product and substrate. [37] This approach is easy but has its own limitations. More resin added to the reactor results in more mass transfer and oxygen

transfer limitation of the substrate with the biocatalyst, affecting its performance. Also, increased stirring rate may lead to cell lysis by shearing.

To avoid this we used a reactor called a "Recycle Reactor". [38] Here, the bioconversions and product extraction were carried out in two separate units. The charged resin was placed as a fixed bed into a column in an external loop. In the bioconversion unit, substrate was added and when the product concentration neared the product inhibition levels the cell broth was passed through the resin by a peristaltic pump. The resin could be reused because the solid phase extraction was performed in the external loop to avoid the continuous and vigorous stirring conditions in the reactor.

Unfortunately, ethyl butytrylacetate is less polar than the hydroxyl product and was thus preferentially adsorbed on to the column, lowering the resin's capacity for the hydroxyl product. Therefore all the substrate added was exhausted prior to the product removal. The medium was then recirculated through the resin column until all the product and substrate were removed from the medium. A new batch of biotransformation with the same biocatalyst was carried out enabling a transition to semi-continuous process from a fed-batch process. The substrate was added at the rate of 15 mM/hr.

Detailed studies of this system can lead to a much more efficient continuous process for the reduction of ethyl butytrylacetate by adding the substrate at limiting rate; under these conditions all substrate is converted to product, which can be continuously concentrated in the extraction unit by adjusting the rate of the cell broth through the external loop.

In Situ Product Removal (ISPR)

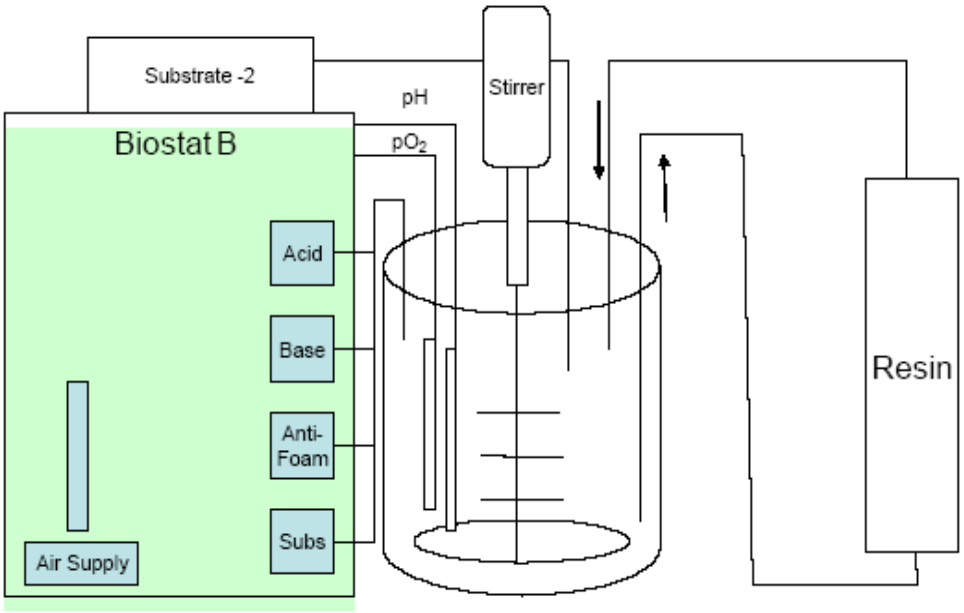


Figure 2-6. Experimental setup for *in situ* product removal

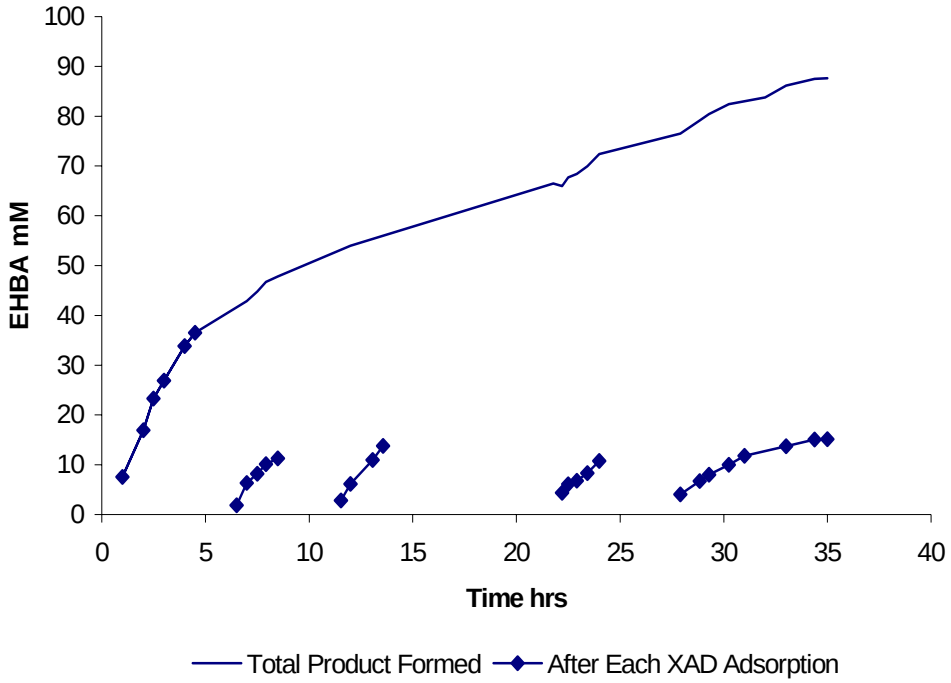


Figure 2-7. *In situ* product removal using an external column packed with resin Amberlite XAD-4

Strategy III: Biphasic reactor (ATPS)

Problems arise when conducting biocatalysis in aqueous medium due to poorly soluble substrate and or products. Low concentrations of the product or substrate, that are hydrophobic can be maintained in the reactor by use of a "Biphasic Reactor" or an "Aqueous Two-Phase System" (ATPS). [39, 40] This can be achieved by addition of an immiscible organic solvent or other non-conventional media like ionic liquids to the reaction mixture which act as a substrate reservoir or a product extractant. [41, 42] The solvent acts as a bulk extractant or sink, which effectively withdraws product or substrate from the aqueous phase, and keeps the effective concentration of these compounds near the biocatalyst at low levels. As products are concentrated in organic phase, they can be easily separated from the biocatalyst suspension and the extractants can be recycled, therefore making the process more cost-effective.

The most important task when using the biphasic reactor system is to find a biocompatible organic solvent. In selection of the auxiliary organic phase in the bioprocess, the most important parameter is the log P value of the organic phase. [43,44] Log P is the value indicating the partition coefficient of the solvent in the octanol-water two-phase system. Microbial whole cells are generally compatible with solvents with log P value greater than 4.

Another factor desirable in the organic phase is a favorable distribution coefficient for the biotransformation product. The organic phase should show high product recovery capacity and high selectivity. These are quantified by the partition coefficient (Kp) and the separation factor (α). Kp is defined as the ratio between product concentration in solvent and its concentration in aqueous medium; the separation factor (α) is the ratio

between partition coefficient of the product and the other contaminant from which we want to isolate the product, e.g. substrate.

We tried two organic phases: hexadecane and bis ethyl hexyl phthalate (BEHP) with BEHP giving better results. [45, 46] BEHP is cheaper and higher boiling than hexadecane, has low flammability and shows no toxicity towards *E.coli* cells. Therefore we carried out our further studies with BEHP, efficiently overcoming product inhibition. The reaction was conducted in a fed-batch two-liquid phase reactor. The reduction was carried out with BEHP as the organic phase with a ratio of 0.5, i.e. the apolar solvent consisted of 50 % of the reactor volume. The reactions were carried out with OD_{600} of 18, 22 and 25 with no significant increase in total final product formation for higher biomass loading. The substrate was added at the rate of 50 mM/hr.

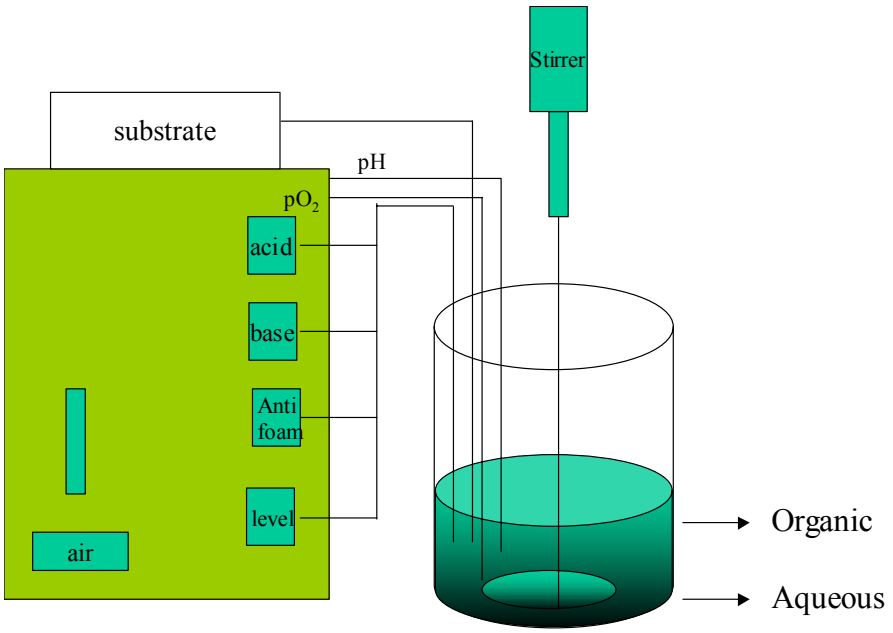


Figure 2-8. Biphasic reactor (aqueous two phase system)

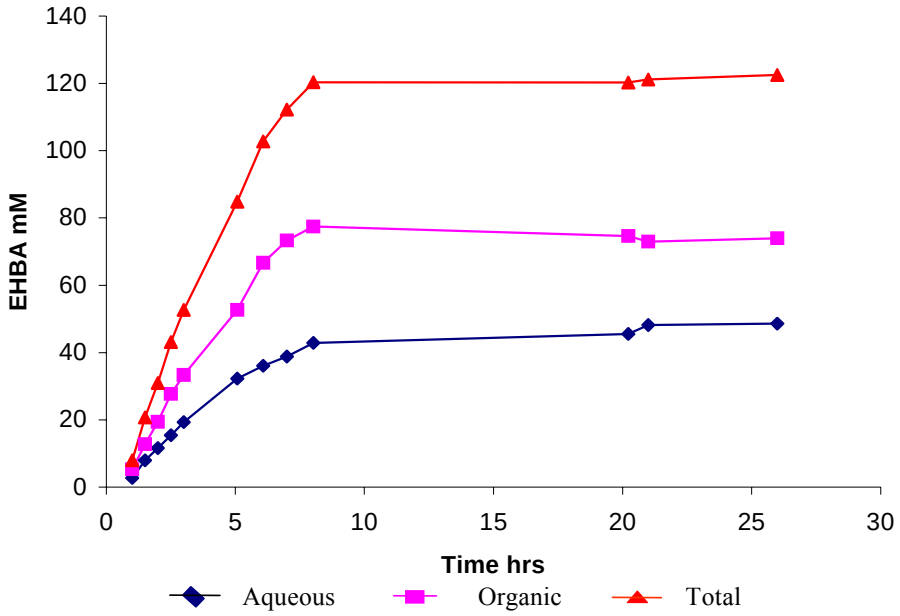


Figure 2-9. Bioconversion in a biphasic reactor containing BEHP as the organic phase

Bioprocess Development for the Reduction of Ethyl butyrylacetate

The bioprocess efficiency for the reduction of ethyl butyryl acetate was increased using techniques like *in situ* product removal and biphasic reactor.

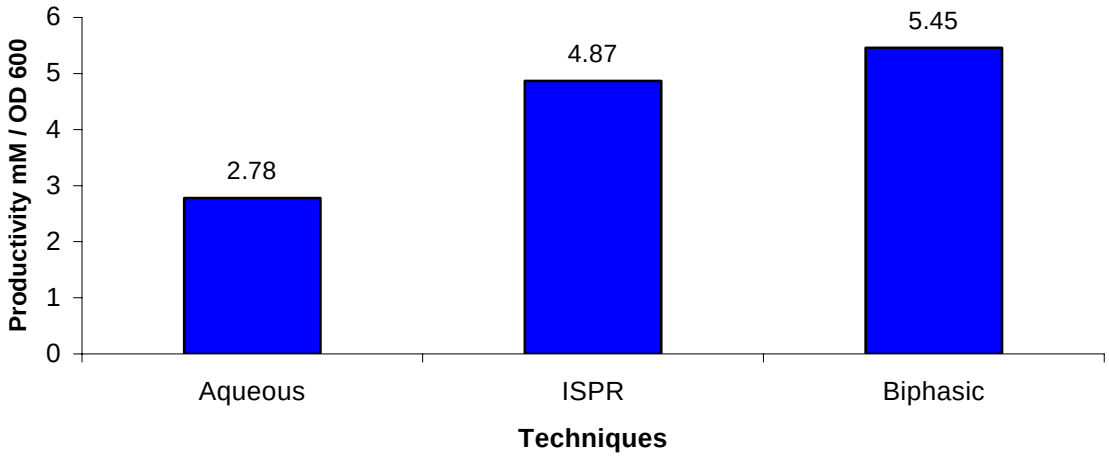


Figure 2-10. Bioprocess development for the reduction of Ethyl Butyryl Acetate

Section II: Bioprocess Development for Reduction of Ethyl 4-chloroacetoacetate

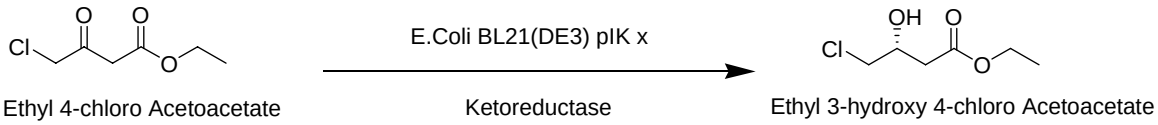


Figure 2-11. Reduction of ethyl 4-chloro acetoacetate

Asymmetric reduction of ethyl 4-chloro acetoacetate leads to the formation of (R) or (S) ethyl 4-chloro 3-hydroxy butanoate (ECHB). Both the (R) and (S) enantiomers are important chiral synthons in the synthesis of various pharmaceuticals and agrochemicals. [47] For example, the R- enantiomer is an important chiral building block for the synthesis of (−)-macrolactin A, L-carnitine and (R)-γ-amino-β-hydroxybutyric acid. [48] On the other hand the S enantiomer is a key chiral intermediate in the enantioselective synthesis of slagenins B and C and in the total synthesis of a class of HMG-CoA reductase inhibitors. [49]

Baker's Yeast reduction of ethyl 4-chloro acetoacetate results in products with low optical purity owing to the presence of forty different reductases of opposing stereoselectivites. [50] An in-house fusion protein library of 20 β-keto ester ketoreductases from *Sacchromyces cerevisae* over expressed in *E.coli* was screened for the reduction of ethyl 4-chloro acetoacetate. A group of yeast enzymes denoted by their genetic codes YJR096w, YDL124w, YHR104w, YGL185c, YAL061w and YOL151w bearing the plasmids pIK-9, pIK-8, pIK-29, pAKS-1, pIK-28 and pIK-3 yielded an enantiomeric excess of >99% to give the optically pure (S) enantiomer. [16]

Biotransformations with Non-growing Cells

The whole cell reductions were carried out with non-growing cells using our standard procedure wherein cells were harvested just before reaching stationary phase were resuspended in non-growing minimal media (M-9 media composition without NH_4Cl) with a 4:1 dilution with an OD_{600} 18. Temperature and dissolved oxygen were kept constant at 30^{o}C and 75 % saturation.

Ethyl 4-chloro-acetoacetate slowly decomposes in aqueous medium to 4-chloroacetoacetic acid which is known to be a toxic compound for *E.coli* cells. [51] This decomposition is accelerated at pH ≥7 and high concentration (the mechanism of decomposition is not known) but no decomposition was observed if the pH was lowered to 6.00.Therefore, a pH of 6.00 was maintained during the bioreduction.

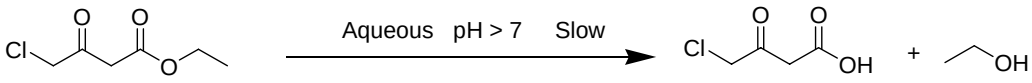


Figure 2-12: Slow decomposition of ECAA at pH ≥ 7 in aqueous medium

Strategies to overcome substrate inhibition

Slow substrate feed

Different rates for substrate feed from 1mM/hr to as low as 60 μM/hr were tested to avoid substrate inhibition. As soon as the substrate is added it is converted to the product, but this prolonged the reaction time and made it an extremely slow process. The reactions were carried out for 24 hrs. Also, product formation never exceeded 18 mM.

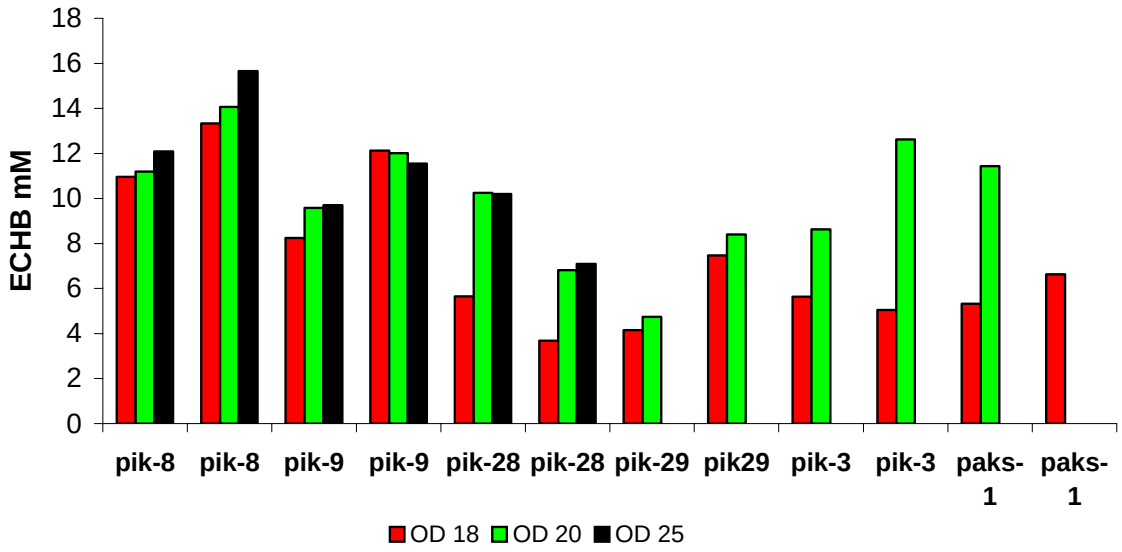


Figure 2-13. Reduction of ECAA by *E.coli* whole cells expressing different Bakers'Yeast reductases

Slow-release biocatalysis

In reactions that show substrate inhibition, the substrate concentration cannot be too high. Absorbing resins have been used for *in situ* product removal to prevent product inhibition and cell toxicity. Similarly, ion exchange resins can absorb a large quantity of substrate and then release it slowly to the reaction solution. [37] This technique is effective in eliminating substrate inhibition. Ten grams of resin Amberlite XAD-16 were preadsorbed with 40mM of ECAA so that the substrate is slowly released into the solution. The slow-release biocatalysis did not work as intended yielding 25mM product formation at much slower rate than without the resin.

Addition of organic solvent to increase the solubility of the ECAA

The solubility of ethyl 4-chloro acetoacetate is very in low in water, and this limits the substrate available to the biocatalyst making the bioprocess inefficient. Organic solvents can be added in small amounts to dissolve the hydrophobic compound and increase its availability to the whole cells. [52] Ethyl 3-hydroxy butyrate (50mM), which

was readily available in our lab from reductions of ethyl acetoacetate by the same biocatalyst, was added as an organic solvent to dissolve ECAA. [53] The addition of organic solvent worked to a smaller extent increasing the product formation to 34 mM.

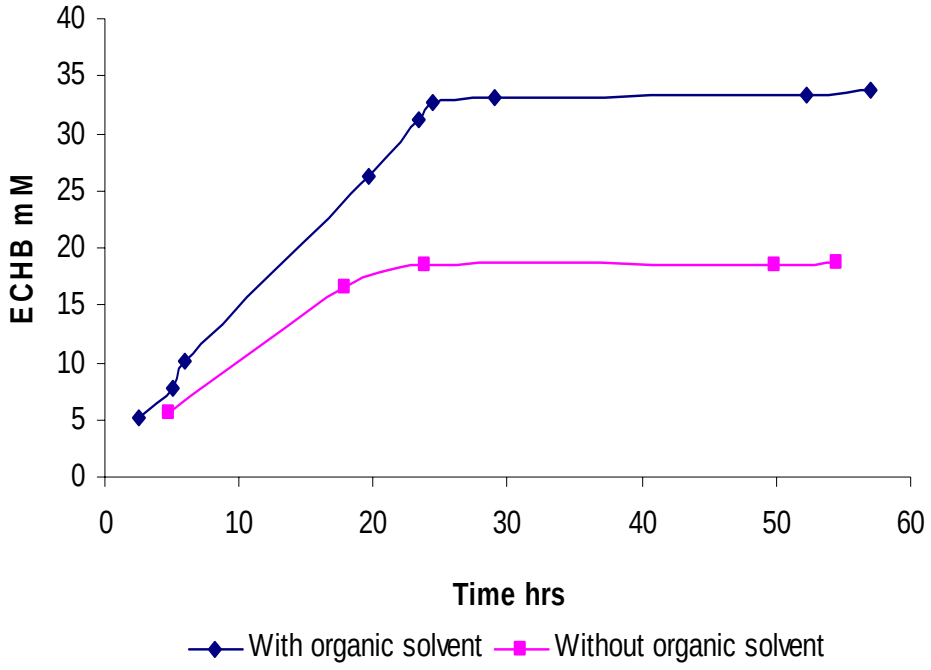


Figure 2-14. Comparison of product formation with and without organic solvent

Addition of surfactants to the whole cells

When whole cells are used, the cell membrane acts as a barrier to the transport of substrate to the enzyme and hampers optimal biotransformation. Different surfactants like Tween 20 and Triton-X have been used to increase the permeability of the cells, facilitating the transport of substrate and product across the cell membrane. [54] We used Tween 20 and Triton-X in 0.5 % w/v in the reactor but product was not formed in greater amount than in the absence of the surfactant.

Further studies are required to understand the effect of ethyl-4 chloroacetoacetate on whole cells.

Biotransformations with Cell-free Extracts:

Since we were not able to increase the bioprocess efficiency with whole cells, we turned to reductions of ECAA with cell free extracts. [55-57] Here the complex network of cofactor regeneration within an active cell breaks down. All our reductases are NADPH dependant and cannot accept NADH as a cofactor. Due to the high cost and relative instability of NADPH, it cannot be added in stochiometric amounts and needs to be regenerated. We tried the coupled-substrate regeneration using isopropanol as an auxiliary substrate but none of our enzymes accepted it as a co-substrate. We then tried the coupled-enzyme regeneration system for NADPH using glucose dehydrogenase (GDH) from *B.subtilis* over expressed in *E.coli* as the second enzyme. It oxidizes glucose to gluconolactone that spontaneously hydrolyses to gluconic acid. This GDH did not accept ECAA as a substrate.

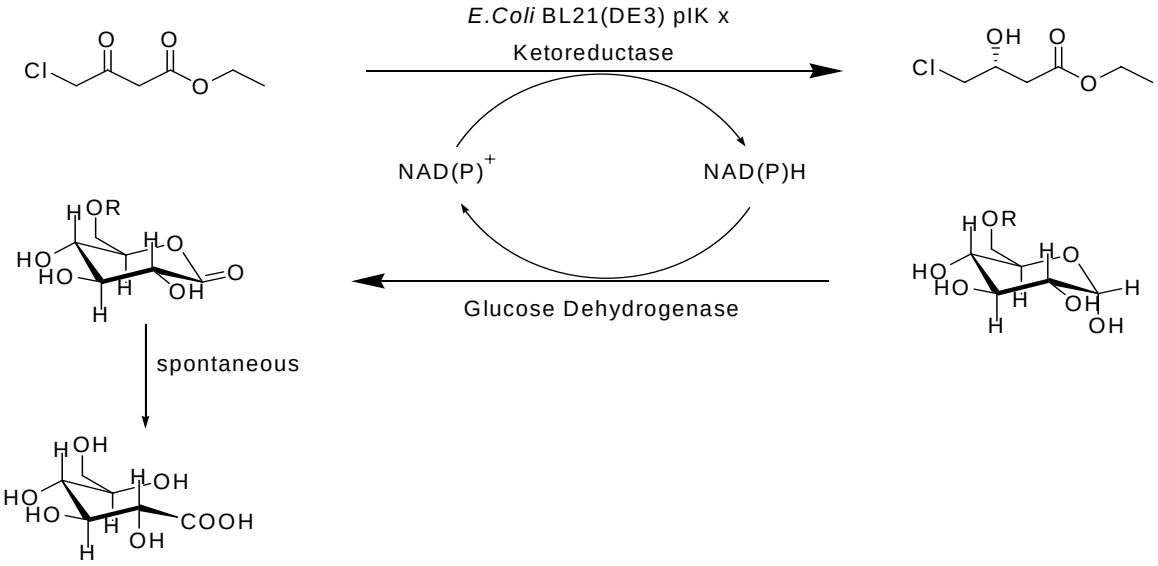


Figure 2-15. Coupled enzyme regeneration of NADPH using glucose dehydrogenase from B.subtilis over expressed in E.coli BL21(DE3) cells for the reduction of ECAA using Bakers' Yeast reductases over expressed in E.coli BL21(DE3)

Cells over expressing YDL124w gene product and glucose dehydrogenase were grown separately in a 4-liter fermentor and were harvested just before they reached the

stationary phase. These cells were lysed using a French Press. These cell-free extracts were then mixed together in M-9 minimal medium (without NH_4Cl) and the biotransformation performed by addition of the substrates ECAA and glucose. The volume of the reactions was kept constant at 50ml in all cases. Different biomass ratios of ketoreductase to GDH, viz. 8:1, 4:1, and 2:1 were applied to find the best ratio to give better productivity. 200 mM substrate was added to the shake flask reactor. Final product concentrations of 100mM were reached.

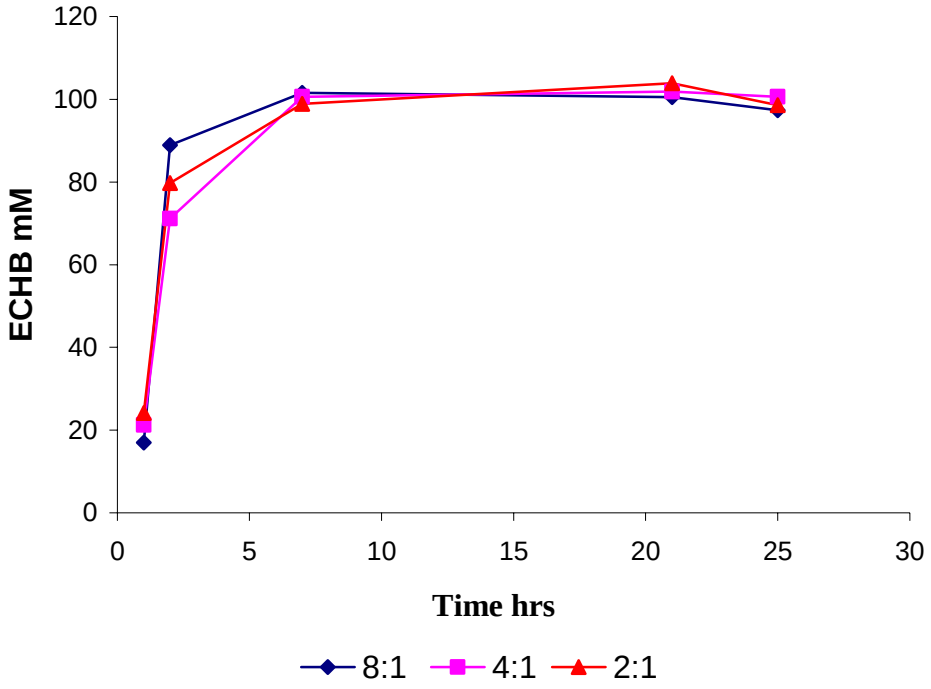


Figure 2-16. Reduction of ECAA with cell free extracts with different ratios of reductase and glucose dehydrogenase (GDH)

A small increase in the final product titer was observed by using higher ratio of ketoreductase but the amount of catalyst consumed was more per mole of product formed.

Biotransformation with cell free extracts under controlled pH conditions

The formation of gluconic acid via gluconolactone from glucose oxidation by GDH used for regeneration of the nicotinamide co-factors caused the pH to drop, making the conditions acidic. The acidic conditions, though good for ECAA (no decomposition at pH 6.00 and lower), are harmful to NADPH causing it to degrade. The bioconversions were carried out under controlled pH conditions at pH 7.00 yielding higher final product concentrations. 200 mM substrate was added to the shake flask reactor. A final product concentration of 160mM was obtained.

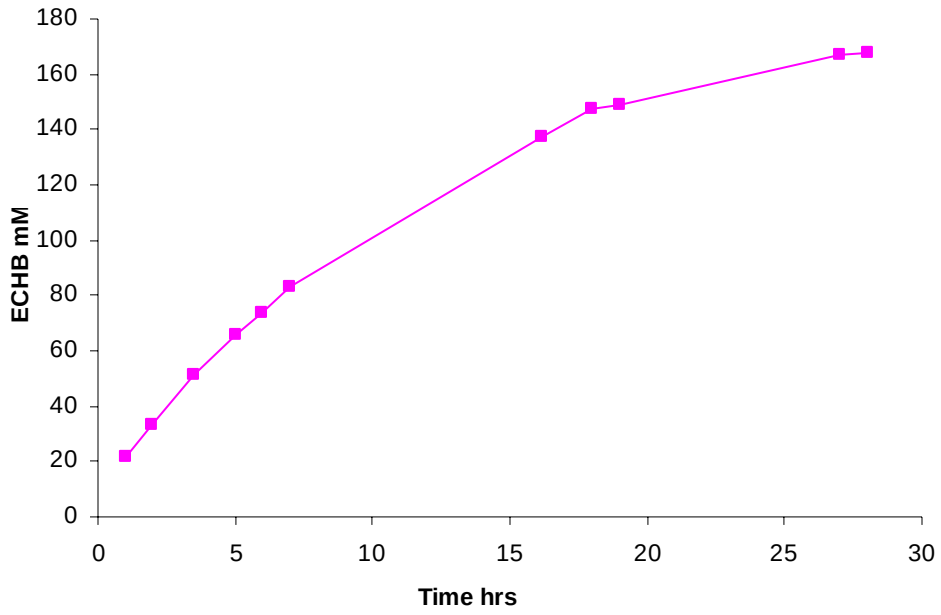


Figure 2-17. Reduction ECAA with cell free extracts under pH controlled conditions

Biotransformation using cell free extract in a two-phase system

ECAA is poorly soluble and unstable at neutral pH in water thus limiting the product formation. To overcome this, an organic solvent is added to the medium so both the product and substrate can be extracted in the organic phase. [58] The bioconversions were carried out with benzene and ethyl acetate as the organic phase and compared with

reductions in the aqueous medium. 200 mM substrate was added to the shake flask reactor.

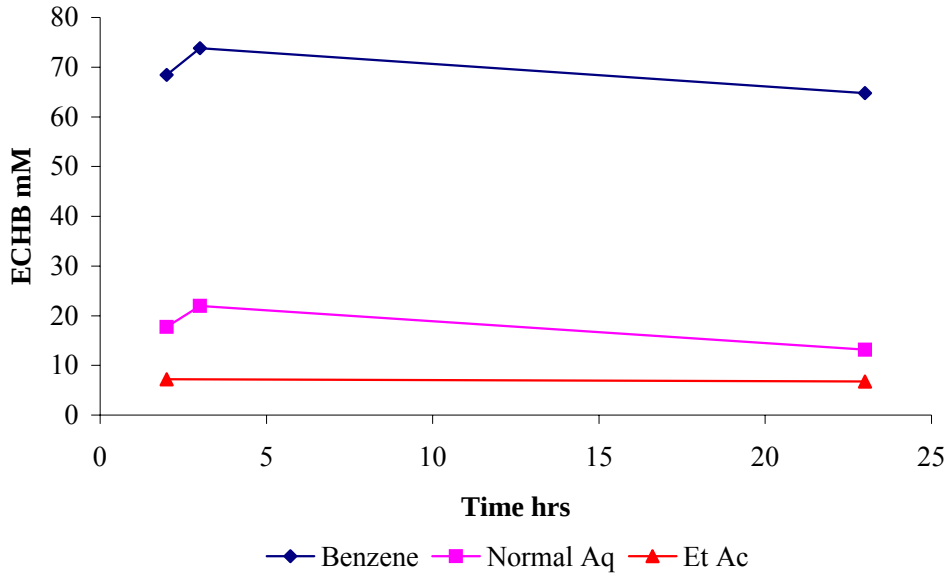


Figure 2-18. Reduction of ECAA with cell free extracts in biphasic conditions

To compare the values of final product concentration reached in each case i.e. whole cells, cell free extracts, pH controlled cell-free extract, two-phase cell free extract the product formation was calculated as product mM per gm of biocatalyst used.

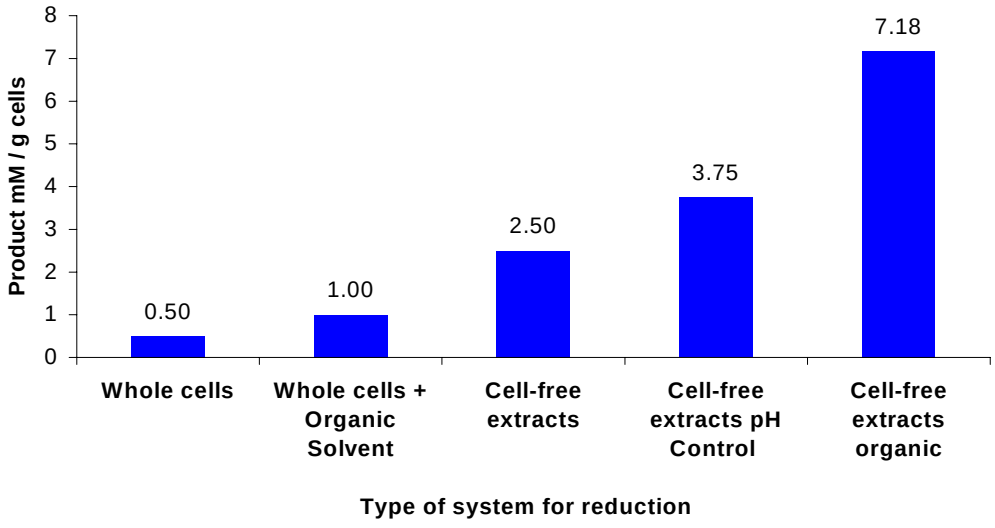


Figure 2-19. Bioprocess development for reduction of ECAA

Section III: Bioprocess Development for Reduction of Ethyl acetoacetate

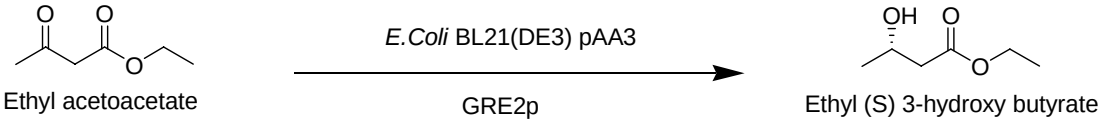


Figure 2-20. Reduction of ethyl acetoacetate

Biotransformation with Non-growing Cells

Reduction of ethyl acetoacetate was done with non-growing *E.coli* BL21(DE3) harboring the plasmid pAA3 over expressing the ketoreductase Gre2p from Baker's Yeast. The reaction was carried out in a reactor with 1L minimal media (M9 without NH_4Cl) yielding an OD_{600} 18 at 30^{o}C, pH 7.00 dissolved oxygen tension 75%. The maximum product formation observed was 285 mM, and it consumed 228 mM glucose producing 1.25mole of NADPH per mole of glucose consumed. Stewart and Walton published a basic study of reduction of ethyl acetoacetate where in they established that NADPH dependent reactions could be carried out under non-growing conditions using glucose-fed bath conditions. [30] It was proposed in this study that the efficiency of the biocatalytic process can be further enhanced and the costs reduced. The central carbon metabolism in *E.coli* proceeds via the Embden-Meyerhoff-Parnas (EMP) pathway (glycolysis), the Phosphate pentose pathway (PPP) and the Entner–Doudoroff pathway (EDP). [59] The EDP pathway links together the "starting" points of the EMP, ED and PPP pathways. The pyruvate formed is decarboxylated and is further metabolized by Krebs cycle.

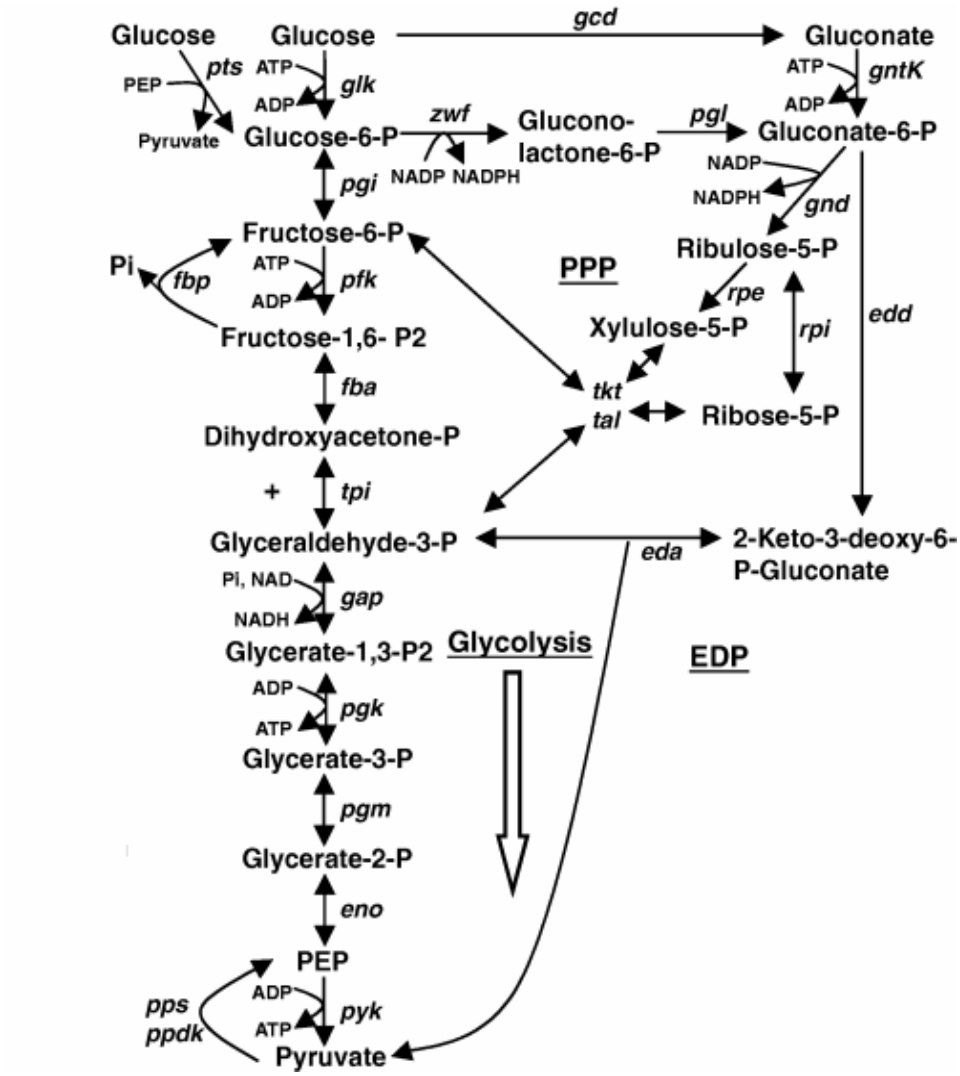


Figure 2-21. Major pathways of glucose metabolism in *E.coli*. Glycolysis (Embden-Meyerhoff-Parnas Pathway), Pentose Phosphate pathway (PPP) and the Entener-Doudoroff pathway (EDP).Gene names are italicized. [60]

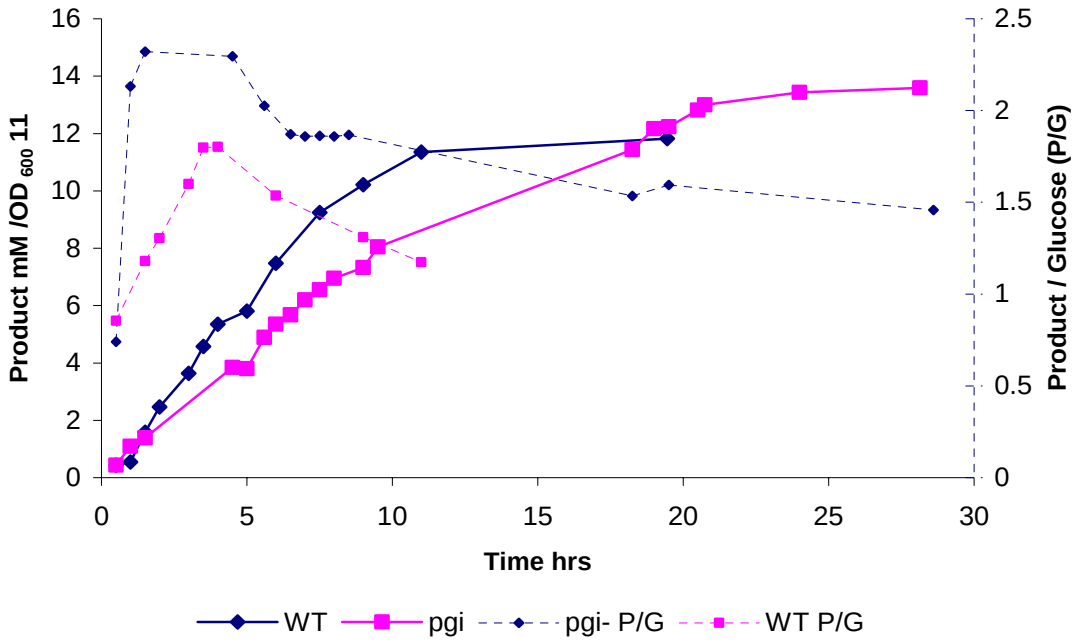
pts,PEP:glucose phosphotransferase system;
glk, glucose kinase;
pgi, phosphoglucose isomerase;
pfk, phosphofructokinase;
fbp, fructose-1,6-bisphosphatase;
fba, fructose-1,6-bisphosphate aldolase;
tpi, triosephosphate isomerase;
gap, glyceraldehyde 3-phosphate dehydrogenase;
pgk, phosphoglycerate kinase;
pgm, phosphoglyceratemutase;
eno, enolase;
pyk, pyruvate kinase;
pps, PEP synthase;
ppdk, pyruvate phosphate dikinase;

gcd, glucose dehydrogenase;
gntK, gluconate kinase;
zwf, glucose-6-phosphate dehydrogenase;
pgl, 6-phosphogluconolactonase;
edd, 6-phosphogluconate dehydratase;
eda, 2-keto-3-deoxy-6-phosphogluconate aldolase;
gnd, 6-phosphogluconate dehydrogenase;
rpe, ribulose-5-phosphate epimerase;
tkt, transketolase;
tal, transaldolase;
rpi, ribose-5-phosphateisomerase transketolase;
tal, transaldolase;
rpi,ribose-5-phosphateisomerase

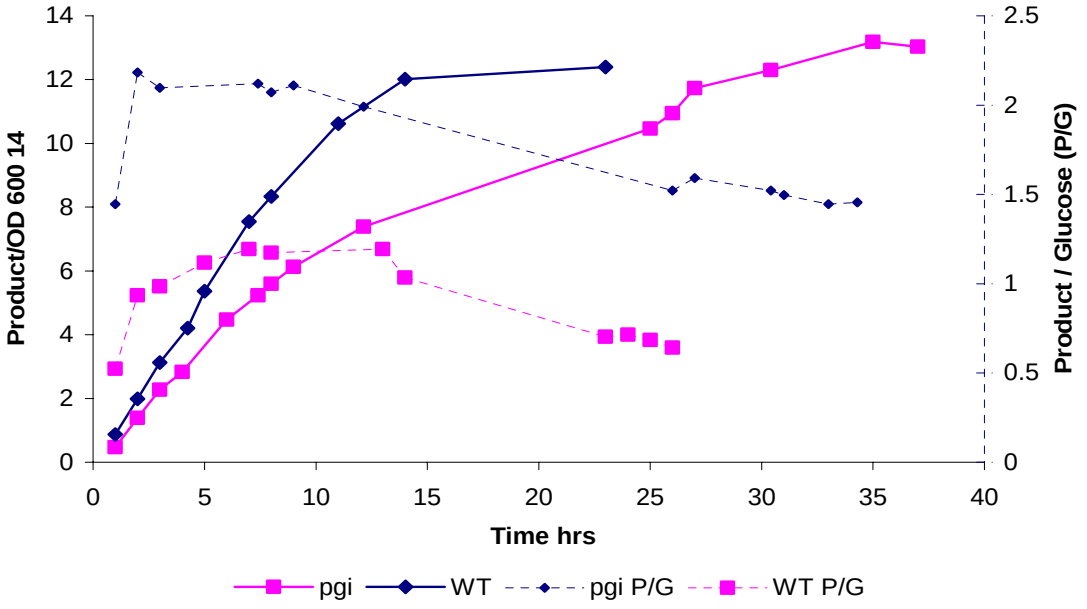
Metabolic Engineering

The tools of metabolic engineering were applied for the optimization of biocatalysts to lower the bioprocess costs. The targeted change of flux rates in the metabolism for the purpose of optimization of product yield is called metabolic engineering. In this case glucose was used as the source of reducing equivalents. In *E. coli*, 80% of the glucose is consumed via the EM pathway. The other 20% is sent via the HMP shunt and consumed through the pentose pathway. [61] The NADPH produced per mole of glucose consumed can be improved by increasing the carbon flux through the pentose phosphate pathway. This could be achieved by over expressing the two NADP+ producing dehydrogenases of this pathway or by knocking out the phosphoglucoisomerase (*pgi*) gene and forcing the carbon flux through the phosphate pentose pathway. [62,63] We selected the later and the strain with the knocked out *pgi* gene was made by Despina Bouigioukou in our lab.

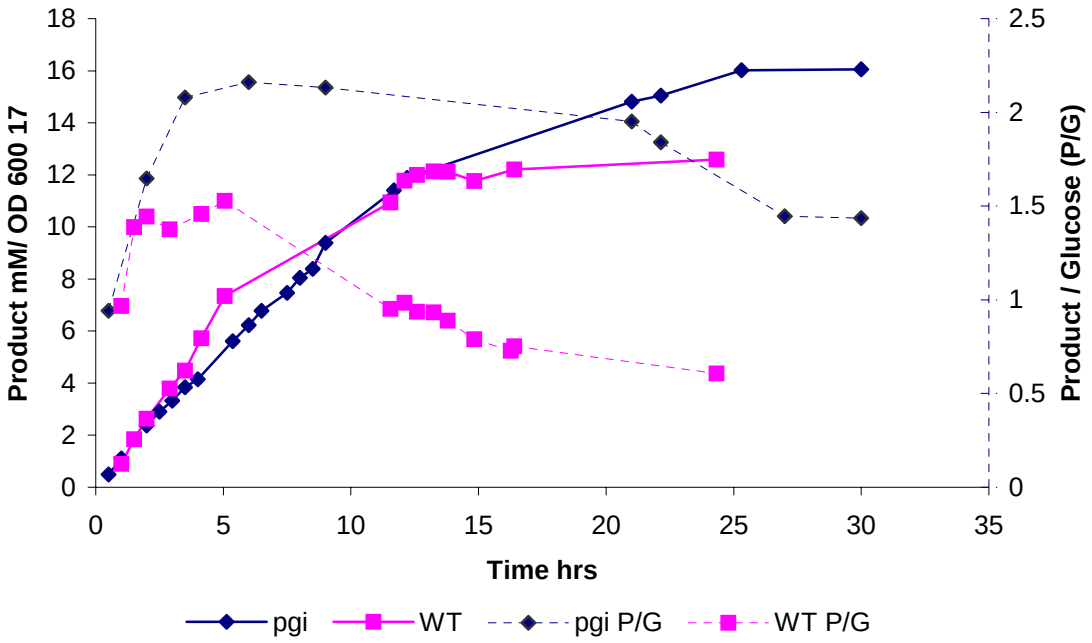
The *pgi* knockouts were compared with the cells with the *pgi* gene (referred to as the wild type) for the NADPH produced per mole of glucose consumed. The biotransformation was carried out with our usual conditions of temperature 30^{o}C, pH 7.00 and dissolved oxygen tension 75%. The reactions were carried out at different biomass loading giving an OD_{600} of 11, 14 and 17. The substrate was fed with a pump at the rate of 20 mM/hr.


Product mM /OD 600 11
Product / Glucose (P/G)
Time hrs
WT
pgi
pgi- P/G
WT P/G

A)


Product/OD 600 14
Product / Glucose (P/G)
Time hrs
pgi
WT
pgi P/G
WT P/G

B)



C)

Figure 2-22. Comparison of wild type and Δpgi knockout for product formation and glucose consumed per mole of product formed. A) OD_{600} = 11 B) OD_{600} = 14 C) OD_{600} = 17.

The delta *pgi* knockout strain was better than the wild type. It produced more NADPH per mole of glucose consumed and the final product concentration per gm of cells was higher than the wild type. So,the efficiency of the bioprocess was increased.

Section IV: Bioprocess Development using Immobilised Cells and Enzymes.

Biocatalyst costs are often important. Immobilization of biocatalysts is a method where catalyst is recycled or its prolonged use leads to significant reduction in catalyst cost. [64] Also, improved performance such as activity, stability, selectivity, and productivity can be achieved by immobilization. Immobilized biocatalysts can be considered as a composite consisting of two essential components: the non catalytic structural component (carrier) which is designed to aid separation and reuse of the catalyst, and the catalytic functional component (enzyme or whole cell), which is designed to convert the desired substrates into desired products. [65] Immobilization can be

either carrier bound or carrier free. The criteria for effective immobilized systems are defined by requirements such as mechanical and chemical stability, high volume activity, operation stability, reusability, easy disposal and safety depending on the non-catalytic and catalytic function of the system. [66] The catalytic functions are responsible for the desired activity, selectivity, substrate specificity, productivity and space-time yields. The non-catalytic functions are dependent on reactor configurations (batch, stirred-tank, column, plug-flow), the type of reaction medium (aqueous, organic, biphasic), the process conditions (temperature, pressure, pH). [67] The selected parameters should facilitate downstream processing and control of the process.

A carrier can be considered as a modifier of the biocatalyst as it is not only the support on which the biocatalyst can be attached but its surface chemistry, pore size and hydrophilicity or hydrophobicity dictates the activity, selectivity and stability of the biocatalyst. [68] A large number of synthetic or natural carriers with different characteristics are available according to the requirements. Enzyme carriers can be of different types like beads, hollow fibers, capsules, films, membranes, sol-gels, mesoporous molecular sieves and inorganic nanocomposites. [66]

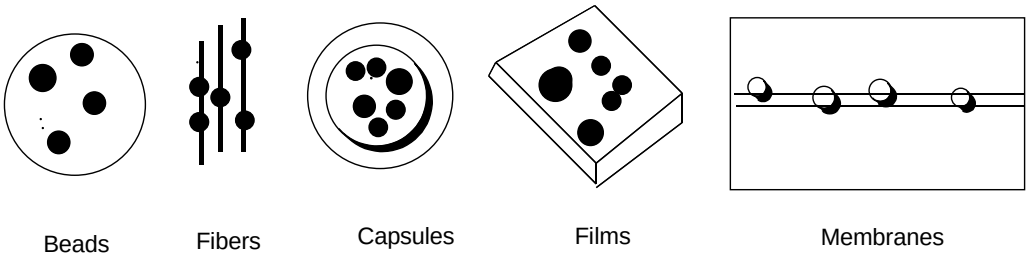


Figure 2-23. Different types of carriers for immobilization of biocatalyst [66]

Bioencapsulations with silica sol-gel glasses, which is biologically inert and imparts better mechanical strength and chemical stability and does not swell in aqueous or organic solvents, is a new and interesting field. [69, 70] This process of immobilization

involves no high temperature and harsh chemical reactions; it has the ability to immobilize biomolecules without modifying their structure or function greatly. Moreover, the porosity can be controlled with selection of proper precursors, modifiers and polymerization conditions.

Sol-gel entrapment techniques often use orthosilicate such as tetramethyl orthosilicate (TMOS), tetraethyl orthosilicate (TEOS) or methyl trimethoxy silane (MTMS) as precursors. [71] In aqueous media, the hydrolysis of precursor results in the formation of an oligomer that is further hydrolyzed to form an aqueous sol. The occurrence of gelation process leads to development of a three dimensional network where the biomolecules are trapped. A major disadvantage of this process is that the high concentration of ethanol or methanol produced during the immobilization process is harmful to the activity of enzymes or cells. Recently a sodium silicate silica based process for sol-gel formation was reported which avoids the formation of alcohol completely. [72]

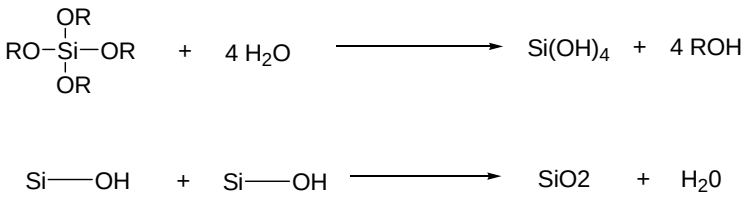


Figure 2-24. Reactions occurring during formation of sol-gel synthesis of silica

We decided to study the immobilization of whole cells within the silica gel glass due to the dependence of our enzymes on NADPH cofactors. Sodium silicate silica and colloidal silica (LUDOX HS40) was used for synthesis of silica gel. Phosphoric acid was added to decrease the pH to 7.0 before adding the whole cells. The gel was air-dried for 75 hrs and then crushed. This crushed silica gel glass containing the biocatalyst was resuspended in non-growing minimal M-9 medium without NH_4Cl for the

biotransformation. The reduction of ethyl acetoacetate was used as the model reaction and *E.coli* BL21(DE3) expressing the enzyme GRE2p harboring the plasmid pAA3 was used for the bioconversions. Different experiments were performed to improve the productivity of the biotransformation using immobilized cells. 50 mM substrate was added in all the reactions. Also, in all the experiments the reusability of the immobilized cells was checked. The immobilized cells were centrifuged and then resuspended in fresh buffer for repeated use.

Optimal Total Si: Buffer Ratio for Better Product Formation.

The ratio of the total silica content to the buffer added with a fixed amount of cell loading yielding gels with different properties was studied .The volume ratios used were 3:1, 2:1, 1:1 respectively. The gelation occurred within one minute.

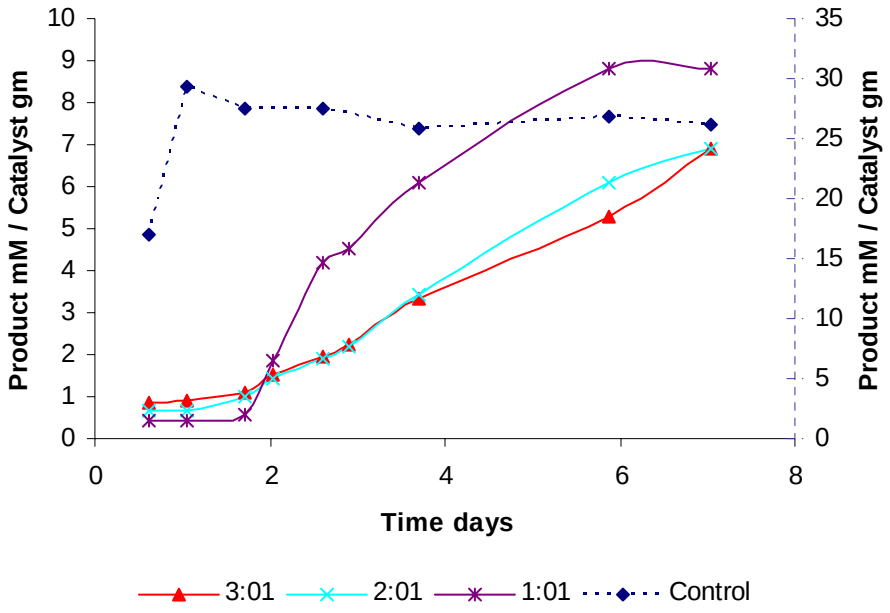


Figure 2-25. Product mM / gm catalyst for the reduction of ethyl acetoacetate using immobilized whole cells in silica gel glass with different Si:Buffer ratio. Secondary axis corresponds to control

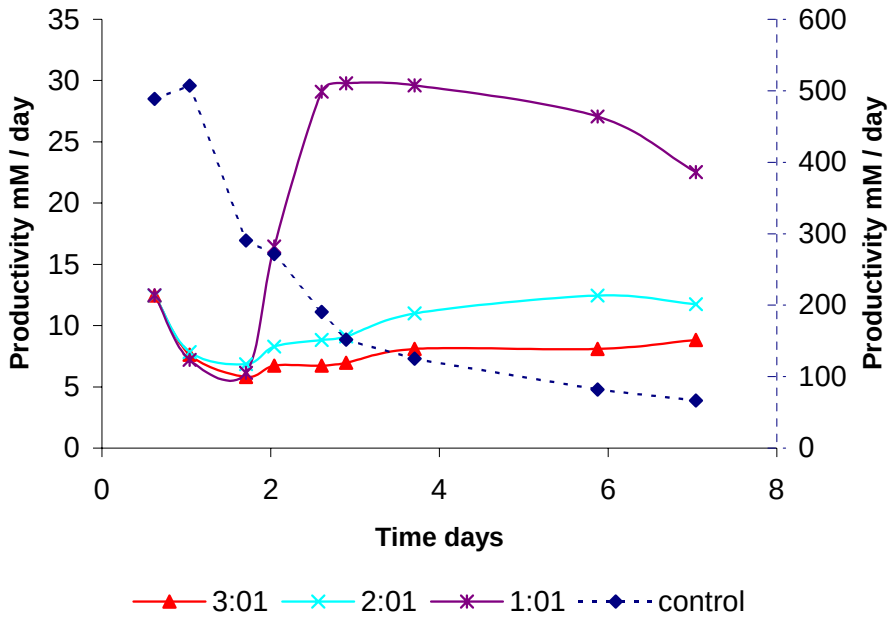


Figure 2-26. Productivity for the reduction of ethyl acetoacetate using immobilized whole cells in silica gel glass with different Si:Buffer ratio**.** Secondary axis corresponds to control.

The immobilized cells were checked for their reusability. The free and immobilized cells were centrifuged and resuspended in a fresh medium and glucose and substrate was added to the system for a new round of bioconversion.

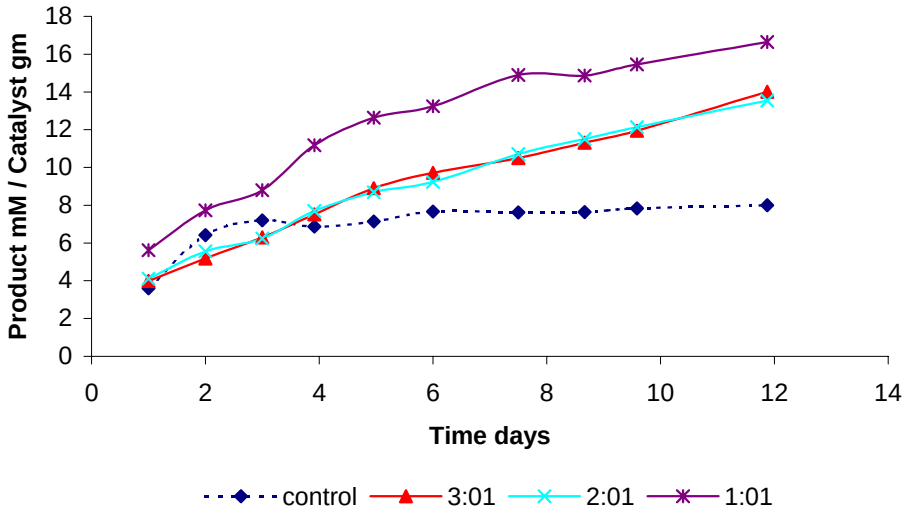


Figure 2-27. Reuse of immobilized cells for the biotransformation with different Si: Buffer ratio

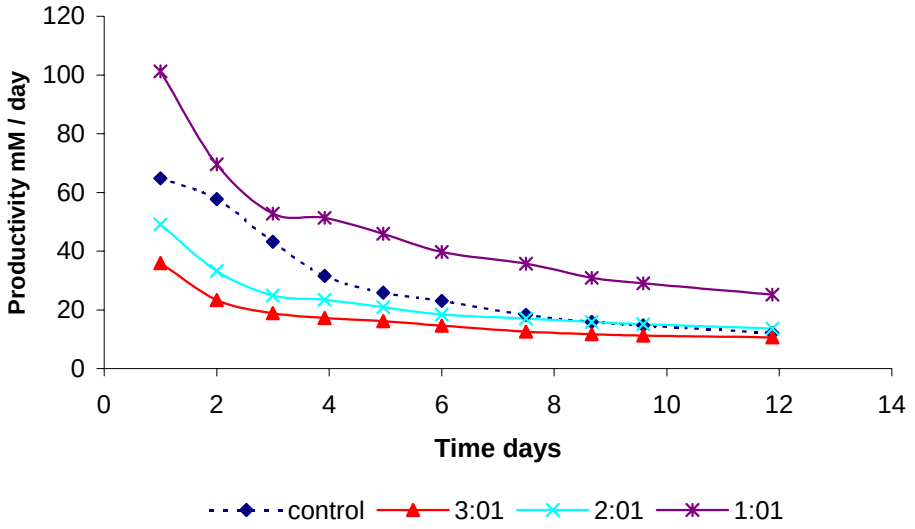


Figure 2-28. Productivity of the reused immobilized cells with different Si:Buffer ratio

These cells were again centrifuged and reused for another round of biotransformation.

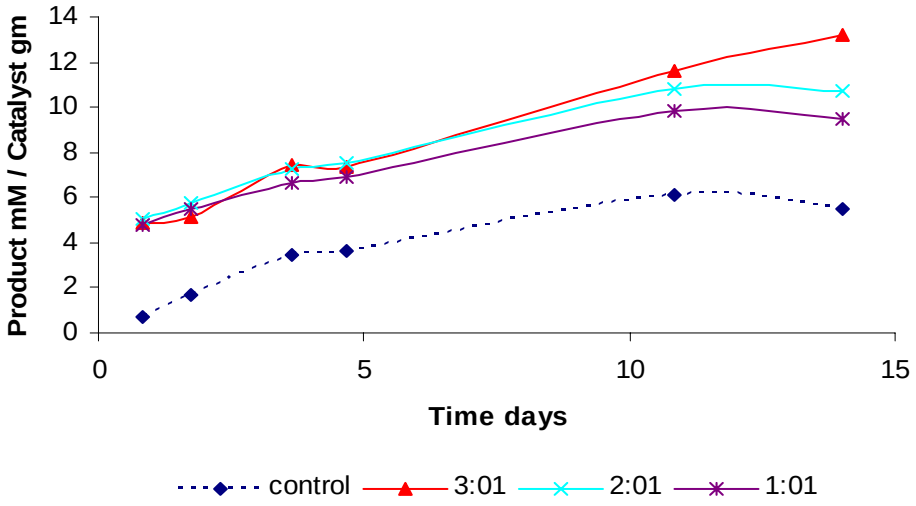


Figure 2-29. 2nd Reuse of immobilized cells with different Si: Buffer ratio

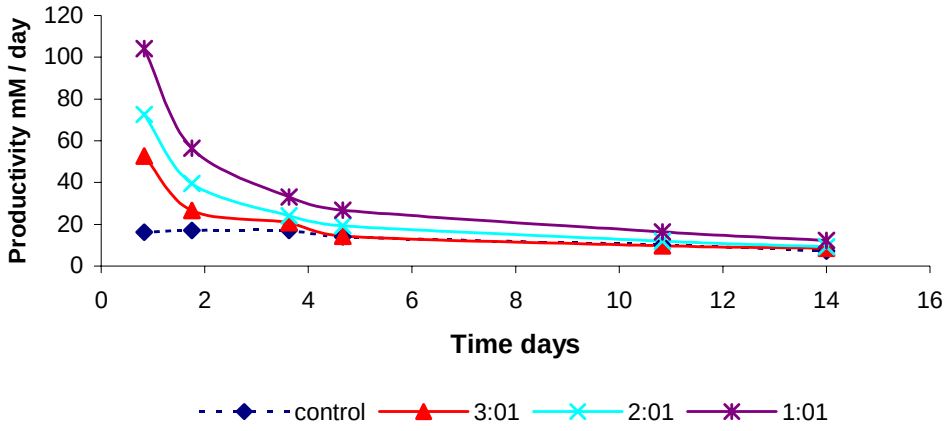


Figure 2-30. Productivity of the immobilized cells after 2nd reuse with different Si: Buffer ratio

For all the above experiments Si: Buffer ratio of 1:1 gave the best results and was kept constant for the next experiments.

Different Composition of the Silica Gel Matrix for Better Product Formation

Once we determined the ratio of total Si:Buffer, we decided to optimize the silica matrix by varying the Si:Ludox ratio. This ratio would the change the composition of the gel and thus affect the characteristics of the matrix affecting the biotransformation.

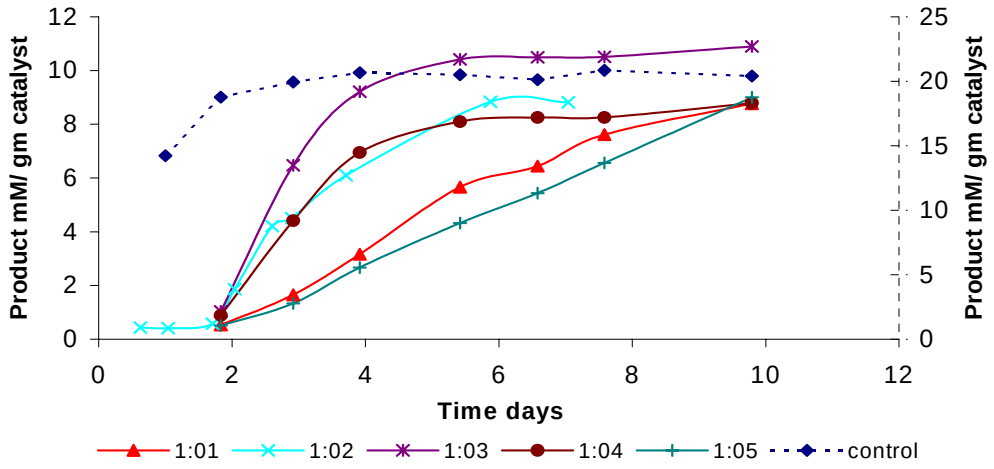


Figure 2-31. Product mM / gm catalyst for the reduction of ethyl acetoacetate using Immobilized whole cells in silica gel glass with different NaSi:Ludox ratio. Secondary axis corresponds to control.

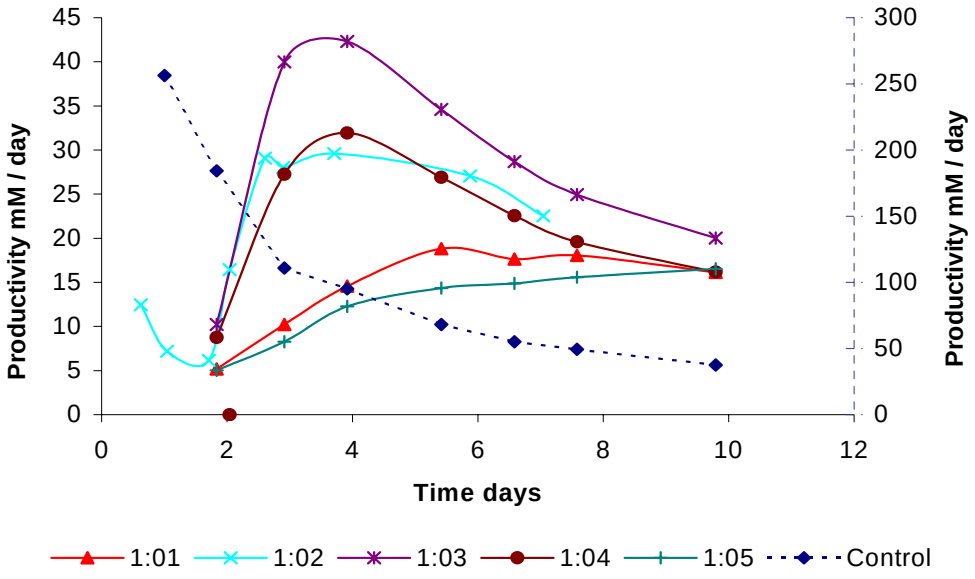


Figure 2-32. Productivity for the reduction of ethyl acetoacetate using immobilized whole cells in silica gel glass with different NaSi:Ludox ratio. Secondary axis corresponds to control.

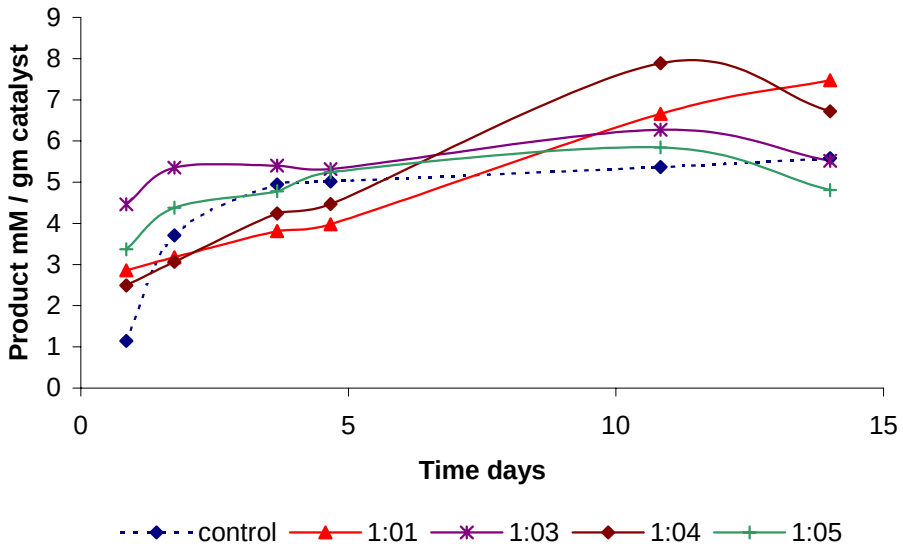


Figure 2-33. Reuse of immobilized cells for the biotransformation with different NaSi:Ludox ratio

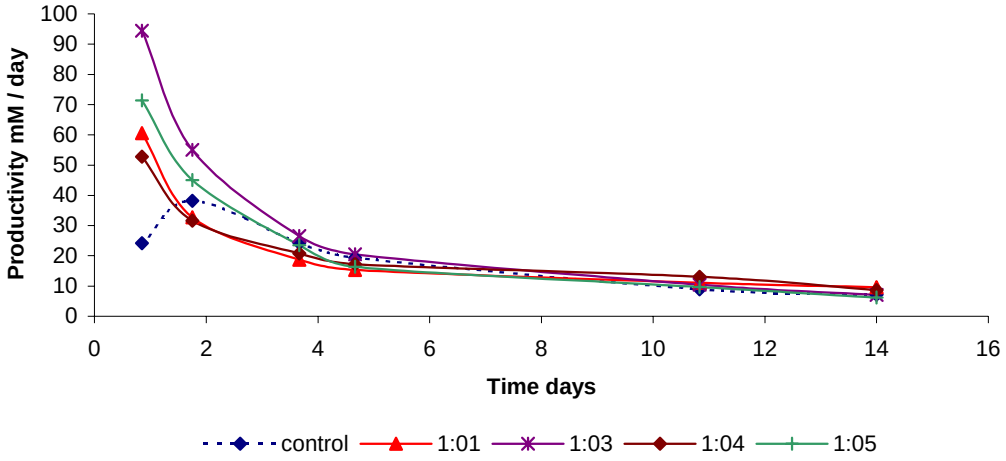


Figure 2-34. Productivity of the reused immobilized cells different NaSi:Ludox ratio

NaSi:Ludox with a ratio 1:3 gave the best results. The gels used for further tests had the Si:Buffer ratio 1:1 and NaSi:Ludox ratio 1:3.

Effect of Amount of Cell Loading on the Product Formation

We studied the effect of different amounts of cell loading in the silica-gel matrix. This was an important parameter affecting the bioconversions. There definitely is an optimum loading of cells beyond which results in lower productivity. The loading amount varied from 0.24 gm to 12 gm of *E.coli* BL21 (DE3) pAA3 (wet weight) in 200ml total volume of the system.

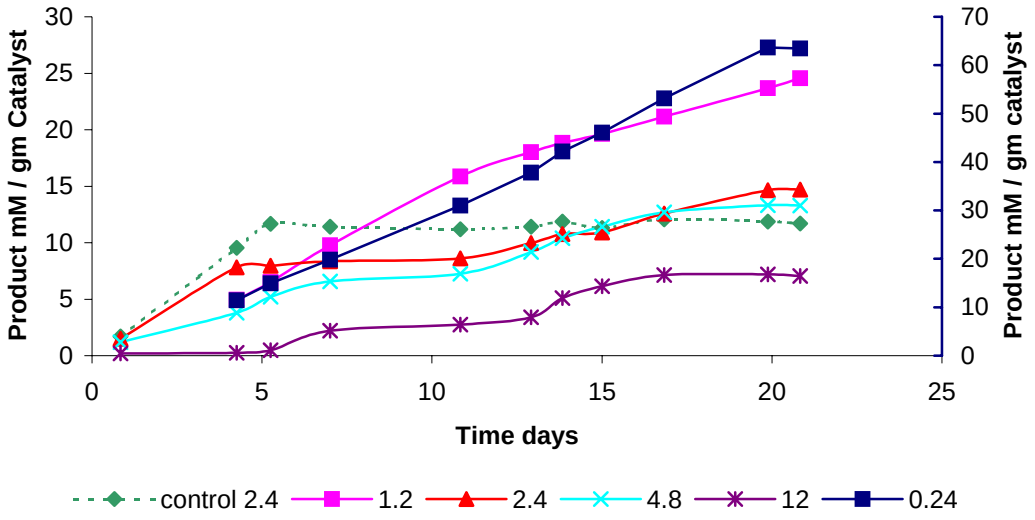


Figure 2-35. Product mM / gm catalyst for the reduction of ethyl acetoacetate using immobilized whole cells in silica gel glass with different amount of cell loading. Secondary axis corresponds to sample with 0.24 g biocatalyst.

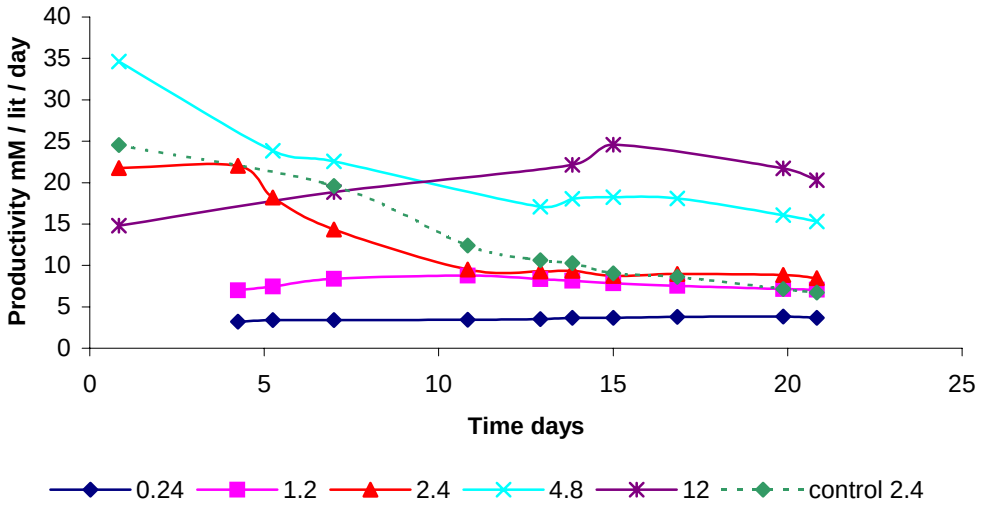


Figure 2-36. Productivity for the reduction of ethyl acetoacetate using immobilized whole cells in silica gel glass with different amount of cell loading.

We also studied the stability of immobilized cells. The immobilized cells stored at room temperature for three weeks showed no deterioration and were perfectly normal. The product formation was exactly the same as in the case of the immobilized cells used

immediately without storing. The free cells cannot be stored at room temperature for more than a day.

All previously mentioned reactions were carried out in shake flask reactors. We tried to use a plug-flow column reactor and were able to get the product, but the product formation was low. We suspect that due to diffusion barrier created by the silica gel glass around the biocatalyst.

Further studies are required to make the silica gel glasses with the right porosity to enhance diffusion and increase the product formation rate. Also, this would enable us to use the immobilized cells in plug-flow column type reactors further increasing the productivity of the system

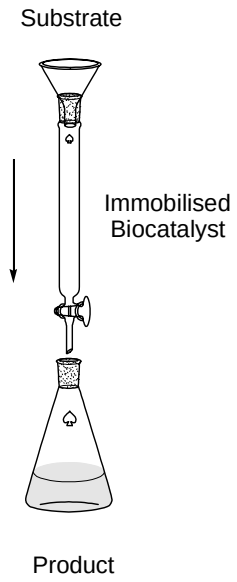


Figure 2-37. Plug –flow column reactor

Conclusion

In conclusion, we have tried to overcome the disadvantages of biocatalysis such as the scale up of reactions, very low space-time yields and product or substrate inhibition, etc. using different methods for each problem.

Production of optically active alcohols using either whole cells or enzymes by the enantioselective reduction of ketones is a very important activity in biocatalysis. We studied the reduction of ethyl butyryl acetate, ethyl 4-chloro acetoacetate and ethyl acetoacetate. Each reduction was accompanied by its own unique set of problems that were overcome using simple bioprocess solutions.

Ethyl butyrylacetate reduction yielding ethyl 3-hydroxy butyrylacetate was limited due to the product inhibition of the biocatalyst. We applied simple techniques like *in situ* product removal using solid resins and enhanced the productivity of the system. Additional increases in the productivity can be achieved by using the *in situ* SFPR technique i.e. the in-situ substrate feeding and product removal techniques wherein the substrate feed is adjusted so that all the substrate added is converted to product which is removed promptly from the reactor. Thus a steady concentration of the product and substrate is maintained at all times, and longer reaction times can be achieved resulting in higher productivities.

Another method that was explored to avoid product inhibition was the use of a Biphasic reactor also known as an Aqueous Two Phase System (ATPS). In this method, we added an organic solvent to the reactor and the product and substrate would partition in the two phases. The total concentration of the inhibitory substance, the product in our case, around the biocatalyst is reduced enabling more product formation. We used Bis-Ethyl-Hexyl Phthalate (BEHP) as the organic phase because it is inexpensive and increased the final product concentration of the product. BEHP also made product recovery easier because most of the product can be removed from the organic phase by

simple distillation as the boiling point of BEHP is very high, allowing for the separation of organic phase and the aqueous phase by centrifugation

Using solvents with better partition rates can optimize the biphasic approach. Ionic liquid solvents are a good example because they are environmentally friendly, have essentially no vapor pressure and can be reused, making them very attractive for biocatalysis.

Reduction of ethyl-4 chloro acetoacetate presented different problems, such as toxicity of the substrate to the whole cells. The amount of substrate tolerated by the whole cells was very low, thus making the use of whole cells unattractive as it slowed the reaction time substantially. The reactions with cell free extracts with a cofactor regenerating system were tried with moderate success. The final product concentration increased from 18 mM to 100 mM. pH control of this reaction system further increased the product formation to 165 mM. Some basic studies regarding biphasic reactions using benzene and ethyl acetate as organic solvents with the cell-free extracts helped increase the rate of reaction. More studies with these biphasic organic reactions with pH and temperature control are required to increase the final product titer and the productivity.

Application of metabolic engineering to further reduce the costs of the bioprocess was successfully achieved. The glucose metabolism of the *E.coli* cells was routed through the pentose phosphate pathway rather than the Krebs cycle, thus increasing the amount of NADPH/glucose consumed. This was achieved by knocking out the phosphoglucoisomerase (*pgi*) gene from the *E.coli*. The engineered *E.coli* consumed less glucose per mol NADPH produced and thus reducing the operating costs of the biotransformation.

Reduction of ethyl acetoacetate was used to confirm the validity of the knockout. The wild type produced 1.25mol NADPH per mole of glucose consumed and the engineered *pgi* knockout produced 2.25mol NADPH per mole of glucose consumed.

Immobilized cell systems wherein the whole cells were encapsulated in silica matrix were studied. This system needs further studies to be developed by altering the porosity of the silica gel glasses formed to enhance the diffusion of the substrate and the product. This system has its advantages regarding the reuse and storage of the biocatalyst. We have thus improved the bioprocess efficiency for the asymmetric reduction of β-keto esters viz. ethyl butyryl acetate, ethyl 4-chloroacetoacetate, and ethyl acetoacetate using various techniques.

CHAPTER 3
EXPERIMENTAL

Materials

Ethyl acetoacetate, ethyl butyrylacetate, ethyl 4-chloro acetoacetate were purchased from Sigma. (R, S) Ethyl 3-hydroxy butyrate, (R, S) ethyl 3-hydroxy 4-chloro acetoacetate, ethyl 3-hydroxy butyrylacetate purchased from Aldrich.

Resins Amberlite XAD 4, 8, 16 purchased from Aldrich and bis-ethyl hexyl phthalate (BEHP) from TCI America.

Gas Chromatography

GC analysis was done on Hewlett-Packard model 5890 GC/FID with a Chirasil-Val and DB17 columns.

Ethyl butyrylacetate was analyzed on a 25m Chirasil-Val column. The temperature program consisted of an initial time and temperature of 2 minutes and 80 ^{o}C, a gradient of 1^{o} C/min until the temperature reached 110^{o} C, and final time of 2 minutes at 180 ^{o}C attained with a rate of 10 ^{o}C / min.

Ethyl 4-chloro acetoacetate was analyzed on a 25m Chirasil-Val column. The temperature program consisted of an initial time and temperature of 2 minutes and 60 ^{o}C, a gradient of 1 ^{o}C/min until a temperature of 90 ^{o}C was reached and then with a gradient of 10 ^{o}C/min final temperature of 180 ^{o}C with a final time 2 minutes.

Ethyl acetoacetate was analyzed on a 30m DB-17 column. The temperature program consisted of an initial time and temperature of 2 minutes and 60 ^{o}C, a gradient of 10 ^{o}C /min until the temperature reached 180 ^{o}C, and a final time of 2 minutes.

Sample Preparation for GC

200 µl of aqueous reaction media was extracted with 600 µl of ethyl acetate containing 1 mM methyl benzoate as the internal standard. After one more extraction with 600-µl ethyl acetate and the two organic phases were combined. Samples were mixed using a vortex mixer. Standard curves for ethyl 3-hydroxy butyrate, ethyl 3-hydroxy 4-chloro acetoacetate, and ethyl 3-hydroxy butyrylacetate were prepared by making aqueous solutions of different concentrations and extracted identically. The starting materials, i.e. the β-keto esters ethyl acetoacetate, ethyl-4chloroacetoacetate and ethyl butyryl acetate decomposed during the GC analysis and were therefore not quantified.

Glucose Assay

The glucose concentration in the reactor was determined offline using a Trinder assay kit commercially available from Diagnostic Chemicals Limited, Canada. The absorbance of the reaction, which consists of 5 µl of the reaction media and 1ml of the Trinder reagent mixed by inversion and incubated at 37 ^{o}C for 15 minutes, was measured at 505 nm. The concentration of the glucose in the reactor was measure by comparing it to the standard reference containing 0.4g/L glucose.

Media Preparation

Cell growth was always in Luria-Bertani (LB) medium. Composition of LB medium: 10g/L tryptone, 5g/L Yeast Extract, 10g/L NaCl. For LB plates 15g/L of agar was used for solidification. The medium was sterilized by autoclave. This medium was supplemented with 4g/L glucose for cell growth. The glucose addition was done with 20 % glucose solution that is autoclaved separately.

Non-growing medium for biotransformation was always M-9 minimal medium that was autoclaved before use. It consisted of di-sodium hydrogen phosphate 12.8 g/L, Potassium di-hydrogen phosphate 3 g/L, NaCl 0.5g/L and NH_4Cl that is omitted to maintain non-growing conditions. 2 mM of $CaCl_2$ and 0.1 mM of $MgSO_4$ and 4g/L glucose are separately sterilized and added to the total volume.

Cell Culturing

The different *E.coli* BL21 (DE3) strain expressing a ketoreductase used for the biotransformation were streaked fresh weekly from a 15% glycerol stock stored at -80 ^oC, onto LB plates with 40 μM kanamycin, incubated overnight until colonies formed. Single colonies were picked to prepare inocula for cell growth. Fresh stock of the appropriate strain was prepared whenever necessary using the correct plasmid via electroporation.

Biotransformations with Non-growing Cells

50ml of LB was inoculated with a single colony of E.coli expressing the required ketoreductase with kanamycin (40 mg/L) and shaken overnight. The preculture was added to 4L of LB media with 4g/L glucose, 40 mg/L kanamycin and 0.25ml of antifoam AF-204 (Sigma) in New Brunswick fermentor. The cells were grown at 37 ^oC until the OD_{600} reached ~1 .The temperature was then lowered to 30 ^oC and the cells were induced with 0.1mM isopropyl thio galactoside (IPTG). The cells were harvested after 6.00hrs.During cell growth the agitation speed was 800 rpm and the aeration rate 1 lit/min was kept constant.

The cells were centrifuged at 6000 rpm for 30minutes and then resuspended in 100ml of M-9 medium without NH_4Cl.For biotransformation, the cell slurry was added to 1L of medium to a final OD_{600} of about 18.

Biotransformation with *In situ* Product Removal External Column

The external *in situ* product removal column contained 200gms of XAD-4 resin packed in a glass column attached to the fermentor. The aqueous reaction media was passed through it when the product neared inhibitory levels. The product free medium was subjected to another cycle of biotransformation.

Biotransformation in Biphasic Reactor

The biotransformation is carried out as usual but with 50% of the aqueous reaction phase is replaced by an organic phase. Hexadecane and Bis-ethyl hexyl phthalate were used as the organic phase. BEHP yielded better results and was used for further studies.

Biotransformation with Cell-free Extracts

The biotransformation carried out with cell free extracts involved cell growth as usual. The cell were lysed using an ultrasonicator or a French press. The results obtained using with either method were similar. The lysed cell extract suspension was centrifuged at 600 rpm for 15 minutes and the supernatant used for bioconversion. The total volume of the shake flask reactor was kept constant at 50 ml.

Biotransformation in Biphasic Medium with Cell-free Extracts

The supernatant obtained from the above-described method was then resuspended with organic solvents forming 50% of the total reactor volume. The organic solvents used were benzene, ethyl acetate. The total volume of the system was constant at 50 ml.

Biotransformation with Immobilized Cells

Cells grown were harvested and washed twice with minimal media M-9 without NH_4Cl. To maintain non-growing conditions they were resuspended in the M-9 minimal media without NH_4Cl and 10 % (by volume) glycerol. An aqueous sol using molar solutions of sodium silicate silica (27 wt % SiO_2 with 14 wt % NaOH from Sigma) and

colloidal silica (Ludox HS 40 from Aldrich) was synthesized. Phosphoric acid was used to maintain a pH of 7.00.The slurry containing *E.coli* (Buffer) was immediately added to the sol. Gelation time varied from instantaneous to 1 minute. The total volume for sol-gel was kept constant at 200ml. This silica gel was air-dried for 75 hrs. The resultant silica gel glass encapsulating the whole *E.coli* cells was crushed with a pestle and mortar and resuspended in minimal media M-9 without NH_4Cl. Different ratios of total Si content and the buffer (Si:Buffer) from 3:1, 2:1, 1:1 were used for the reaction. Once this parameter was fixed then the composition of the gel was varied by varying the silica ratio of sodium silicate silica with the colloidal silica Ludox (HS 40) viz. 1:1, 1:2, 1:3, 1:4 and 1:5 yielding gels with different characteristics. The immobilised cells with Si:Buffer ratio 1:1 and the composition of gel with Si:Ludox ratio 1:3 gave best results. The effect of loading of cells onto the silica matrices was studied by loading different amount of cells in the system. The wet weight of the cells loaded on the silica matrix was 0.24 gm, 1.2gm, 2.4gm, 4.8gm, and 12 gm. The biotransformation was carried out in a shake flask reactor by resuspending the crushed silica gel glass in 50 ml M-9 media without NH_4Cl and adding 50mM of ethyl acetoacetate as a substrate and glucose for recycling the cofactors. Centrifuging the reaction mixture and resuspending the catalyst in a fresh medium tested reusability of the immobilized cells for undergoing an additional round of biotranformations.

Product Recovery

In case of whole cell reactions the substrate was converted to product completely and then the aqueous reaction media was continuously extracted with ethyl acetate in a continuous extractor. The arrangement was as in the figure.

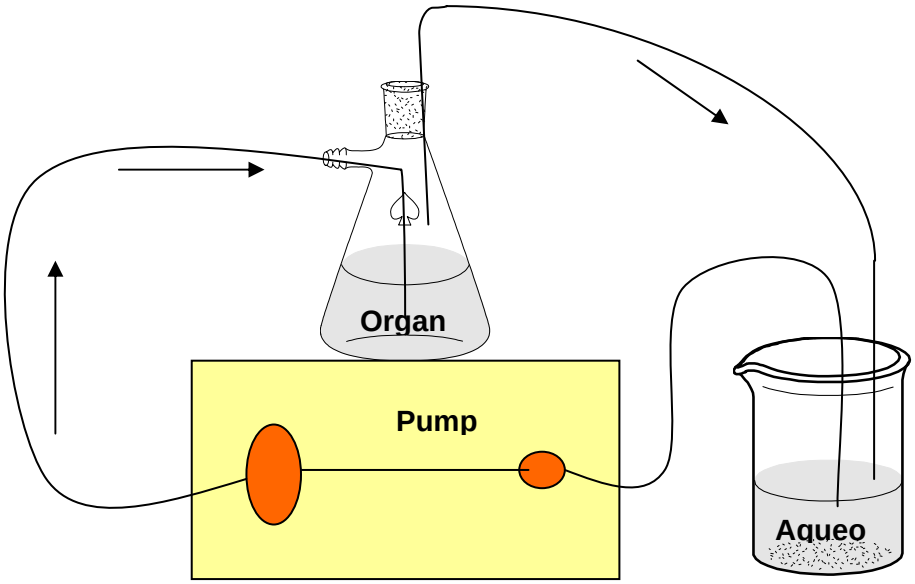


Figure 3-1. Experimental set-up for continuous extraction for product recovery

The ethyl acetate was concentrated and removed over a rotary evaporator leaving the pure product behind. Distillation was used to separate product and substrate if present. In the case of the ISPR column, passing acetone through the column and the acetone evaporating the acetone over a rotary evaporator washed out the product adsorbed on the column. In case of a biphasic system involving BEHP, the aqueous and the organic phase were separated using centrifugation and the product from BEHP was extracted by distillation. Product from the aqueous phase was recovered using the usual method.

In the case of product recovery from cell-free extract systems, $(NH_4)_2SO_4$ was added to the medium to precipitate the proteins and the product then extracted using chloroform from the reaction mixture. In the case of biphasic reactions with cell-free extracts, the organic phase was removed using a separatory funnel and concentrated giving the product, which was pooled with the product obtained from the aqueous medium using the above-described method.

LIST OF REFERENCES

(1) Schulze, B.; Wubbolts, M. G. *Curr. Opin. Biotechnol.* **1999**, *10*, 609-615.

(2) Panke, S.; Held, M.; Wubbolts, M. *Curr. Opin. Biotechnol.* **2004**, *15*, 272-279.

(3) Liese, A.; Villela F. M. *Curr. Opin. Biotechnol.* **1999**, *10*, 595-603.

(4) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. *Nature* **2001**, *409*, 258-268.

(5) Schoemaker, H. E.; Mink, D.; Wubbolts, M. G. *Science* **2003**, *299*, 1694.

(6) Straathof, A. J. J.; Panke, S.; Schmid, A. *Curr. Opin. Biotechnol.* **2002**, *13*, 548-556.

(7) Webb, N. C. *Enzyme Nomenclature 1992: Recommendations of the NCIUBMB on the Nomenclature and Classification of Enzymes*; Academic Press: San Deigo, 1992; pp 24-154

(8) Chartrain, M.; Greasham, R.; Moore, J.; Reider, P.; Robinson, D.; Buckland, B. *J. Mol. Catal. B: Enzymatic* **2001**, *11*, 503-512.

(9) Burton, S. G. *Trends in Biotechnology* **2003**, *21*, 543-549.

(10) Prelog, V. *Pure Appl. Chem.* **1964**, *9*, 119-130.

(11) Robinson, J.A.; Rete, J. *Stereospecificity in Organic Chemistry and Enzymology*; Weinheim VCH, 1982; pp 112-180

(12) Nakamura, K.; Yamanaka, R.; Matsuda, T.; Harada, T. *Tetrahedron: Asymmetry* **2003**, *14*, 2659-2681.

(13) Csuk, R. G.; Brigitte I. *Chem. Rev.,* **1991**, *91*, 49-97.

(14) Stewart, J. D.; Kayser, M. M *Enzyme Technologies for Pharmaceutical and Biotechnological Applications*; Marcel Dekker: New York, 2001; pp 175-208

(15) Martzen, M. R.; McCraith, S. M.; Spinelli, S. L.; Torres, F. M.; Fields, S.; Grayhack, E. J.; Phizicky, E. M. *Science* **1999**, *286*, 1153-1155.

(16) Kaluzna, I. A.; Matsuda, T.; Sewell, A. K.; Stewart, J. D. *J. Am. Chem. Soc.* **2004**, *126*, 12827-12832.

(17) Faber, K. *Biotransformations in Organic Chemistry*; Springer-Verlag: Berlin, 2000; pp 57-73.

(18) Sybesma W.F.H, Jongejan J.A, Pronk J.T, Heignen J.J. *Biocatal. Biotrans.* **1998**, *16*, 95-134.

(19) Donk, H. Z. *Curr. Opin. Biotechnol.* **2003**, *14*, 583-589.

(20) Kula, W. H. *Eur. J. Biochem.* **1989**, *184*, 1-13.

(21) Chenault, H. K.;Simon, E. S.; Whitesides, G. M. *Biotechnol. Genet. Eng. Rev.* **1988**, *6*, 221-270.

(22) Chenault, H. K.; Whitesides, G. M. *Appl. Biochem. Biotechnol.* **1987**, *14*, 147-197.

(23) Birgit, K; Wolfgang, S.; Mateja, P.; Walter, G.; Kurt, F.; Wolfgang, K. B. *Biotechnol. Bioeng.* **2004**, *86*, 55-62.

(24) May, O.; Gröger, H. *Specialty Chemicals* **2004**, *24*, 24-25.

(25) Rollmann, C. G.; Hummel, H. W; Bommarius, Andreas; Drauz, K. WO2004085636: Germany, 2004.

(26) Wong, C. H.; Drueckhammer, G. D.; Henri, M. S. *J. Am. Chem. Soc.* **1985**, *107,* 4028-4031.

(27) Wong, C. H.; Gordon, J.; Cooney, C. L.; Whitesides, G. M. *J. Org. Chem.* **1981**, *46*, 4676-4679.

(28) Kometani, T.; Yoshii, H.; Matsuno, R. *J. Mol. Catal. B: Enzymatic* **1996**, *1*, 45-52.

(29) Johanson, T.; Katz, M.; Gorwa-Grauslund, M. F. *FEMS Yeast Research* **2005**, *5*, 513-525.

(30) Walton, A. Z.; Stewart, J. D. *Biotechnol. Prog.* **2004**, *20*, 403-411.

(31) Freeman, A.; Woodley, J. M.; Lilly, M. D. *Nat Biotech* **1993**, *11*, 1007-1012.

(32) Raffer, N.; Heidersdorf, U.; Kretzers, I.; Sprenger, G. A.; Raeven, L.; Takors, R. *Bioproc. Biosys. Eng.* **2004**, *26*, 239-248.

(33) Yang, L.; Wei, D.; Zhang, Y. *J. Chem. Tech. & Biotechnol.* **2004**, *79*, 480-485.

(34) Stark, D.; von Stockar, U. *In Situ Product Removal (ISPR) in Whole Cell Biotechnology During the Last Twenty Years*; Adv. in Biochem. Eng./Biotechnol., Springer: Berlin/Heidelberg, 80 ed., 2003; pp 149-175.

(35) Nakamura, K.; Fuji, M.; Ida, Y. *J. Chem. Soc., Perkin Trans. 1* **2000**, 3205 - 3211.

(36) Lye, G. J.; Woodley, J. M. *Trends in Biotechnology* **1999**, *17*, 395-402.

(37) Houng, J. Y.; Liau, J. S. *Biotechnology Letters* **2003**, *25*, 17-21.

(38) Hilker, I.; Alphand, V.; Wohlgemuth, R.; Furstoss, R. *Adv. Synth. Catal.* **2004**, *346*, 203-214.

(39) Wubbolts, M. G.; Favre-Bulle, O.; Witholt, B. *Biotechnol.Bioeng.* **1996**, *52*, 301-308.

(40) Kwon, Y. J.; Kaul, R.; Mattiasson, B. *Biotechnol.Bioeng.* **1996**, *50*, 280-290.

(41) Cull, J. D. H.; Vargas-Mora, V.; Seddon, K. R.; Lye, G.J. *Biotechnol.Bioeng.* **2000**, *69*, 227-233.

(42) Panke, S.; Wubbolts, M. G.; Schmid, A.; Witholt, B. *Biotechnol.Bioeng.* **2000**, *69*, 91-100.

(43) Straathof, A. J. J. *Biotechnol. Prog.* **2003**, *19*, 755-762.

(44) Laane, C.; Boeren, S.; Vos, K.; Veeger, C. *Biotechnol.Bioeng.* **1987**, *30*, 81-87.

(45) Bühler, B.; Bollhalder, I.; Hauer,B; Witholt, B; Schmid, A. *Biotechnol.Bioeng.* **2003**, *82*, 833-842.

(46) Bühler, B.; Bollhalder, I.; Hauer,B; Witholt, B; Schmid, A. *Biotechnol.Bioeng.* **2003**, *81*, 683-694.

(47) Shieh, W.; Gopalan, A. S.; Sih, C. J. *J. Am. Chem. Soc.* **1985**, *107*, 2993-2994.

(48) Zhou, B.N.; Gopalan, A. S.; VanMiddlesworth, F.; Shieh, W.; Sih, C. J. *J. Am. Chem. Soc.* **1983**, *105*, 5925-2926.

(49) Patel, R. N.; McNamee, C. G.; Banerjee, A.; Howell, J. M.; Robison, R. S.; Szarka, L. J. *Enz. Microbiol. Tech.* **1992**, *14*, 731-738.

(50) Kita, K.; Kataoka, M.; Shimizu, S. *J. Biosci. Bioeng.* **1999**, *88*, 591-598.

(51) Shimizu, S.; Kataoka, M.; Katoh, M.; Morikawa, T.; Miyoshi, T.; Yamada, H. *Appl Environ Microbiol.* **1990**, *56*, 2374–2377.

(52) R. León, Fernandes, P.; Pinheiro, H. M.; Cabral, J. M. S. *Enz. Microbiol. Technol.* **1998**, *23*, 483-500.

(53) Chin-Joe, I.; Straathof, A.J.J.; Pronk, J.T.; Jongejan, J.A.; Heijnen, J.J. *Biocatal. Biotrans.* **2002**, *20*, 337-345.

(54) Gong, P. F.; Xu, J. H.; Shen, D.; Liu, Y.Y. *Appl. Microbiol. Biotechnol.* **2002**, *58*, 728 - 734.

(55) Yamamoto, H.; Kobayashi, Y. *Appl. Microbiol. Biotechnol.* **2003**, *61*, 133-139.

(56) Liu, Y.; Xu, Z.; Jing, K.; Jiang, X.; Lin, J.; Wang, F.; Cen, P. *Biotechnol. Lett.* **2005**, *27*, 119-125.

(57) Shimuzu, S.; Kataoka, M.; Kita, K. *Ann NY Acad Sci* **1998**, *864*, 87-95.

(58) Rotthaus, O.; Krüger, D.; Demuth, M.; Schaffner, K. *Tetrahedron* **1997**, *53*, 935-938.

(59) Munoz-Elias, E. J.; McKinney, J. D. *Cellular Microbiol.* **2006**, *8*, 10-22.

(60) Fraenkel, D. G. *Glycolysis. Escherichia coli and Salmonella: Cellular and Molecular Biology.* ASM Press: Washington, DC, 1996, pp 189-198

(61) Fischer, E.; Sauer, U. *Eur J Biochem* **2003**, *270*, 880-891.

(62) Lim, S. J.; Jung, Y. M.; Shi, H. D.; Lee, Y. H. *J. Biosci.Bioeng.* **2002**, *93*, 543-549.

(63) Mascarenhas, D.; Ashworth, D. J.; Chen, C. S. *Appl Environ Microbiol.* **1991**, *57*, 2995–2999.

(64) Ferreira, B. S.; Fernandez, P.; Cabral, J. M. S. *Multiphase Bioreactor Design*; Taylor & Francis: London, 2001, pp 85-114.

(65) Klibanov, A. M. *Science* **1983**, *219*, 722-727.

(66) Hartmeier, W. *Immobilized Biocatalysts: An Introduction*; Springer-Verlag: Berlin, 1988; pp 1-35.

(67) Mosbach, K. *Immobilized Enzymes and Cells*; Academic Press: Orlando, 1987; pp 1-584.

(68) Cao, L. In *Carrier-bound Immobilized Enzymes: Principles, Application and Design.* WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, 2005, pp 1-52.

(69) Livage, J.; Coradin, T.; Roux, C. *J. Phys.: Condens. Matter* **2001**, R673-R691.

(70) Nassif, N.; Bouvet, O.; Rager, M. N.; Roux, C.; Coradin, T.; Livage, J. *Nat. Mat.* **2002**, *1*, 42–44.

(71) Coiffier, A.; Coradin, T.; Roux, C.; Bouvet; O.; Livage, J. *J. Mater. Chem.* **2001**, *11*, 2039 - 2044.

(72) Bhatia, R. B.; Brinker, C. J.; Gupta, A. K.; Singh, A. K. *Chem. Mater.* **2000**, *12*, 2434-2441.

BIOGRAPHICAL SKETCH

Parag Anil Parekh is from Pune, India. He is a graduate of the Fergusson College, Pune, with a Master of Science in organic chemistry. He joined the National Chemical Laboratory as a Research Assistant. He was assigned to a project sponsored by Department of Biotechnology, Government of India, investigating the oxidation of polyunsaturated fatty acids. He joined the Department of Chemistry at the University of Florida to advance his career gaining expertise in the field of bioprocess chemistry.