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Enzymatic Bioprocess Development for *R*-PAC: an Intermediate for Ephedrine/Pseudoephedrine Production

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A thesis submitted in fulfilment
of the requirements for the degree of
Doctor of Philosophy

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May, 2002

CERTIFICATE OF ORIGINALITY

I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

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Vanessa Mary Sandford

For mum and dad

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Abstract

R-Phenylacetylcarbinol (*R*-PAC) is the key chiral precursor for the pharmaceutical compounds ephedrine and pseudoephedrine. Pyruvate decarboxylase (PDC) catalyzes the decarboxylation of pyruvate to 'active acetaldehyde' which is further ligated to benzaldehyde forming *R*-PAC.

Detailed studies of the isolated enzyme process using *Candida utilis* PDC have identified irreversible enzyme inactivation as a major factor limiting *R*-PAC formation. In this study it was shown that rapid enzyme inactivation occurred in benzaldehyde emulsions (> 60 mM), with significantly less enzyme inactivation in benzaldehyde solutions (< 60 mM). Product *R*-PAC and by-product acetoin also contribute significantly to enzyme inactivation. *R*-PAC did not inhibit initial reaction rates or final yields of *R*-PAC formation up to 153 mM *R*-PAC.

An aqueous-organic two-phase system was designed, focusing initially on the effect of a range of organic solvents on enzyme stability and *R*-PAC formation in this system. Octanol and nonanol were found to be suitable organic phase solvents, allowing high levels of *R*-PAC formation per unit of enzyme.

Octanol was further evaluated in a two-phase reactor. The system consisted of an octanol phase containing benzaldehyde at high concentrations, which partitioned into the aqueous phase at low concentrations not exceeding 60 mM. Partially purified PDC in the aqueous phase catalyzed the biotransformation of pyruvate contained in this phase and benzaldehyde to form *R*-PAC. Continuous extraction of *R*-PAC and by-product acetoin into the octanol phase further minimized inactivation of pyruvate decarboxylase.

The mode of operation was an important factor in the two-phase biotransformation. With rapidly stirred emulsion operation the rate of *R*-PAC formation and enzyme inactivation were greater than with slowly stirred phase separated operation. The amount of product per amount of enzyme was significantly greater with phase-separated operation, however scale-up of this system was more difficult.

Under optimized reactor conditions, 940 mM (141 g/l) *R*-PAC was achieved in the octanol phase with an additional 127 mM (19 g/l) in the aqueous phase in 49 hours. Significant activity remained after 50 hours to possibly support further product formation with additional substrate feeding and pH control. Product formation was increased significantly in comparison to the maximum of 300 mM (45 g/l) *R*-PAC achieved in a benzaldehyde emulsion batch reactor, and significantly exceeded previously published maximum concentrations of 167-200 mM (25-28 g/l) *R*-PAC.

Publications

Papers

Rosche, B., Leksawasdi, N., **Sandford, V.**, Breuer, M., Hauer, B. and Rogers, P. (2002). Enzymatic (R)-phenylacetylcarbinol production in benzaldehyde emulsions. Applied Microbiology & Biotechnology. Submitted for publication.

Rosche, B., **Sandford, V.**, Breuer, M., Hauer, B. and Rogers, P. L. (2001). Enhanced production of *R*-phenylacetylcarbinol (*R*-PAC) through enzymatic biotransformation. Journal of Molecular Catalysis B-Enzymatic. Submitted for publication.

Rosche, B., **Sandford, V.**, Breuer, M., Hauer, B. and Rogers, P. (2001). Biotransformation of benzaldehyde into (*R*)-phenylacetylcarbinol by filamentous fungi or their extracts. Applied Microbiology & Biotechnology, 57(3): 309-315.

Patent application

Sandford, V., Rosche, B., Rogers, P., Hauer, B. and Breuer, M. (2001).
Process for production of *R*-phenylacetylcarbinol by an enzymatic process in a two-phase system. DE 10142574.0. BASF Aktiengesellschaft.

Poster and abstract publications

Sandford, V., Rosche, B., Breuer, M., Hauer, B. and Rogers, P. (2002). Development of a two-phase enzymatic reactor for *R*-PAC production. Australian Biotechnology Association Fermentation and Bioprocessing Conference: Emerging Science and Technologies, Melbourne April 18-19.

Rosche, B., **Sandford, V.**, Breuer, M., Hauer, B. and Rogers, P. (2002). Enhanced production of (*R*)-phenylacetylcarbinol through enzymatic biotransformation. Australian Biotechnology Association Fermentation and Bioprocessing Conference: Emerging Science and Technologies, Melbourne April 18-19.

Sandford, V., Rosche, B., Breuer, M., Hauer, B. and Rogers, P. (2001). Production of *R*-phenylacetylcarbinol in a two-phase reactor by partially purified pyruvate decarboxylase from *Candida utilis*. Biotrans2001 Conference, Darmstadt, September 2-7.

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Rogers, P., Rosche, B., **Sandford, V.**, Breuer, M. and Hauer, B. (2001). Enhanced production of *R*-phenylacetylcarbinol (*R*-PAC) through enzymatic biotransformation. Lecture presented by Professor Peter Rogers at Biotrans2001 Conference, Darmstadt, September 2-7.

Sandford, V., Rosche, B. and Rogers, P. (2001). Enhanced production of *R*-phenylacetylcarbinol (*R*-PAC) through enhanced biotransformation. 23rd Symposium on Biotechnology for Fuels and Chemicals, Colorado, USA, May 6-10.

Sandford, V., Rosche, B. and Rogers, P. (2000). Production of phenylacetylcarbinol by partially purified *Candida utilis* pyruvate decarboxylase. Australian Biotechnology Association Conference, Brisbane, Australia, July 2-7.

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Aims of this Study

The research presented and discussed in this thesis extends from previous studies of *R*-phenylacetylcarbinol (*R*-PAC) for ephedrine/pseudoephedrine production by our group using partially purified pyruvate decarboxylase (PDC) from *Candida utilis* by Shin (1994) and Chow (1998). In our recent research, *R*-PAC production from *Rhizopus javanicus* PDC has been evaluated with the development of an improved benzaldehyde emulsion reactor (Rosche *et al.* 2002a), which is further examined for PDC from *Candida utilis* in this thesis. *R*-PAC production is currently being further characterized also using a mathematical model developed by Chow (1998).

The specific aims of this investigation which are associated with the latter studies are:

(1) to develop standard protocols for the production and monitoring of *Candida utilis* PDC in a bioreactor, and for recovery of PDC for further investigations.

(2) to evaluate *R*-PAC production using PDC isolated from *Candida utilis*, with particular focus on ensuring reproducibility of product formation and PDC stability with different enzyme batches. This evaluation will establish protocols for monitoring enzyme stability under reaction conditions.

(3) to determine the most critical factors influencing enzyme inactivation during the biotransformation. This evaluation will concentrate on determining the inactivating influences of substrate benzaldehyde, product *R*-PAC and by-products acetoin and acetaldehyde.

(4) to design and develop various enzyme bioreactors for enhanced production of *R*-PAC and minimization of enzyme inactivation using the knowledge established in analysis of enzyme stability in part (3).

Based on an improved understanding of the factors affecting enzymatic *R*-PAC formation it is anticipated that an improved bioprocess will be developed to enhance *R*-PAC production. This should provide a biotransformation system that could be applied to whole-cells or isolated pyruvate decarboxylase from various microorganisms.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The following literature review outlines the important aspects of *R*-PAC production for use in the manufacture of ephedrine/pseudoephedrine. The traditional method of *R*-PAC production is the pyruvate decarboxylase (PDC) mediated biotransformation of benzaldehyde and pyruvate. This literature review focuses on *R*-PAC formation using whole-cell mediated catalysis and *R*-PAC formation in cell-free preparations, outlining the major critical factors influencing the biotransformations. The various strategies taken to optimize *R*-PAC formation are also discussed and include improvements made to the properties of pyruvate decarboxylase through mutation and enzyme modification and various approaches to process design.

In this literature study, the main challenges associated with enzyme-mediated catalysis in other biotransformations are highlighted, with a review of recent advances in biocatalysis including directed evolution techniques, the use of supercritical fluids and ionic liquids, and most significantly the use of organic solvent systems. Each approach is discussed with regards to the major challenge of enzyme instability in these systems, with potential application for improving *R*-PAC synthesis in cell-free systems.

1.2 Research Status

Previous research by our group has focused on production of *R*-PAC using fermenting whole-cells of *Candida utilis* with various reactor designs including batch, fed batch and a three stage process designed to maximize *R*-PAC formation by generating biomass, accumulating pyruvate and by inducing pyruvate decarboxylase activity. These approaches are further outlined in Section 1.5.4.2.

By-product formation and low final concentrations of *R*-PAC in the whole cell system directed further studies to *R*-PAC formation using isolated pyruvate decarboxylase in cell-free biotransformations. Initial research using *C.utilis* PDC by Shin (1994), Shin and Rogers (1996a,b) and summarized by (Rogers *et al.* 1997) showed that increased levels of *R*-PAC could be achieved compared to the traditional yeast based fermentation process, however the biotransformations were possibly limited by enzyme inactivation. Chow *et al.* (1995) and Chow (1998) quantified enzyme inactivation due to the substrate benzaldehyde and established an initial mathematical model to describe the complexities of the biotransformation.

This model is currently being further developed by Lewsawasdi (unpublished results), with the aim of establishing an optimized fed batch reactor, with possible further application to optimization of a two-phase enzyme-based bioreactor.

A comprehensive screen of various yeast and fungi has identified several other strains possessing PDC capable of producing *R*-PAC (Rosche *et al.* 2001, 2002b). Detailed studies of *R.javanicus* PDC selected from this screen has led to the development of an improved system for *R*-PAC formation in a benzaldehyde emulsion process (Rosche *et al.* 2002a). Despite significant improvements in PDC stability, the loss of enzyme activity during the benzaldehyde emulsion biotransformation remained a significant factor preventing further large increases in *R*-PAC concentrations (Rosche *et al.* 2002a). The developments made in the study (i.e. pH control and the use of MOPS buffer for enzyme stabilization) will be applied to PDC from *C.utilis* for further process development.

Whole-cells, partially purified PDC and crude extracts are currently being compared by our group for their ability to produce *R*-PAC, with the aim being to establish a cost effective process that can be easily applied to industry.

Identification of the most critical factors limiting *R*-PAC formation by partially purified PDC from *Candida utilis* should allow the development of a cell-free enzymatic bioreactor which maximizes *R*-PAC formation per amount of catalyst with possible further application to whole-cell systems and crude extracts to minimize enzyme costs.

1.3 Biotransformation Trends

Generation of biopharmaceuticals and fine chemicals by harnessing the reactions of microbial cells and their enzymes is of increasing importance to the pharmaceutical industry worldwide.

The regio- and in particular stereo-specificity of enzyme catalyzed reactions allows the synthesis of enantiomerically pure compounds such as ephedrine/pseudoephedrine for use as pharmaceutical intermediates, overcoming the need to purify chemically synthesized racemic mixtures into the respective isomers. The industry is growing rapidly with chiral drug sales reaching $146 billion USD in 2000 and expected to rise an average of 8 % per year thereafter (Stinson 2000).

Several companies now specialize in the production of a range of enantiomerically pure amines, alcohols, esters and intermediates based on amino acids and carbohydrates. This growing supply of intermediates provides a solid base for drug development from more easily obtainable starting materials (Stinson 2000). Biocatalysis provides the basis for compounds that include anti-cancer, anti-viral, anti-psychotic, anti-arthritic, cholesterol lowering calcium channel blockers and many others (Zaks 2001). Biocatalysis has also found application in several other industries including bioremediation, food, detergent and herbicide industries (Schulze and Wubbolts 1999; Zaks 2001).

1.4 Ephedrine/Pseudoephedrine

1.4.1 Introduction

Ephedrine and pseudoephedrine are alkaloids, which occur naturally in several plant members of the genus *Ephedra* (order *Gnetales*, family *Ephedraceae*). There are over 40 species of *Ephedra* containing varying levels of ephedrine and pseudoephedrine depending on plant variety and growth conditions. Dried preparations of *Ephedra* named 'Ma Huang' have been used for over 500 years in Chinese medicine as a herbal remedy for asthma, coughs, colds, headaches, fever, and allergies. Species used to prepare herbal 'Ma Huang' are *E. major*, *E. equisetina* and *E.sinica*. The active ingredient ephedrine was first isolated in 1887 by a Japanese scientist named Nagai. International interest in the therapeutic potential of the compound increased when Chen and Schmidt studied the pharmacological effects of ephedrine in 1930 (Tang 1996).

1.4.2 Medical Uses

The medicinal properties of the herbal remedy 'Ma Huang' are attributed to the major components ephedrine and its isomer, pseudoephedrine. Both of ephedrine and pseudoephedrine are the active compounds in preparations used to treat asthma, and nasal decongestion associated with influenza and allergies (Tang 1996).

These compounds are sympathomimetic agents that stimulate both α and β-adrenergic receptors, acting on the same site as adrenalin. The primary influence of ephedrine is on the respiratory and cardiovascular system (Dollery 1991). Other effects of ephedrine include central nervous system stimulation, relaxation of gastrointestinal muscles, increased oxygen consumption and accelerated metabolic rate (Tang 1996).

There has been increasing interest in obesity control using ephedrine in combination with other compounds such as guarana or caffeine. However there are serious safety and efficacy concerns arising from its use at concentrations required to promote weight

loss (Pasquali *et al.* 1987; Pasquali *et al.* 1992; Astrup *et al.* 1992a,b; Tang 1996; Boozer *et al.* 2001).

Other reported uses of ephedrine include increasing muscle strength in patients afflicted by an autoimmune neuromuscular disorder called myasthenia gravis (Tang 1996), and prevention of blood pressure drop after spinal anesthesia in surgery (Bhagwanjee *et al.* 1990; Ayorinde *et al.* 2001).

1.4.3 Production of Ephedrine/Pseudoephedrine

There are three methods for producing ephedrine and pseudoephedrine, involving direct extraction from the genus *Ephedra*, chemical synthesis and a combination of biological and chemical synthesis.

1.4.3.1 Extraction from Plant

The *Ephedra* plant species are small evergreen shrubs with several alkaloids including ephedrine, pseudoephedrine, norephedrine and norpseudoephedrine contained within the green branches of the plant. The alkaloid content of the stems, roots, flowers and fruit is minimal. The ratio of alkaloids varies with plant species, growth conditions and harvest time, yielding typically 0.5 % to 2.5 % alkaloids of which 30-90 % is ephedrine/pseudoephedrine. The dried preparations are sold directly as herbal remedies or the ephedrine/pseudoephedrine compounds are further extracted (Tang 1996).

There are several methods for extraction and purification of ephedrine and pseudoephedrine from plant material (Yamasaki *et al.* 1973; Sorenson and Spenser 1988). Ephedrine has also been produced in small amounts by callus tissue culture of *Ephedra gerardiana* (Ramawat and Arya 1979).

1.4.3.2 Chemical Synthesis

Chemical synthesis of ephedrine can be achieved involving several complex chemical synthesis stages. Initial synthesis of 1-phenyl propane-1,2-dione is achieved by decomposition of isonitrosoethylphenyl ketone or by oxidation of ethyl phenyl ketone. The second step involves the condensation of 1-phenyl-1,2-propanedione with methylamine resulting in a mixture of ephedrine and pseudoephedrine. Ephedrine and pseudoephedrine readily form crystalline salts with optically active mandelic acid. Three recrystallizations were needed to obtain optically pure preparations of ephedrine and pseudoephedrine in high yield (Coles *et al.* 1929; Manske and Johnson 1929a,b).

1.4.3.3 Combined Enzymatic and Chemical Synthesis

Ephedrine and its isomer pseudoephedrine can be produced using combined biological and chemical synthesis. The first biological stage of synthesis involves the decarboxylation of pyruvate, followed by the addition of the resultant 'active' acetaldehyde to benzaldehyde to form the intermediate phenylacetylcarbinol of which the (*R*) enantiomer is required for further synthesis. This step of production is catalyzed enzymatically by whole cells of yeast, fungi and bacteria containing pyruvate decarboxylase activity. *R*-PAC is recovered from the fermentation broth or enzyme-mediated reaction by solvent extraction, followed by removal of the solvent under reduced pressure. The second chemical step involves the reductive amination of *R*-PAC to ephedrine and pseudoephedrine. In this step *R*-PAC is hydrogenated in the presence of a platinum catalyst, with methylamine solution added gradually to form ephedrine. Ephedrine may be further converted to pseudoephedrine (Nebesky *et al.* 1978; Breuer and Hauer 2001). This approach to *R*-PAC formation is shown in Figure 1.1.

Shukla and Kulkarni (1999) have described the recovery of *R*-PAC from fermentation broth using extraction with diethylether, followed by separation of contaminating components using several column chromatography stages. The purity of *R*-PAC recovered was 82.5 % with a yield of 90 %.

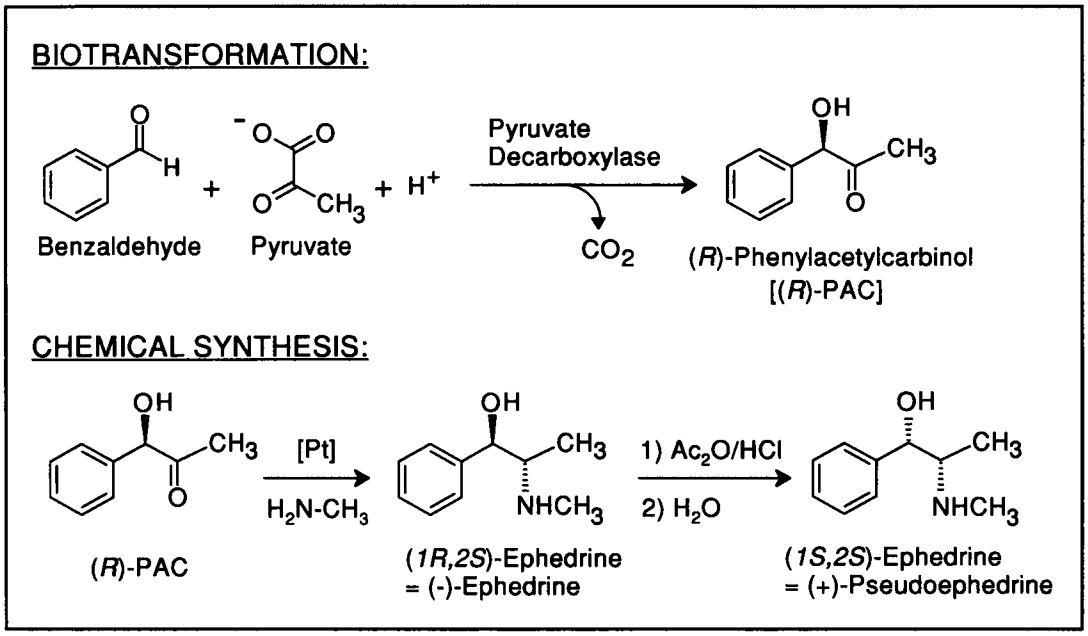


Figure 1.1: Combined enzymatic and chemical synthesis (Rosche *et al.* 2001c).

1.5 Biotransformation to Synthesize Phenylacetylcarbinol

1.5.1 Pyruvate Decarboxylase Enzyme

Pyruvate decarboxylase utilizes thiamine pyrophosphate to cleave carbon-carbon bonds adjacent to a keto group. The enzyme has been shown to form a wide range of subsequent acyloins by joining the decarboxylated α-keto acid to various aldehydes (Crout *et al.* 1991).

Several other enzymes such as benzylformate decarboxylase, transketolase, pyruvate oxidase, indole-3-pyruvate decarboxylase, phenyl pyruvate decarboxylase, pyruvate dehydrogenase, 1-deoxyxylulose-5-phosphate synthase and formate transketolase also

use thiamine pyrophosphate as a coenzyme and are capable of carbon-to-carbon bond cleavage followed by condensation of the cleaved compound to an added accepter to form carbon-to-carbon bonds (Muller *et al.* 1993; Iding *et al.* 1998; Schorken and Sprenger 1998). Pyruvate decarboxylase is the only enzyme reported to form *R*-phenylacetylcarbinol from the decarboxylation of pyruvic acid with subsequent carboligation to benzaldehyde or by the direct carboligation of acetaldehyde and benzaldehyde.

1.5.1.1 Production of Pyruvate Decarboxylase in Yeast

Induction of pyruvate decarboxylase (PDC) activity is strongly dependent on cell metabolism and growth environment. The metabolic rates of cells depend on yeast species, carbon sources, physiochemical factors and culture media. Glucose and oxygen concentrations have a large impact on sugar metabolism.

Fermentation characteristics of *Candida utilis*

Candida utilis is a facultative fermentative Crabtree-negative yeast. It exhibits low fermentation capacity when grown aerobically in glucose, but is induced to a higher fermentative state when exposed to glucose anaerobically. In *C.utilis* culture, PDC levels are associated with the rate of catabolic flux in the anaerobic utilization of glucose to the main fermentation product ethanol. Increased PDC activity and production of ethanol closely parallel increased rates of fermentation. High levels of PDC were obtained in shake flask culture under oxygen limited conditions (Van Urk *et al.* 1989). Sims and Barnett (1991) confirmed that PDC activity appears to be the rate limiting enzyme in anaerobic fermentation rather than the activities of glycosidase or alcohol dehydrogenase in 6 other yeast strains including *Candida viswanathii.*

In *Candida utilis* continuous culture, Weusthuis *et al.* (1994) showed that for 3 g/l dry weight biomass, fully respiratory conditions were achieved with an oxygen flow greater than 30 mmol/l/h. Below this, the biomass yield decreased and alcoholic fermentation began, with simultaneous respiration and fermentation. The pyruvate decarboxylase activity in this range increased with decreasing oxygen flow. Below an oxygen flowrate

of 4.3 mmol/l/h, the glucose consumption decreased with a corresponding reduction in growth rate.

Residual levels of PDC were found when *C.utilis* is grown on glucose under fully respiratory conditions. PDC production continued during logarithmic growth while stationary phase cells exhausted of glucose produced 60 % less PDC with low carbon dioxide evolution rates. *C.utilis* grown under aerobic conditions on non-fermentable carbon compounds such as acetate and ethanol experienced a lag in PDC production in addition to lower final levels of activity when transferred to a glucose medium under anaerobic conditions (Franzblau and Sinclair 1983).

C.utilis grown in continuous culture under growth limiting conditions (low glucose) showed low rates of oxygen uptake and carbon dioxide production, which did not vary when the oxygen supply rate was altered. At high glucose levels, both oxygen uptake rate and carbon dioxide production rate increased with oxygen concentration (Moss *et al.* 1969).

Rogers *et al.* (1997) reported that changing to more fermentative conditions by reducing aeration and stirrer speed induced higher levels of PDC activity. It was reported that cultures of *C.utilis* produced a maximum specific decarboxylase activity of 0.9 U/mg protein over 18-20 h with glucose pulse feeding.

Chow (1998) studied PDC activity levels in continuous culture with controlled oxygen delivery. These studies indicated that the level of aeration, controlled by stirrer speed and oxygen flowrate, greatly affected the decarboxylase activity in cells of *C.utilis*. The decarboxylase activity was found to peak sharply over a narrow range of air flowrates.

1.5.1.2 Genetics of Pyruvate Decarboxylase Production

Yeast

Pyruvate decarboxylase genes have been cloned and sequenced from various yeasts including *Saccharomyces cerevisiae* (brewer's and baker's yeast), *Kluyveromyces marxianus*, *Pichia stipitus*, *Kluyveromyces lactis*, *Zygosaccharomyces bisporus* and *Hanseniaspora uvarum*. Depending on the yeast from which the enzyme was isolated, either one (haploid yeast) or two (diploid brewer's yeast) types of enzyme subunits were found (Holloway and Subden 1993; Bianchi *et al.* 1996; Konig 1998; Neuser *et al.* 2000).

In *Saccharomyces cerevisiae*, nucleotide sequences of six genes have been found in a diploid strain, all of which play a role in PDC expression. Three of these genes have been identified as structural genes, *pdc*1, *pdc*5 and *pdc*6. These structural genes have 82 % identical amino acid sequences. Wild type cells produce 85 % of PDC protein from *pdc*1 translation and 15 % from *pdc*5, with *pdc*6 expressed at very low levels. Autoregulation of gene expression exists, with deletion of the *pdc*1 increasing the expression of the *pdc*5. PDC activity is undetectable in mutants lacking both *pdc*1 and *pdc*5. Mutants lacking *pdc*6 show no reduction in specific enzyme activity and deletion of *pdc*6 did not change the phenotype or specific activity of mutants lacking either *pdc*1 or *pdc*5 (Konig 1998). The *pdc*6 gene is weakly expressed, with slightly higher expression during yeast growth in ethanol medium. Deletion of the *pdc*6 caused a reduction in decarboxylase activity when grown on ethanol . Other genes *pdc*2, *pdc*3 and *pdc*4 are thought to encode for regulatory functions. The *pdc*2 gene product was shown by Hohmann (1993) to be involved in the regulation of *pdc*1 and *pdc*5 expression at the transcriptional level. The *pdc*3 gene product is thought to be involved in a postranslational modification step (Killenbergjabs *et al.* 1996).

Three distinct isoforms (α_4,β_4,$\alpha_2\beta_2$) of PDC have been identified from diploid *S.cerevisiae* PDC by SDS-PAGE analysis. The α_4 isoform consists of 4 identical subunits of approximately 57,000 molecular weight each. The β_4 isoform consists of 4 identical subunits of approximately 59,000 molecular weight each. The $\alpha_2\beta_2$ isoform

consists of two α subunits and two β subunits. The three isoforms are mechanistically indistinguishable, with similar Km, Vmax, Hill coefficients and stability. The *pdc*1 gene product has been expressed in *E.coli* and resulted in functional expression of an α_4 homotetrameric enzyme (Candy and Duggleby 1998).

Purification of pyruvate decarboxylase from a haploid yeast strain expressing only one gene (*pdc*1) for PDC was achieved by Killenbergjabs *et al.* (1996). The enzyme resulting from this expression was homotetrameric with a molecular mass of each subunit being 62,000 and had similar kinetic properties to PDC1 from the diploid strain, *Saccharomyces cerevisiae*. Expression of *pdc*1 and *pdc*5 in *E.coli* also was shown to give identical homotetrameric structures. It has been proposed that the various isoforms are the result of proteolytic digestion of the larger subunit. This assumption has been supported by the expression of only homotetrameric PDC of the larger subunit in a strain of *S.cerevisiae* that was protease deficient (Ullrich and Leube 1986). To challenge this assumption, analysis of amino acid content of the α and β polypeptide chains by Zehender and Ullrich (1985) showed that differences in the composition indicated isoenzyme formation. There is some controversy as to whether the different sized enzyme subunits result from isoenzyme production or proteolytic digestion, with isoenzymes identified in some strains of yeast but not in others. A gene encoding the second smaller subunit has not been identified at this stage. The proposed different isoforms have shown little difference with respect to the kinetics of acetaldehyde production from pyruvate, but have shown some variation in stability. The $\alpha_2\beta_2$ arrangement from brewer's yeast was reported to stabilize the enzyme towards both proteolytic degradation and enzyme inactivation, in comparison to the homotetrameric *S.cerevisiae* expressed in *E.coli* (Konig 1998). Additional differences have been identified in the level of acetoin production for the different isoforms (Stivers and Washabaugh 1993).

K.lactis was found to have one gene expressing pyruvate decarboxylase activity in comparison to multiple genes detected in other yeasts.

Two genes Ps*pdc*1 and Ps*pdc*2, encoding for PDC have been isolated from *Pichia stipitis*. At the amino acid and nucleotide level Ps*pdc*1 gene shows 70 % and 63 %

similarity respectively to the Sc*pdc*1 gene with the Ps*pdc*2 gene being less conserved (68 % and 62 % similarity respectively). In relation to each other the two genes are 72 % similar in amino acid sequence and 70 % similar at the nucleotide level. The two *P.stipitus* proteins diverge as far from each other as they do from other known yeast PDC sequences, which suggests that they may play different roles in *P.stipitus* metabolism. A 27 amino acid sequence (amino acids 264-290) was found in PDC from *P.stipitus* that is not present in other known PDC's. The new structure has a similar tertiary structure in the region close to Cys221 which is important for substrate activation in the tertiary structure and may explain the different kinetic regulation of *P.stipitus* and *S.cerevisiae* activities. Based on the three dimensional structure of PDC from *S.cerevisiae* and *H.uvarum*, the loop in the tertiary structure may also form an amphipathic α helix on the surface of the molecule (Lu *et al.* 1997).

Bacteria

Pyruvate decarboxylase genes have been cloned and sequenced from three bacterial sources, *Zymomonas mobilis*, *Sarcina ventriculi* and *Acetobacter pasteurianus* (Konig 1998; Chandra *et al.* 2001; Talarico *et al.* 2001).

Only one structural gene was found for pyruvate decarboxylase from *Zymomonas mobilis*, which results in a homotetramer. The proportion of ordered secondary structures like α-helices and β-sheets is higher than in yeast PDC and may explain the high stability of this enzyme. The arrangement and nature of amino acid side chains at the thiamine diphosphate binding site in the active centre is the same as in yeast, except for His-113 and Tyr-290 (Konig 1998). The dimers are rectangularily arranged at 80 degrees to each other resulting in a densely packed tetramer. All active sites are enclosed in the crystal structure by a C-terminal helix. The C-terminal helix may change its shape thereby opening up the thiamine diphosphate binding site. This movement may play an important role in the catalytic cycle (Konig 1998). In comparison to yeast, *Z.mobilis* PDC had a similar decarboxylation activity optimum at pH 6-6.5, however the enzyme retains its tetrameric structure outside this pH range, even with the removal of cofactors above pH 7.5. The resulting enzyme structure is more stable than yeast PDC in aqueous solution losing little activity over 24 hours at 30 °C (Konig 1998).

The largest difference between *Z.mobilis* PDC and other PDC's is that the enzyme is not subject to allosteric substrate activation. A hyperbolic dependence of reaction rate on pyruvate concentration occurs in comparison to a sigmoidal dependence in yeast (Konig 1998). In addition, *Z.mobilis* PDC differs from yeast with respect to its Km values for both pyruvate and benzaldehyde. A study of *S.carlsbergensis* PDC showed that the Km for pyruvate was 1.2 mM in comparison to 0.6 mM for *Z.mobilis* (30 °C, pH 6.0). The Km values for benzaldehyde were 50 mM and 125 mM for *S.carlsbergensis* and *Z.mobilis PDC* respectively (30 °C, pH 6.0). Shin (1994) determined the Km for *Candida utilis* PDC to be 2.4 mM for pyruvate (25 °C, pH 6.0) and 42 mM for benzaldehyde (4 °C, pH 7.0). These Km values reflect the ability of yeast PDC to produce higher levels of *R*-PAC from benzaldehyde and pyruvate despite the higher decarboxylase activity of the bacterial PDC.

Acetobacter pasteurianus, an obligately oxidative bacterium uses the PDC enzyme in the conversion of pyruvate to acetaldehyde during the oxidative metabolism of lactate to acetate. The translated PDC sequence was found to be most similar to *Z.mobilis* PDC (Chandra *et al.* 2001).

Sarcina ventriculi PDC was found to be most similar to PDC's from fungi and higher plants. The recombinant *S.ventriculi* enzyme displays sigmoidal kinetics, suggesting that it is subject to activation by the substrate pyruvate, a property which is typical for yeast and plant pyruvate decarboxylases. This property is in contrast to that of *Z.mobilis* PDC, which displays Michaelis-Menten kinetics. In addition, *S.ventriculi* PDC shows a Km value for pyruvate of 2.8 mM (pH 6.0, 25°C), which is similar to that of fungal and plant PDC's and higher than that of *Z.mobilis* (Lee and Langston-Unkefer 1985).

Plant

Several plant PDC genes have been sequenced from the orange, zea mays, bean seeds, *Oryza sativa*, sweet potato roots and wheat germ (Oba and Uritani 1975; Raymond *et al.* 1979; Lee and Langston-Unkefer 1985; Leblova and Martinec 1987; Zehender *et al.* 1987; Rivoal *et al.* 1990).

Like yeast systems, three structural genes for pyruvate decarboxylase have been found in germinating pea seeds of *P.sativum*. The purified enzyme contained two subunits of differing types with molecular masses of 63 kDa and 65 kDa. The enzyme was found to be more stable in aqueous solution in comparison to brewers yeast, but similar in its substrate activation properties. *P.sativum* PDC was shown to have a higher affinity for the substrate pyruvate in comparison to yeast PDC. PDC from plants show complex oligiomeric structures with tetramers, octamers and hexadecamers in equilibrium in aqueous solution.

Filamentous fungi

Pyruvate decarboxylase (PDC) forms subcellular filaments in vegetative cells of *Neurospora crassa*, *Neurospora tetraesperma*, *Podospora anserina* and *Sordaria macrospora* (Alvarez *et al.* 1993; Thompson-Coffe *et al.* 1999). In *Neurospora crassa* it has been shown that these PDC filaments consist of homopolmers of a 59 kDA polypeptide. Glucose medium was shown to be a strong inducer of PDC filaments as shown by mRNA and activity levels. *N.crassa* PDC shows a higher homology with bacterial *Z.mobilis* PDC rather than with yeast PDC (Alvarez *et al.* 1993).

Production of *R*-PAC by extracts from several other filamentous fungi indicates the presence of PDC in these strains (Rosche *et al.* 2001, 2002a). Production of *R*-PAC by these strains is discussed in Section 1.5.1.1.

1.5.1.3 Structure of Pyruvate Decarboxylase

The crystal structures of pyruvate decarboxylase have been determined for the yeasts *Saccharomyces cerevisiae* and *Saccharomyces uvarum* and also for the bacterium *Zymomonas mobilis* (Dyda *et al.* 1993; Arjunan *et al.* 1996; Dobritzsch *et al.* 1998).

The crystal structure of PDC has been determined from *S.cerevisiae* using the known amino acid sequence. This structural data provided detailed analysis of protein folding, dimer and tetramer assembly, active sites and also knowledge of tetramer assembly upon substrate activation (Arjunan *et al.* 1996; Lu *et al.* 1997; Furey *et al.* 1998). The

crystal structure determined for *S.cerevisiae* was found to be isomorphous to that determined for *S.uvarum* (Dyda *et al.* 1993). Several different crystal structures have been determined upon binding of substrate analogues which attempt to lock the enzyme into an active form. These different modes of tetramer assembly suggest that the flexibility of the enzyme may have implications in catalysis and regulation (Lu *et al.* 1997; Furey *et al.* 1998).

The enzyme structure determined for pyruvate decarboxylase from *Zymomonas mobilis* identified a similar overall tetramer assembly, active sites and cofactor binding sites. Large differences were found in both the interfacial binding areas between subunits and the packing of the subunits. Smaller structural differences were found in the active site in comparison to the yeast enzyme (Dobritzsch *et al.* 1998).

Subunit structure

In yeast, each PDC subunit consists of a single polypeptide of 563 amino acids in three domains named α, β, and γ. All domains consist of an arrangement of α helixes, β sheets and β strands. A complete subunit of all three domains forms a triangular structure with a cavity at the centre. The β domain is known to be more flexible than the other domains.

Dimer structure

Two subunits bind tightly together between residues in the α and γ domains. Binding of the cofactor magnesium occurs between subunits, with the phosphate end of thiamine pyrophosphate (TPP) binding strongly to magnesium in the γ domain. The aminopyrimidine end of TPP forms an additional association to the γ domain and the α domain of the other subunit. The active site therefore forms at the TPP binding site between subunits with the TPP cofactor buried deeply in a 'V' conformation, which is important for chemical interaction and efficient catalysis. There are two active sites per dimer and four in the overall tetramer.

Tetramer structure

Two dimers associate weakly to form a tetrameric structure, creating an active PDC molecule. The overall structure can be summarized as a dimer of dimers, containing four subunits, four active centers and four bound TPP and Mg^{2+} molecules with a molecular mass of approximately 250 000 Da. The tetrameric structure is shown in Figure 1.2.

A total of 440 water molecules are located in the tetramer, playing important roles in enzyme crosslinking, cofactor binding, hydration layer and reaction mechanisms (Arjunan *et al.* 1996).

By comparison, the *Z.mobilis* PDC contains 2569 water molecules (Dobritzsch *et al.* 1998). Length and orientation of the three domains are different in *Z.mobilis* PDC, with large differences in the amino acid sequences resulting in structural variation in comparison to yeast. The active site of the enzyme is less accessible to solvent molecules due to an increase in amino acid chain length which spans the active site. The contact area between the subunits is larger and includes salt bridges and hydrogen bonds, which results in a more tightly associated dimer-to-dimer interaction than seen in the yeast structure.

The structure of brewer's yeast PDC in an activated state has been determined by crystallizing the enzyme in the presence of the substrate analogue pyruvamide (Lu *et al.* 1997). The dimer packing structure was altered, with an increased angle between dimers resulting in a tetramer that has an increased dimer-to-dimer interaction. Only two of the four active sites were accessible in the activated form, with more studies necessary to confirm these structural changes (Lu *et al.* 1997; Furey *et al.* 1998).

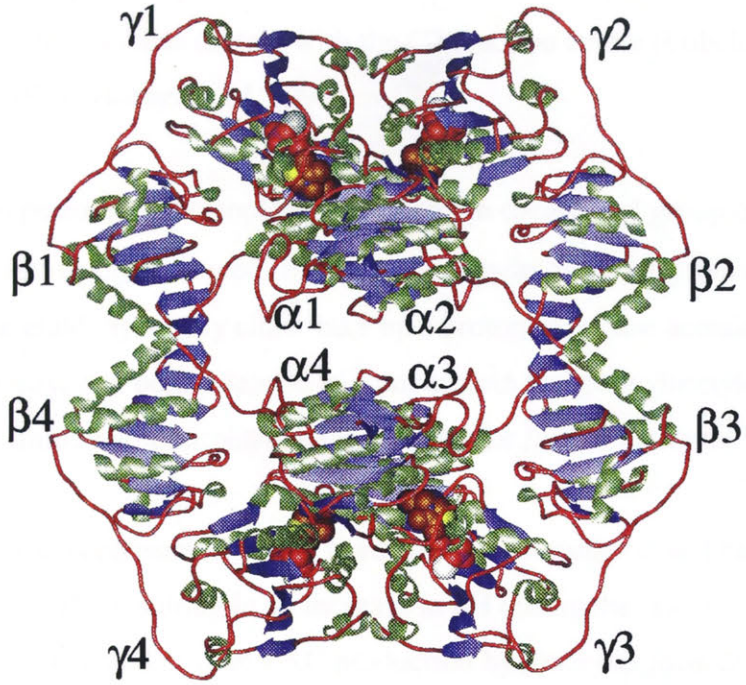


Figure 1.2: Ribbon diagram of a complete PDC tetramer in crystal form, viewed down the crystallographic two-fold axis. TPP and Mg^{2+} are included with a space filling representation (Furey *et al.* 1998).

1.5.2 Decarboxylation and Carboligation Reaction Mechanisms

Lobell and Crout (1996) have carried out molecular modeling studies on *S.uvarum* and *S.cerevisiae* PDC to deduce the reaction mechanisms of decarboxylation and carboligation. Pyruvate decarboxylase catalyses the decarboxylation of pyruvate and subsequent formation of phenylacetylcarbinol by the action of the coenzyme thiamine pyrophosphate (TPP), shown in Figure 1.3. The α-carbon of pyruvate cannot maintain a stable negative charge needed for decarboxylation and TPP serves to catalyze the reaction, with its main role in proton/electron transfer for the decarboxylation process. The pyridyl nitrogen N1', C2 and the 4' amino group are important residues for coenzyme function during decarboxylation and carboligation.

Catalysis begins with extraction of the proton from C2-H in the thiazolium ring, transferring it to the N1' atom in the pyrimidine ring of TPP. The positive charge at the N1' residue is delocalized to the 4' amino group which makes this group able to act as a proton donor in subsequent steps. The 'V' conformation of enzyme bound TPP allows the 4' amino group to associate closely with the C2 reaction centre (Lobell and Crout 1996; Kern *et al.* 1997; Hubner *et al.* 1998).

The C2 carbanion perfoms a nucleophilic attack on the α-carbonyl group of pyruvate to form an α-carbanion-enamine named 'active acetaldehyde' following release of carbon dioxide. Active acetaldehyde may either take up a proton to release acetaldehyde or attack free acetaldehyde or benzaldehyde to form acetoin and phenylacetylcarbinol respectively. Thiamine pyrophosphate is regenerated for further catalysis.

An overall net proton is consumed during pyruvate decarboxylation and carboligation (Lobell and Crout 1996), resulting in an increase in pH during the reaction as shown by Rosche *et al.* (2002a) in studies of *R*-PAC production by *Rhizopus javanicus*.

Pyruvate decarboxylase from *Z.mobilis*, yeast, and filamentous fungi produce *R*-phenylacetylcarbinol in high enantiomeric excess (Pohl 1997; Rosche *et al.* 2001; Rosche 2002a,b).

By-product acetoin formation may result in a mixture of (*R*) and (*S*) enantiomers. Wheat germ and yeast PDC produce acetoin with an enantiomeric excess of 50 % for the (*R*) enantiomer. *Z.mobilis* produces predominantly the (*S*) configuration of acetoin (Lobell and Crout 1996; Iding *et al.* 1998).

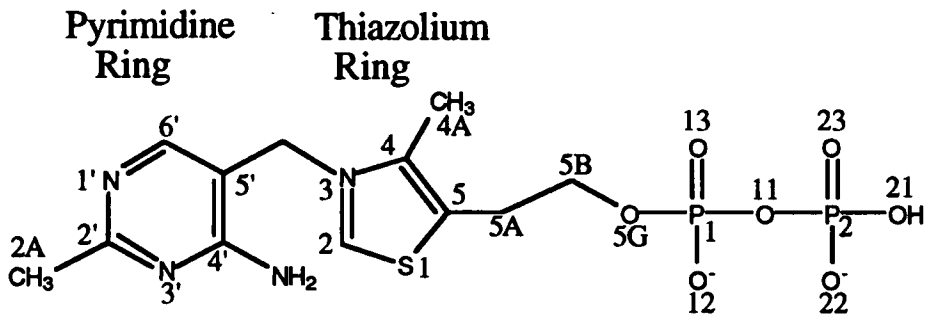


Figure 1.3: Thiamine diphosphate (Furey *et al.* 1998).

1.5.2.1 By-Product Formation

There are several by-products of the fermentation process which are outlined in Figure 1.4. Benzyl alcohol formation from benzaldehyde is a result of oxidoreductase activity (i.e. alcohol dehydrogenase). Benzyl alcohol formation is not seen in purified pyruvate decarboxylase or in cell-free extracts, as active whole cells are required to regenerate NADH necessary for the reduction. Benzaldehyde may be oxidized additionally to benzoic acid on exposure to oxygen.

Acetoin formation is a pyruvate decarboxylase mediated reaction in which 2 moles of pyruvate are converted stoichiometrically firstly to acetaldehyde and then to acetoin. It is reported that acetoin formation occurs due to the build up of excess level of acetaldehyde in the reaction medium (Bringer-Meyer and Sahm 1988).

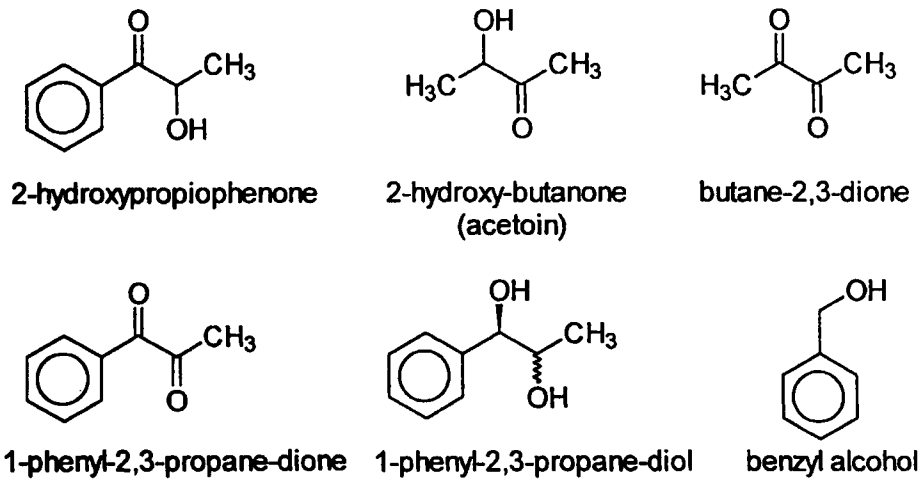


Figure 1.4: By-product formation in the pyruvate decarboxylase catalyzed formation of *R*-phenylacetylcarbinol from pyruvate or acetaldehyde and benzaldehyde (Iwan *et al.* 2001).

1.5.3 Enzyme Modification and Mutation

Pyruvate decarboxylase has been chemically modified and mutated by both site directed and directed evolution techniques for the purpose of studying enzyme reaction mechanisms and for improving stability and activity.

1.5.3.1 Chemical Modification

Pyruvate decarboxylase from brewers yeast was crosslinked using bifunctional reagents. It was found that crosslinking the enzyme using dimethyl glutarimidate and methyl propionimidate in the absence of pyruvate at low ionic strength resulted in an enzyme with a blocked activation mechanism which remained inactive when incubated with pyruvate. Removal of crosslinks restored enzyme activity. Crosslinking with dimethyl adipimidate and dimethyl suberimidate in the presence of high concentrations of pyruvate resulted in permanently activated enzyme. The lag phase of product formation disappeared completely (Konig *et al.* 1990).

The impact of intra subunit crosslinking on enzyme stability was investigated by Dobritzsch *et al.* (1996). The stability of the enzyme at 40 °C when modified with dimethyl adipimidate increased three fold in comparison to the native enzyme.

Pyruvate decarboxylase from brewer's yeast was modified using a N-hydroxysuccinimide ester of an amylose derivative. Thirty of the 68 amino groups were modified and resulted in improved thermostability of the enzyme. The optimum decarboxylation temperature increased from 35 °C to 40 °C, with the enzyme retaining 80 % of its activity when incubated at 45 °C for 20 minutes, in comparison to only 20 % in the unmodified enzyme (Ohba *et al.* 1995).

The effect of chemical modification on *R*-phenylacetylcarbinol formation has not been reported in literature.

1.5.3.2 Mutation

Several amino acids have been exchanged by site-directed mutagenesis in *Saccharomyces cerevisiae* and *Zymomonas mobilis* PDC to determine important residues for activity, regulation and stability (Pohl 1997; Liu *et al.* 2001; Sergienko and Jordan 2001; Wang *et al.* 2001a).

1.5.3.3 Mutation for Improved Carboligase Activity

Z.mobilis PDC has demonstrated superior stability and decarboxylase activity in comparison to *S.cerevisiae* PDC, however the carboligase activity is much less efficient in comparison to the yeast. Several mutations of *Z.mobilis* PDC using both site-directed mutagenesis and directed evolution techniques have been used to improve the efficiency of the carboligase reaction.

A tryptophan residue at position 392 is located at the deep cleft leading to the active site and it was hypothesized by Bruhn *et al.* (1995) that this residue might block access of benzaldehyde to the active site. Mutation in PDC from *Z.mobilis* of this residue to

alanine, by site-directed mutagenesis techniques increased the formation of *R*-PAC from pyruvate and benzaldehyde by a factor of four. A 50 % decrease in decarboxylase activity of the wild type enzyme was noted. Urea denaturation experiments showed that enzyme stability was reduced in comparison to the wild type enzyme (Bruhn *et al.* 1995). Enzyme stability towards the substrate acetaldehyde was improved for mutant enzymes W392M and W392I, with tryptophan replaced by methionine and isoleucine respectively. These enzymes also combine high stability towards acetaldehyde with maximal carboligase activities (Iwan *et al.* 2001).

Significant amino acid sites that affect the size of the substrate binding site and the enantioselectivity of the product *R*-PAC have been determined by Pohl *et al.* (1998) to be isoleucine residues I472 and I476. The stereochemistry of *R*-PAC formation was impaired with mutant PDCI476E, which produced *S*-PAC in comparison to *R*-PAC by the wild type enzyme. Glutamate residue E473 was found to influence enzyme activity, with no catalytic activity detected in mutants with this residue exchanged for other amino acids.

Successive mutations of *Z.mobilis* PDC by directed evolution error prone PCR techniques improved the specific carboligase activity and stability of the enzyme towards acetaldehyde. In addition saturation mutagenesis at position 392 produced several mutants with altered specific carboligase activity and stability towards acetaldehyde. However these mutants did not lead to an improvement in *R*-PAC production (Breuer and Hauer 2001).

1.5.4 *R*-PAC Formation using Whole Cells

1.5.4.1 Microorganisms for *R*-PAC Formation

Production of *R*-PAC has been reported for several yeasts, bacteria and filamentous fungi. In general it is accepted that pyruvate decarboxylase in each of these organisms is responsible for *R*-PAC formation, with no other enzyme catalyzing its formation. In an early study, initial rates and final yields of *R*-PAC production by 38 strains of yeast mostly from the genera *Saccharomyces*, *Candida* and *Pichia* were evaluated.

High initial rates of production were followed by rapid cessation in production. The results showed that initial rate measurement did not accurately reflect the overall performance of the yeasts. The highest final level of 6.3 g/l *R*-PAC was achieved with *Saccharomyces carlsbergensis*. Other promising strains were *Hansenula* sp. and *Candida* sp. producing maximum *R*-PAC concentrations of 5.9 g/l and 2.8 g/l respectively (Netrval and Vojtisek 1982).

R-PAC formation has been observed using whole mycelia of six strains of filamentous fungi (*Rhizopus javanicus* NRRL 13161 and NRRL 2871, *Rhizopus oryzae* NRRL 6201 and NRRL 1501, *Aspergillus oryzae* NRRL 694 and, *Aspergillus tamarii* NRRL 429). Very low *R*-PAC concentrations between 0.2-0.7 mM were formed in 12 hours in shake flask culture at 30 °C (Rosche *et al.* 2001). Production of *R*-PAC by fermenting *R.javanicus* was achieved using stepwise feeding of benzaldehyde. In 8 hours, 2.9 g/l *R*-PAC was produced, with large losses of benzaldehyde to benzylalcohol formation (Rosche *et al.* 2001).

Torulopsis glabrata is reported to produce up to 30 g/l *R*-PAC, with lower concentration of 6.83 g/l achieved with *Torulopsis delbrueckii* (Miyata 2000; Shukla and Kulkarni 2001).

R-PAC production by *Candida utilis* has been extensively studied by Shin (1994), Shin and Rogers (1995) and Rogers *et al.* (1997). Further details are provided in Section 1.4.5.2

1.5.4.2 Reactor Designs for *R*-PAC Formation using Whole Cells

R-PAC has been produced in a batch manner with benzaldehyde delivered at the beginning of the biotransformation, or after a period of cell growth and enzyme induction. This method has been widely employed in screening protocols. Batch production is hindered by cell toxicity due to benzaldehyde and loss of benzaldehyde to the byproduct benzyl alcohol. Many different approaches including fed batch, cell immobilization, aqueous-organic two phase systems and membrane recycle have been investigated for the development of a process to minimize these effects.

A wide range of polymeric materials were evaluated by Nikolova and Ward (1994) as support matrices for *R*-PAC formation from baker's yeast. Cells immobilized using ENT-4000 produced 0.56 g/l *R*-PAC in comparison to 0.98 g/l achieved using free cells. The change in level of benzyl alcohol formation in comparison to *R*-PAC was effected by the different properties of the support.

Aqueous organic two-phase systems have been evaluated to deliver higher levels of sparingly soluble substrate benzaldehyde. It was demonstrated by Nikolova and Ward (1992a,c) that whole cells of bakers yeast adsorbed onto celite could produce *R*-PAC in a range of organic solvents with 0.5-20 % moisture content. The highest level of *R*-PAC formation was achieved with hexadecane with 10 % moisture, in a 6 hour process yielding 0.45 mg/ml *R*-PAC. Damage to the cell structure was found to be a limiting factor with other solvents examined. Smallridge *et al.* (2001b) demonstrated that *R*-PAC could be synthesized from benzaldehyde and pyruvic acid in organic solvent by non-fermenting cells of *Saccharomyces cerevisiae*. An aqueous phase consisting of 0.6-1.2 ml buffer per gram of yeast was present, however only 1 g/l *R*-PAC was produced. It has also been demonstrated that *R*-PAC can be synthesized in petroleum spirit using lactic acid as the acyloin precursor. Lactic acid was oxidized to pyruvic acid, which was condensed with benzaldehyde to form *R*-PAC. The process resulted in lower by-product formation with easier product recovery (Laurence *et al.* 2001).

Production of *R*-PAC from pyruvate and benzaldehyde by concentrated cells of *Saccharomyces cerevisiae* has been achieved in liquid and supercritical carbon dioxide and in liquid petroleum gas. Increased yields of up to 84 % *R*-PAC on benzaldehyde fed were achieved in comparison to conventional fermentation that achieves approximately 70 %. Complete inhibition of benzyl alcohol by-product formation was achieved (Smallridge *et al.* 2001a).

A membrane bioreactor was investigated by Liew *et al.* (1995) for continuous production of *R*-PAC using *Candida utilis*. High growth rates and biomass concentrations were achieved, however this contributed to reduced permeate flux. The addition of 3 ml/h of benzaldehyde for 17 hours in a 3.3 litre bioreactor resulted in significant cell death. *R*-PAC levels achieved in this system were not reported.

β-cyclodextrans that were added to the fermentation medium of immobilized *Saccharomyces cerevisiae* were found to reduce the toxic effect of benzaldehyde and stimulate cell growth. Stimulated cell growth was thought to be due to the reduced toxic effect of benzaldehyde in this system. An increase in the initial rate of *R*-PAC production occurred in comparison to the control with no β-cyclodextran added, with a maximum final *R*-PAC level of 12 g/l achieved in 12 hours. The control produced a maximum of 9 g/l in a 24 hour process. The most promising result from this study was that the theoretical yield of *R*-PAC based on fed benzaldehyde was increased from 57 % in the control to 77 % in the process incorporating β-cyclodextran (Mahmoud *et al.* 1990a).

Mahmoud *et al.* (1990b) designed a batch reactor using alginate immobilized cells of *Saccharomyces cerevisiae*. It was found that the toxic effect of benzaldehyde was reduced, with correspondingly more *R*-PAC produced than free cells. A maximum of 5.5 g/l *R*-PAC was achieved in 9 hours. The minimization of benzaldehyde toxicity and immobilization of cells allowed repeated use of the cells over 7 cycles spanning 200 hours. Continuous feeding of benzaldehyde promoted higher production of *R*-PAC in comparison to sequential, intermittent doses. 10 g/l *R*-PAC was achieved over 6 hours feeding 0.8 ml/h of benzaldehyde in a 0.5 litre bioreactor (Mahmoud *et al.* 1990c).

In comparison to Mahmoud *et al.* (1990b,c), *Candida utilis* cells that were immobilized in calcium alginate achieved 15.2 g/l *R*-PAC in 22 hours (Shin and Rogers 1995). The respiratory quotient was controlled between 5-7 with benzaldehyde maintained at a concentration of 2 g/l. It was also found that cell immobilization improved cell resistance to benzaldehyde toxicity. It was demonstrated that cessation in *R*-PAC production was due to depletion of pyruvate and loss of metabolic activity. However the productivity of the continuously operated process was only 0.6 g/l/h of *R*-PAC and was hindered by low pyruvate decarboxylase activities, mass transfer limitations and loss of benzaldehyde to benzyl alcohol. Park and Lee (2001) also assessed *R*-PAC production by calcium alginate encapsulated *Saccharomyces cerevisiae* and confirmed the protective effect of immobilization against benzaldehyde toxicity.

A three stage bioreactor process was reported by Rogers *et al.* (1997) that consisted of a fully aerobic stage designed to increase biomass. A second partially fermentative stage with additional glucose feeding accumulated pyruvate and induced PDC. A third continuous biotransformation stage incorporated controlled benzaldehyde feeding to reduce toxicity to cells and maximize *R*-PAC formation. Low level glucose feeding was performed in stage three to ensure yeast metabolic activity. Respiratory Quotient was used to control the metabolic state of the cells of *Candida utilis* in stage 2. In this system 10.6 g/l *R*-PAC was achieved 300 hours after benzaldehyde feeding commenced and the maximum productivity was 0.44 g/l/h.

Batch and fed batch biotransformation using the yeast *Torulaspora delbrueckii* for *R*-PAC production has been reported recently (Shukla and Kulkarni 2001). A 24 hour old culture of the yeast was suspended into the biotransformation medium supplemented with glucose and peptone and also containing benzaldehyde and/or acetaldehyde. With batch operation, 3.31 g/l *R*-PAC was achieved in 2 hours from 6 g/l benzaldehyde fed initially. Addition of 0.6 % acetaldehyde increased *R*-PAC levels to 4.5 g/l. Acetaldehyde acts as a substrate for alcohol dehydrogenase in preference to benzaldehyde, thereby reducing levels of benzyl alcohol formation, a method also used by Shukla *et al.* (2001). Semi-continuous feeding benzaldehyde and acetaldehyde increased *R*-PAC levels to 6.83 g/l. The advantage of this approach was that cell mass could be reused up to 9 times when benzaldehyde and acetaldehyde levels were maintained at low levels.

Shukla *et al.* (2001) evaluated the biotransformation of benzaldehyde to *R*-PAC by whole cells of *Saccharomyces cerevisiae*. The gas liquid transfer coefficient in the fermenter (k_La) was used as the critical parameter when scaling up from shake flask culture to a 5 litre fermentor. At the larger scale, more cell mass was achieved which resulted in a greater yield of *R*-PAC. Correlations for the mass transfer coefficients were developed for both cell growth and biotransformation phases, and were used to determine impeller speed and air flow rates for the larger scale. Higher initial *R*-PAC production rates and lower benzyl alcohol formation were achieved in 5 litre operation in comparison to shake flask culture, although final concentrations were similar at both scales. After 2 hours, 4.6 g/l *R*-PAC was achieved at the 5 litre scale in comparison to 3.3 g/l in the shake flask from an initial benzaldehyde level of 6 g/l. Whole cell

bioreactors for production of significant concentrations of *R*-PAC are compared in Table 1.1.

Table 1.1: Whole cell bioreactors for production of significant concentrations of *R*-PAC.

Catalyst	Process	*R*-PAC (g/l)	Productivity (g/l/h)
S.cerevisiae (Mahmoud *et al.*, 1990a)	β-cyclodextrans added to fermenting immobilized cells	12.0	24.0
S.cerevisiae (Mahmoud *et al.*, 1990b)	Calcium alginate immobilized cells with slow benzaldehyde feeding	10	40
Fermenting cells of *C.utilis* (Shin and Rogers 1995)	Cells immobilized in calcium alginate with slow benzaldehyde feeding	15.2	16.6
C.utilis (Rogers *et al.* 1997)	Continuous three stage bioreactor for generation of biomass, induction of pyruvate decarboxylase activity and formation of *R*-PAC with controlled benzaldehyde feeding	10.6	0.44
Free-cells of *C.utilis* (Rogers *et al.* 1997)	Three stage fed batch bioreactor	22.0	1.6
24 hour old culture of *T.delbrueckii* (Shukla and Kulkarni 2001)	Semi-continuous feeding of benzaldehyde and acetaldehyde	6.83	33.0
Free-cells of *T.glabrata* (Miyata 2000)	Pyruvate accumulation phase, followed by enzyme induction and *R*-PAC formation.	30.2	-

1.5.5 *R*-PAC Formation by Crude extracts/Cell-Free Preparations of Pyruvate Decarboxylase

R-PAC formation using whole cell catalysis is limited by the loss of benzaldehyde substrate to benzyl alcohol by the action of oxidoreductases requiring NADH (i.e. alcohol dehydrogenase). This results in reduced yields of *R*-PAC on benzaldehyde consumed and limits the level of *R*-PAC obtainable.

By isolating the enzyme from the cell, the ability to regenerate NADH is lost and the action of the oxidoreductases is therefore removed. The loss of benzaldehyde to by product benzyl alcohol is eliminated. However, it is necessary to supply pyruvate or acetaldehyde to the enzyme when it has been removed from the cell.

1.5.5.1 Sources of Pyruvate Decarboxylase for Cell Free *R*-PAC Formation

R-PAC has been produced using cell free extracts of bacteria, yeast and fungi as described below.

Pyruvate decarboxylase from *Zymomonas mobilis* and *Saccharomyces carlsbergensis* partially purified by gel filtration were shown by Bringer-Meyer and Sahm (1988) to produce phenylacetylcarbinol from benzaldehyde and pyruvate. *Z.mobilis* showed five times more decarboxylase activity than the yeast, but produced 4-5 times less phenylacetylcarbinol. This was attributed to a lower affinity of *Z.mobilis* for the hydrophobic substrate benzaldehyde. The efficiency of the *R*-PAC formation from benzaldehyde and pyruvate was improved by site directed mutagenesis of *Z.mobilis* PDC as described by Bruhn *et al.* (1995). Mutated purified PDC from *Z.mobilis* showing improved stability to acetaldehyde is capable of synthesizing *R*-PAC from acetaldehyde and benzaldehyde in an enzyme membrane reactor (Iwan *et al.* 2001).

Crout *et al.* (1991) showed that yeast PDC could catalyze a wide range of acyloins from aliphatic, aromatic and heterocyclic aldehydes. *R*-PAC was produced from pyruvate

and benzaldehyde and identified by GC-MS and NMR. Young and Ward (1989) also produced *R*-PAC from baker's yeast PDC using homogenized cells supplemented with benzaldehyde and cofactors magnesium and thiamine pyrophosphate. Partially purified PDC and immobilized PDC from *Candida utilis* have been shown by Rogers *et al.* (1997) to produce high levels of *R*-PAC from pyruvate and benzaldehyde.

Biotransformation of pyruvate and benzaldehyde to *R*-PAC was achieved using crude extracts from 14 strains of filamentous fungi (Rosche *et al.* 2001). *R*-PAC was produced in all 14 strains with high enantiomeric excess. The most promising strains were *Rhizopus javanicus* and *Fusarium* sp. *R*-PAC production with *R.javanicus* PDC is further described in Section 1.5.5.2.

An extensive screen of crude extracts from a range of yeast species by Rosche (2002b), identified that *R*-PAC production from benzaldehyde and pyruvate and the stability of carboligase activity towards acetaldehyde was the most promising with crude extracts from *Candida* sp. No *R*-PAC was produced from benzaldehyde and acetaldehyde by any of the cell free extracts. It has been speculated that this activity might be specific to prokaryotic PDC.

1.5.5.2 Factors Affecting *R*-PAC Formation by Cell Free Preparations of Pyruvate Decarboxylase

Effect of benzaldehyde and *R*-PAC on enzyme stability

Shin (1994) showed that *Candida utilis* PDC activity was drastically reduced with increasing benzaldehyde concentration and was diminished more gradually in the presence of *R*-PAC. The toxicity of benzaldehyde was 2.5 times higher than that of *R*-PAC on a molar basis, in a concentration range of 50-200 mM.

A kinetic model was developed for the deactivation of *Candida utilis* PDC by benzaldehyde emulsions in the range of 100-300 mM benzaldehyde (Chow *et al.* 1995; Chow 1998). Sensitivity analyses showed that the enzyme decay constant (k_d) had a significant effect on *R*-PAC production (Chow 1998).

The deactivation effect of benzaldehyde, *R*-PAC and benzyl alcohol on enzyme activity has also been noted for PDC isolated from *Saccharomyces cerevisiae* (Bringer-Meyer and Sahm 1988; Long and Ward 1989).

Effect of acetaldehyde on *R*-PAC formation and enzyme stability

A decrease in initial reaction rates and final concentrations of *R*-PAC occurred in the presence of increasing levels of free acetaldehyde. In the presence of 50 mM acetaldehyde, an 85 % reduction in initial reaction rate and an 18 % reduction in final *R*-PAC level was seen after bioconversion of 70 mM pyruvate and 70 mM benzaldehyde by 7 U/ml decarboxylase activity from *Candida utilis* at 4 °C (Shin 1994; Shin and Rogers 1996b).

Inhibition of baker's yeast PDC decarboxylation rates by acetaldehyde has been reported also by Juni (1961). Inhibition of decarboxylation was reversible upon removal of acetaldehyde. It was demonstrated that acetaldehyde was inhibitory to pyruvate decarboxylation, but not an inactivating influence on the enzyme.

High levels of irreversible enzyme inactivation due to acetaldehyde have been reported for *Zymomonas mobilis* PDC (Bruhn *et al.* 1995; Goetz *et al.* 2001; Iwan *et al.* 2001).

Effect of reaction temperature on *R*-PAC formation.

A comparison of biotransformation temperatures by *Candida utilis* PDC by Shin (1994) and Shin and Rogers (1996b), determined that *R*-PAC production was maximal at 4 °C in comparison to reactions at 10 °C and 25 °C. Final *R*-PAC concentration after an 8 hour reaction period at 4 °C was higher compared to the reaction at 10 °C, however by-product acetoin and acetaldehyde levels were slightly higher at 10 °C. At 25 °C, final *R*-PAC levels were lower and byproduct acetaldehyde formation was significantly higher.

Initial *R*-PAC production rates by *Rhizopus javanicus* PDC were higher at 23 °C in comparison to 6 °C, however higher final *R*-PAC concentrations were achieved at 6 °C (Rosche *et al.* 2002a).

Effect of ethanol on enzyme stability and *R*-PAC formation

The use of organic cosolvent ethanol in the reaction medium by Shin (1994) and Shin and Rogers (1996b), was shown to increase initial rates of *R*-PAC formation by *Candida utilis* PDC in a phosphate buffer at 4 °C up to a level of 3 M ethanol. The initial rate achieved in the presence of 3 M ethanol was 140 % of the control with no ethanol added. It was also found that ethanol had a positive effect on enzyme stability up to 3 M, with an optimum concentration of 2 M for maintenance of enzyme activity.

Addition of ethanol to the reaction medium (0.05-2 M) did not have a beneficial effect on *R*-PAC formation by *Rhizopus javanicus* PDC (Rosche *et al.* 2002a).

Effect of pH on *R*-PAC and by-product formation

R-PAC production by *Candida utilis* PDC was strongly dependent on the pH of the reaction medium. *R*-PAC production was optimal at pH 7.0, with by-product acetaldehyde production optimal at pH 6.0. Quantitative comparison of reaction rates showed that the rate of *R*-PAC production at pH 7.0 was three times higher than at pH 6.0. Acetaldehyde production at pH 7.0 was half that at pH 6.0 (Shin 1994; Shin and Rogers 1996b).

A similar trend for *R*-PAC formation was noted for *Z.mobilis* PDC when pyruvate and benzaldehyde were carboligated to *R*-PAC. An optimum for carboligation (*R*-PAC formation) occurring at pH 7.0, with an optimum for the decarboxylation of pyruvate occurring at pH 5.0 (Iwan *et al.* 2001). The pH optimum for PDC-catalyzed carboligation of acetaldehyde and benzaldehyde was in the range of pH 6.5-7.0 (Goetz *et al.* 2001). Similarly, *R*-PAC formation was optimum at pH 6.5-7.0 for *Rhizopus javanicus* PDC, with a dramatic decrease in reaction rates above pH 7.0 (Rosche *et al.* 2002a).

Effect of enzyme activity, benzaldehyde concentration and ratio of pyruvate to benzaldehyde on *R*-PAC formation.

Initial *R*-PAC formation rates determined for *Candida utilis* PDC by Shin (1994) and Shin and Rogers (1996b) were greatly dependent on decarboxylase activity and benzaldehyde concentration. The reactions were conducted with equimolar ratios of pyruvate to benzaldehyde concentration in phosphate buffer containing 2 M ethanol at pH 7.0 and 4 °C. The highest initial reaction rate was achieved with 120 mM of substrates with a starting decarboxylase activity of 10 U/ml, however the highest final *R*-PAC concentration was achieved with 150 mM substrates at 7 U/ml decarboxylase activity. At 10 U/ml decarboxylase activity, pyruvate was converted into by product acetaldehyde in preference to *R*-PAC. Final *R*-PAC yields as a function of the molar ratios of pyruvate to benzaldehyde showed that an increase in molar ratio was accompanied by an increase in yield of *R*-PAC on benzaldehyde added. At ratios above 1.5, the final yield based on benzaldehyde added was not increased further and remained constant at close to 100 %. The higher fraction of pyruvate was presumably converted to by-products or remained in excess concentration in the reaction medium (Shin 1994; Shin and Rogers 1996b).

A study of *Rhizopus javanicus* PDC showed that *R*-PAC formation increased with carboligase activity reaching a maximum of 50 g/l from 7.8 U/ml in 28 hours at 6 °C from 406 mM benzaldehyde and 600 mM pyruvate in 2 M MOPS buffer. The yield of *R*-PAC per amount of enzyme reached a maximum at 1-3 U/ml and decreased significantly at higher levels of carboligase activity (Rosche *et al.* 2002a).

Effect of pH and buffer species on enzyme stability

The stability of *Candida utilis* PDC which was purified by ammonium sulfate precipitation and gel filtration was evaluated by Shin (1994) and Shin and Rogers (1996b) in a variety of buffers. Enzyme stability was strongly dependent on buffer species and presence of cofactor thiamine pyrophosphate (TPP). The enzyme was most stable in sodium citrate and phosphate buffers supplemented with 30 μM TPP, with a half-life of 28 hours at 25 °C and pH 6. Enzyme inactivation was greater in the absence of TPP. This loss of activity is consistent with previous reports which established that

the heterologous *Saccharomyces cerevisiae* PDC tetramer ($\alpha_2\beta_2$) dissociated into dimers and lost activity upon the release of cofactors TPP and magnesium ions. This dissociation was predominantly a function of pH but was strongly affected also by buffer species and appeared to be more evident in Tris-Cl buffers in comparison to phosphate and citrate buffers (Gounaris *et al.* 1971). The stability of *Saccharomyces cerevisiae* PDC was affected by the pH of the buffer medium and incubation temperature. Within the pH range of 5.5-6.5, the enzyme preparation was the most stable. Enzyme inactivation was rapid at pH values above 7 and below 5.5. Optimum stability was achieved at pH 6 in a phosphate buffer at 4 °C, with a half life of 110 hours in comparison to 28 hours at 25 °C (Gounaris *et al.* 1971).

It has been previously reported by Hopmann (1980), Konig *et al.* (1993) and Gounaris *et al.* (1971) that binding of cofactor TPP is strongly pH dependent. In yeast PDC, the tetramer dissociates with loss of cofactors into an unreactive dimer below pH 6.0, exists as a reactive tetramer between pH 6.0- 8.0, and as an unreactive dimer above pH 8.0. These pH ranges have been found to vary according to buffer species (Gounaris *et al.* 1971). The dimers could be reconstituted in the presence of Mg^{2+} and excess TPP by adjusting the pH to 6.5. The activity recovered depended on the length of time exposed to pH 8.0.

PDC from *Z.mobilis* exhibited low stability in buffers with pH less than 5.5 and greater than 8.5, while it was more stable in neutral and weakly alkaline buffers in the presence of cofactors (Pohl *et al.* 1995).

Stability PDC from *Rhizopus javanicus* increased 7-fold with an increase of MOPS concentration from 0.05 M to 2 M. The half life was 13.5 hours in 2 M MOPS at pH 7 and 4 °C with an initial enzyme carboligase activity of 10 U/ml. 2 M MOPS had been selected for buffering the reaction at pH 7.0 with high initial substrate concentrations (Rosche *et al.* 2002a).

1.5.5.3 Reactor Design for *R*-PAC Formation using Cell Free Extracts of Pyruvate Decarboxylase

The kinetics of the biotransformation using partially purified PDC from *Candida utilis* has been described, with benzaldehyde delivered as an emulsion in a batch reactor. In an 8 hour process, 191 mM (28.6 g/l) *R*-PAC was achieved from 200 mM benzaldehyde and 400 mM pyruvate using 7 U/ml decarboxylase activity. Enzyme inactivation due to benzaldehyde and *R*-PAC and product inhibition due to *R*-PAC and acetaldehyde were identified as the main factors limiting further product formation. Incorporation of the ethanol as a cosolvent in this batch reactor increased initial reaction rates (Shin and Rogers 1995). In contrast, for *Rhizopus javanicus* PDC it was found that addition of ethanol had no beneficial impact on initial rates or final yields of *R*-PAC formation (Rosche *et al.* 2002a).

Further developments using *Candida utilis* PDC immobilized in spherical polyacrylamide beads improved enzyme stability towards benzaldehyde. Despite improvements in enzyme stability, less *R*-PAC was produced than in the free enzyme with similar substrate concentrations. Immobilized enzyme was exposed to higher levels of benzaldehyde up to 300 mM forming 181 mM (27.1 g/l) *R*-PAC from 450 mM pyruvate in 14-20 hours. A reduction in the yield of *R*-PAC on pyruvate indicated high by-product formation. It was suggested that mass transfer of benzaldehyde to the enzyme was limited by the immobilization matrix (Shin 1994; Shin and Rogers 1995).

A continuous process for *R*-PAC formation based on immobilized PDC was further developed by Shin and Rogers (1996a). The immobilized enzyme was contained in a glass column and fed low levels of pyruvate and benzaldehyde. The continuous biotransformation with 100 mM pyruvate and 50 mM benzaldehyde at space-time of 8 hours resulted in 30 mM (4.5 g/l) *R*-PAC at 3.7 mM/h productivity and 60 % molar conversion yield based on inlet benzaldehyde.

A continuously operated enzyme membrane reactor for synthesis of *R*-PAC from acetaldehyde and benzaldehyde has been described by Goetz *et al.* (2001) and Iwan *et al.* (2001). Enzyme was retained in a polypropylene reactor by a semi-permeable

membrane, allowing residual substrates and products to pass through. Mutant *Z.mobilis* pyruvate decarboxylase showing improved stability and activity over the wild type enzyme achieved 81 g *R*-PAC/ (L.day) from a solution containing 50 mM of both aldehyde substrates. The reactor was operated for 40 days without significant enzyme inactivation. *R*-PAC formation from pyruvate and benzaldehyde in this system achieved 27.4 g *R*-PAC/ (L.day). A cascade of enzyme membrane reactors achieved an increase in *R*-PAC concentration in comparison to a single enzyme membrane reactor. The final *R*-PAC concentration achieved with a single EMR was 3.3 g/l, with a 22 % yield of *R*-PAC on substrates fed. A two-stage cascade EMR was used to increase the yield to 45 %.

Enzymatic cell-free systems for *R*-PAC production are summarized and compared in Table 1.2.

Table 1.2: Enzyme-based cell-free systems for *R*-PAC production

Catalyst	Process	*R*-PAC (g/l)	Productivity (g/l/h)
Candida utilis PDC partially purified immobilized (Shin 1994).	Emulsion batch reactor using substrates pyruvate and benzaldehyde.	27.1	1.6
Candida utilis PDC partially purified immobilized (Shin 1994).	Emulsion batch reactor operated continuously using substrates pyruvate and benzaldehyde.	4.5	0.6
Candida utilis PDC partially purified (Shin 1994).	Emulsion batch reactor using substrates pyruvate and benzaldehyde.	28.6	3.6
Rhizopus javanicus PDC partially purified (Rosche *et al.* 2001)	Emulsion batch reactor using substrates pyruvate and benzaldehyde, with optimized buffer conditions for improved stability and pH control.	50.6	1.7
Mutant *Z.mobilis* PDC crude extract (Goetz *et al.* 2001; Iwan *et al.* 2001)	Continuously operated enzyme membrane reactor (EMR) using acetaldehyde and benzaldehyde as substrates	3.3	3.4
Mutant *Z.mobilis* PDC purified (Goetz *et al.* 2001; Iwan *et al.* 2001)	Continuously operated EMR using pyruvate and benzaldehyde as substrates	3.3	1.1

1.6 Advances in Biocatalysis

Despite the specificity of enzyme-mediated reactions, several of the following problems have been identified as general limitations of industrial biocatalysis using either whole microorganisms or isolated enzymes.

- Poor substrate and/or product solubility.
- Instability of products. Products may be further metabolized by competing reactions.
- Enzyme mediated reactions are usually inhibited by their own substrates and products, which can severely limit the productivity of a process.
- Enzymes can be unstable in an industrial environment due to elevated temperatures, toxicity of substrates and/or reaction media.
- Many enzymes require expensive cofactors, which can be difficult to obtain in bulk quantities as well as presenting a large cost barrier.

The most significant and widespread problem in enzyme catalysis is the instability of enzymes once they are removed from their cellular environment. In the past decade there has been several large advances in the area of biocatalysis to minimize this problem. The developments of enzyme immobilization, chemical modification, catalysis in non-aqueous media and more recently directed evolution techniques have in some cases improved the stability of catalysts, expanded their substrate ranges, overcome operational difficulties with insoluble substrates and enhanced several other characteristics which have improved the transfer of laboratory biocatalysis to a larger scale manufacturing environment (Zaks 2001).

1.6.1 Directed Evolution

The application of native enzymes as industrial biocatalysts has been increased due to improved understanding of enzyme stability and activity in non-conventional media. Tailoring enzyme properties for use in these reaction media has been achieved by both site-directed mutagenesis and directed-evolution techniques.

Directed evolution techniques have been successfully used to improve the operational capabilities of many enzymes. Advances in gene shuffling made by companies such as Diversa and Maxygen have led to techniques where multiple properties of an enzyme can be optimized simultaneously and rapidly. In comparison to site-directed mutagenesis, no structural knowledge of the enzyme is required (Arnold 2000; Tobin *et al.* 2000). Directed evolution techniques have improved some enzymes to give increased thermostability, increased stability in solvents and increased enantiomeric excess of the product (Iverson and Breaker 1998; Schmidt-Dannert and Arnold 1999).

1.6.2 Non-Aqueous Media

The use of hydrophobic substrates in enzyme catalysis has seen the development of reaction media designed to improve solubility of these compounds thereby allowing increased productivity. It has been shown that enzymes are active in a wide range of reaction media including aqueous media containing cosolvents, enzymes in pure organic solvent two-phase systems, reverse micelles, ionic liquids and supercritical fluids.

1.6.2.1 Organic Cosolvent Addition

Water miscible organic solvents have been incorporated into aqueous reaction media as means to increase enzyme stability and improve substrate solubility. Enzymes can maintain activity or may increase activity in the presence of these solvents until a particular concentration is reached, above which enzyme inactivation begins. The inactivation may be reversible or irreversible and the concentration of solvent at which inactivation begins is strongly dependent on the enzyme and solvent (Khmelinitsky *et al.* 1991b; Gupta *et al.* 1997).

Enzyme immobilization and chemical crosslinking have been shown to improve enzyme stability in miscible solvents (Sobolov *et al.* 1994; Tyagi *et al.* 1999).

1.6.2.2 Monophasic Organic Solvent Systems

Enzyme catalysis has been achieved in organic solvents where there is no aqueous phase present. The role of enzyme-associated water, nature of biocatalyst preparation and properties of the organic solvent are important considerations for enzyme stability and activity in these systems. Due to the absence of a water phase, these systems are predominately used where substrates and products are hydrophobic in nature and therefore soluble in organic solvents. This approach can also be used to limit water-dependent competing side reactions and has been successful in shifting thermodynamically unfavourable reverse hydrolysis reactions e.g. the formation of esters.

Immobilization of enzymes on glass beads or celite and modification of enzymes for improved solubility in organic solvents has improved the diffusional limitations of this reaction medium (Dordick 1989; Yamane *et al.* 1990; Jene *et al.* 1997).

Maintenance of a water layer around the enzyme is important for activity in monophasic solvent systems (Dordick 1989). Lyophilization of proteins in trehalose, sorbitol, sucrose, anionic detergents and simple non-buffer salts can enhance the hydration level and flexibility of the macromolecules while also providing a stabilizing matrix. Lyoprotectants and salts affect the structure of the solvent that surrounds the enzyme, inducing positive effects on stability and activity (Dabulis and Klibanov 1993; Skrika-Alexopoulos and Freedman 1993; Triantafyllou *et al.* 1997).

1.6.2.3 Reverse Micelles

Reverse micelles are ternary structures of nanometer-sized water droplets containing enzyme which are dispersed in an organic phase by the action of a surfactant. The mixtures are optically transparent on the macroscopic level, however a microenvironment exists in which the enzyme activity and stability is dependent on an array of factors including surfactant type, water activity or hydration level, pH,

temperature and size of the reverse micelles (Sanchez-Ferrer and Garcia-Carmona 1994; Ballesteros *et al.* 1995; Adlercreutz 1996).

The use of reverse micelles in enzyme catalysis is well documented and widely used as a common strategy in situations where water minimization is critical and where substrates and/or products are hydrophobic.

Enzyme molecules in reverse micelles are stabilized by preventing direct contact of the enzyme with the organic solvent. The interior of the reverse micelle provides a favourable environment for enzyme activity and large interfacial area to improve reaction rates. In one example of their use, lipases catalyze the hydrolysis of triacylglycerols *in vivo* and are capable of catalyzing the reversal synthetic reactions of esterification as well as transesterification (Ballesteros *et al.* 1995; Carvalho and Cabral 2000).

1.6.2.4 Ionic Liquids

Ionic liquids are salts that are liquid at room temperature and have variable chemical and physical properties influenced by simple structural modifications to the cations or anions. These allow the solvent to be tailored for a particular process where solubility of reactants or products is an issue. Ionic solvents have no measurable vapour pressure, which makes them ideal replacements for situations where volatile toxic organic solvents are not desirable (Cull *et al.* 2000). Reactions in ionic liquids show some advantages including increased product yields and in some cases an increase in enantioselectivity (Kaftzik and Kragl 2001).

1.6.2.5 Supercritical Fluids

A supercritical fluid is defined as any substance existing above its critical temperature and critical pressure. In the critical region, the substance exhibits properties of both a gas and a liquid, with one phase present. Around the critical point, a large range of physical properties are achievable through variation of temperature and pressure. These

properties are used to manipulate reaction rates and solubility of substrates and products in catalysis.

Supercritical fluid technology offers a chance to combine product formation and recovery in a single operation through manipulation of process temperature and pressure (Hartmann *et al.* 2001).

Numerous enzymatic reactions have been investigated in supercritical carbon dioxide. The water content of the system greatly influences reaction rates, enzyme stability, enantioselectivity and stereoselectivity in lipase catalyzed reactions (Dumont *et al.* 1993; Glowacz *et al.* 1996; Gandhi *et al.* 2000; Al-Duri *et al.* 2001). Enzyme supports have been investigated to provide improvement in enzyme stability and to allow ease of recovery and reuse of the enzyme (Martins *et al.* 1994; Capewell *et al.* 1996). Enzyme stability and activity can be affected by changes in fluid density, pressurization/depressurization cycling and carbamate formation (Kamat *et al.* 1995; Capewelll *et al.* 1996; Lozano *et al.* 1996).

1.6.2.6 Aqueous-Organic Two-Phase Systems

Aqueous organic two-phase systems consist of an aqueous component and a separate distinct organic solvent phase that is immiscible with the aqueous phase. The volume ratio of aqueous phase to organic phase can be varied according to reaction requirements. The two-phase system may consist of mainly a water phase or mainly an organic solvent (Biselli *et al.* 1995).

Two-phase systems have been widely used to deliver sparingly soluble and/or toxic substrates, allowing higher levels of starting materials available for conversion. The continuous extraction of product into the organic phase can reduce end-product inhibition and drive thermodynamically unfavourable reverse hydrolysis reactions to completion. The containment of substrates and products in the organic phase away from the enzyme in the aqueous phase can protect the biocatalyst from inactivation if these substances interfere with enzyme stability (Biselli *et al.* 1995). Selection of a suitable organic solvent is critical to both enzyme compatibility and extraction/delivery

of substrates/products. A single solvent or a mixture of various solvents may be used to achieve effective partitioning and limit the toxic effect of solvent on the enzyme (Bruce and Daugulis 1991).

The operational stability of biocatalysts has been improved using established techniques of chemical modification, immobilization and by directed evolution and traditional mutation techniques. Recent developments in cell immobilization using celite, porous glass, sol-gels, polyvinyl alcohol cryogel beads and nanoporous materials have provided catalysts which are more robust to the organic solvents and harsh conditions in two-phase systems (Plieva *et al.* 2000; Xin *et al.* 2000; Wang *et al.* 2001).

Organic-aqueous two-phase systems offer many advantages for implementation in continuous processes. The continuous extraction of product into the organic phase can provide a concentrated product stream making downstream recovery more efficient and cost effective (Bruce and Daugulis 1991; Xin *et al.* 2000).

1.6.2.7 Factors Influencing Enzyme Catalysis in Non-Conventional Media

Solvent compatibility

There have been several attempts to predict enzyme behaviour in solvent systems. Several enzyme properties including stability, activity and inhibition have been correlated to parameters such as denaturation capacity, hydrophobicity (measured by $\log_{10}P$) and polarity index of the organic solvents. No overall parameter can be used generally for all proteins, with correlations applying in some instances and not in others (Budde and Khmelinitsky 1999; Rodakiewicz-Nowak *et al.* 2000; Watanabe *et al.* 2000). In general the activity and stability of enzymes in organic solvents depend strongly on the solvent used and whether or not a predictive pattern can be established (Ghatorae *et al.*, 1994b; Bauer *et al.*, 1999; Budde and Khmelinitsky, 1999).

The partition coefficients (P) of various solvents are defined as the partitioning of a given solvent between water and 1-octanol in a two-phase system.

$P = [Solvent]_{octanol} / [Solvent]_{water}$

Lanne *et al.*(1987) found a correlation between the hydrophobicity of the solvent and the activity retention of the biocatalyst. In general it was found that solvents with $\log_{10}P$ values greater than 4 exhibited high degrees of biocompatibility in terms of activity retention. Solvents with $\log_{10}P$ values less than 2 were found to inactivate enzymes. However, this correlation could not be generally applied to all enzymes. The correlation has been used for aqueous monophasic media saturated with organic cosolvents, monophasic pure solvent systems and two-phase systems. Modified insoluble catalase dispersed in pure organic solvents with $\log_{10}P$ values ranging from 1 to 2.8 showed increasing activity with increasing $\log_{10}P$ (Jene *et al.* 1997). Ghatorae *et al.* (1994b) demonstrated that $\log_{10}P$ could be used to predict the pattern of interfacial inactivation for lipase and chymotrypsin, however no trend of interfacial inactivation with solvent $\log_{10}P$ was found for urease. The measured effect for dissolved solvent without interfacial inactivation was also correlated to $\log_{10}P$ for lipase, however chymotrypsin and urease exhibited no patterns. In general the correlations were enzyme specific with no overall correlation found for any one class of enzyme.

The polarity index and denaturation capacities have been used also to select solvents for use in miscible, monophase cosolvent systems. Gupta *et al.* (1997) showed that denaturation of polyphenol oxidase, peroxidase, acid phosphatase and trypsin show good stability in solvents with polarity indexes above 5.8. Enzymes exposed to 50 % v/v solutions of solvents in aqueous media retained most of their activity after 48 hours. Solvents with polarity indices less than 5.1 were shown to completely inactivate enzyme in less than 4 hours. Denaturation capacity has been proposed by Khmelinitsky *et al.* (1991) as a predictor of enzyme stability in a monophasic aqueous environment with organic cosolvents. The denaturation capacity is a parameter calculated on the basis of a thermodynamic model of protein denaturation taking into account the threshold concentration and physiochemical properties of the organic solvent.

Enzyme inactivation

Enzyme inactivation in non-aqueous media is a disadvantage of these systems. Enzymes are designed by nature for maximum stability and activity in an aqueous cell

environment. Exposure of the enzyme to solvents other than water can lead to irreversible enzyme inactivation, a problem that can limit the application of enzymes for industrial synthesis of chiral compounds.

The stability of the enzyme is affected by the amount of solvent dissolved in the aqueous phase in direct contact with the enzyme, with additional significant enzyme inactivation at liquid/liquid and gas/liquid interfaces.

Solvents that are miscible with water have the ability to remove water molecules from the protein which may be essential for stability and activity. In aqueous solution, the protein is surrounded by a hydration shell of water molecules non-covalently bound to the protein by hydrogen bonding. This water shell is critical for enzyme activity and stability. Organic solvents displace the water molecules and can cause a dramatic change in the protein structure and function. The effect of this interaction can either enhance activity or denature the protein (Khmelinitsky *et al.* 1991; Ó Fágáin 1997).

Enzyme inactivation at the aqueous/organic interface is a significant consideration in two-phase systems, reverse micelles and supercritical fluids. Additional inactivation in supercritical fluid systems has been attributed to the shear effect that proteins experience at gas/liquid interfaces (Kamat *et al.* 1995). Improvement in understanding and quantification of interfacial inactivation is one of the main challenges of enzyme industrial catalysis.

It is known that protein in the aqueous phase can be attracted to the immiscible organic solvent where it can adsorb and form a monolayer at the interface, which may alter the structure of the enzyme leading to inactivation. The hydrophobic active site of the enzyme may orient towards the hydrophobic solvent. This association has been found to be irreversible with chymotrypsin in emulsions of octadecane and, reversible upon desorption when lysozyme and ribonuclease were exposed to decane (Ghosh and Bull 1962; Cecil and Louis 1970). Emulsions of protein and solvent can occur spontaneously with gentle agitation, with the degree of emulsification dependent on the protein concentration. Emulsification occurs when protein adsorption at the interface lowers the surface free energy between the liquid phases. The rate limiting factor in the

formation of emulsions appears to be the adsorption of the protein rather than the break up of organic solvent (Cecil and Louis 1970).

Ghatorae *et al.* (1994a) designed a liquid-liquid bubble column apparatus for quantification of the dissolved and interfacial solvent effect on enzyme stability. The apparatus allowed exposure of enzyme solutions to water immiscible organic solvents with a known total interfacial area with defined exposure time. Exposure of ribonuclease, chymotrypsin, urease and lipase to n-butanol, isopropylether, 2-octanone, n-hexane indicated that inactivation was dependent on enzyme and solvent combinations. Ribonuclease was relatively stable to interfacial inactivation with all solvents, while lipase was inactivated by less polar solvent/aqueous interfaces and chymotrypsin by more hydrophobic interfaces. Urease was inactivated by some interfaces although no general trend was observed. Study of interfacial inactivation in this way gave a clear distinction between dissolved and interfacial effects. A significant conclusion for all enzymes was that inactivation was proportional to the total area of interface exposed and not to the time of exposure to droplets (Ghatorae *et al.* 1994a). A Lewis cell reactor consisting of a cylindrical vessel with two compartments separated by an interfacial plate containing a central hole that can be altered to allow contact between the two phases has been used also to quantify interfacial inactivation (Baldascini *et al.* 1999).

Several other enzymes such as hydroxynitrile lyases, pancreatic lipases, β-galactosidase, and epoxide hydrolase have been shown also to inactivate at solvent/aqueous interfaces (Gargouri *et al.* 1995; Okahata and Mori 1998; Baldascini *et al.* 1999; Bauer *et al.* 1999; Hickel *et al.* 2001).

In summary, this literature review has described factors affecting *R*-PAC formation by whole-cells and cell free extracts using various organisms in a range of different processes. The studies reported in literature highlight that *R*-PAC formation in the cell-free enzymatic system is challenged mainly by stability of the pyruvate decarboxylase catalyst. A review of recent advances in the area of catalysis has shown that enzyme catalysis in organic solvent systems can enhance product formation though improved process design.

Experimental strategy

The following proposed experimental strategy will be implemented to achieve the objectives outlined in the Aims of this Study (see page 1).

1) Production and recovery of pyruvate decarboxylase (PDC) from *Candida utilis*

- Series of fermentations for PDC production under controlled conditions.
- Evaluation of PDC recovery methods, including cell breakage, solvent precipitation and freeze-drying.
- Selection of an optimal procedure for recovery of PDC from *C.utilis*.

2) Characterization of the factors affecting enzyme stability and *R*-PAC production with partially purified PDC from *C.utilis*

- Ensuring reproducible *R*-PAC formation for each batch of enzyme.
- Determination of enzyme inactivation caused by benzaldehyde, *R*-PAC and by-products acetaldehyde and acetoin.
- Determination of possible inhibition by *R*-PAC.

3) Development of an enzymatic reactor that minimizes PDC inactivation and maximizes *R*-PAC formation

- Evaluation of *R*-PAC production in a benzaldehyde emulsion reactor (Rosche *et al.* 2002a).
- Development of an aqueous-organic two-phase reactor for *R*-PAC production, involving selection of the most appropriate solvent based on enzyme stability and *R*-PAC formation.
- Evaluate and compare the kinetics of *R*-PAC production in the benzaldehyde emulsion and two-phase systems, with preliminary two-phase system scale-up experiments.

4) Comparison of enzymatic processes with the traditional commercial process based on the key criteria:

- *R*-PAC concentration and productivity.
- Yields of *R*-PAC based substrate utilized.

CHAPTER 2

MATERIALS AND METHODS

2.1 Strain

Candida utilis yeast strain #70940 was obtained from the University of New South Wales Culture Collection in the Department of Microbiology (World Directory of Culture Collections No.248). The freeze dried stock culture was regenerated in malt liquid medium (Medium 4) prior to storage on malt agar slopes (Medium 5) for a maximum of six months.

2.2 Chemical and Protein Sources

Table 2.1 lists all important chemicals and protein sources. All other chemicals, which are not listed in Table 2.1 were analytical grade reagents.

Table 2.1: Sources of important chemicals and proteins.

Sigma-Aldrich Corp., USA.
Sigma chemicals
Cocarboxylase (thiamine pyrophosphate chloride), alcohol dehydrogenase from baker's yeast, MOPS free acid (3-[N-morpholino]propanesulfonic acid), pyruvate decarboxylase from brewers yeast, benzoic acid (free acid), citric acid (free acid anhydrous), pyruvic acid standard solution 0.04 mg/ml, MES free acid (2-(N-Morpholino)ethanesulfonic acid monohydrate)

Fluka chemicals Pyruvic acid (sodium salt), acetoin, pyruvic acid, acetaldehyde, methyl-tert-butylether (MTBE)
Pierce Chemical Company, USA Coomassie® plus protein assay reagent, albumin standard (2 mg/ml in 0.9 % NaCl + 0.05 % sodium azide)
BDH Chemicals Pty., Ltd., Aust. Magnesium sulfate (heptahydrate), octanol, butanol, pentanol, nonanol, heptane, nonane, dodecane, methylcyclohexane
Roche Diagnostics Corp., Germany Lactate dehydrogenase from rabbit muscle, NADH (disodium salt grade 2)
AJAX chemicals, Aust. Benzaldehyde, hexane
May and Baker Pty. Ltd., Aust. Toluene
Oxoid Unipath Ltd., Eng. Yeast extract
BASF AG, Germany Phenylacetylcarbinol

2.3 Media and Buffer Compositions

Medium 1: agar plate medium

30 g/l glucose
5 g/l yeast extract
2 g/l $(NH_4)_2SO_4$
2 g/l KH_2PO_4
1 g/l $MgSO_4.7H_2O$
15 g/l agar
no pH adjustment was made.

Medium 2: pre-seed and seed culture medium

10 g/l glucose
5 g/l yeast extract
2 g/l $(NH_4)_2SO_4$
2 g/l KH_2PO_4
1 g/l $MgSO_4.7H_2O$
200 mM MES
Adjusted to pH 6.0 at 25 °C with 4 M KOH

Medium 3: 30 litre and 5 litre batch culture medium

90 g/l glucose
10 g/l yeast extract
10 g/l $(NH_4)_2SO_4$
3 g/l KH_2PO_4
2 g/l $Na_2HPO_4.2H_2O$
1 g/l $MgSO_4.7H_2O$
0.05 g/l $CaCl_2.H_2O$
Adjusted to pH 5.5 with H_3PO_4/NaOH

Medium 4: malt liquid culture medium

3 % (w/v) Cornwall malt syrup

Medium 5: malt agar slope medium

3 % (w/v) Cornwall malt syrup
2 % (w/v) agar

Buffer 1: phosphate reaction buffer

40 mM KH_2PO_4

2 M ethanol

1 mM $MgSO_4.7H_20$

1 mM thiamine pyrophosphate

Adjusted to pH 7.0 at 4 °C with 4 M KOH.

Buffer 2: citrate carboligase assay dilution buffer

200 mM citric acid

2 mM thiamine pyrophosphate

20 mM $MgSO_4.7H_20$

Adjusted to pH 6.4 at 25 °C with 4 M KOH

Buffer 3: citrate decarboxylase assay buffer

200 mM citric acid

2 mM thiamine pyrophosphate

20 mM $MgSO_4.7H_20$

Adjusted to pH 6.0 at 25 °C with 4 M KOH

Buffer 4: citrate collection buffer (for gel filtration)

400 mM citric acid

4 mM thiamine pyrophosphate

40 mM $MgSO_4.7H_20$

Adjusted to pH 6.0 at 25 °C with 4 M KOH

Buffer 5: MES buffer

50 mM MES
1.5 M ethanol
20 mM $MgSO_4.7H_20$
1 mM thiamine pyrophosphate
Adjusted to pH 7.0 at 4 °C with 4 M KOH.

Buffer 6: MOPS biotransformation buffer

2 M MOPS
1 mM thiamine pyrophosphate
1 mM $MgSO_4.7H_20$
Adjusted to pH 7.0 at 4 °C with 4 M KOH

Buffer 7: MOPS two phase system reaction buffer

2.5 M MOPS
1 mM thiamine pyrophosphate
1 mM $MgSO_4.7H_20$
Adjusted to pH 6.5 at 4 °C with 4 M KOH

2.4 Fermentation: Enzyme Production

2.4.1 Media Preparation and Sterilization

All media was sterilized at 121 °C and 125 kPa for 20 minutes. All hardware were sterilized at 121 °C and 125 kPa for 30 minutes. All shake flask media were sterilized with all components combined together at final concentrations in the flask.

Media components for all bioreactors were prepared in Reverse Osmosis (RO) water and sterilized as four separate solutions as indicated in Table 2.2 and Table 2.3. After sterilization, the solutions were stored at 4 °C until required. The addition of seed

culture as described in Section 2.4.2 and 2.4.3, adjusted the final working volumes of the 5 litre and 30 litre bioreactors to 3.5 and 20 litres respectively.

Table 2.2: Medium components for the 5 litre bioreactor.

Component	Volume (l)	Concentration (g/l)
glucose	1.00	315
yeast extract	0.70	50
KH_2PO_4 / $Na_2HPO_4.2H_2O$	0.75	14/9.33
$(NH_4)_2SO_4$ / $MgSO_4.7H_2O$ / $CaCl_2.H_2O$	0.70	50 / 5 / 0.25
total medium volume	3.15	

Table 2.3: Medium components for the 30 litre bioreactor.

Component	Volume (l)	Concentration (g/l)
glucose	3.0	600
yeast extract	2.0	100
KH_2PO_4 / $Na_2HPO_4.2H_2O$	1.5	40 / 26.7
$(NH_4)_2SO_4$ / $MgSO_4.7H_2O$ / $CaCl_2.H_2O$	2.0	100 / 10 / 0.5
total medium volume	12.5	

2.4.2 Growth of Pre-Seed and Seed

Candida utilis from a malt agar slope (medium 5) was used to inoculate a fresh agar plate (medium 1), which was incubated for 3 days at 30 °C. A pure single colony from the plate culture was transferred to a 500 ml baffled shake flask containing 150 ml of pre-seed medium (medium 2). The pre-seed was shaken at 180 rpm for 12 hours at 30 °C.

2.5 ml of pre-seed was transferred into 1 litre baffled flasks containing 250 ml of seed medium (medium 2). The 5 litre bioreactor required 2 flasks (350 ml), while the 30 litre required 6 flasks (1.5 litre). The flasks were shaken at 250 rpm for 10 hours at 30 °C to obtain approximately 3 g/l dry weight of *Candida utilis* in the exponential phase of

growth. The culture from all flasks were combined and used to inoculate the 5 or 30 litre bioreactor.

2.4.3 Bioreactor Operation

2.4.3.1 5 Litre Bioreactor Culture

A Biostat B, 5 litre bioreactor (Braun,Germany) was used to grow yeast for the production of pyruvate decarboxylase enzyme. A schematic diagram of the bioreactor is shown in Figure 2.1.

The 5 litre bioreactor consisted of a double jacketed borosilicate glass baffled vessel with a concave bottom section. The top was sealed with a stainless steel top plate containing ports for measuring probes, agitator, medium addition, pH adjustment, inlet air, condenser and sampling. A control unit containing digital PID controllers was used to monitor and control temperature, stirrer speed, pH, air flowrate and dissolved oxygen concentration. The unit contained the thermostat system, installations required for power, air flowmeter and peristaltic pumps for antifoam, alkali and acid addition. Monitoring and storage of process data, and remote control were performed via the control unit, which was linked to a desktop computer.

Prior to sterilization of the bioreactor, the pH probe (Mettler Toledo 404-DPAS-SC-K8S/325, Switzerland) and Ingold oxygen sensor (Mettler Toledo, ∅ 12 mm, Switzerland) were calibrated using the control unit calibration routine before being mounted into the vessel. The pH probe was calibrated with pH 4 and 7 calibration solutions. The dissolved oxygen probe was calibrated in percent oxygen saturation using Ingold zeroing gel (Mettler Toledo, Switzerland) for 0 % and air saturated water (obtained by bubbling compressed air into a beaker of water) for 100 % oxygen.

After calibration, the pH and dissolved oxygen probes were attached to the top plate. The air inlet line and air outlet lines were fitted with a 0.2 μm PTFE filter, ∅ 50 mm (Advantec, Japan). All lines into the vessel for medium and corrective solution addition (acid, alkali, antifoam) were unclamped with all open connections covered with cotton

wool and aluminium foil. All unused ports were sealed with stainless steel closures. The water jacket was filled and 100 ml of RO water was placed into the vessel. The bioreactor was autoclaved at 121 °C and 125 kPa for 40 minutes. Bottles and tubing for acid, alkali and antifoam addition were sterilized empty. Concentrated acid and alkali were not sterilized. Antifoam was autoclaved separately.

After sterilization, the vessel was cooled to room temperature. Corrective solutions were added to bottles aseptically. The temperature, pH and dissolved oxygen probes, air inlet line, corrective solution lines (acid, alkali, antifoam) and cooling water lines were attached to the control unit. The agitator was connected to the stirrer shaft.

All sterilized and cooled medium components as described in Section 2.4.1 were combined aseptically into a 5 litre flask with sidearm and connection lines to allow addition of medium to the bioreactor. The volume was adjusted to 3.15 litres with sterile RO water to account for volume losses that may have occurred during sterilization of medium components. The flask containing the medium was connected aseptically to the bioreactor and the medium drained into the vessel using gravity. The temperature of the medium was adjusted to 28 °C using the bioreactor temperature controls with an air flowrate of 2 ml/min to ensure positive pressure. When the temperature had equilibrated, the pH of a media sample was measured externally and compared with the bioreactor pH probe. Adjustments were made according to the variation. The 100 % dissolved oxygen point was recalibrated by saturating the medium with oxygen using compressed air at 2 ml/min and stirring at 500 rpm.

350 ml of seed inoculum as described in Section 2.4.2 was transferred aseptically into a sterile 1 litre flask with sidearm connection line to enable attachment to the bioreactor. The inoculum was drained into the bioreactor by gravity.

Post inoculum addition, the stirrer speed was set to 500 rpm with an airflow of 2 l/min representing 0.57 vvm. Samples were taken according to Section 2.4.6.

When the Respiratory Quotient (RQ) reached a value of approximately 1.0 after 4.5 hours of operation, the remote RQ controller was turned on and controlled with a setpoint of 1.1. At this point the cell concentration was 2 g/l dry weight. When the cell

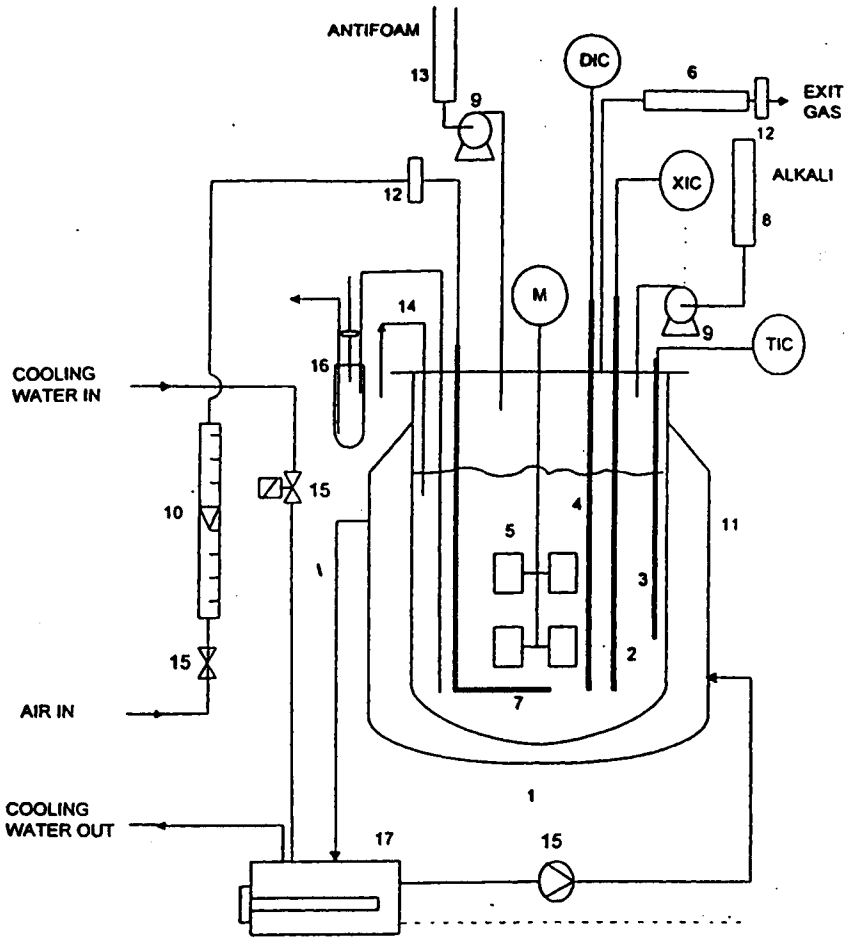
concentration reached 8 g/l after 8.5 hours, the RQ setpoint was increased to 4.0 to induce more fermentative conditions. An RQ of approximately 4.0 was maintained approximately for the duration of the fermentation. When the glucose level reached close to 10 g/l, the bioreactor was stopped and cells harvested according to Section 2.5.1.

2.4.3.2 30 Litre Bioreactor Culture

For production of increased quantities of yeast and enzyme, a 30 litre Biostat C bioreactor was used (Braun, Germany). A photo of the bioreactor and associated equipment is shown in Figure 2.2.

The 30 litre bioreactor consists of a jacketed stainless steel culture vessel mounted in a frame to support the piping, vessel and thermostat system. The vessel was sealed with a stainless steel top plate containing ports for an agitator, medium addition, corrective solutions (acid, alkali and antifoam), pressure relief valve, inlet air and filter and exhaust cooler. A digital measurement and control unit containing PID controllers and installations for power was used to monitor and control temperature, stirrer speed, pH, air flowrate and dissolved oxygen concentration. The unit contained peristaltic pumps for antifoam, alkali and acid addition. Monitoring and storage of process data, and remote control of the vessel were performed via the control unit, which was linked to a desktop computer.

The thermostat system was comprised of a closed loop hot water system with two heat exchangers for cooling water and steam. Air supply was controlled automatically with an online mass flow controller. Air entering the vessel passed through a membrane filter mounted on top of the vessel. An agitator consisted of three Rushton turbines driven by a 900 W motor connected to the stirrer shaft mounted on the top plate. The pH probe, oxygen sensor and temperature probe were mounted in side ports in the lower vessel wall. A sample port was located at the base of the vessel and could be sterilized *in situ*. Septum ports were used for connection of medium solutions, inoculum and corrective solutions (acid, alkali and antifoam).



1.	Fermentor	12.	Air filter
2.	pH probe	13.	Antifoam reservoir
3.	Temperature sensor	14.	Inoculum port
4.	DO probe	15.	Valve
5.	Agitator	16.	Sampling system
6.	Condensor	17.	Thermostat
7.	Air sparger		
8.	Alkali reservoir	M	Motor
9.	Peristaltic pump	DIC	DO indicator & control
10.	Rotameter	XIC	pH indicator & control
11.	Water jacket	TIC	Temperature indicator & control

Figure 2.1: A schematic diagram of the 5 litre bioreactor (Chow 1998).

Prior to sterilization of the bioreactor, the pH probe (Mettler Toledo 405-DPAS-SC-K8S/120, Switzerland) and Ingold oxygen sensor (Mettler Toledo, Switzerland) were calibrated using the control unit calibration routine before being mounted into the vessel. The pH probe was calibrated with pH 4 and 7 calibration solutions. The dissolved oxygen probe was calibrated in percent oxygen saturation using Ingold zeroing gel (Mettler Toledo, Switzerland) for 0 % and air saturated water (obtained by bubbling compressed air into a beaker of water) for 100 % oxygen.

After calibration, the pH and oxygen sensors were inserted into side entry ports at the bottom of the vessel. The water jacket and bioreactor vessel were filled with RO water for the *in situ* sterilization cycle. All unused ports and septum ports were sealed with blind closures. The relief valve and inlet air filter were set to sterilization positions. The bioreactor was sterilized at 121 °C for 1 hour using the inbuilt sterilization cycle. Bottles, tubing and septum connectors for acid, alkali and antifoam addition were sterilized empty. Concentrated acid and alkali were not sterilized. Antifoam was sterilized separately.

After sterilization, the vessel containing sterile RO water was cooled to 28 °C. Corrective solutions were added to bottles aseptically. The temperature probe, pH probe, oxygen sensor, and corrective solution lines (acid, alkali, antifoam) were attached to the control unit and bioreactor. Relief valve and air inlet filter were set to fermentation mode. The condenser for cooling bioreactor exhaust gas was connected to the cooling water lines.

After RO water in the vessel had cooled, the volume was drained to 10 litres through the sterile sample line. All sterilized and cooled medium components as described in Section 2.4.1 were combined aseptically into two 5 litre flasks with sidearm and septum connection lines and were connected aseptically to the bioreactor. Medium was drained into the vessel using gravity. The medium was adjusted to 28 °C using the bioreactor temperature controls with a sterile air flowrate of 2 ml/min to ensure positive pressure. When the temperature had equilibrated, a sample of medium was taken from the bioreactor and the pH measured externally and compared with the bioreactor pH probe. Adjustments were made according to the variation. The 100 % air saturation point was recalibrated by saturating the medium with air at 2 ml/min and stirring at 500 rpm. The

pH of the medium in the bioreactor was adjusted to 5.5 using the bioreactor control unit and online corrective solutions. The volume was adjusted to 18.5 litres with additional sterile RO water.

1.5 litres of seed inoculum as described in Section 2.4.2 was transferred aseptically into a 2 litre flask with sidearm and septum connection line to enable attachment to the bioreactor. The inoculum was drained into the bioreactor by gravity.

Post inoculum addition, the stirrer speed was set to 500 rpm with an airflow of 10 l/min representing 0.5 vvm. Samples were taken according to Section 2.4.6.

When the RQ reached approximately 1.0 after 4.5 hours of operation, the remote RQ controller was turned on and controlled with a setpoint of 1.0. At this point the cell concentration was 2 g/l dry weight. When cell concentration reached 12 g/l after 8.5 hours, the RQ setpoint was increased to 4.0 to induce more fermentative conditions. An RQ of approximately 4.0 was maintained for the duration of the fermentation. When the glucose level reached close to 10 g/l the bioreactor was stopped and cells harvested according to Section 2.5.1.

2.4.4 Bioreactor Control and Data Collection

Bioreactor control and raw data collection were performed using the integrated digital control unit, combined with remote computer control using MFCS/win version 1.1 software (Braun, Germany).

For the initial 4.5 hours of operation, the bioreactor was controlled via the integrated digital control unit operated at pH 5.5, 500 rpm, 10 l/min airflow, and a temperature of 28 °C. Setpoints were entered and viewed on the control unit display. The raw data was viewed and collected on a computer.

After 4.5 hours, the control unit was placed in remote operation mode and the RQ was controlled via the computer using programmed algorithms and calculations and proportional integral control.

Figure 2.2: 30 litre bioreactor and associated equipment.

2.4.5 Bioreactor RQ Control

Respiratory Quotient (RQ) control of bioreactors was established using MFCS/win version 1.1 software (Braun, Germany).

Online carbon dioxide and oxygen exhaust gas concentrations were used to calculate the RQ from CER and OUR calculations. The RQ value was compared to a setpoint RQ value that was entered offline. The difference between the setpoint and the measured value was calculated. A new stirrer setpoint was calculated from proportional integral control equations based on control constants. The new stirrer set point was transferred to the stirrer via the control unit. If the measured RQ value was higher than the setpoint, the stirrer speed was increased to improve oxygen transfer to medium and cells. If the RQ value was lower than the setpoint, the stirrer speed was decreased to lower oxygen availability to cells.

Calculation of Respiratory Quotient (RQ)

$$RQ = \frac{\text{Carbon dioxide Evolution Rate (CER)} \quad \text{(mmol/l/h)}}{\text{Oxygen Uptake Rate (OUR)} \quad \text{(mmol/l/h)}}$$

RQ was estimated from the composition of the inlet and outlet gases as measured by the on-line oxygen and carbon dioxide gas analyzers. A mass balance was used to calculated CER and OUR, using a balance on inert nitrogen to solve the unknown parameters.

The OUR and CER are calculated from the difference between the rate of oxygen or carbon dioxide entering and exiting the bioreactor divided by the volume of the bioreactor. The following equation variables are defined as;

OUR	=	Oxygen uptake rate (mmol/l/h)
CER	=	Carbon dioxide evolution rate (mmol/l/h)
V	=	Volume of bioreactor (l)
F_i	=	Inlet gas flowrate (l/h)

F_o	=	Outlet gas flowrate (l/h)
$\% O_{2,I}$	=	Inlet composition of oxygen (%)
$\% O_{2,o}$	=	Outlet composition of oxygen (%)
$\% CO_{2,I}$	=	Inlet composition of carbon dioxide (%)
$\% CO_{2,o}$	=	Outlet composition of carbon dioxide (%)
$\% N_{2,i}$	=	Inlet composition of nitrogen (%)
$\% N_{2,o}$	=	Outlet composition of nitrogen (%)

$$OUR = \frac{[(F_i * \% O_{2,i}) - (F_o * \% O_{2,o})]}{V} \quad \text{(mmol/l/h)} \quad \text{with } O_{2,i} = 20.9\ \% \qquad \textbf{(1)}$$

$$CER = \frac{[(F_o * \% CO_{2,o}) - (F_i * \% CO_{2,i})]}{V} \quad \text{(mmol/l/h)} \quad \text{with } CO_{2,i} = 0.03\ \% \qquad \textbf{(2)}$$

Both the volume of the bioreactor (V) and then inlet flowrate (F_i) were entered as online-variables. $\% CO_{2,o}$ and $\% O_{2,o}$ were measured from exit gas analysis.

A mass balance using the nitrogen gas flowrate and composition, allows the expression of the flowrate out of the bioreactor as an expression of the inlet flowrate.

$$F_i * \% N_{2,i} = F_o * \% N_{2,o} \qquad \text{with } N_{2,i} = 79.07\ \%$$

Therefore $F_o = F_i * (\% N_{2,i}) / (\% N_{2,o})$

where $\% N_{2,o} = 100 - \% O_{2,o} - \% CO_{2,o}$

therefore $F_o = F_i\,(79.07)/(100 - \% O_{2,o} - \% CO_{2,o})$ **(3)**

Substitution of **(3)** into **(1)** and **(2)** allows for the two following algorithms that can be solved using exit gas analysis, allowing for the calculation of RQ.

$$OUR = \frac{[\,20.9 - (79.07 * \% O_{2,o})\,]}{(100 - \% O_{2,o} - \% CO_{2,o})} * \frac{(F_i)}{(V * K_{OUR})}$$

$$CER = \left[\frac{(79.07 * \% CO_{2,o})}{(100 - \% O_{2,o} - \% CO_{2,o})} - 0.03 \right] * \frac{(F_i)}{(V * K_{CER})}$$

Two factors, K_{OUR} and K_{CER} are needed for conversion of units.

$$K_{OUR} = \frac{1.41\ g}{l} * \frac{60\ min}{h} * \frac{mol}{32\ g} * \frac{1}{100\ \%} * \frac{1000\ mmol}{mol}$$

K_{OUR} = 26.44 min*mmol/l/h

$$K_{CER} = \frac{1.95\ g}{l} * \frac{60\ min}{h} * \frac{mol}{44\ g} * \frac{1}{100\ \%} * \frac{1000\ mmol}{mol}$$

K_{CER} = 26.59 min*mmol/l/h

2.4.6 Bioreactor Sampling

For each timepoint, a 10 ml sample was taken from the bioreactor for supernatant, optical density and cell dry weight analysis. 1 ml of culture was pipetted into a 1.5 ml centrifuge tube and centrifuged for 5 minutes at 12000 rpm (11600 RCF) using a 5415C centrifuge (Eppendorf, USA). The supernatant was analyzed for ethanol, pyruvate and glucose concentrations. The remaining 9 ml of sample was used to determine the cell dry weight and optical density. For enzyme activity determination a further 25 ml of sample was taken and immediately chilled on ice for enzyme activity determination. The above samples were taken and analyzed in duplicate. A summary of the sampling procedure is shown in Figure 2.3.

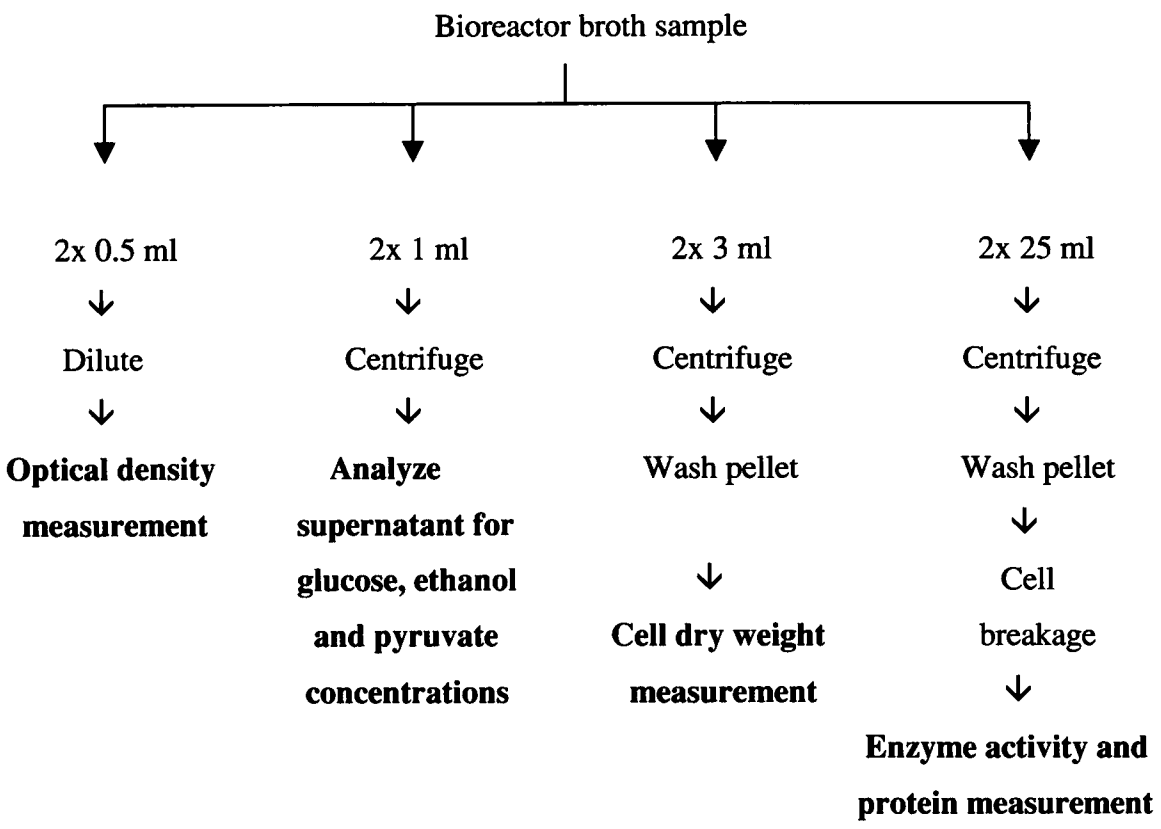


Figure 2.3: Sampling procedure for bioreactor analysis.

2.5 Enzyme Recovery

2.5.1 Cell Harvesting

The total bioreactor broth was placed in the -20 °C freezer and quickly chilled to 4 °C. The broth was centrifuged in 1 litre batches at 7000 rpm (12227 RCF) for 5 minutes using a JLA rotor and an Avanti J-20 centrifuge (Beckman, USA) that was chilled to 4 °C. The supernatant was discarded and additional bioreactor broth was added on top of the cell pellet and centrifuged at the above conditions. This process was repeated until cells were separated. The cell pellet was washed 2 times in cold RO, centrifuging at the above conditions between washes. The cells were finally resuspended in citrate buffer (Buffer 2) to 60 g/l dry weight and frozen at -20 °C.

2.5.2 Cell Breakage

Cells in citrate buffer (Buffer 2) at 60 g/l dry weight were defrosted in a 30 °C water bath and stored at 4 °C before being frozen in liquid nitrogen 3 times, with defrosting at 30 °C in between freezing.

The freeze/thawed cells were placed in a blender with 0.5 mm glass beads (Biospec, USA) at a ratio of 1 volume beads to 1 volume cells in buffer. The cells were blended for two 1 minute intervals with a 5 minute incubation on ice in between blends. The cell broth was centrifuged at 12000 rpm (23435 RCF) using a GSA rotor and a Sorvall RC-5B centrifuge (Dupont, USA) chilled at 4 °C for 15 minutes. The supernatant (crude extract) was frozen at -20 °C.

2.5.3 Enzyme Precipitation and Preparation

Pyruvate decarboxylase enzyme was recovered from the crude extract by acetone precipitation in the range of 40-50 % (v/v) acetone assuming additive volumes. The protocol was adapted from a general solvent precipitation method described by Scopes (1994).

The crude extract was defrosted by incubation in a 30 °C water bath, and stored at 4 °C until precipitation. Acetone was chilled to -10 °C in a salt/iced water bath. For adjustment of crude extract to 40 % (v/v) acetone, 666 ml of chilled acetone was added per litre of crude extract with slow constant stirring. The crude extract/acetone mixture was incubated at -10 °C for 15 minutes. The mixture was centrifuged at 7000 rpm (7974 RCF) for 5 minutes at 0 °C using a GSA rotor in a Sorvall RC-5B centrifuge (Dupont, USA). The supernatant was adjusted to a final acetone level of 50 % (v/v), 200 ml of acetone was added per litre of supernatant. The solution was incubated in the -10 °C water bath for 15 minutes before centrifuging at 10000 rpm (16274 RCF) for 5 minutes at 0 °C. The pellet was incubated on ice with compressed air blown over the pellet for 1 hour to remove residual acetone. The pellet was stored at -20 °C before

being freeze dried for 24 hours. After freeze drying the enzyme was ground to a fine powder with a mortar and pestle before being stored at -20 °C.

2.5.4 Freeze Drying

The acetone precipitated enzyme pellet was freeze dried using a freeze drier (FD3, Dynavac, Aust) with the chamber at -50 °C and 0.1 mbar for 24 hours.

2.6 Characterization of Factors Affecting Enzyme Stability and *R*-PAC Formation

2.6.1 Enzyme Stability in the Presence of Benzaldehyde Emulsions and Solutions in Phosphate Buffer

For evaluation of enzyme stability in the presence of benzaldehyde, 500 µl of enzyme at 14 U/ml in phosphate buffer (Buffer 1) was mixed with 500 µl of double strength benzaldehyde in the same buffer. The sample was vortexed for 3 seconds before being turned continuously on a vertical rotary wheel at 4 °C. The enzyme sample was gel filtered at a specific exposure time and the residual carboligase activity was determined. A control of enzyme without exposure to benzaldehyde was included. Ten vials were evaluated for each benzaldehyde concentration, and the error was calculated as the standard deviation from the mean of residual carboligase activities.

Benzaldehyde solutions were created by vortexing double strength benzaldehyde in buffer at 30 °C until it was completely dissolved, ensuring no benzaldehyde droplets remained.

Benzaldehyde emulsions were created by vortexing double strength benzaldehyde in buffer at 30 °C forming an homogenous mixture of droplets dispersed in buffer. To make a 50 mM benzaldehyde emulsion, 100 mM of benzaldehyde was vortexed at

30 °C and combined with an equal volume of enzyme before the benzaldehyde had fully dissolved.

2.6.2 Enzyme Stability in the Presence of Protease Inhibitors, Bovine Serum Albumin (BSA) and MOPS Buffer

Protease inhibitors

500 µl of enzyme at 14 U/ml in phosphate buffer (Buffer 1) was mixed with 500 µl of the same buffer containing the following protease inhibitors:

- Complete (Boehringer Mannheim, Germany) - 1 tablet in 12.5 ml buffer
- Pepstatin A (Boehringer Mannheim, Germany) - 1.4 µg/ml

The mixtures were incubated on ice for 4 days. The starting enzyme level was therefore 7 U/ml containing protease inhibitors to half of the concentrations listed above. A control consisting of 500 µl of enzyme at 14 U/ml in phosphate buffer was mixed with an additional 500 µl buffer. After 4 days, the carboligase activity was measured.

Bovine serum albumin (BSA)

500 µl of enzyme at 14 U/ml in phosphate buffer (Buffer 1) was mixed with 500 µl of the same buffer containing differing levels of BSA according to Table 2.4. A control for each batch consisted of 500 µl of enzyme at 14 U/ml in phosphate buffer mixed with 500 µl of additional buffer. The initial enzyme level was 7 U/ml with approximately 6.0 mg/ml protein in each sample. After 4 days on ice, the carboligase activity was measured directly.

MOPS buffer

1 ml of enzyme at 7 U/ml in MOPS buffer (Buffer 6) was incubated on ice. After 4 days, the carboligase activity was measured.

Table 2.4: Concentration of BSA solutions added to each batch of enzyme for evaluation of enzyme stability.

Batch #	1	2	3	4
Enzyme level in double strength enzyme solution (U/ml)	14.0	14.0	14.0	14.0
Protein level in double strength enzyme solution (mg/ml)	6.4	7.8	12.0	3.2
BSA solution (mg/ml) mixed 1:1 with 14 U/ml enzyme solution to adjust all protein levels to 6 mg/ml.	5.6	4.2	0	8.8

2.6.3 Enzyme Stability in MOPS Buffer Exposed to 50 mM Benzaldehyde

500 µl of enzyme at 14.4 U/ml in MOPS buffer (Buffer 6) was mixed with 500 µl of 100 mM benzaldehyde solution in MOPS buffer (Buffer 6). The sample was vortexed for 3 seconds before being turned continuously on a vertical rotary wheel at 4 °C. Residual carboligase activity was determined without filtration with a 20 times dilution for each time point. A control of enzyme exposed to no benzaldehyde was included. The initial enzyme level was 7.2 U/ml prior to exposure to 50 mM benzaldehyde delivered as a solution.

The 100 mM benzaldehyde solution was created by vortexing benzaldehyde in buffer at 30 °C until it was completely dissolved, ensuring no benzaldehyde droplets remained.

2.6.4 Measuring the Effect of *R*-PAC on Enzyme Stability

Preparation of *R*-PAC

R-PAC was recovered from the biotransformation broth after conversion of pyruvate and benzaldehyde by partially purified *Candida utilis* PDC. This preparation was used to determine the effect of *R*-PAC on reaction inhibition and enzyme stability.

A substrate solution was prepared by mixing 3.2 ml of MOPS buffer (Buffer 6), 19.2 ml of 1.25 M pyruvate in MOPS buffer (Buffer 6) and 1.64 ml of benzaldehyde (97.4 % w/v). The mixture was vortexed to disperse the emulsion. The biotransformation was started with the addition of 16 ml of enzyme in MOPS buffer (Buffer 6) at 17.5 U/ml carboligase activity. The reaction was stirred at 4 °C for 160 hours. The total reaction volume was 40 ml, with initial substrate concentrations of 406 mM benzaldehyde and 600 mM pyruvate.

The following additions were made to ensure complete conversion of substrates:

At 24 hours 0.33 g of enzyme powder was added to 1.0 ml of MOPS buffer (Buffer 6), vortexed and incubated on ice for 30 min and 0.5 ml of the supernatant was added to the reaction. The pH was adjusted to 7.0 with concentrated H_2SO_4.

At 72 hours, 354 mM pyruvate remained unconsumed. Benzaldehyde was added to adjust the concentration to 240 mM. Enzyme was added as described above at the 24 hour timepoint. The pH was adjusted to 7.0 with concentrated H_2SO_4.

At 120 hour 56.8 mM pyruvate remained unconsumed and 30 mM benzaldehyde was added with additional enzyme as described at the 24 hour timepoint. The pH was adjusted to 7.0 with concentrated H_2SO_4.

After 160 hours all of the fed pyruvate was consumed, with residual benzaldehyde and no remaining carboligase activity. To recover *R*-PAC the sample was centrifuged to remove precipitated protein and the supernatant was extracted with an equal volume of

methyl-tert-butylether (MTBE). The MTBE was removed under reduced pressure by placing 0.5 ml samples in a vacuum centrifuge for 30 minutes. The samples were resuspended into 100 µl MOPS buffer (Buffer 6) and combined. Fourty percent of *R*-PAC in the biotransformation broth was recovered in this process. Analysis of the *R*-PAC sample was is shown in Table 2.5. Analysis of the HPLC chromatogram showed that there was no indication of other by products other than a small peak at 5.85 min as a shoulder peak of *R*-PAC.

Table 2.5: Concentration of all components in the *R*-PAC sample recovered from the biotransformation.

Protein (mg/ml)	0
R-PAC	342.6 mM
Benzaldehyde (mM)	6.6 mM
Acetoin (mM)	3.5 mM
Acetaldehyde (mM)	0
Pyruvate (mM)	0

Effect of *R*-PAC on enzyme stability

For the evaluation of enzyme stability in the presence of *R*-PAC, concentrated *R*-PAC solution and MOPS buffer (Buffer 6) were combined as shown in Table 2.6. Concentrated enzyme was added, the vial vortexed before being turned continuously on a vertical rotary wheel at 4 °C for 168 hours (7 days). Control (a) consisted of enzyme in MOPS buffer (Buffer 6) containing no added *R*-PAC. The level of acetoin and benzaldehyde that was present in the stability evaluation in the presence of 274 mM *R*-PAC was 2.8 mM and 5.3 mM respectively. Control (b) therefore consisted of enzyme combined with MOPS buffer containing 3.5 mM acetoin and 6.6 mM benzaldehyde to adjust the final concentration of acetoin and benzaldehyde to 2.8 mM and 5.3 mM respectively. Residual carboligase activities were determined after gel filtration to remove *R*-PAC.

Table 2.6: Solutions for evaluation of enzyme stability in the presence of *R*-PAC.

Final *R*-PAC concentration (mM)	342.6 mM *R*-PAC solution (µl)	MOPS buffer (Buffer 6) (µl)	Enzyme at 35 U/ml carboligase activity (µl)
274	400	0	100
206	300	100	100
137	200	200	100
69	100	300	100
34	50	350	100
0 (a) and 0(b)	0	400*	100

* Control 0 (b), MOPS buffer contained 2.8 mM acetoin and 5.3 mM benzaldehyde.

2.6.5 Measuring the Effect of Acetaldehyde on Enzyme Stability

500 µl of enzyme at 14 U/ml in MES buffer (Buffer 5) was mixed with 500 µl of 60 mM acetaldehyde in the same buffer. The mixture was vortexed for 3 seconds and incubated for 15 hours at 4 °C. A control consisted of 500 µl of enzyme at 14 U/ml in MES buffer mixed with 500 µl of MES buffer. The initial enzyme level was 7 U/ml prior to exposure to 30 mM acetaldehyde. After 15 hours the carboligase activity was determined after gel filtration to remove acetaldehyde.

2.6.6 Measuring the Effect of Acetoin on Enzyme Stability

400 µl enzyme at 17.5 U/ml carboligase activity in MOPS buffer (Buffer 6) was mixed with additional buffer, followed by buffer containing acetoin, according to Table 2.7. Each mixture was vortexed for 3 seconds before being turned continuously on a vertical rotary wheel at 4 °C for 250 hours. Remaining carboligase activities were determined after gel filtration to remove acetoin.

Table 2.7: Solution combinations for evaluation of enzyme stability in the presence of acetoin.

Acetoin level (mM)	Volume of enzyme at 16.3 U/ml (µl)	Volume of Buffer 6 (µl)	Volume of 100 mM acetoin (µl)
5	400	550	50
20	400	400	200
40	400	200	400
50	400	100	500
60	400	0	600
0 (Control)	400	600	0

2.6.7 Measuring the Effect of Inhibition Due to *R*-PAC

The following solutions were combined according to Table 2.8. Control (a) consisted of enzyme combined with substrates with no added *R*-PAC. Control (b) was similar to control (a), with the addition of acetoin and benzaldehyde to account for the possible effect of these compounds on *R*-PAC formation. For control (b), enzyme was combined with solution 3 that contained 2.2 mM acetoin and 4.0 mM benzaldehyde. The final concentrations of acetoin and benzaldehyde in the control reaction were therefore 1.6 mM and 3.0 mM respectively and reflected levels present in the highest concentration of *R*-PAC (153.6 mM).

Table 2.8: Solution combinations for evaluation of reaction inhibition due to *R*-PAC.

Final concentration of *R*-PAC (mM)	Solution 1[a] (µl)	Solution 2[b] (µl)	Solution 3[c] (µl)
153.6	100	200	100
115.0	100	150	150
76.7	100	100	200
30.7	100	40	260
0(a)	100	0	300
0(b)	100	0	300

[a] Solution 1: Enzyme at 28 U/ml carboligase activity in MOPS buffer (Buffer 6) containing 50 mM benzaldehyde as a solution.

[b] Solution 2: 50 mM benzaldehyde, 100 mM pyruvate and 306.8 mM *R*-PAC (*R*-PAC was diluted directly from the solution prepared in Section 2.6.2, with the diluted sample containing 3.2 mM acetoin and 5.9 mM benzaldehyde).

[c] Solution 3: 50 mM benzaldehyde and 100 mM pyruvate

2.6.8 *R*-PAC Formation in Benzaldehyde Emulsion Batch Reactions

The methods detailed in Section 2.6.8 were established by Rosche *et al.* (2002a) and were used in combination with methods established in this thesis.

2.6.8.1 Variation of Substrate Levels

Pyruvate, MOPS buffer (Buffer 6) and benzaldehyde (97.4 % [w/v]) were combined in 1.5 ml vials according to Table 2.9. The vials were placed on ice. Each vial was vortexed for 5 seconds prior to enzyme addition to emulsify benzaldehyde. Each reaction was started with the addition of 200 µl enzyme at 17.5 U/ml carboligase activity. Vials were vortexed for 3 seconds before being turned continuously on a vertical rotary wheel at 4 °C. The starting enzyme level was 7 U/ml carboligase activity. Reactions were stopped after 18 hours by diluting samples 20 times with 10 % [w/v] trichloroacetic acid. Samples were centrifuged to remove precipitated protein before analyzing the supernatant.

A blank was included that contained substrates without enzyme addition. The amount of benzaldehyde and pyruvate lost over 20 hours was measured.

Table 2.9: Substrate, buffer and enzyme combinations for benzaldehyde emulsion reactions. Pyruvate and enzyme were dissolved in MOPS buffer (Buffer 6).

Benzaldehyde:pyruvate (mM)	MOPS Buffer 6 (µl)	1.25 M pyruvate (µl)	Benzaldehyde (µl)	Enzyme (µl)
102/150	235	60	5.13	200
203/300	170	120	10.26	200
254/375	137	150	12.82	200
304/450	105	180	15.39	200
406/600	40	240	20.52	200
406/600 - blank	240	240	20.52	0

2.6.8.2 Variation of Enzyme Level

For evaluation of *R*-PAC formation with various enzyme levels, 240 µl of 1.25 M pyruvate in MOPS buffer (Buffer 6), 20.52 µl of 97.4 % [w/v] benzaldehyde, and 40 µl of MOPS buffer were combined in 1.5 ml vials. The vials were placed on ice. Prior to enzyme addition each vial was vortexed for 5 seconds to emulsify benzaldehyde and pyruvate. Reactions were started with the addition of 200 µl of 2.5 times concentrated enzyme. Vials were vortexed for 3 seconds before being turned continuously on a vertical rotary wheel at 4 °C. The starting enzyme levels were 0.7, 1.0, 1.2, 1.6, 2.1, 2.7, 3.1, 4.1, 5.2, 7.3, 9.4, and 10.4 U/ml. Reactions were stopped after 40 hours by diluting the total reaction volume 20 times with 10 % [w/v] trichloroacetic acid. Each sample was centrifuged to remove precipitated protein before analyzing the supernatant.

2.6.8.3 Full Profile of Benzaldehyde Emulsion Batch Reactions

240 µl of 1.25 M pyruvate in MOPS buffer (Buffer 6), 20.5 µl of 97.4 % benzaldehyde, and 40 µl of MOPS buffer were combined in 1.5 ml vials. The vials were placed on ice. Prior to enzyme addition each vial was vortexed for 3 seconds to emulsify benzaldehyde. Reactions were started with the addition of 200 µl of 17.5 U/ml enzyme.

Vials were vortexed for 3 seconds before being turned continuously on a vertical rotary wheel at 4 °C

Four vials were sampled for each timepoint. The entire contents of two vials were diluted 20 times in 10 % [w/v] trichloroacetic acid. The samples were centrifuged to remove precipitated protein and the supernatant analyzed. Samples from the remaining two vials were gel filtered to remove substrates and products before being assayed for carboligase activity.

2.7 Two-Phase System

2.7.1 Effect of Organic Phase Solvents on Enzyme Stability in the Two-Phase System

Into a 4 ml glass vial (12 mm internal diameter), 1.08 ml of each organic solvent was layered on top of 1.08 ml of enzyme at 7 U/ml carboligase activity in MOPS buffer (Buffer 6). The bottom aqueous phase was stirred magnetically with a 12 mm length magnetic stirrer. Phase separation was maintained at all times with minimal disturbance at the interface. A control of 1.08 ml enzyme at 7 U/ml was stirred under the same conditions without organic solvent. The ratio of organic phase to aqueous phase was one, with a ratio of interfacial surface area to solvent volume of 1.05:1 (cm^2/cm^3). Duplicate vials were assayed for each solvent tested.

To 200 µl of carboligase assay solution, 190 µl citrate buffer (Buffer 2) was added and mixed. A 10 µl sample taken from the aqueous phase using a 25 µl glass syringe (SGE, Aust) was added to the assay solution and the carboligase activity determined. A 20 times dilution as described above was used for each time point to ensure that residual organic solvent concentrations in the assay were the same with each sample.

2.7.2 Effect of Organic Phase Solvents on *R*-PAC Production in the Two-Phase System

Into a 4 ml glass vial (12 mm internal diameter), 1.08 ml of 1.8 M benzaldehyde in each organic solvent was layered on top of 0.83 ml of sodium pyruvate in MOPS buffer (Buffer 6). Phase separation was maintained at this stage. The reaction was started with the addition of 0.25 ml enzyme at 31.6 U/ml carboligase activity in MOPS buffer (Buffer 6) into the aqueous phase. Directly after enzyme addition, the reaction was stirred rapidly forming an emulsion. The initial concentration of enzyme was 7.3 U/ml with starting substrate levels of 1.43 M pyruvate and 1.8 M benzaldehyde.

For benzaldehyde as organic phase solvent, 1.08 ml of benzaldehyde was combined directly with pyruvate prior to enzyme addition as described above. A control consisted of MOPS buffer (Buffer 6) substituted for enzyme addition.

After 72 hours, a sample from the organic phase was analyzed for *R*-PAC and benzaldehyde by capillary gas chromatography. A 25 µl sample from the aqueous phase was taken with a glass syringe (SGE, Aust) and mixed with 475 µl 10 % [w/v] trichloroacetic acid. The sample was vortexed and centrifuged to remove precipitated protein before being stored at –20 °C prior to analysis. The supernatant was analyzed for *R*-PAC and benzaldehyde by HPLC, and pyruvate by the spectrophotometric assay.

2.7.3 Determination of Partition Coefficients in the Octanol Two-Phase System

1.08 ml of octanol containing *R*-PAC, benzaldehyde, acetoin or acetaldehyde was layered on top of 1.08 ml of MOPS buffer (Buffer 6). The bottom aqueous phase was stirred magnetically with a 12 mm length magnetic stirrer. Phase separation was maintained at all times. Each flask was stirred for 24 hours before sampling from each phase to determine component concentrations. Organic phase concentrations of *R*-PAC, benzaldehyde, acetaldehyde and acetoin were determined by capillary gas

chromatography. Aqueous phase *R*-PAC and benzaldehyde were determined by HPLC. Aqueous phase acetoin was determined by GC and acetaldehyde and pyruvate by spectrophotometric assay. Carboligase activity was determined from *R*-PAC production analyzed by HPLC.

2.7.4 Enzyme Inactivation due to Benzaldehyde in the Octanol Two-Phase System

1.08 ml of octanol containing various concentrations of benzaldehyde were layered on top of 1.08 ml of enzyme at 7 U/ml carboligase activity in MOPS buffer (Buffer 6). The two phases were stirred rapidly to form an emulsion. Stirring was initiated directly after the organic phase was added to the enzyme.

Two controls were included:

1. enzyme exposed to octanol only
2. enzyme stirred alone without octanol or benzaldehyde.

After 3 hours, a 500 µl sample was taken, centrifuged at 4 °C for 3 seconds to separate the phases. The enzyme sample was diluted 20 times for carboligase activity determination. 200 µl of double strength carboligase assay substrate solution (see Section 2.8.9.2) was mixed with 190 µl citrate buffer (Buffer 2). A 10 µl sample taken from the aqueous phase using a 25 µl glass syringe (SGE, Aust) was added to the assay solution and the carboligase activity was determined. A 20 times dilution as described above was used for each time point to ensure that residual organic solvent concentrations in the assay were the same with each sample.

2.7.5 *R*-PAC Formation in MOPS Buffer Saturated with Octanol

MOPS buffer (Buffer 6) was combined with an equal volume of octanol, vortexed and stirred slowly at 6 °C for 48 hours to allow the phases to separate. The aqueous MOPS phase saturated with octanol was recovered for use in the following experiment.

A double strength enzyme solution at approximately 14 U/ml carboligase activity was prepared by dissolving 0.33 g of acetone precipitated enzyme powder into 10 ml of MOPS buffer (Buffer 6) saturated with octanol. A control was prepared in the same manner by dissolving enzyme into MOPS buffer (Buffer 6) that had not been saturated with octanol. The enzyme solutions were incubated on ice for 3 hours. After 3 hours the enzyme solutions were centrifuged to remove solids and the carboligase activities were determined after gel filtration to remove octanol and also with a 40 times dilution without octanol removal. The activity determined by these two approaches showed the same initial starting activities of 13.7 U/ml, with a protein concentration of 9.6 mg/ml.

Double strength substrate solutions were prepared by dissolving 0.41 g (150 mM) pyruvate and 0.27 g (100 mM) of benzaldehyde into 25 ml of MOPS buffer (Buffer 6) saturated with octanol. The substrate solution for the control was prepared in the same manner using MOPS buffer (Buffer 6) that had not been saturated with octanol. These solutions were incubated at 30 °C to fully dissolve the benzaldehyde. The substrate concentrations were analyzed and confirmed to be 100 mM benzaldehyde and 150 mM pyruvate.

To begin the reaction, 2.5 ml of enzyme at 4 °C was combined with 2.5 ml of buffer containing substrates. The solutions were stirred at 4 °C. Reactions were performed in duplicate. Samples were taken over several timepoints by combining 50 µl of sample with 5 µl of 100 % (w/v) TCA, vortexing and adding a further 945 µl RO water. The samples were analyzed for *R*-PAC, with final timepoints also measured for benzaldehyde, acetoin and acetaldehyde. The residual carboligase activities were determined at the final timepoint after gel filtration to remove containing substances.

2.7.6 *R*-PAC Production in the Rapidly Stirred Emulsion Two-Phase System with Octanol as Organic Solvent

2.7.6.1 Effect of Benzaldehyde Concentration

Into a 4 ml glass vial (12 mm internal diameter), 1.08 ml of benzaldehyde at various concentrations in octanol was layered on top of 0.83 ml of 1.87 M sodium pyruvate in MOPS buffer (Buffer 7). Phase separation was maintained at this stage. The reaction was started with the addition of 0.25 ml enzyme into the aqueous phase at 30.3 U/ml carboligase activity in MOPS buffer (Buffer 7). Directly after enzyme addition, the reaction was stirred rapidly forming an emulsion. The initial concentration of enzyme was therefore 7 U/ml with a starting pyruvate level of 1.43 M with various initial benzaldehyde concentrations.

After 40 hours a 500 µl sample was centrifuged for 3 seconds at 4 °C. A sample was taken from the organic phase, stored at –20 °C to be analyzed for benzaldehyde and *R*-PAC by capillary GC. A 25 µl sample taken from the aqueous phase using a glass syringe (SGE, Aust.) was added to 475 µl of 10 % [w/v] trichloroacetic acid. The sample was vortexed and centrifuged to remove precipitated protein before being stored at –20 °C prior to analysis for pyruvate by spectrophotometric assay and *R*-PAC and benzaldehyde by HPLC.

2.7.6.2 Effect of Enzyme Concentration

Into a 4 ml glass vial (12 mm internal diameter), 1.08 ml of 1.5 M benzaldehyde in octanol was layered on top of 0.83 ml of 1.87 M sodium pyruvate in MOPS buffer (Buffer 7). Phase separation was maintained at this stage. The reaction was started with the addition of 0.25 ml enzyme at various carboligase activities into the aqueous phase. Directly after enzyme addition, the reaction was stirred rapidly forming an emulsion. The initial substrate concentrations were 1.5 M benzaldehyde and 1.43 M pyruvate with various enzyme levels.

After 40 hours a 500 µl sample was centrifuged for 3 seconds at 4 °C. A sample was taken from the organic phase and stored at –20 °C prior to analysis of benzaldehyde and *R*-PAC by capillary GC. A 25 µl sample taken from the aqueous phase using a glass syringe (SGE, Aust.) was added to 475 µl of 10 % [w/v] trichloroacetic acid. The sample was vortexed and centrifuged to remove precipitated protein before being stored at –20 °C prior to analysis of pyruvate by spectrophotometric assay and *R*-PAC and benzaldehyde by HPLC.

2.7.6.3 Kinetic Evaluation and Scale-Up of *R*-PAC Formation in the Fully Stirred Two-Phase System

To a 150 ml glass vessel (5.6 cm in diameter) 52 ml of 1.5 M benzaldehyde in octanol was added to 40 ml of 1.87 M sodium pyruvate in MOPS buffer (buffer 7) at 4 °C. Phase separation was maintained at this stage. To begin the reaction, 12 ml of enzyme at 36.8 U/ml carboligase activity in MOPS buffer (Buffer 7) was added to the aqueous phase. Immediately after enzyme addition the reaction was stirred rapidly forming an emulsion. The starting conditions were therefore 1.5 M benzaldehyde in octanol, 1.43 M pyruvate and 8.5 U/ml carboligase activity.

At each time point a 500 µl sample was taken and immediately centrifuged for 3 seconds at 4 °C. A sample was taken from the organic phase and stored at -20 °C prior to analysis for *R*-PAC, benzaldehyde, acetaldehyde and acetoin by capillary GC.
A 25 µl sample taken from the aqueous phase using a glass syringe (SGE, Aust) was added to 475 µl of 10 % [w/v] trichloroacetic acid. The sample was vortexed and centrifuged to remove precipitated protein before being stored at –20 °C prior to analysis of pyruvate and acetaldehyde by spectrophotometric assays, *R*-PAC and benzaldehyde by HPLC, acetoin by GC. The pH was estimated by pH paper (Merck, USA) in the aqueous phase.

2.7.7 *R*-PAC Production in the Phase Separated Two-Phase System with Octanol as Organic Solvent

2.7.7.1 Effect of Enzyme Concentration

Into a 4 ml glass vial (12 mm internal diameter), 1.08 ml of 1.5 M benzaldehyde in octanol was layered on top of 0.83 ml of 1.87 M sodium pyruvate in MOPS buffer (Buffer 7). Phase separation was maintained. The reaction was started with the addition of 0.25 ml enzyme in MOPS buffer (Buffer 7) at various carboligase activities into the aqueous phase. Directly after enzyme addition, the bottom aqueous phase was stirred slowly with a magnetic stirrer with minimal disturbance at the interface.

A 200 µl sample was taken from the organic phase and analyzed for benzaldehyde and *R*-PAC by capillary GC. A 200 µl sample was taken from the aqueous phase using a glass syringe (SGE, Aust.). To 25 µl of the aqueous sample, 475 µl of 10 % [w/v] trichloroacetic acid was added. The sample was vortexed and centrifuged to remove precipitated protein before being stored at –20 °C prior to analysis of pyruvate by spectrophotometric assay, *R*-PAC and benzaldehyde by HPLC and acetoin by GC.

2.7.7.2 Kinetic Evaluation and Scale-Up of *R*-PAC Formation in the Phase Separated Two-Phase System

To a 150 ml glass vessel (5.6 cm in diameter) 52 ml of 1.5 M benzaldehyde in octanol was added to 40 ml of 1.87 M sodium pyruvate in MOPS buffer (Buffer 7) at 4 °C. Phase separation was maintained at this stage. To begin the reaction, 12 ml of enzyme in MOPS buffer at 16.5 U/ml in one vial and at 3.9 U/ml carboligase activity in a second vial was added into the aqueous phase. Immediately after enzyme addition, the bottom aqueous phase was stirred slowly with a magnetic stirrer with minimal disturbance at the interface. The starting conditions were therefore 1.5 M benzaldehyde in octanol, 1.43 M pyruvate and with 0.9 U/ml carboligase activity in one flask and 3.8 U/ml carboligase activity in a second flask.

A 200 µl sample was taken from the organic phase and stored at –20 °C prior to analysis of *R*-PAC, benzaldehyde, acetaldehyde and acetoin by capillary GC. A 200 µl sample was taken from the aqueous phase. 475 µl of 10 % [w/v] trichloroacetic acid was added to 25 µl of the aqueous phase sample. The sample was vortexed and centrifuged to remove precipitated protein before being stored at –20 °C prior to analysis of pyruvate and acetaldehyde by spectrophotometric assays, *R*-PAC and benzaldehyde by HPLC, acetoin by GC. Estimation of pH in the aqueous phase was by pH paper (Merck, USA).

2.8 Methods of Analysis

2.8.1 Organic Phase *R*-PAC and Benzaldehyde by Capillary Gas Chromatography

Concentrations of phenylacetylcarbinol and benzaldehyde in the organic phase were determined by gas chromatography using a capillary column operated with split injection. Concentrations of each component were determined by comparing peak areas with a set of standards. A sample chromatogram and calibration curves for all compounds are shown in Figure A1 (a) and (b), Appendix A.

The following operating conditions were used:

Varian Star 3400 gas chromatograph, with chromatograms recorded and peak areas calculated using a Spectra Physics SP 4600 integrator

Column: 100 % dimethylpolysiloxane capillary column (Chrompack Cp-sil 5B, 50 m length x 0.25 mm internal diameter, 0.12 µm phase thickness, Varian Inc, Aust.)
Carrier gas and average linear velocity: Nitrogen at 0.98 ml/min (30 psi constant head pressure)
Split ratio: 120
Detector: Flame ionization detector (FID) with hydrogen (20 ml/min) and air (300 ml/min).
Injection volume: 1 µl

Injection temperature: 180 °C
Detection temperature: 180 °C
Oven temperature: 130 °C for 2.9 min, increased to 150 °C at 50 °C/ min. Held at 150 °C for 2 min.
Total run time: 10 min.

2.8.2 Organic Phase Acetoin by Capillary Gas Chromatography

Acetoin in the organic phase was determined by gas chromatography using a capillary column operated with split injection. The concentration was determined by comparing the peak area with a standard curve. A sample chromatogram and calibration curve is shown in Figure A2(a) and (b), Appendix A.

The following operating conditions were used:

Varian Star 3400 gas chromatograph, with chromatograms recorded and peak areas calculated using a Spectra Physics SP 4600 integrator.

Column: 100 % dimethylpolysiloxane capillary column (Chrompack Cp-sil 5B, 50 m length x 0.25 mm internal diameter, 0.12 μm phase thickness, Varian Inc, Aust.)
Carrier gas and average linear velocity: Nitrogen at 0.66 ml/min (20 psi constant head pressure)
Split ratio: 150
Detector: Flame ionization detector (FID) with hydrogen (20 ml/min) and air (300 ml/min).
Injection volume: 1 μl
Injection temperature: 180 °C
Detection temperature: 180 ° C
Oven temperature: 100 ° C for 5.0 min, increased to 150 ° C at 50 ° C/ min. Held at 150 ° C for 2 min.
Total run time: 10 min.

2.8.3 Organic Phase Acetaldehyde by Capillary Gas Chromatography

Acetaldehyde in the organic phase was determined by gas chromatography using a capillary column operated with split injection. The concentration was determined by comparing the peak area with a standard curve. A sample chromatogram and calibration curve is shown in Figure A2 (a) and (b), Appendix A.

The following operating conditions were used:

Varian Star 3400 gas chromatograph, with chromatograms recorded and peak areas calculated using a Spectra Physics SP 4600 integrator

Column: 100 % dimethylpolysiloxane capillary column (Chrompack Cp-sil 5B, 50 m length x 0.25 mm internal diameter, 0.12 μm phase thickness, Varian Inc, Aust.)
Carrier gas and average linear velocity: Nitrogen at 0.66 ml/min (20 psi constant head pressure)
Split ratio: 150
Detector: Flame ionization detector (FID) with hydrogen (20 ml/min) and air (300 ml/min).
Injection volume: 1 μl
Injection temperature: 100 °C
Detection temperature: 100 ° C
Oven temperature: 80 ° C for 4.0 min, increased to 150 ° C at 50 ° C/ min. Held at 150 ° C for 8 min.
Total run time: 15 min.

2.8.4 Aqueous Phase *R*-PAC and Benzaldehyde by High Performance Liquid Chromatography (HPLC)

Reverse phase HPLC was used to analyze for aqueous phase concentrations of phenylacetylcarbinol and benzaldehyde. The method was modified by Rosche *et al.* (2001) according to the established protocol of Breuer (1997). Concentrations of each component were calculated by comparison of peak areas with a set of standards. A sample chromatogram and calibration curves for all three compounds are shown in Figure A3 (a) and (b), Appendix A.

The following operating conditions were used:

Data captured and processed using Delta 5.0 chromatography software (Digital Solutions, Pty Ltd., Aust.)

Column: Alltima™ C8 (5 μm partical size, 150 mm length, 4.6 mm internal diameter, Alltech Associates Pty., Ltd., Aust)
Guard column: All-guard™ (5 μm particle size, 7.5 mm length, 4.6 mm internal diameter, Alltech Associates Pty., Ltd., Aust.)
Detection: U.V absorbance at 283 nm
Mobile phase: 32 % (v/v) acetonitrile; 0.5 % (v/v) acetic acid; MilliQ water
Flowrate: 1.0 ml/min isocratic operation
Run time: 20 min.
Injection volume: 5 μl

2.8.5 Aqueous Phase Acetoin by Gas Chromatography

Aqueous phase concentration of acetoin was determined by gas chromatography. Acetoin concentration was determined by comparison of peak areas with a set of standards. A sample chromatogram and calibration curve is shown in Figure A4 (a) and (b), Appendix A.

The following operating conditions were used:

Packard 427 gas chromatograph with chromatograms recorded and peak areas calculated using a Spectra Physics SP 4600 integrator.

Column: Stainless steel column containing 10 % carbowax® on chromsorb ® W-AW (80-100 μm mesh range, 3.6 m length, 3.2 mm internal diameter, Alltech Associates Pty., Ltd., Aust.)
Carrier gas and flowrate: Nitrogen at 12 ml/min (head pressure of 4 kp/cm^2)
Detector: Flame ionization detector (FID) with hydrogen (20 ml/min) and air (300 ml/min).
Injection volume: 3 μl
Injection temperature: 160 °C
Detection temperature: 160 °C
Oven temperature: 110 °C isothermal operation.
Total run time: 20 min.

2.8.6 Ethanol by Gas Chromatography

Solid phase gas chromatography was used to determine ethanol concentrations in fermentation samples. Ethanol concentration was determined by comparison of peak areas with a set of standards. A sample chromatogram and calibration curve is shown in Figure A5 (a) and (b), Appendix A.

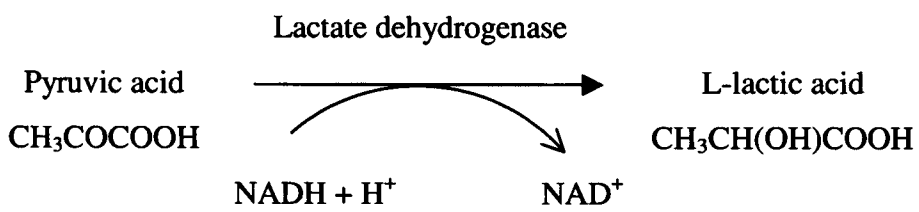
The following operating conditions were used:

Packard 427 gas chromatograph with chromatograms recorded and peak areas calculated using a Spectra Physics SP 4600 integrator

Column: Glass column containing Porapak® type Q (100-120 µm mesh range, 1 m length, 2.35 mm internal diameter, Alltech Associates Pty., Ltd., Aust.)
Carrier gas and flowrate: Nitrogen at 30 ml/min (head pressure of 2.8 kp/cm^2)
Detector: Flame ionization detector (FID) with hydrogen (20 ml/min) and air (300 ml/min).
Injection volume: 3 µl
Injection temperature: 220 °C
Detection temperature: 220 °C
Oven temperature: 180 °C isothermal operation.
Total run time: 6 min.

2.8.7 Pyruvic Acid (Pyruvate)

Pyruvic acid was determined spectrophotometrically according to an established method of Roche (Germany). Pyruvic acid is reduced to lactic acid by the enzyme lactate dehydrogenase with reduced nicotinamide-adenine dinucleotide (NADH) according to the following reaction.



The amount of NADH oxidized is stoichiometrically proportional to the amount of pyruvic acid present in the sample. The amount of NADH consumed is monitored by the decrease in absorbance at 340 nm.

Buffer: 0.75 M Triethanolamine, 7.5 mM EDTA, pH 7.6 adjusted with 5 M NaOH
NADH Solution: 6 mM NADH, 120 mM $NaHCO_3$
L-LDH: lactate dehydrogenase
Pyruvic acid standard: 0.04 mg/ml
Sample range: 0.02-0.5 g/l pyruvic acid

To quantify pyruvic acid, a cuvette containing 250 µl of buffer was combined with 500 µl of reverse osmosis (RO) water, 25 µl of NADH solution and 25 µl of sample. After mixing of the cuvette contents by inversion, the initial absorbance at 340 nm was measured (A1). 5 µl of L-LDH enzyme was added to begin the reaction. After 8 minutes the final absorbance at 340 nm was measured (A2). A blank of RO water and a positive control using a pyruvic acid standard solution were substituted for the 25 µl of sample. The amount of pyruvic acid in the sample was calculated as follows:

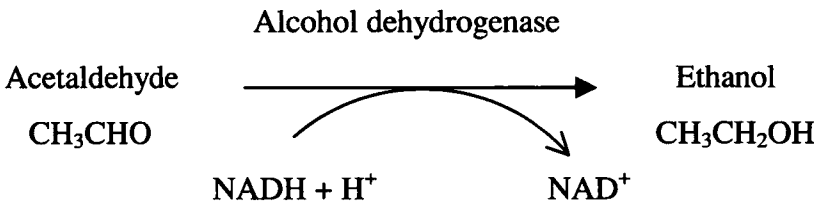
$$C = \frac{V_t * MW * (A1-A2)}{\varepsilon * d * v_s}$$

C = Concentration of pyruvic acid (g/l)
V_t = Final volume (805 µl)
v_s = Sample volume (25 µl)
MW= Molecular weight of pyruvic acid (88.11)
d = length of light path (1 cm)
ε = Extinction coefficient of NADH at 340 nm
= 6300 ($l.mol^{-1}.cm^{-1}$)

$$\text{Concentration} = 0.4502 * [(A1-A2)_{sample} - (A1-A2)_{blank}] \quad (g/l)$$

2.8.8 Acetaldehyde in the Aqueous Phase

Acetaldehyde was determined spectrophotometrically according to the following method. Acetaldehyde is reduced to ethanol by the enzyme alcohol dehydrogenase with reducing agent nicotinamide-adenine dinucleotide (NADH) according to the following reaction.



The amount of NADH oxidized is stoichiometrically proportional to the amount of acetaldehyde present in the sample. The amount of NADH consumed is monitored by the decrease in absorbance at 340 nm.

Buffer: 200 mM citric acid, pH 7.0 at 25 °C adjusted with 4 M KOH
NADH Solution: 18 mM NADH
Alcohol dehydrogenase: 10 U/ml
Sample range: 0.07-0.1 g/l

To quantify acetaldehyde, a cuvette containing 940 µl of buffer was mixed with10 µl of NADH solution and 50 µl of sample. The initial absorbance at 340 nm was measured (A1). 2 µl of alcohol dehydrogenase enzyme was added to begin the reaction. After 8 minutes the final absorbance at 340 nm was measured (A2). A blank of reverse osmosis (RO) water and a positive control using an acetaldehyde standard solution were substituted for the 50 µl of sample. The buffer concentration sufficiently maintained the pH at 7.0 for samples containing 10 % TCA [w/v]. The amount of acetaldehyde in the sample was calculated as follows:

$$C = \frac{V_t * MW * (A1-A2)}{\varepsilon * d * v_s}$$

C = Concentration of acetaldehyde (g/l)

V_t = Final volume (1002 µl)

v_s = Sample volume (50 µl)

MW = Molecular weight of acetaldehyde (44.1)

d = Length of light path (1 cm)

ε = Extinction coefficient of NADH at 340 nm

= 6300 ($l.mol^{-1}.cm^{-1}$)

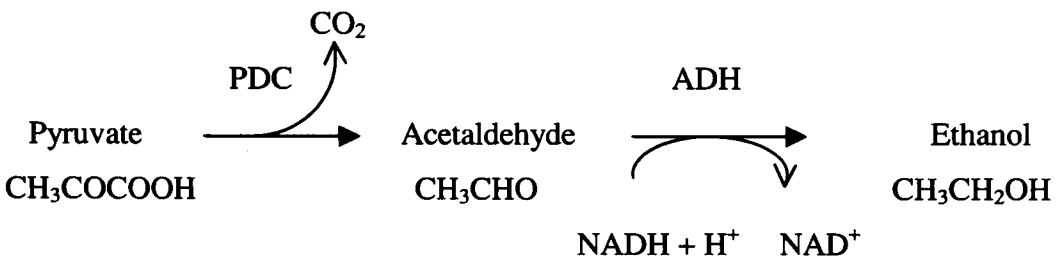
Concentration = $0.1403 * [(A1-A2)_{sample} - (A1-A2)_{blank}]$ (g/l)

2.8.9 Enzyme Activity

2.8.9.1 Decarboxylase Activity

One unit of decarboxylase activity converts 1.0 µmol of pyruvate to acetaldehyde per minute at 25 °C and pH 6.0.

Assay was performed according to an established protocol of Sigma (USA). Pyruvate was decarboxylated to acetaldehyde and carbon dioxide by pyruvate decarboxylase (PDC) in the presence of cofactors, thiamine pyrophosphate and magnesium ions. Acetaldehyde formed was quantified by reduction to ethanol by the action of alcohol dehydrogenase (ADH) and reducing agent nicotinamide-adenine dinucleotide (NADH) according to the following reaction.



The rate of NADH consumption is monitored by the decrease in absorbance over time at 340 nm. The amount of NADH oxidized is stoichiometrically proportional to the amount of pyruvate consumed in the sample. The reaction mixture consisted of the following components described in Table 2.10.

Table 2.10: Components of the decarboxylase assay.

	Reference	Substrate blank	Test sample
Citrate buffer at 28.5 °C (Buffer 3)	2.90 ml	2.80 ml	2.70 ml
1 M sodium pyruvate in RO water at 25°C	100 µl	100 µl	100 µl
6.4 mM NADH in RO water on ice	0	50 µl	50 µl
ADH in RO water on ice 200 U/ml	0	50 µl	50 µl
Enzyme sample on ice (0.3-0.6 U/ml in Buffer 3)	0	0	100 µl

All reagents were mixed directly into a cuvette and placed into a spectrophotometer with a temperature controlled cell maintained at 25 °C. The reference mixture was used to zero the spectrophotometer. The decrease in absorbance of substrate blank and test sample were measured over 2 minutes at 340 nm, with the rate of decrease calculated over the linear period from 0.5 to 2 minutes. The activity was calculated as follows;

$$C = \frac{\Delta A/t * V_t}{\varepsilon * d * v_s}$$

$\Delta A/t$ = Change in absorbance (ΔA) over time (t)

C = Pyruvate concentration converted to acetaldehyde (µmol)

V_t = Final volume (3.0 ml)

v_s = Sample volume (0.1 ml)

d = Length of light path (1 cm)

ε = Extinction coefficient of NADH at 340 nm

= 6.3 ($ml.\mu mol^{-1}.cm^{-1}$)

PDC (U/ml) = 4.76 * ΔA/time

2.8.9.2 Carboligase Activity

One unit of carboligase activity is defined as one micromole of *R*-PAC produced per minute at 25 °C and pH 6.4. The following method was modified according to an established protocol of Breuer (1997).

Double strength carboligase assay substrate solution

200 mM citric acid
3.0 M ethanol
2 mM thiamine pyrophosphate (TPP)
2 mM $MgSO_4.7H_2O$
80 mM benzaldehyde
200 mM sodium pyruvate
pH 6.4 at 25 °C adjusted with 4 M KOH

A 50 ml solution was made up as follows: citric acid and magnesium sulfate were dissolved in 30 ml RO water. The pH was adjusted to 6.0 with 4 M KOH. The solution was then cooled to 4 °C. Ethanol, sodium pyruvate and TPP were then fully dissolved. Benzaldehyde was weighed into the solution and stirred at room temperature until the benzaldehyde was fully dissolved. The pH and final volume adjustments were then made. 200 µl aliquots of the assay solution was dispensed into 1.5 ml polypropylene tubes (Eppendorf, USA) and frozen at –20 °C for a maximum of 2 weeks.

For carboligase activity determination, the above 200 µl aliquots of solution were defrosted at 25 °C for 10 minutes and vortexed. The enzyme sample was diluted in citrate buffer (buffer 3) to between 0.1-0.5 U/ml carboligase activity. 200 µl of this enzyme sample was mixed with 200 µl of the above double strength assay substrate solution, vortexed and incubated at 25 °C for 20 minutes. Combining of sample and substrate in this way results in a 2 fold dilution factor. The initial substrate concentration in the assay was therefore 40 mM benzaldehyde and 100 mM pyruvate. After 20 minutes, the reaction was terminated by the addition of 40 µl of 100 % (w/v) trichloroacetic acid, resulting in a 1.1 fold dilution. The sample was centrifuged at

12000 rpm (11600 RCF) for 5 minutes in an Eppendorf (USA) 5415C centrifuge to remove precipitated protein. The supernatant was analyzed for *R*-PAC by HPLC. Commercial PDC from brewer's yeast was used as a positive control. The commercial preparation yielded in 28 U/ml carboligase activity.

The activity was calculated as follows;

$$\text{Activity (U/ml)} = \frac{\mu\text{mol}}{\text{ml.min}}$$

$$= \frac{\text{mM } R\text{-PAC} * \text{Dilution factor}}{20 \text{ min}}$$

The dilution factor accounts of sample dilution, TCA addition (x1.1) and substrate addition to sample (x2).

$$\text{Dilution factor} = \text{sample dilution factor} * 1.1 * 2$$
$$= 2.2 * \text{sample dilution factor}$$

Activity (U/ml) = 0.11 * (mM *R*-PAC) * (sample dilution factor)

2.8.9.3 Determination of Enzyme Activity in Samples Containing Interfering Substances

Enzyme activity cannot be determined in the presence of substances that interfere directly with the decarboxylation and carboligation assays. Therefore, micro chromatography columns containing Bio-gel P6 packing material in sodium chloride, sodium citrate buffer (Biorad, Aust) were used to remove small molecular weight compounds according to the following protocol.

The column was vortexed for 5 seconds to resuspend settled gel. The column cap and drainage tip were removed and the column placed into a collection tube. The column was allowed to stand for 5 minutes, draining excess buffer into a collection tube.

The drained buffer was discarded prior to centrifuging column and collection tube at 4000 rpm (1300 RCF) for 4 minutes in an Eppendorf (USA) 5415C centrifuge. After centrifugation, the column was placed into a new collection tube containing 55 µl of citrate collection buffer (Buffer 4). 75 µl of enzyme sample containing interfering substances was pipetted onto the top of the column. The column and collection tube containing buffer were centrifuged at 4000 rpm (1300 RCF) for 1 minute in an Eppendorf (USA) 5415C centrifuge. The combined volume of enzyme recovered and collection buffer was approximately 110 µl. The collection tube was vortexed and placed on ice for 20 minutes to allow gel filtered enzyme to reassociate with cofactors. Then the carboligase activity was measured. If *R*-PAC was present in the original sample, the residual *R*-PAC level was determined in the gel filtered sample. The residual *R*-PAC level was accounted for in the carboligase assay.

2.8.10 Protein Concentration Determination

Soluble protein was determined using Pierce reagent (Pierce, USA) Coomassie® protein assay solution. This method is based on the previously developed Bradford colorimetric method for protein quantification (Bradford 1976). 750 µl of assay reagent at 25 °C was mixed with 25 µl of sample directly into a disposable cuvette. The mixture was incubated at room temperature for 5 minutes before measuring the absorbance at 595 nm. An estimation of soluble protein was made by direct comparison to a bovine serum albumin standard solution. The assay linearity was between 0.1-1.0 mg/ml protein as shown in the standard curve Figure A6, Appendix A.

2.8.11 Glucose Determination

Glucose concentrations in fermentation samples were determined using a Glucose Analyzer, Model YSI 2300 Stat Plus (Yellow Springs Instruments Co, USA).

Quantification is based on the oxidation of glucose by the immobilized enzyme glucose oxidase to produce glucose - δ - lactone and hydrogen peroxide. The hydrogen

peroxide is oxidized at the platinum anode and the current produced is measured between the two electrodes.

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Glucose - } \delta \text{ - lactone} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{Platinum anode}} 2H^+ + O_2 + 2e^-$$

$$2AgCl + 2e^- \xrightarrow{\text{Silver cathode}} 2Ag + 2Cl^-$$

2.8.12 Biomass Determination

2.8.12.1 Cell Dry Weight

3 ml of culture broth was pipetted into a pre-weighed 5 ml glass culture tube and centrifuged at 4000 rpm for 15 minutes. The supernatant was discarded and the cell pellet washed twice with RO water, with the sample centrifuged between washes as above. The washed cell pellet was placed into an oven for 24 hours at 105 °C. The dried tubes were placed in a desiccator to cool before measuring the final weight of the tube. The dry weight was calculated as follows:

$$\text{Cell dry weight (g/l)} = \frac{\text{weight of tube with sample after drying (g)-weight of empty tube initially (g)}}{\text{volume of sample added to tube (l)}}$$

2.8.12.2 Optical Density

The optical density of the culture broth was measured at 660 nm using a Shimadzu UV 1201 UV-Vis spectrophotometer. The culture broth was diluted in RO water between 0.1-0.4 absorbance. The correlation between optical density and cell dry weight is shown in Figure A7 in Appendix A.

2.8.13 PDC Activity Analysis from Bioreactor Samples

The following method was used to analyze for decarboxylase activity, carboligase activity and protein concentration of samples from the 5 and 30 litre fermentation.

The 25 ml sample was centrifuged at 10000 rpm (14476 RCF) for 5 minutes at 4 °C using a SA-600 rotor in a Sorvall RC-5B centrifuge (Dupont, USA). The supernatant was discarded after centrifuging. The cell pellet was washed twice in cold RO water and centrifuged as above. The pellet was resuspended to 10 g/l dry weight in citrate buffer (buffer 2) by optical density correlation. The exact cell dry weight of this sample was determined.

To break open the cells, 2 ml of cells in buffer were freeze thawed in liquid nitrogen three times, with defrosting at 30 °C in between freeze thawing. This extract was transferred to a 5 ml glass bottle containing 2 ml of cold 0.5 mm glass beads (Biospec, USA). The mixture was vortexed for five 1 minute intervals with 5 minute rests on ice in between vortexing. A 1 ml sample of the broken cells was centrifuged at 12000 rpm (16090 RCF) in a chilled centrifuge for 5 minutes. The supernatant was analyzed for decarboxylase activity, carboligase activity and protein concentration as described previously in Section 2.8.10.

2.8.14 Exit Gas Analysis

The fermentation exhaust gas was analyzed for oxygen and carbon dioxide content. These values were subsequently used for respiratory quotient (RQ) calculation and control.

The exhaust gas was passed from the fermentor through a dehumidifier (model DH 1052G, Komatsu Electronics Inc, Japan) before being analyzed for oxygen content by an oxygen gas analyzer (model 1420B, Servomex Ltd UK) and a carbon dioxide gas analyzer (model 1410B, Servomex Ltd UK).

Oxygen is quantified by utilizing its ability to be attracted into a magnetic field. A magneto-dynamic measuring cell produces a current which is proportional to the oxygen concentration. Carbon dioxide is quantified using dual wavelength, single beam infrared technology.

Calibration of the zero point for both analyzers was performed using nitrogen. A gas mix consisting of 4.97 % CO_2 and 10.0 % O_2 was used to calibrate the positive response. For all calibrations and exhaust analysis, the gas flowrate was maintained at 5 ml/min at ambient temperature.

2.8.15 Enantiomeric Excess (EE) Determination

HPLC was used to analyze for relative concentrations of both (R) and (S) enantiomers of phenylacetylcarbinol according to the established protocol of Breuer (1997). Comparison of relative peak areas of both enantiomers was used to calculate the enantiomeric excess. A sample chromatogram is shown in Figure A8, Appendix A.

The sample was prepared by vortexing 200 µl of the aqueous sample with 200 µl of ethyl acetate. After separating the aqueous and organic phases by centrifugation, 150 µl of the organic phase containing extracted *R*-PAC was placed into a 1.5 ml tube (Eppendorf, USA). The sample was placed in the rotary vacuum centrifuge for 15

minutes to remove ethyl acetate. The sample was resuspended in 300 µl hexane, vortexed and centrifuged to remove solids. The supernatant was analyzed by the following HPLC method.

The following operating conditions were used:

Column: Chiralcel OD (10 µm particle size, 25 cm length, 4.6 mm internal diameter, Diacel Chemical industries Ltd., USA)
Detection: U.V. absorbance at 283 nm
Mobile phase: 950 ml hexane, 50 ml isopropanol and 1 ml formic acid
Flowrate: 0.8 ml/min isocratic operation
Run time: 30 minutes
Injection volume: 1 µl

2.8.16 Gas Chromatography - Mass Spectrometry (GC-MS)

GC-MS operation and method development was performed by Charlton (2000). Separation of compounds for mass spectrometry was performed using gas chromatography using a capillary column operated in splitless mode. The mass spectrometry was performed on a Hewlett-Packard model 5971A Mass Selective Detector. The mass spectrometer was configured for scan mode (50-600 amu).

The following gas chromatography operating conditions were used:

Hewlett Packard model 5890 Series II gas chromatograph

Column: 14 % cyanopropylphenyl polysiloxane (Solgel-1, 30 m length, 0.32 mm internal diameter, 0.25 µm phase thickness; SGE Pty., Ltd., Aust.)
Injection volume: 1 µl performed in splitless mode (1.5 min) into a deactivated glass liner at constant head pressure of 5 psi.
Injection temperature: 280 °C and 300 °C at the interface

Oven temperature: 50 °C for 1.5 min, ramped at 20 °C/min to 300 °C. The mass spectrometry was performed on a Hewlett-Packard model 5971A Mass Selective Detector. The mass spectrometer was configured for scan mode (50-600 amu).

2.9 Equations for Calculations

2.9.1 Batch Fermentation Calculations and Parameters

Yield of cell mass on substrate

$$Y_{biomass/glucose} = \frac{\text{Biomass produced}}{\text{Glucose consumed}} \quad (g/g)$$

Yield of product on cell mass

$$Y_{product\ (P)\ /biomass} = \frac{\text{Product formed}}{\text{Biomass produced}} \quad (g/g)$$

where product (P) was ethanol or pyruvate

Specific growth rate

$$\mu = 1/x \,.\, dx/dt = \ln (x_t/x_i)/t \qquad (h^{-1})$$

Where x_t and x_i are the biomass concentrations at time (t) and at initial time (i) respectively calculated during the exponential growth period.

Respiratory Quotient

$$\text{Respiratory quotient (RQ)} = \frac{\text{carbon dioxide evolution rate (CER)}}{\text{oxygen uptake rate (OUR)}}$$

Enzyme production during the fermentation

Specific enzyme production

$$= \frac{\text{Enzyme activity in crude extract (U/ml)}}{\text{Cell concentration in sample (g dry weight/ml)}} \quad \text{(U/g)}$$

Specific enzyme activity

$$\text{Specific activity} = \frac{\text{Activity (U/ml)}}{\text{Protein concentration (mg/ml)}} \quad \text{(U/mg)}$$

2.9.2 Biotransformation Calculations and Parameters

$$\text{pyruvate} + \text{benzaldehyde} \xrightarrow{\text{PDC}} R\text{-PAC} + CO_2$$

$$2\ \text{pyruvate} \xrightarrow{\text{PDC}} 2\ \text{acetaldehyde} + CO_2 \xrightarrow{\text{PDC}} \text{acetoin}$$

1 mol of pyruvate is converted to 1 mol *R*-PAC

1 mol benzaldehyde is converted to 1 mol *R*-PAC

1 mol pyruvate is converted to 1 mol acetaldehyde

2 mol pyruvate are converted to 2 mol acetaldehyde which forms 1 mol acetoin

Molar conversion yields

$$Y_{R\text{-PAC/benzaldehyde}} = \frac{\text{Amount of } R\text{-PAC formed (mol)}}{\text{Amount of benzaldehyde consumed (mol)}} \times 100 \quad (\%)$$

$$Y_{R\text{-PAC/pyruvate}} = \frac{\text{Amount of } R\text{-PAC formed (mol)}}{\text{Amount of pyruvate consumed (mol)}} \times 100 \quad (\%)$$

$$Y_{acetaldehyde/pyruvate} = \frac{\text{Amount of acetaldehyde formed (mol)}}{\text{Amount of pyruvate consumed (mol)}} \times 100 \quad (\%)$$

For calculation of the yield of acetoin on pyruvate utilized, the stoichiometric conversion of 2 moles of pyruvate to 1 mole of acetoin is included in the calculation to allow a mass balance on pyruvate from all yield calculations.

$$Y_{acetoin/pyruvate} = \frac{\text{Amount of acetoin formed (mol)} \times 2}{\text{Amount of pyruvate consumed (mol)}} \times 100 \quad (\%)$$

Specific R-PAC production

$$\text{Specific } R\text{-PAC production} = \frac{R\text{-PAC produced (mg/ml)}}{\text{activity (U/ml)}} \quad (\text{mg } R\text{-PAC/U})$$

$$\text{Specific } R\text{-PAC productivity} = \frac{\text{Specific } R\text{-PAC production}}{\text{Time (h)}} \quad (\text{mg } R\text{-PAC/U/h})$$

Log (P)

Where P= Partition coefficient = $[x]_{organic} / [x]_{aqueous}$

$$\text{Log (P)} = \log_{10} (P)$$

Where $[x]_{organic}$ = organic phase concentration of component

$[x]_{aqueous}$ = aqueous phase concentration of component

CHAPTER 3

PRODUCTION OF PYRUVATE DECARBOXYLASE

3.1 Introduction

Induction of pyruvate decarboxylase production in *Candida utilis* has been achieved in anaerobic and semi anaerobic environments. The respiratory quotient (RQ) has been identified as an important parameter for the control of the metabolic state of yeast. It has been shown that as RQ increases, production of pyruvate decarboxylase also increases (Chow 1998). Work by Weusthuis *et al.* (1994) showed that the availability of oxygen to *C.utilis* had a dramatic effect on PDC activity. This data was used to demonstrate a strong correlation between RQ and PDC activity at RQ values lower than 50, and relative insensitivity at higher RQ's (Chow 1998).

Fermentations for the production of partially purified pyruvate decarboxylase from the strain used in this study have been achieved previously by Shin (1994) and Chow (1998). Both studies identified variation in the properties of enzyme between different batches of partially purified enzyme. Yields of *R*-PAC and enzyme stability in the presence of benzaldehyde varied between batches of enzyme. It was not known if fermentation conditions or purification conditions were responsible for these variations. Evaluating yields of *R*-PAC on substrates consumed and stability of the enzyme preparation, followed by reproducible production of these characteristics, is an important requirement for the establishment of an industrial process.

The objectives of this Chapter are:

- to induce pyruvate decarboxylase production in *Candida utilis* under fermentative conditions and to evaluate RQ control for enzyme induction at the 5 litre and 30 litre scale,

- to recover pyruvate decarboxylase from whole cells, providing a storage stable enzyme preparation for further characterization. This preparation is to be used for investigation of enzyme characteristics and *R*-PAC production in subsequent chapters,

- to investigate the differences existing between various batches of enzyme, with a view to understanding the enzyme production protocol.

3.2 Bioreactors

The aim of this section was to produce pyruvate decarboxylase under controlled conditions that were based on the recommended findings by Shin (1994) and Chow (1998). An RQ level of 4 was recommended by Chow (1998) as sufficient to fully induce PDC production. The emphasis was to induce pyruvate decarboxylase activity in several bioreactors, each operated under similar conditions to ensure reproducibility. Bioreactors were not subsequently optimized to maximize pyruvate decarboxylase activity.

Pyruvate decarboxylase from *Candida utilis* was produced in 5 litre and 30 litre controlled bioreactors with working volumes of 3.5 litre and 20 litre respectively. The bioreactors were controlled at 28 °C and pH 5.5 according to the protocols outlined in Section 2.4.3.

The 5 and 30 litre bioreactors were controlled at an RQ setpoint of 1 for the first 8.5 hours. After 8.5 hours the bioreactor was controlled at an approximate RQ setpoint of 4.

For all bioreactors, the following trends were observed:

During the initial 8.5 hours, consumed glucose was converted to biomass, with small amounts of ethanol produced. An increase in the rate of ethanol production occurred after the RQ setpoint was increased to 4 at 8.5 hours. The accompanied increase in carbon dioxide evolution rate (CER) and a decrease in oxygen uptake rate (OUR) confirmed a cessation in fully respiratory growth and change to fermentation. The measured RQ was oscillatory after the RQ setpoint was changed to 4, however the measured RQ settled to a final value of 4 ± 1 by the end of the fermentation. This suggests that the proportional/integral control constants could be improved.

The pyruvate levels accumulated outside the cells were low, with 1.4 g/l produced in the 5 litre bioreactor, and 2.7-2.9 g/l in the 30 litre bioreactor at the end of fermentation.

In all cases, pyruvate decarboxylase production increased under more fermentative conditions with RQ controlled at approximately 4. In all cases the specific activity, activity per cell dry weight and activity per volume of fermentation broth increased after the RQ was increased.

Final concentrations of biomass, ethanol, pyruvate, as well as specific growth rates and enzyme levels are compared in Table 3.1. Raw data showing growth profiles, CER, OUR, RQ, dissolved oxygen and enzyme levels are shown in Sections 3.2.1, 3.2.2, and 3.2.3.

3.2.1 5 Litre Bioreactor

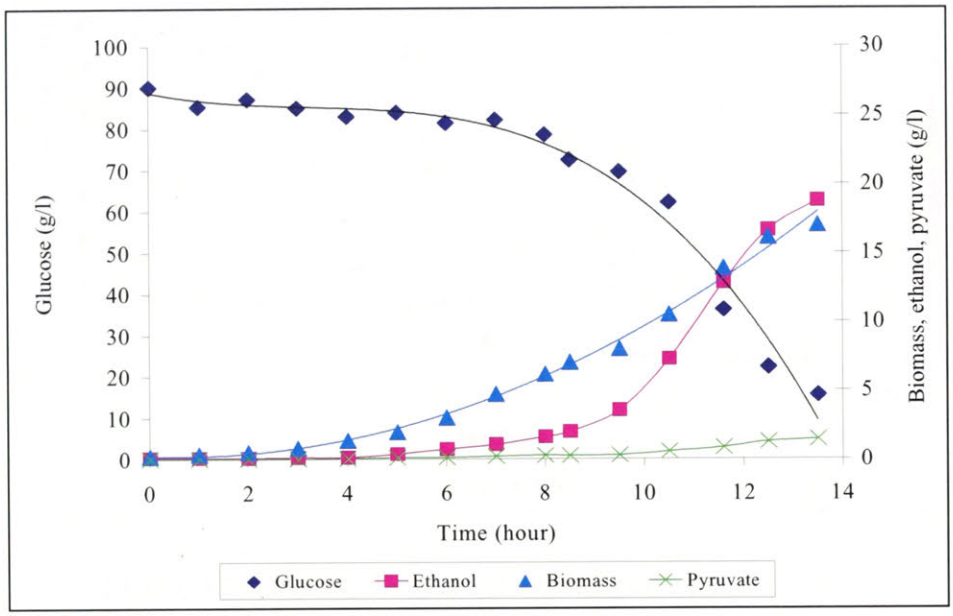


Figure 3.1: Profile for the 5 litre bioreactor.

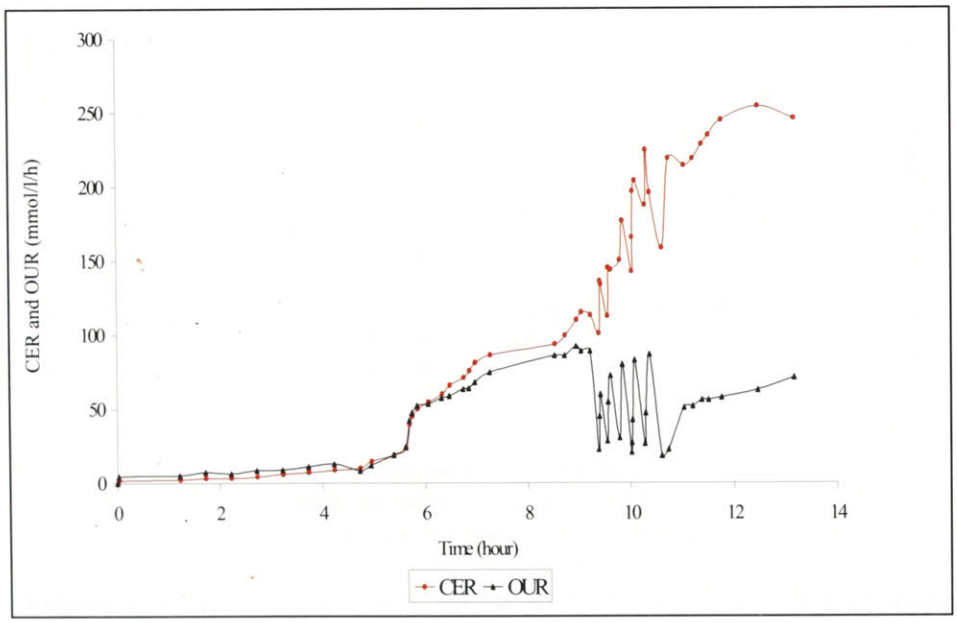


Figure 3.2: CER and OUR for the 5 litre bioreactor.

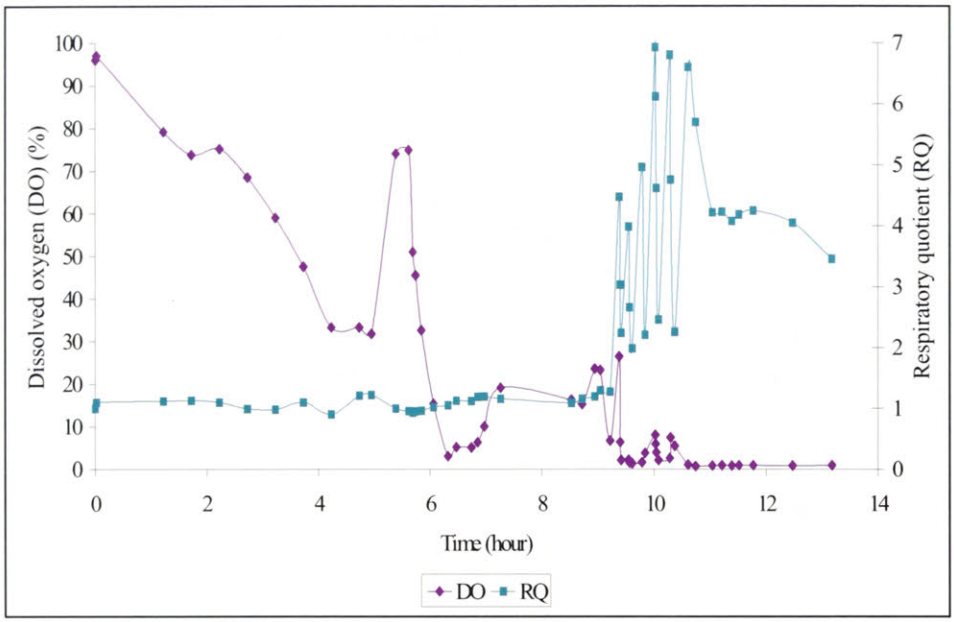


Figure 3.3: Dissolved oxygen (DO) and respiratory quotient (RQ) for the 5 litre bioreactor.

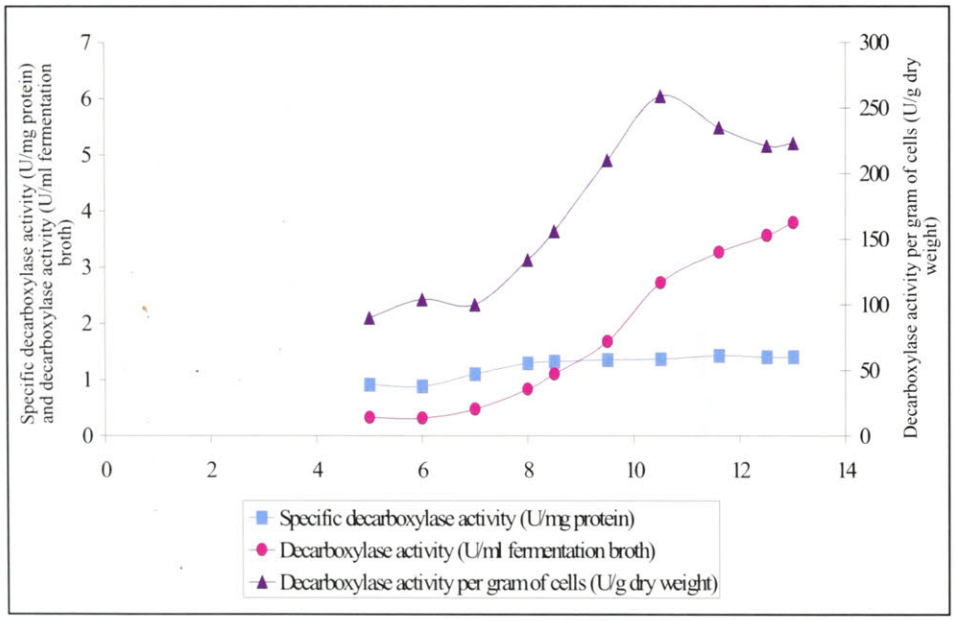


Figure 3.4: Pyruvate decarboxylase production in the 5 litre fermentation.

3.2.2 30 Litre Bioreactor: Batch (a)

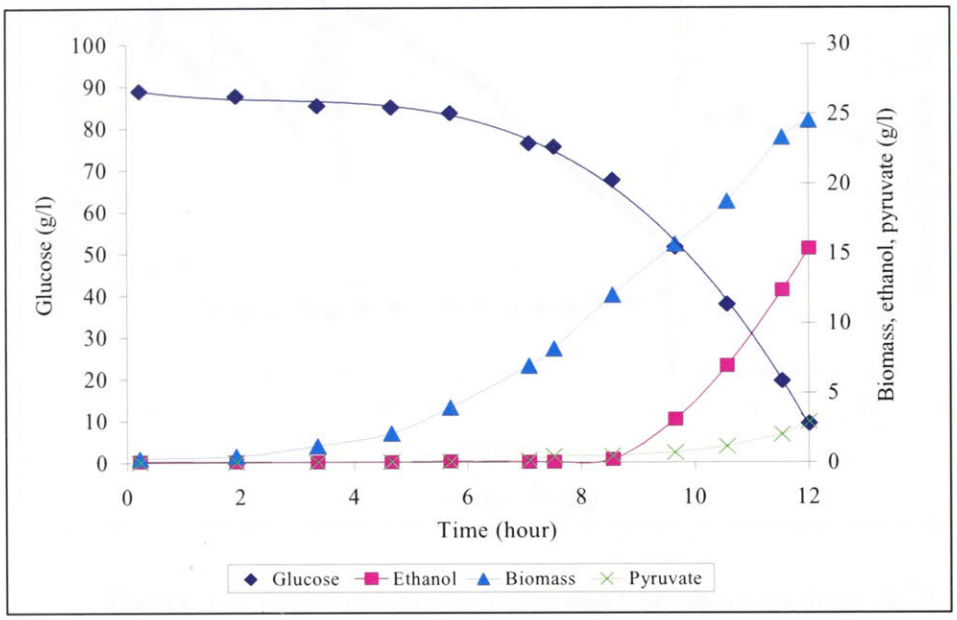


Figure 3.5: Profile for the 30 litre bioreactor: batch (a).

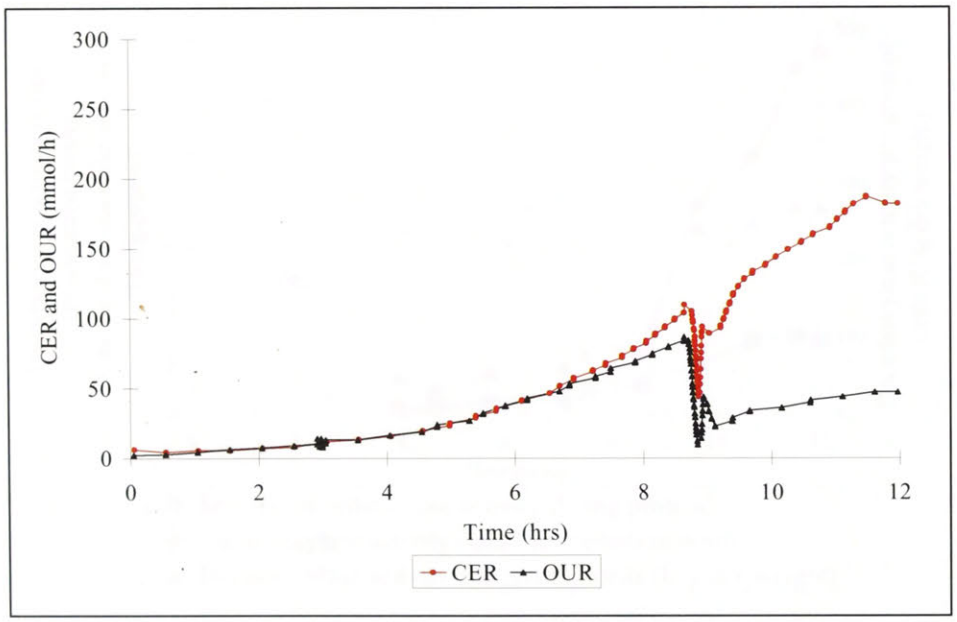


Figure 3.6: CER and OUR for the 30 litre bioreactor: batch (a).

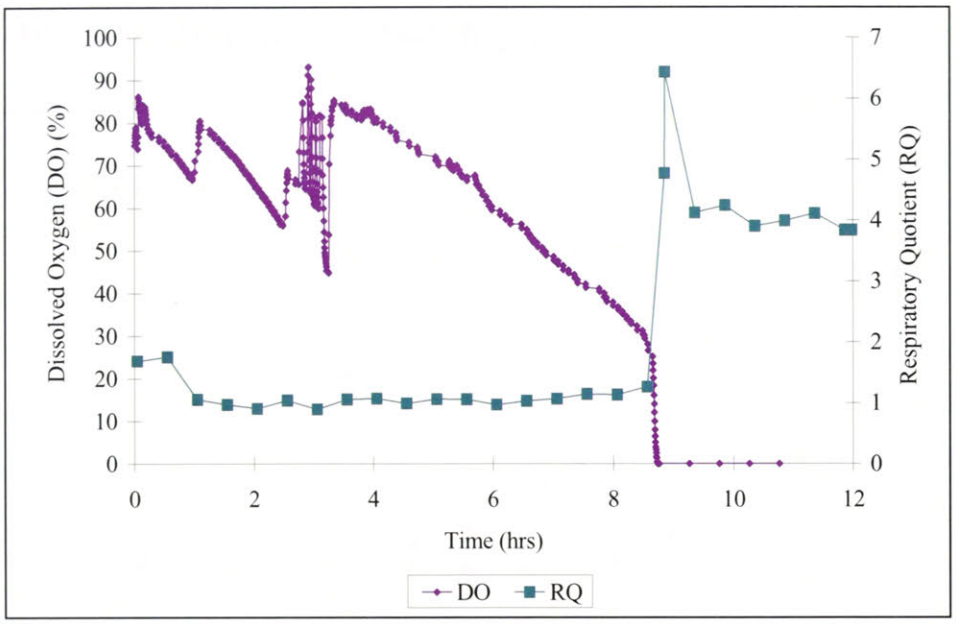


Figure 3.7: Dissolved oxygen (DO) and respiratory quotient (RQ) for the 30 litre bioreactor: batch (a).

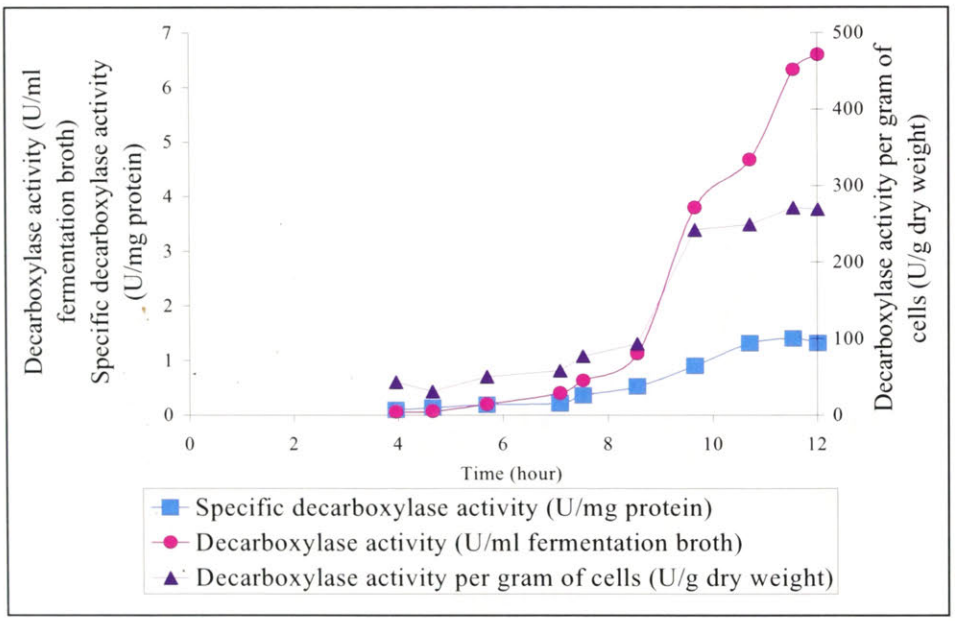


Figure 3.8: Enzyme production during the 30 litre bioreactor: batch (a).

3.2.3 30 Litre Bioreactor: Batch (b)

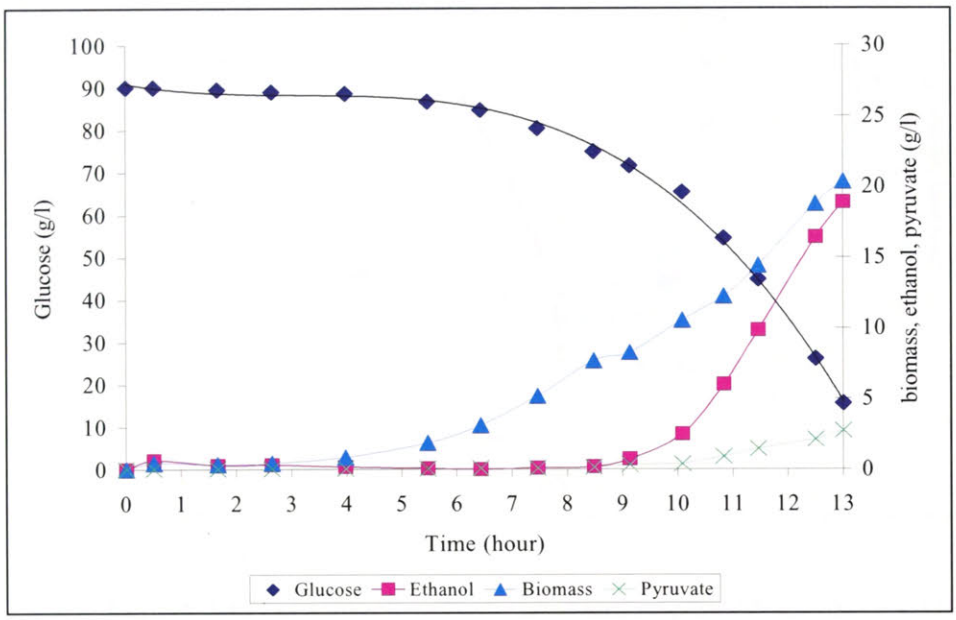


Figure 3.9: Profile for the 30 litre bioreactor: batch (b).

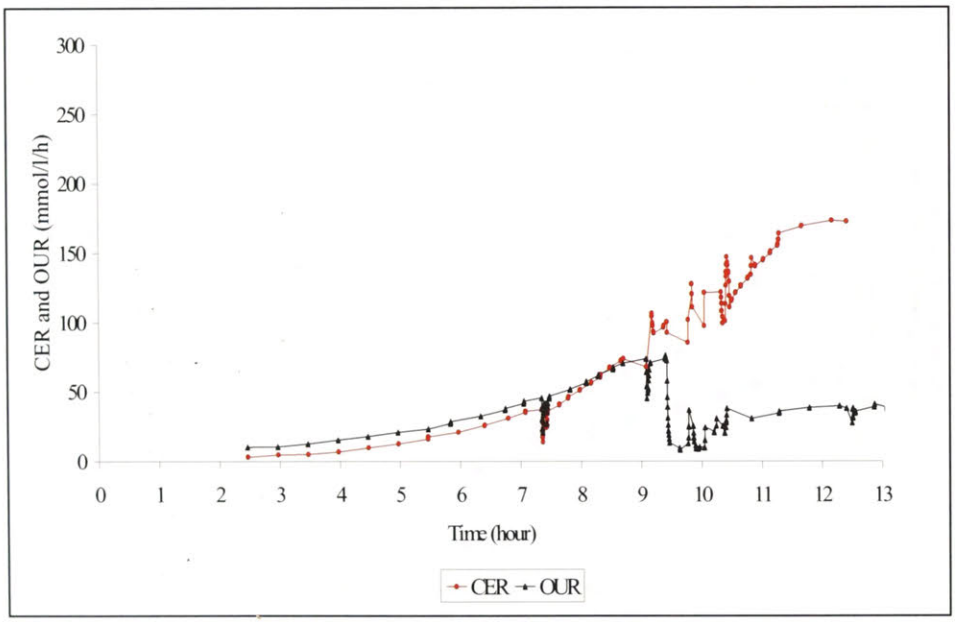


Figure 3.10: CER and OUR for 30 litre bioreactor: batch (b).

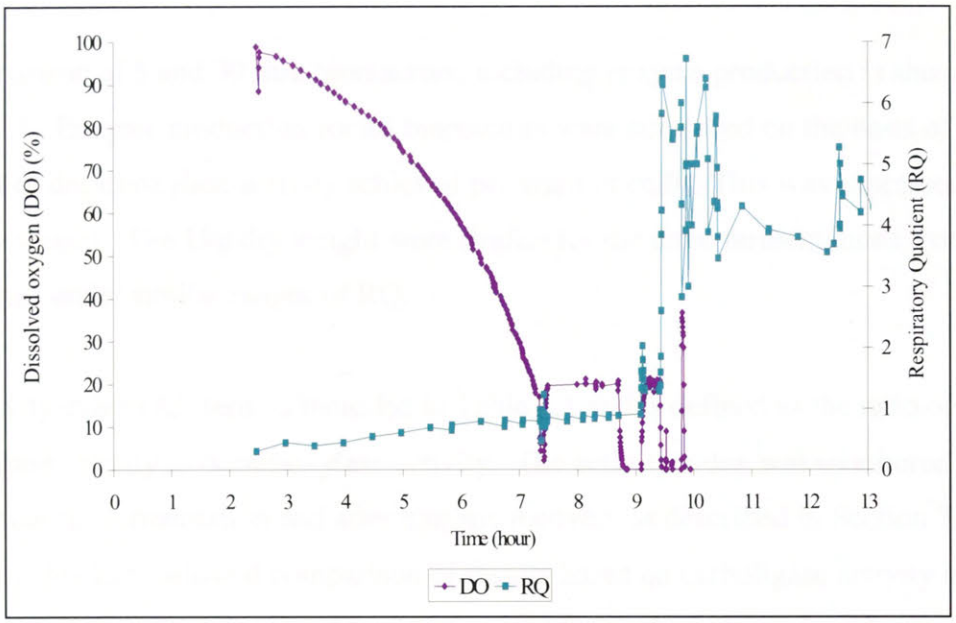


Figure 3.11: Dissolved oxygen (DO) and respiratory quotient (RQ) for the 30 litre bioreactor: batch (b).

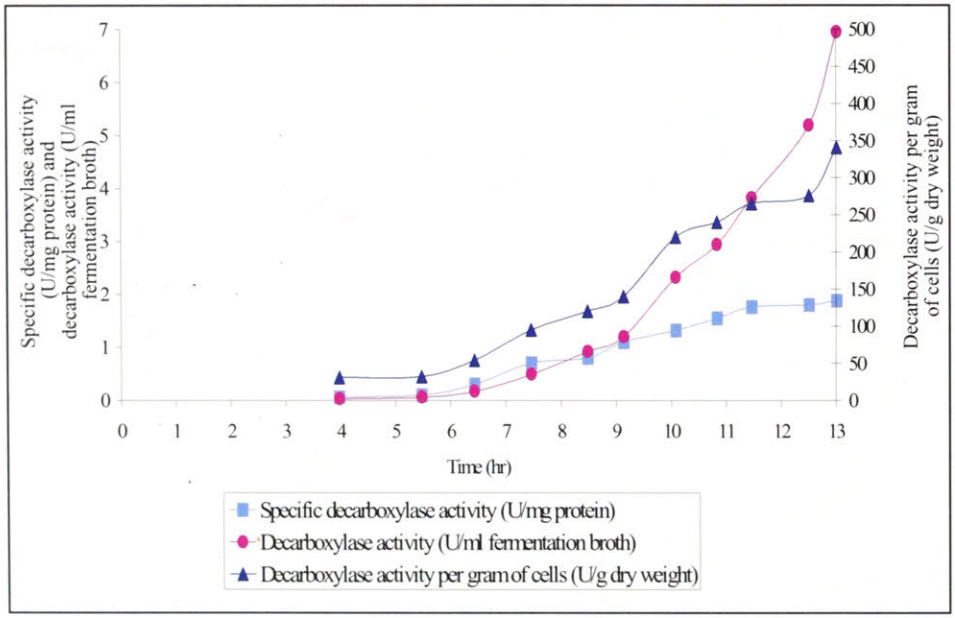


Figure 3.12: Enzyme production in the 30 litre bioreactor: batch (b).

3.2.4 Comparison of Bioreactors

A comparison of 5 and 30 litre bioreactors, including enzyme production is shown in Table 3.1. Enzyme production for all bioreactors were compared on the basis of the amount of decarboxylase activity achieved per gram of cells. This was expressed as U/g dry weight. The U/g dry weight were similar for the three fermentations that were performed under similar ranges of RQ.

An activity index (AI) term is included in Table 3.1 and is defined as the ratio of carboligase activity to decarboxylase activity. The activity index was monitored throughout the fermentation and after enzyme recovery as described in Section 3.3. The activity index term allowed comparison of results based on carboligase activity in this thesis to those results determined in literature and in previous theses based on decarboxylase activity.

The carboligase activity determination was used in this study as a definitive assay for measuring *R*-PAC formation. By comparison, the decarboxylase activity assay only quantifies pyruvate decarboxylation to acetaldehyde as described in Materials and Methods Section 2.8.9.

Table 3.1: Comparison of yields of biomass, ethanol and pyruvate and enzyme production for each fermentation.

	5 litre (a)	30 litre (a)	30 litre (b)
RQ control conditions	1.1-4	1-4	1-4
Batch #	1	2	4
Final biomass (g/l dry weight)	17.0	24.5	20.4
Final ethanol (g/l)	18.8	15.4	18.9
Final pyruvate (g/l)	1.40	2.90	2.70
Specific growth rate μ (h^{-1})	0.43	0.46	0.51
Yield $_{(biomass/glucose)}$ (g/g)	0.23	0.30	0.27
Yield $_{(ethanol/glucose)}$ (g/g)	0.25	0.19	0.25
Yield $_{(pyruvate/glucose)}$ (g/g)	0.02	0.04	0.04
Decarboxylase activity (U/ml fermentation broth)	3.80	6.60	6.95
Carboligase activity (U/ml fermentation broth)	0.95	2.20	1.74
Specific decarboxylase activity (U/mg protein)	1.40	1.30	1.88
Specific carboligase activity (U/mg protein)	0.35	0.43	0.47
Activity index (AI)*	0.25	0.33	0.25
U decarboxylase/g dry weight biomass	223	269	341
U carboligase/g dry weight biomass	56	89.8	86

*Activity index (AI): Carboligase activity/ decarboxylase activity

3.3 Enzyme Recovery

The aim of this section is to establish methods for recovery of pyruvate decarboxylase from whole cells of *Candida utilis* harvested from the bioreactor. The emphasis was to recover pyruvate decarboxylase in high yield and to produce a stable preparation of enzyme that could be easily redissolved and evaluated in various reaction buffers. Recovery of the enzyme from the crude extract using precipitation was evaluated using various concentrations of ethanol, acetone and ammonium sulfate.

3.3.1 Cell Breakage

Cell breakage was achieved with a combination of freeze thawing and blending with glass beads. Cells were adjusted to 60 g/l cell dry weight, freeze thawed three times in liquid nitrogen followed by blending with glass beads. Detailed methodology is described in Section 2.5.2.

3.3.2 Enzyme Precipitation

3.3.2.1 Ethanol Precipitation

Pyruvate decarboxylase was inactivated by concentrations of ethanol above 35 % (v/v). Significant precipitated enzyme could not be recovered as shown by the low levels of decarboxylase activity detected in the precipitate (Figure 3.13). The recovery of protein shown in Figure 3.14, demonstrates that protein was irreversibly denatured and could not be redissolved. The calculated yield and purification factors for several fractions are shown in Table 3.2. The purification factors are acceptable, however the yield of enzyme was low and therefore ethanol precipitation is not suitable for enzyme recovery.

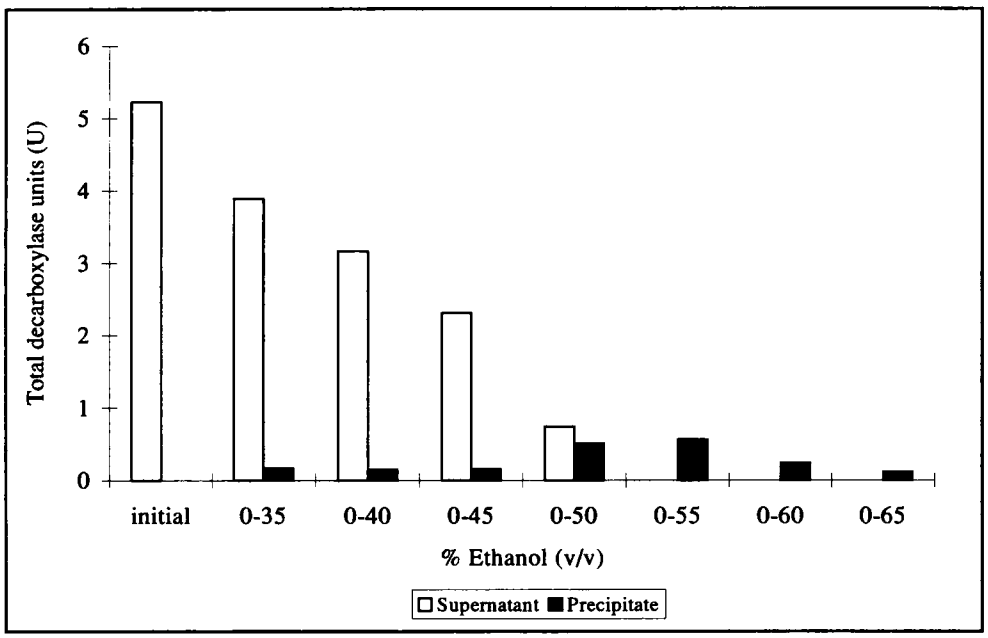


Figure 3.13: Effect of ethanol concentration on enzyme precipitation.

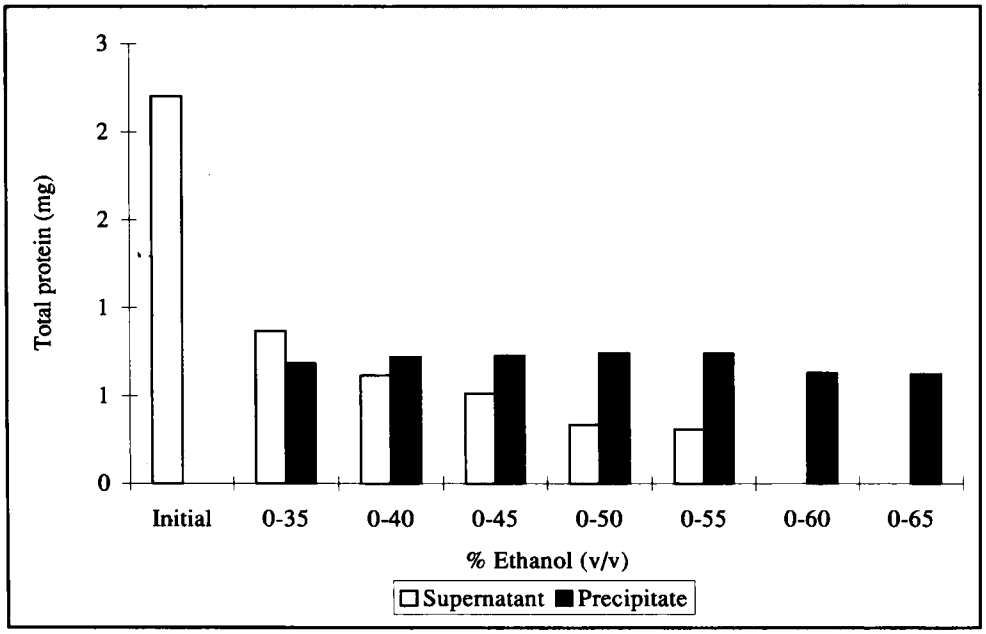


Figure 3.14: Effect of ethanol concentration on protein precipitation.

Table 3.2: Yields and purification factors for pyruvate decarboxylase for different ethanol fractions.

Ethanol fraction (% v/v)	Enzyme yield (%)	Enzyme purification factor
35-55	7.5	1.7
40-55	7.8	9.3
45-55	7.6	9.1
50-55	1.1	2.7

3.3.2.2 Acetone Precipitation

Pyruvate decarboxylase was precipitated by acetone as demonstrated in Figure 3.15. The amount of protein recovered is shown in Figure 3.16. The calculated enzyme yield and purification factors for several fractions are shown in Table 3.3. Pyruvate decarboxylase was recovered in the precipitate with a suitable yield of approximately 68 % with a purification factor between 2.6-4.6. Although the 45-50 % (v/v) acetone fraction has a higher purification factor, the 40-50 % fraction was chosen for further experiments due to the possibility that additional protein may help stabilize the enzyme against denaturation.

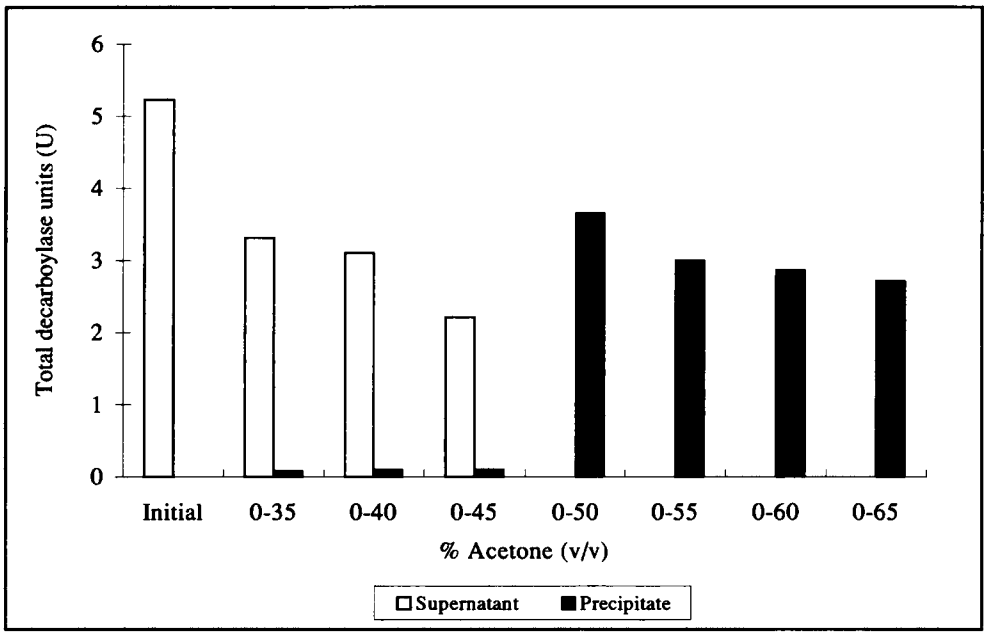


Figure 3.15: Effect of acetone concentration on enzyme precipitation.

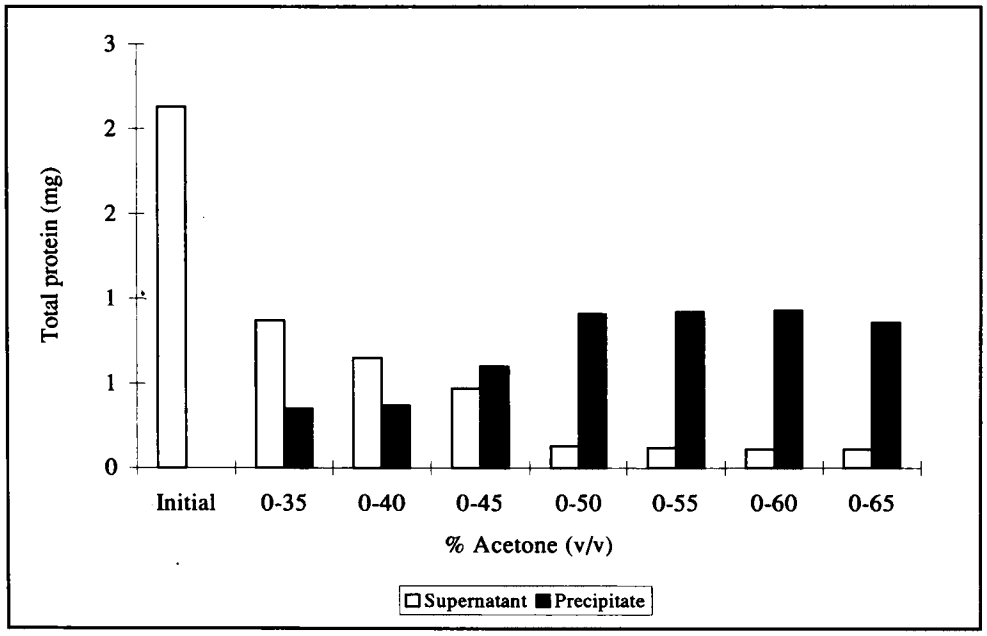


Figure 3.16: Effect of acetone concentration on protein precipitation.

Table 3.3: Yields and purification factors for pyruvate decarboxylase for different acetone fractions.

Acetone fraction (% v/v)	Enzyme yield (%)	Enzyme purification factor
35-50	68.3	2.6
40-50	67.8	2.6
45-50	67.8	4.6

3.3.2.3 Ammonium Sulfate Precipitation

Pyruvate decarboxylase was precipitated by ammonium sulfate as demonstrated in Figure 3.17. The amount of protein recovered is shown in Figure 3.18. The calculated enzyme yields and purification factors for several fractions are shown in Table 3.4. The 35-50 % ammonium sulfate fraction was selected for further experiments due to the high recovery and purification factor of 66.9 % and 6.2 respectively. The enzyme precipitate was stored in a concentrated ammonium sulfate solution at 4 °C (3.2 M ammonium sulfate, 5 % (v/v) glycerol, 20 mM magnesium sulfate, 2 mM thiamine pyrophosphate, 200 mM citric acid, pH 6.5 adjusted with 4 M KOH at 4 °C).

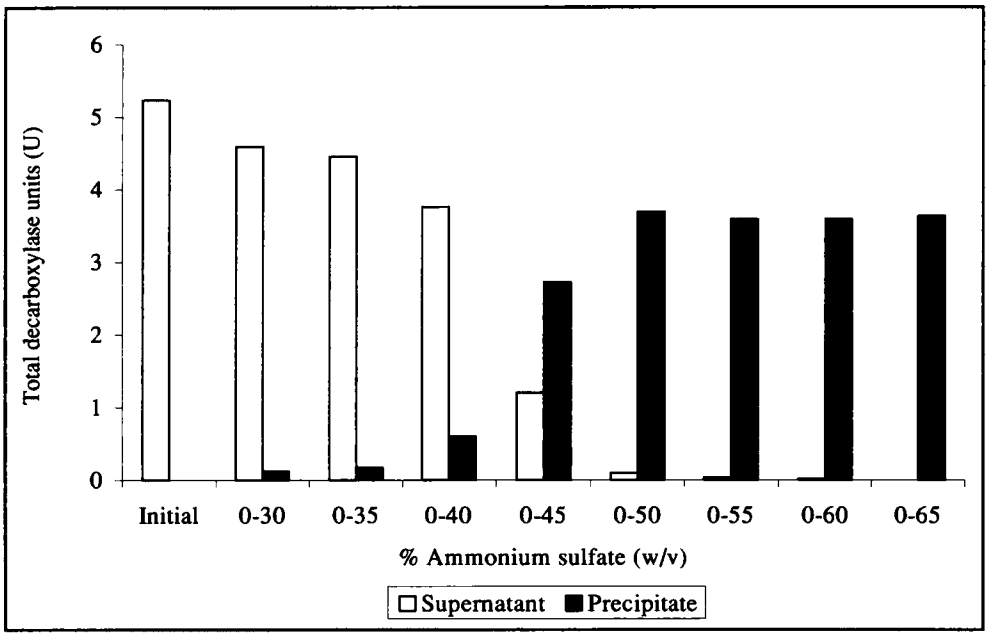


Figure 3.17: Effect of ammonium sulfate concentration on enzyme precipitation.

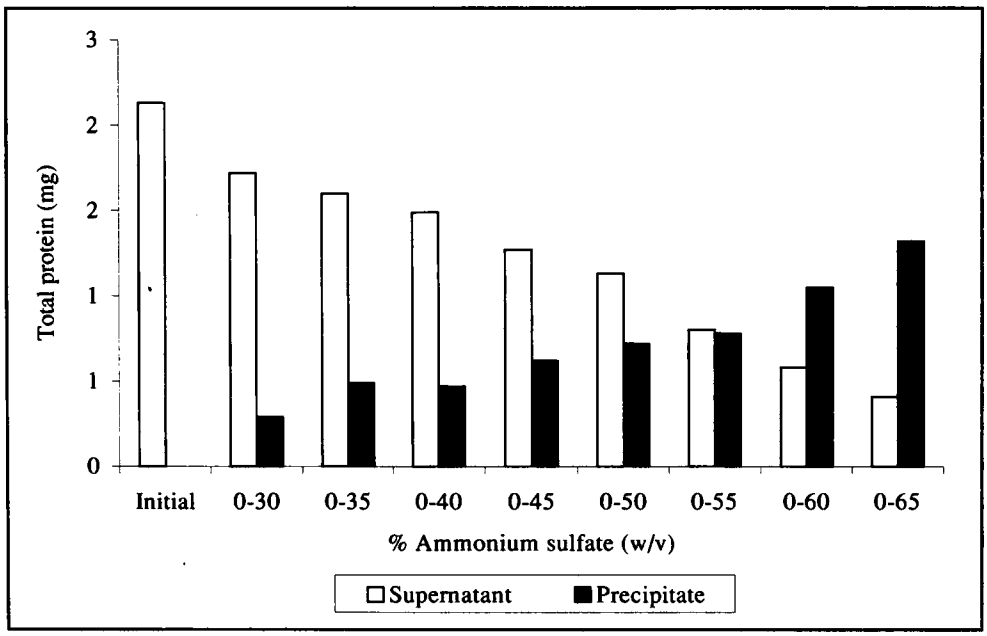


Figure 3.18: Effect of ammonium sulfate concentration on protein precipitation.

Table 3.4: Yields and purification factors for pyruvate decarboxylase for different ammonium sulfate fractions.

Ammonium sulfate fraction (% w/v)	Enzyme yield (%)	Enzyme purification factor
30-50	68.8	3.4
35-50	66.9	6.2
40-50	59.3	5.1
45-50	19.1	4.1

3.3.2.4 Comparison of Precipitation Methods

A comparison of precipitation methods in Table 3.5 indicates that pyruvate decarboxylase was recovered from the crude extract by acetone and ammonium sulfate precipitation with relatively high yields and acceptable purification factors. Ethanol precipitation resulted in a low yield of enzyme, suggesting that the enzyme was irreversibly inactivated by ethanol above 35 % (v/v). The storage stability of acetone and ammonium sulfate precipitated enzyme was evaluated as shown in Table 3.6. The stability of the ethanol precipitate was not evaluated due to the low yield of enzyme.

The freeze-dried acetone precipitated powder was found to be stable at –20 °C for at least three years and was convenient for evaluation of the enzyme in various buffers. Therefore the acetone precipitation procedure was selected for future preparation of pyruvate decarboxylase.

Table 3.5: Comparison of enzyme precipitation methods for recovery of pyruvate decarboxylase.

	Ethanol	Acetone	Ammonium sulfate
Fraction (%)	35-55 (v/v)	40-50 (v/v)	35-50 (w/v)
Initial units of decarboxylase activity (U)	5.2	5.2	5.2
Initial specific activity (U/mg protein)	2.2	2.5	2.1
Final units of decarboxylase activity (U)	0.4	3.6	3.5
Final specific decarboxylase activity (U/mg protein)	7.8	7.8	15.2
Purification factor	3.5	3.1	6.2
Yield (%)	7.6	69.4	66.9

Table 3.6: Comparison of enzyme storage stability for acetone and ammonium sulfate precipitated preparations.

	Acetone	Ammonium sulfate
Fraction (%)	40-50 (v/v)	30-55 (w/v)
Storage conditions	Acetone precipitated freeze dried powder at -20 °C	Ammonium sulfate suspension at 4 °C
Stability	Stable for at least 3 years at -20 °C	Lost 16 % decarboxylase activity over 11 days at 4 °C

3.3.3 Properties of Enzyme Batches

Characteristics of pyruvate decarboxylase purified from each of the fermentations discussed in Chapter 3 are shown in Table 3.7. A comparison of crude extracts with partially purified enzyme shows that a change in activity index (carboligase activity/decarboxylase activity) occurred for batches 2, 3 and 4. In batch #2 there was a large increase in activity index from 0.33 in the crude extract to 2.07 after partial purification. It was not known what influenced this change and if it occurred during acetone precipitation or freeze drying of the enzyme. It is important to note that the enzyme preparations also differ from each other in terms of specific activities. The effect of these differences on enzyme stability and *R*-PAC formation will be examined in Chapter 4.

Included in the table is enzyme harvested from an additional 5 litre bioreactor conducted by Wong (unpublished results). Medium and process conditions were the same as in the fermentations documented in this Chapter. The fermentation in this latter experiment was controlled at an RQ of 1 for the initial 10 hours and then increased to an RQ of 10 with an additional 50 g/l glucose feed (Wong, unpublished results). After 20 hours, the cells were harvested and pyruvate decarboxylase recovered from the cells by the same methods as for the other fermentations documented in this Chapter.

Table 3.7: Characteristics of partially purified enzyme batches from each fermentation.

Batch	#1 5 litre (a)	#2 30 litre (a)	#3 5 litre (b) *	#4 30 litre (b)
Specific carboligase activity of crude extract (U/mg)	0.35	0.33	0.8	0.47
AI ** of crude extract	0.25	0.25	0.33	0.25
Specific carboligase activity of partially purified enzyme (U/mg)	2.2	1.8	1.2	4.5
AI** of partially purified enzyme.	0.25	2.07	0.19	0.45

* Fermentation by Wong (unpublished results)

** Activity index (AI) = carboligase/ decarboxylase activity

3.4 Discussion and Conclusions

Pyruvate decarboxylase production was induced using RQ control. A respiratory growth phase with an RQ of approximately 1, allowed rapid generation of biomass at high yields. A second phase that was fermentative, was achieved by increasing the RQ setpoint to approximately 4. With system stabilization the measured RQ settled with an average value of 4 ± 1. Carbon dioxide evolution rates increased in this phase indicating a change from respiration to fermentative metabolism. Pyruvate decarboxylase levels increased rapidly in this fermentation stage. The measured RQ fluctuated from the setpoint, indicating a need to improve the proportional/integral control settings. For improvement of future RQ process control, a reduction in the air flowrate during the fermentative phase may make the system less sensitive to changes in the stirrer speed that is used to regulate oxygen transfer to the cells. In addition, online modification of control constants may also improve process control.

Pyruvate levels did not exceeding 2.9 g/l in all fermentations conducted in this set of experiments. This emphasizes one of the difficulties which occur in using whole cell catalysis for the production of *R*-PAC. High levels of *R*-PAC formation are restricted

by the availability of the substrate pyruvate for the biotransformation. Rogers *et al.* (1997) showed that cessation of *R*-PAC formation by whole cells of *Candida utilis* was due to pyruvate depletion accompanied by declining pyruvate decarboxylase activity.

The average enzyme level achieved in the three experiments controlled from RQ 1-4 was 278 units of decarboxylase activity per gram dry weight of cells. This was comparable with the 280 U/g dry weight reported by Chow (1998) at an RQ of 4.4 in the fermentation phase.

Experiments in this study show that enzyme levels were still increasing at the end of the fermentation. This suggests that additional glucose feeding may improve final enzyme yields. This was demonstrated previously by Wong (unpublished results), who achieved elevated enzyme levels in comparison to the data presented in this Chapter. Cells from the latter experiments were incorporated into the study to determine how this modification to the bioreactor operation may impact on enzyme properties and *R*-PAC formation. This batch was examined further in Chapter 4, along with other enzyme batches.

A comparison of enzyme precipitation methods established that pyruvate decarboxylase could be recovered from the crude extract in high yield by acetone precipitation. In addition the freeze dried acetone precipitated pellet could be stored with no loss of activity for at least three years. No changes in enzyme characteristics occurred during storage.

It was reported previously by Chow (1998) that an ammonium sulfate preparation stored at –20 °C showed loss of activity after 6 months of storage with additional changes to the specific activity and *R*-PAC production characteristics. This reported activity loss is consistent with the present findings for an ammonium sulfate precipitated enzyme preparation.

Enzyme recovered from the different experiments showed variation in specific activities and activity indices. In three batches, a change in the activity index occurred as a result of partial purification of the enzyme. It is not known if these changes occurred during acetone precipitation or freeze drying. The differences in each batch provide an

opportunity to evaluate enzyme stability and *R*-PAC formation from batches of enzyme showing varying characteristics. Chapter 4 evaluates *R*-PAC production and enzyme stability using each of the various enzyme preparations described in this Chapter. It is important to determine if these differences have a significant influence on *R*-PAC production and if they need to be investigated further.

CHAPTER 4

FACTORS AFFECTING ENZYME STABILITY AND *R*-PAC FORMATION WITH PARTIALLY PURIFIED PDC

4.1 Introduction

Production of *R*-PAC via biotransformation of benzaldehyde and pyruvate by whole cells of *Candida utilis* is currently limited by the following factors:

- by-product formation: loss of substrate benzaldehyde to the by-product benzyl alcohol by the action of oxidoreductases such as alcohol dehydrogenase.

- toxic effect of benzaldehyde which causes a significant reduction in cell viability, resulting in cessation of pyruvate production, and thereby limiting *R*-PAC formation. *R*-PAC production is also hindered by a decrease of *in vivo* pyruvate decarboxylase activity due to the inhibitory and inactivating effect of benzaldehyde (Rogers *et al.* 1997).

To overcome these limitations, cell-free extracts and partially purified pyruvate decarboxylase (PDC) have been investigated as catalysts for *R*-PAC formation (Shin 1994; Shin and Rogers 1996a,b; Goetz *et al.* 2001; Iwan *et al.* 2001; Rosche *et al.* 2001; Rosche *et al.* 2002a,b,c). Removing PDC from the cell has prevented regeneration of NADH/NADPH that is required for reduction of benzaldehyde to benzylalcohol, resulting in improved yields of *R*-PAC from supplied benzaldehyde (Shin and Rogers 1996a,b; Rosche *et al.* 2002a). Increased *R*-PAC concentrations have been achieved by

feeding high levels of pyruvate and benzaldehyde to the enzyme (Shin and Rogers 1996b; Rogers *et al.* 1997; Rosche *et al.* 2002a). However the efficiency of the cell-free process is affected by the high cost of substrate pyruvate, additional costs in enzyme recovery and/or preparation of a cell-free extract and inactivation of the enzyme during the biotransformation. For development of a cell-free industrial process, the most significant challenge is therefore to increase the amount of product per amount of enzyme catalyst while also increasing molar yields on substrates.

Candida utilis PDC was selected for further studies due to its superior stability and carboligase activity relative to other yeast strains that were examined by Rosche *et al.* (2002b). It has been shown in previous studies of *Candida utilis* PDC that significant enzyme inactivation occurred during the biotransformation, which was attributed to the substrate benzaldehyde (Chow *et al.* 1995; Chow 1998). Rising pH during the biotransformation was found to further limit *R*-PAC production, and was overcome by including high concentration of MOPS buffer (Rosche *et al.* 2002a). The reproducibility of *R*-PAC formation from partially purified *C.utilis* PDC was also highlighted as an important issue for establishing an industrial process (Chow *et al.* 1995).

The objective of this Chapter is to investigate enzyme inactivation and to evaluate *R*-PAC production using partially purified PDC from *Candida utilis*. Improved understanding of the limitations of the biotransformation can be used to improve *R*-PAC production in the cell-free process. The following aspects will be investigated to achieve this aim:

- investigation of enzyme stability under reaction conditions.

- investigation of different batches of partially purified enzyme to determine the reproducibility of enzyme stability and *R*-PAC production from batch to batch.

- characterization of enzyme inactivation due to substrate, product and by-products of the biotransformation. Investigation of inhibition of the biotransformation due to *R*-PAC.

4.2 Factors Affecting Enzyme Stability

In the studies of Shin (1994) and Chow (1998) it was found that loss of decarboxylase activity was rapid in the presence benzaldehyde, with some indication of variable inactivation between batches of enzyme. Improved understanding of PDC inactivation in the presence of benzaldehyde is required to enhance *R*-PAC production. In addition Shin (1994) and Chow (1998) established that *R*-PAC contributed less significantly to inactivation of pyruvate decarboxylase.

The effect of acetoin on enzyme stability has not been reported in literature and will be evaluated in this Chapter.

Shin (1994) evaluated *R*-PAC formation with up to 600 mM pyruvate present at the beginning of the biotransformation. No inhibition of initial reaction rates occurred with high levels of pyruvate, which may indicate that inactivation of PDC by pyruvate would not be a significant consideration for these studies. PDC inactivation due to pyruvate was not measured in this Chapter, as enzyme incubation with pyruvate would have resulted in the direct conversion of pyruvate to acetaldehyde and acetoin, thereby influencing PDC inactivation

Zymomonas mobilis PDC catalyzes the direct carboligation of free acetaldehyde and benzaldehyde to *R*-PAC. In this case, acetaldehyde was found to significantly inactivate PDC (Goetz *et al.* 2001; Iwan *et al.* 2001), therefore a detailed analysis of inactivation by acetaldehyde would be important as it is a substrate delivered at high concentration. For yeast PDC however, the enzyme cannot use free acetaldehyde as a substrate for *R*-PAC formation (Rosche *et al.* 2002b). Only small concentrations of acetaldehyde accumulated after the biotransformation of pyruvate and benzaldehyde (Shin 1994). Acetaldehyde is further reacted to acetoin by the action of PDC preventing its accumulation. Acetaldehyde has been shown by Shin (1994) to significantly inhibit initial rates of *R*-PAC formation and was further reported by Juni (1961) to have very little inactivating effect on PDC activity. In this Chapter acetaldehyde is considered a minor influence on the reaction. The stability of partially purified PDC from *C.utilis* was evaluated in the presence of acetaldehyde for comparison to PDC from *Z.mobilis*.

4.2.1 Effect of Benzaldehyde on Enzyme Stability

Shin (1994) and Chow (1998) evaluated *R*-PAC formation from partially purified *Candida utilis* PDC using a phosphate buffer, which was also used initially in these studies to allow comparison between results. Section 4.2.1 evaluates enzyme inactivation due to benzaldehyde, with particular focus on achieving partially purified PDC showing reproducible characteristics in terms of enzyme inactivation and *R*-PAC production.

4.2.1.1 Comparison of Enzyme Stabilities in the Presence of Benzaldehyde Solutions and Emulsions

Benzaldehyde is sparingly soluble, and formed an emulsion above a concentration of 60 mM (6.4 g/l) at 4 °C in the phosphate reaction buffer (40 mM KH_2PO_4, 2 M ethanol, 1 mM Mg^{2+}, 1 mM TPP, pH 7 at 4 °C). At concentrations less than 60 mM (6.4 g/l), benzaldehyde existed initially as an emulsion, and gradually formed a solution as it slowly dissolved. Enzyme stability in the presence of benzaldehyde was evaluated with benzaldehyde delivered as either a solution or as an emulsion.

In the following investigation, enzyme at 7 U/ml carboligase activity in phosphate buffer (40 mM KH_2PO_4, 2 M ethanol, 1 mM Mg^{2+}, 1 mM TPP, pH 7 at 4 °C) was exposed to benzaldehyde solutions at 30 mM (3.2 g/l) and 50 mM (5.3 g/l), and benzaldehyde emulsions at 50 mM (5.3 g/l), 75 mM (8.0 g/l) and 100 mM (10.6 g/l). After 9 hours, the remaining carboligase activity was determined after gel filtration to remove benzaldehyde. The presence of a benzaldehyde emulsion did not affect recovery of the enzyme from the gel filtration columns. The calculated errors represent variations in stability for the same preparations of enzyme in 10 different vials and were calculated as standard deviations from a mean. Detailed methodology is described in Section 2.6.1.

Figure 4.1 shows that enzyme inactivation rates were rapid when the enzyme was exposed to benzaldehyde emulsions. Reproducible levels of inactivation could not be achieved with the same preparation of enzyme in benzaldehyde emulsions.

By comparison, the enzyme was significantly more stable in the presence of a benzaldehyde solution. The error between vials was significantly reduced to within ±5 % or less in comparison to benzaldehyde emulsions. The enzyme retained 95 % of its initial activity after 9 hours when exposed to 50 mM (5.3 g/l) benzaldehyde delivered as a solution. When 50 mM (5.3 g/l) benzaldehyde was delivered as an emulsion, 51 % of the initial activity was lost with large errors between samples.

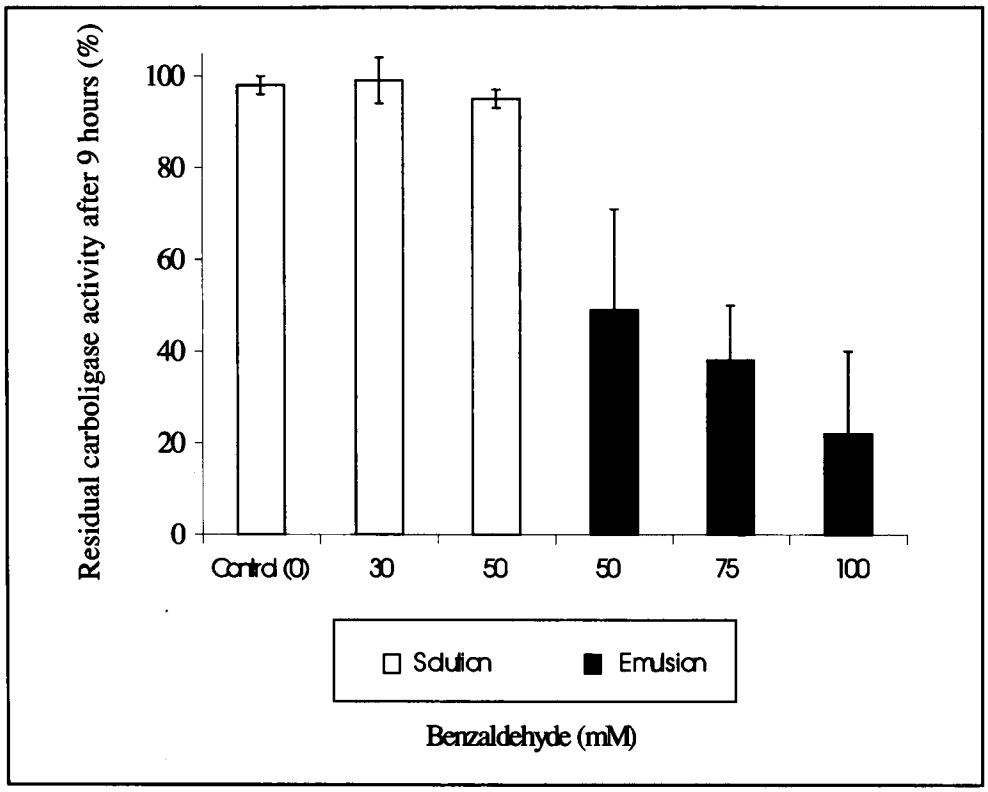


Figure 4.1: Enzyme stability when exposed to benzaldehyde emulsions and solutions (7 U/ml carboligase activity, 9 hours, 40 mM KH_2PO_4, 2 M ethanol, 1 mM Mg^{2+}, 1 mM TPP, pH 7.0 at 4 °C). Error bars indicate the calculated standard deviation from the mean derived from 10 samples.

4.2.1.2 Comparison of Enzyme Stabilities from Four Different Enzyme Preparations

An important finding from Section 4.2.1.1, was that one preparation of enzyme showed variable levels of inactivation when exposed to benzaldehyde emulsions, however reproducible levels of inactivation could be achieved by limiting enzyme exposure to only benzaldehyde solutions. In the following investigation, four different preparations of enzyme obtained from each of the four bioreactors discussed in Chapter 3, were evaluated in 50 mM (5.3 g/l) benzaldehyde solutions to determine if the long-term stability of pyruvate decarboxylase is consistent between different batches of enzyme. Table 4.1 shows that enzyme preparations with different specific activities and activity indices exhibited different stabilities over 4 days in phosphate buffer when incubated without benzaldehyde present. Differences in pyruvate decarboxylase stability were also noted in the same buffer containing a solution of 50 mM benzaldehyde. Each batch exhibited different stability characteristics, which may affect the reproducibility of *R*-PAC formation with different batches of enzyme.

Table 4.1: Comparison of PDC stabilities for different preparations of enzyme (7 U/ml carboligase activity, 40 mM KH_2PO_4, 2 M ethanol, 1 mM Mg^{2+}, 1 mM TPP, pH 7.0 at 4 °C, 4 days). Five litre (a,b) and 30 litre (a,b) bioreactors are described in Chapter 3.

Batch	#1 5 litre (a)	#2 30 litre(a)	#3 5 litre (b)*	#4 30 litre(b)
Specific carboligase activity (U/mg)	2.2	1.8	1.2	4.5
Protein concentration (mg/ml)	3.2	3.9	5.8	1.6
Activity Index**	0.25	2.07	0.19	0.45
Residual carboligase activity after 4 days at 4 °C (%)				
Control (no benzaldehyde present)	53	88	99	72
50 mM (5.3 g/l) benz. solution	24	61	84	22

* Fermentation conducted by Wong (unpublished results) as described in Chapter 3.

** Activity Index = carboligase/decarboxylase activity

4.2.1.3 The Effect of Protease Inhibitors, Protein Addition and Buffer Type on the Stability of Enzyme Batches Without Exposure to Benzaldehyde

This section investigates reasons for the variation in enzyme stability between different batches of enzyme prepared in the same manner.

(A) Effect of proteases

Differing levels of proteases in each batch of enzyme may contribute to variable losses of activity. A commercially prepared mixture of protease inhibitors (Complete EDTA-free, Roche Germany) was added to prevent the action of serine and cysteine proteases excluding metal dependent proteases. Metalloprotease inhibitors were not included, as they would bind the magnesium ion cofactor. These results established that protease inhibitor addition did not have a significant positive impact on enzyme stability as shown in Figure 4.2.

(B) Effect of protein level and specific activity

Each enzyme preparation had differing specific activities, and therefore protein concentrations at 7 U/ml carboligase activity varied in each preparation as shown in Table 4.1. Protein concentration is known to have an influence on enzyme stability, with enzyme protein levels of 1-2 mg/ml prone to inactivation (Scopes 1994; Ó Fágáin 1997). Bovine serum albumin (BSA) addition is often useful for stabilizing proteins at low concentrations (Scopes 1994; Ó Fágáin 1997). In the present experiments each preparation was supplemented with additional BSA to adjust the protein concentration and specific activity to equal that of batch 3, the most stable preparation. Initial carboligase activities were 7 U/ml, with protein levels adjusted to 6mg/ml. Protein addition had a positive affect on batch 1 and 4, but little or no effect on batch 2 as shown in Figure 4.2. The stabilities of different batches were not identical despite protein addition.

(C) Effect of buffer species

The effect of buffer species on enzyme stability was evaluated in this study, using a MOPS buffer that would also be capable of buffering the biotransformation. Previously a MOPS buffer used for pH control during the biotransformation was found to stabilize PDC from *Rhizopus javanicus* (Rosche *et al.* 2002a). In was reported that the stability of PDC from *Rhizopus javanicus* increased 7 fold with an increase in MOPS concentration from 0.05 M to 2 M (Rosche *et al.* 2002a). Enzyme stability was evaluated in a concentrated MOPS buffer, which was viscous and may mimic the effect of high protein concentrations. Initial enzyme concentrations were 7 U/ml in MOPS buffer (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7 at 4 °C). Figure 4.2 shows that all batches exhibited very similar levels of inactivation in concentrated MOPS buffer, with an average retained activity of 96.3 %.

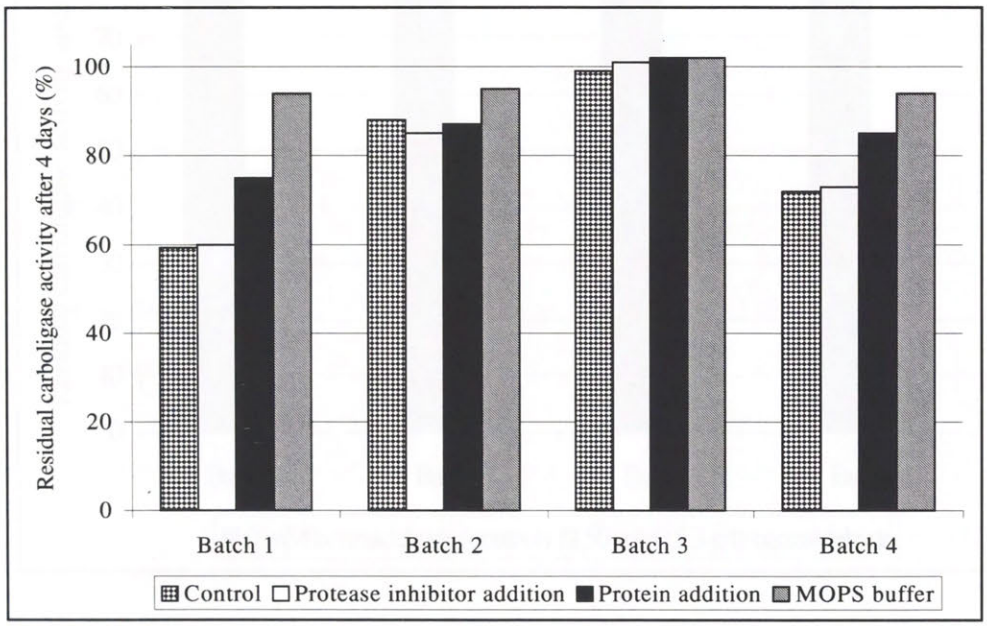


Figure 4.2: Effect of additives on enzyme stability in buffer not exposed to benzaldehyde at 7 U/ml for 4 days. Enzyme stability was evaluated in a phosphate buffer (40 mM KH_2PO_4, 2 M ethanol, 1 mM Mg^{2+}, 1 mM TPP, pH 7.0 at 4 °C) for the control vial and for evaluation of enzyme stability in the presence of protease inhibitors and BSA. MOPS buffer (2 M MOPS, 1 mM Mg^{2+}, 1 mM TPP, pH 7.0 at 4 °C) was used to determine the influence of this buffer on enzyme stability in the remaining vial.

Long-term enzyme stability in the presence of benzaldehyde was evaluated in MOPS buffer to determine if each batch of enzyme exhibits identical levels of inactivation. Initial carboligase levels were 6.7 U/ml for each batch, exposed to 50 mM (5.3 g/l) benzaldehyde solution for 4 days at 4 °C. Figure 4.3 shows that all batches exhibit similar levels of inactivation when exposed to 50 mM (5.3 g/l) benzaldehyde solution. The average level of residual carboligase activity for all batches was 50.5 %. The use of MOPS buffer has successfully adjusted each enzyme preparation to show identical stability characteristics regardless of specific activity or activity index of the preparation.

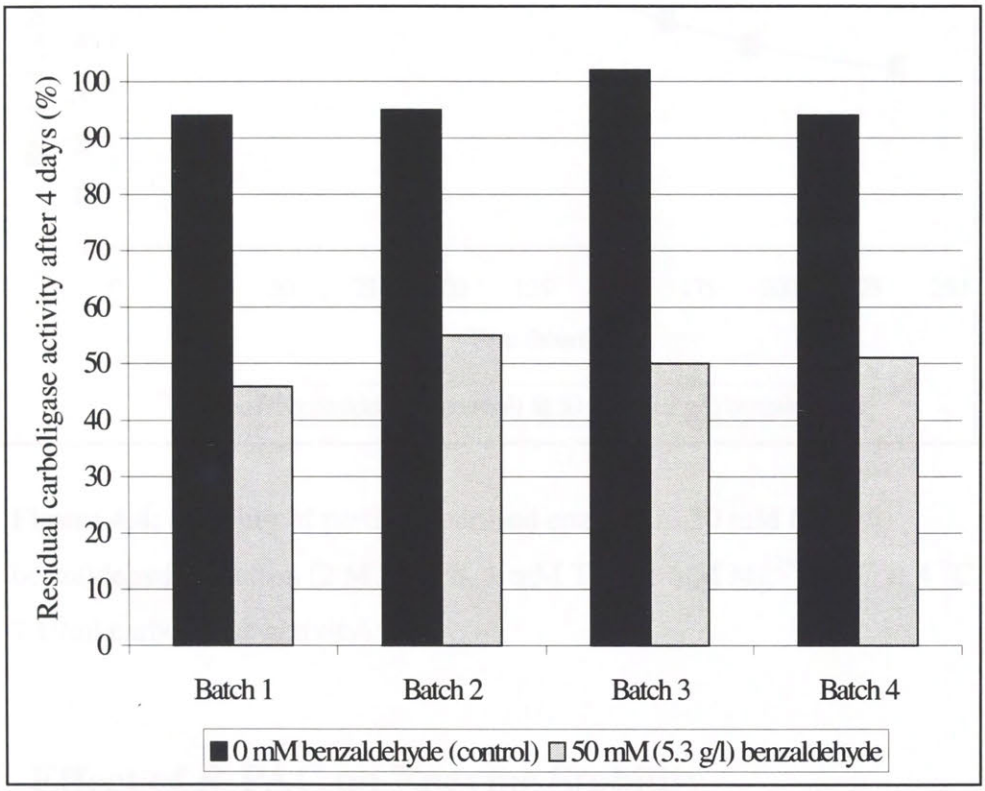


Figure 4.3: Enzyme stability in the presence of 50 mM (5.3 g/l) benzaldehyde solution for four batches of enzyme (7 U/ml, 2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, 4 days).

The profile of enzyme inactivation over a longer time period was evaluated in MOPS buffer with and without exposure to benzaldehyde. Batch #2 was used for all further experiments described in this Chapter and its stability was considered representative of

all other batches. Figure 4.4 shows that the half-life of PDC exposed to 50 mM (5.3 g/l) benzaldehyde solution was 138 hours. The control exposed to no benzaldehyde lost 15 % of its activity over this time.

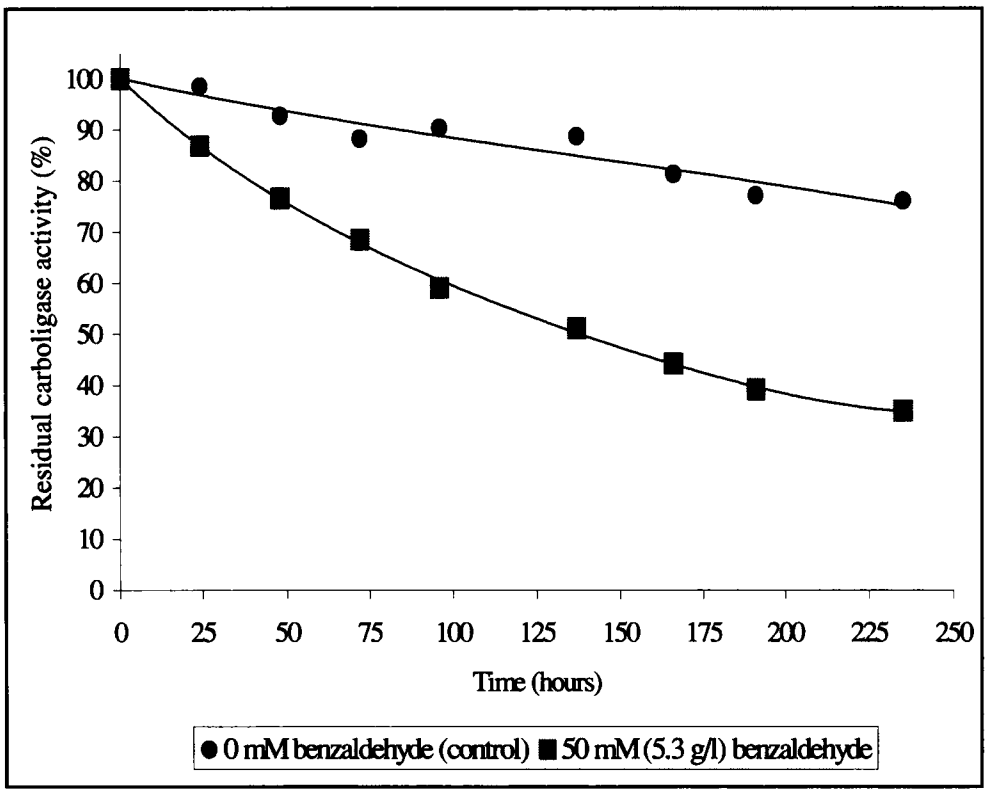


Figure 4.4: Stability of partially purified enzyme in 50 mM (5.3 g/l) benzaldehyde solution (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7 at 4 °C, 7 U/ml carboligase activity).

4.2.2 Effect of *R*-PAC on Enzyme Stability

The effect of *R*-PAC on enzyme stability was evaluated at 7 U/ml carboligase activity over 7 days with various concentrations of *R*-PAC from 34 mM to 274 mM in MOPS buffer (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7 at 4 °C). The remaining carboligase activity was determined at each timepoint after gel filtration to remove *R*-PAC. Detailed methodology is described in Section 2.6.4.

The profile of enzyme inactivation over 7 days of exposure to *R*-PAC is shown in Figure 4.5. Enzyme inactivation increased with increasing *R*-PAC concentration as

shown in a comparison of residual activities after 7 days of exposure in Figure 4.6. These results demonstrate that *R*-PAC has a significant inactivating effect on pyruvate decarboxylase.

Control (a) consisted of enzyme incubated in buffer without exposure to *R*-PAC, and retained 85 % of its activity. Control (b) consisted of enzyme incubated in buffer containing low concentrations of benzaldehyde and acetoin that were also present in the *R*-PAC sample, and retained 84 % of its initial activity over the 7 day period. The final concentrations of acetoin and benzaldehyde in control (b) were 2.8 mM and 5.3 mM respectively and reflected levels present in the highest concentration of *R*-PAC (274 mM).

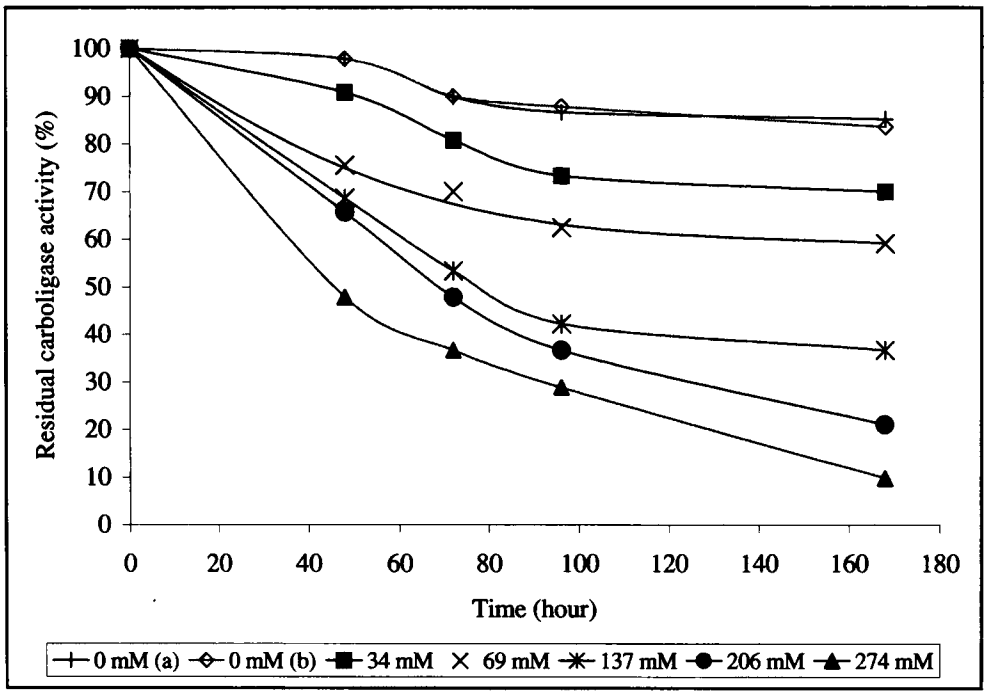


Figure 4.5: Profile of enzyme inactivation after exposure to various concentrations of *R*-PAC (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, 7 U/ml carboligase activity).

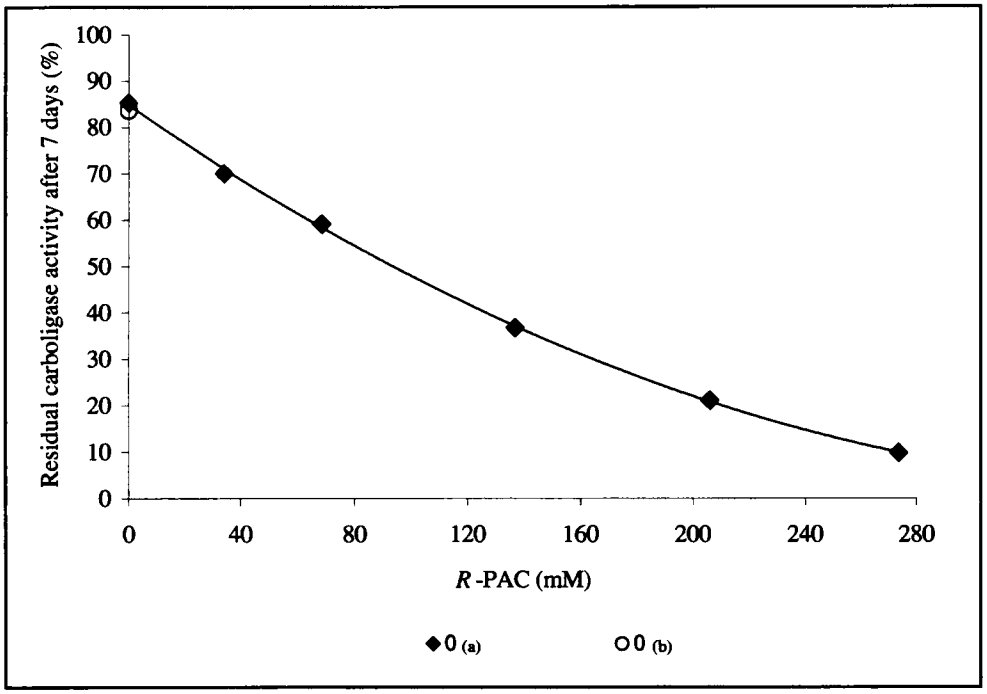


Figure 4.6: Effect of *R*-PAC on enzyme stability (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, 7 U/ml carboligase activity, 7 days).

4.2.3 Effect Of Acetaldehyde On Enzyme Stability

Enzyme stability towards the by-product acetaldehyde was evaluated in MES buffer (50 mM MES/KOH, 1 mM TPP, 20 mM Mg^{2+}, 1.5 M ethanol, pH 7.0 at 4 °C). Enzyme stability was evaluated in MES buffer to allow comparison between *C.utilis* PDC and *Z.mobilis* PDC. The remaining carboligase activity was determined after 15 hours exposure to 30 mM (1.3 g/l) acetaldehyde with a starting activity of 7 U/ml. The remaining carboligase activity was determined after gel filtration to remove acetaldehyde. No loss of acetaldehyde by evaporation occurred over 15 hours. Detailed methodology is described in Section 2.6.5.

After 15 hours, 98 % of the initial carboligase activity remained. The control without exposure to acetaldehyde retained 100 % of its activity over this time. The enzyme exhibited good stability towards the by-product acetaldehyde.

4.2.4 Effect of Acetoin on Enzyme Stability

The effect of by-product acetoin on enzyme stability was evaluated in a MOPS buffer (2 M MOPS, 1 mM TPP, 1mM^{2+}, pH 7.0 at 4 °C). Enzyme at a concentration of 7 U/ml was exposed to various concentrations of acetoin from 5 mM (0.44 g/l) to 60 mM (5.3 g/l). The remaining carboligase activity was determined after gel filtration to remove interfering substances. Detailed methodology is described in Section 2.6.6.

The profile of enzyme inactivation is shown in Figure 4.7. For all acetoin levels tested, an initial rapid drop in activity occurred over the first 24 hours. The inactivation rate slowed after 75 hours. There was a general trend of increasing inactivation with increasing acetoin level Figure 4.8. A control of enzyme not exposed to acetoin retained 85 % of its initial activity over 240 hours. No acetoin was lost during the incubations, indicating that it was not further degraded or converted by enzymes in the protein solution. This data indicates that acetoin production contributes significantly to enzyme inactivation at the higher concentrations.

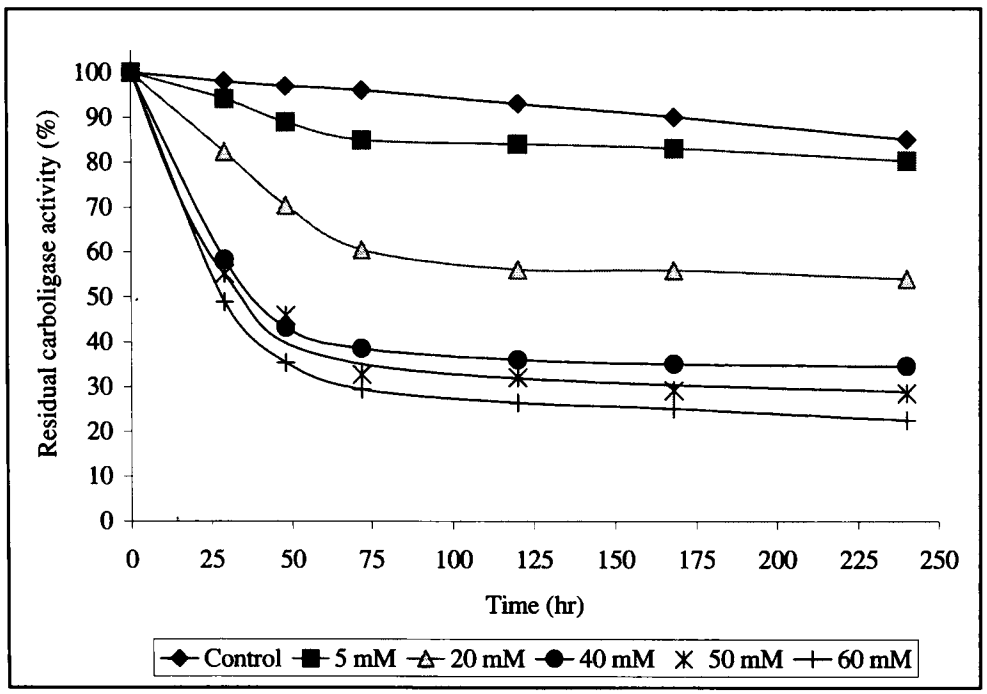


Figure 4.7: Effect of acetoin on enzyme stability (2 M MOPS, 1 mM TPP, 1 mM^{2+}, pH 7.0 at 4 °C, 7 U/ml carboligase activity).

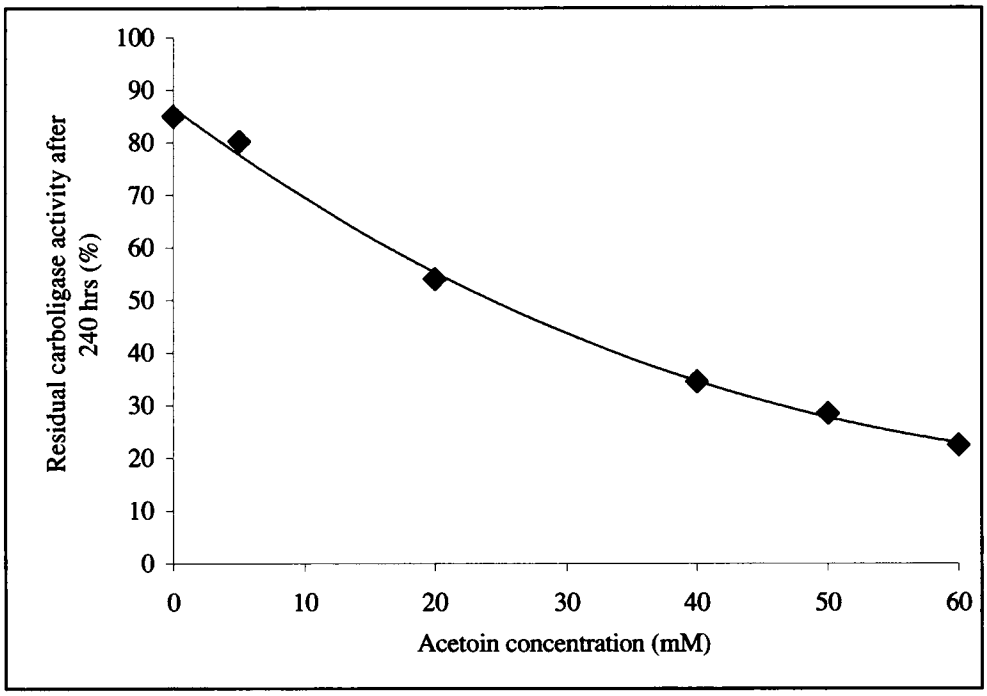


Figure 4.8: Effect of acetoin level on enzyme stability after 240 hours of exposure (2 M MOPS, 1 mM TPP, 1 mM^{2+}, pH 7 at 4 °C, 7 U/ml carboligase activity, 240 hours).

4.3 Factors Affecting *R*-PAC Formation

In Section 4.3, the effect of product inhibition and substrate and enzyme levels on *R*-PAC formation were investigated.

Previously Chow (1998) compared model simulations of the biotransformation with experimental data and thereby predicted that the biotransformation was affected by inhibition due to *R*-PAC formation. In this Section the effect of *R*-PAC on initial rates and final concentrations of *R*-PAC is established experimentally using *R*-PAC produced by partially purified *Candida utilis* PDC. Knowledge of this effect could assist in designing an enzymatic reactor to maximize *R*-PAC production.

In Chapter 3 it was shown that all batches of partially purified PDC exhibited different specific activities and activity indices. In Section 4.2.1.3 it was shown that all batches

exhibited similar stability in the presence of benzaldehyde when evaluated in a MOPS buffer. The effect of these differences on *R*-PAC formation is investigated in this section using each batch of partially purified PDC.

The effect of substrate and enzyme concentrations is also investigated to identify the limitations of *R*-PAC formation in a simple benzaldehyde emulsion batch reactor.

4.3.1 Inhibitory Effect of Product *R*-PAC

Inhibition of the biotransformation due to the product *R*-PAC was evaluated under reaction conditions with benzaldehyde delivered as a solution. *R*-PAC formation was measured from 50 mM (5.3 g/l) benzaldehyde and 75 mM (6.6 g/l) pyruvate at 4 °C and pH 7.0 with 7 U/ml carboligase activity in MOPS buffer (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C). The highest concentration of *R*-PAC evaluated was 153.6 mM due to the limited solubility of *R*-PAC in MOPS buffer in the presence of substrates. Detailed methodology is described in Section 2.6.7.

The profile of *R*-PAC formation in the presence of varying concentrations of *R*-PAC is shown in Figure 4.9. There was no evidence of inhibition of initial reaction rates or final *R*-PAC concentrations with up to 153.6 mM *R*-PAC.

Control (a) represents the biotransformation with no added *R*-PAC. Control (b) was similar to control (a), with the addition of acetoin and benzaldehyde to account for the possible effect of these compounds on *R*-PAC formation. The final concentrations of acetoin and benzaldehyde in control (b) reaction were therefore 1.6 mM and 3.0 mM respectively and reflected levels present in the highest concentration of *R*-PAC (153.6 mM).

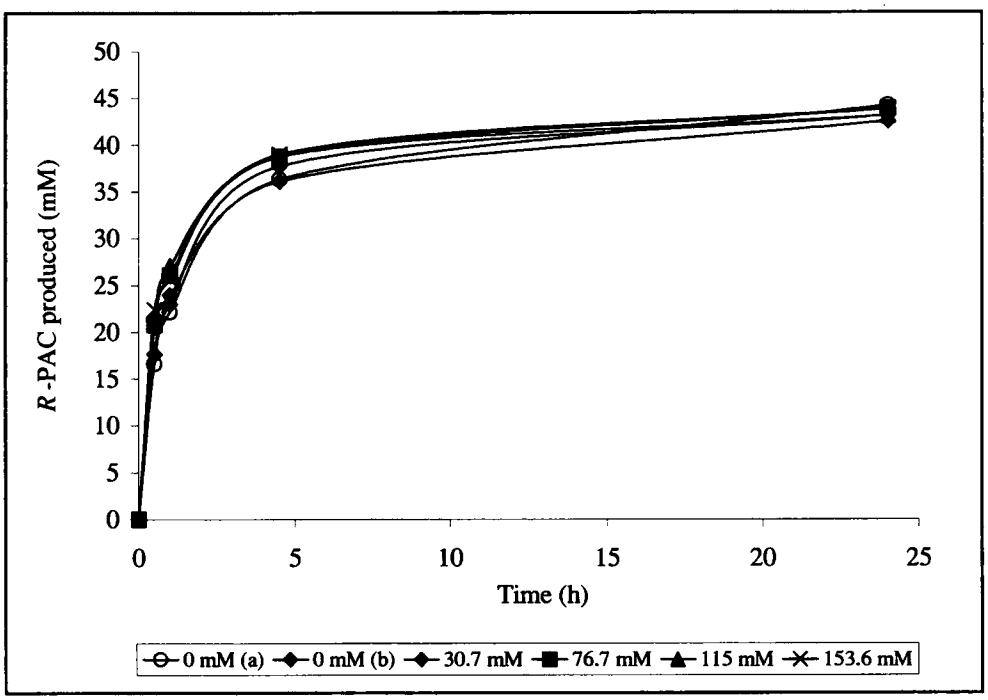


Figure 4.9: Formation of *R*-PAC in the presence of varying concentrations of added *R*-PAC. (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, 7 U/ml carboligase activity, 50 mM benzaldehyde solution and 75 mM pyruvate).

4.3.2 Effect of Activity Index and Specific Activity

R-PAC production from four enzyme batches with variable activity indices and specific activities was evaluated at approximately 7 U/ml carboligase activity in MOPS buffer (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C) with starting substrate levels of 406 mM (43.1 g/l) benzaldehyde and 600 mM (52.9 g/l) pyruvate. *R*-PAC and acetoin formation were measured after 18 hours. Detailed methodology is described in Section 2.6.8.

Table 4.2 shows that all four batches of enzyme produced the same final concentration *R*-PAC despite large differences in activity index and specific activity. Molar yields of *R*-PAC on substrates consumed were comparable between the four batches. Acetoin by-product formation varied between batches and could have been dependent on the activity index of the enzyme preparation. A lower level of acetoin was formed with pyruvate decarboxylase with a higher activity index. Future experiments evaluating

R-PAC production were conducted with batch #2, which was considered representative of all batches in terms of both stability and *R*-PAC formation. The emulsion formed in the 2M MOPS buffer was observed to be more stable in comparison to the previously used phosphate buffer.

These results show that *R*-PAC production is not influenced by the specific activity or the activity index of the preparation under these conditions. The activity index however may influence by-product acetoin production. Subsequent biotransformations reported in this Chapter were conducted using batch #2, which showed identical stability and *R*-PAC formation to the other batches, however formed the least acetoin. The Enantiomeric Excess (EE) of *R*-PAC was high with an average EE value of 95.7 %.

Table 4.2: *R*-PAC production from batches of enzyme exhibiting different activity indices and specific activities (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, 406 mM benzaldehyde, 600 mM pyruvate, 18 hours, 4 °C). Five litre (a,b) and 30 litre (a,b) bioreactors are described in Chapter 3.

Batch	#1 5 litre (a)	#2 30 litre (a)	#3 5 litre (b)*	#4 30 litre (b)
Carboligase (U/ml)	7.2	7.0	6.8	7.2
Activity index**	0.25	2.07	0.19	0.45
Specific activity (U/mg)	2.2	1.8	1.2	4.5
R-PAC after 18 hours (g/l)	40.5	39.4	39.2	40.6
R-PAC after 18 hours (mM)	270	263	261	271
Yield $_{(R\text{-PAC/benzaldehyde})}$ (%)***	76.2	76.2	75.4	79.1
Yield $_{(R\text{-PAC/pyruvate})}$ (%)***	62.2	70.4	64.7	65.6
Acetoin (mM)	30.8	1.9	29.2	8.9
Enantiomeric excess (%)	95.5	95.8	95.7	95.8

* Fermentation conducted by Wong (unpublished results) as described in Chapter 3.

** Activity index = carboligase activity/ decarboxylase activity

*** Yields are based on moles of *R*-PAC formed per mole amount of benzaldehyde or pyruvate utilized.

4.3.3 Effect of Substrate Concentration

In this section, the effect of benzaldehyde and pyruvate concentration on *R*-PAC formation is investigated. There is no reported evidence in literature of enzyme inactivation due to the substrate pyruvate. Investigation of inhibition of *R*-PAC formation by pyruvate has shown that no inhibition occurs with up to 200 mM pyruvate (Leksawasdi, unpublished results). In addition Shin (1994) showed that no inhibition of initial reaction rates occurred with up to 600 mM pyruvate present at the beginning of the biotransformation. Therefore the impact of substrate concentration on *R*-PAC in this section is attributed primarily to the effect of benzaldehyde.

The effect of benzaldehyde concentration on *R*-PAC formation was investigated at 4 °C and pH 7.0, with 7 U/ml carboligase activity in MOPS buffer (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C). *R*-PAC formation was measured after 18 hours. The ratio of pyruvate to benzaldehyde for each reaction was 1.5. Detailed methodology is described in Section 2.6.8.1.

R-PAC formation as a function of benzaldehyde concentration is shown in Figure 4.10. *R*-PAC formation increased with substrate level and plateaued at 267 mM (40 g/l) with initial substrate concentrations of 406 mM (43.1 g/l) benzaldehyde and 600 mM (52.9 g/l) pyruvate. At this maximum, the biotransformation was not substrate limited with 61.3 mM (6.5 g/l) benzaldehyde and 229.8 mM (20.3 g/l) pyruvate remaining.

Pyruvate and benzaldehyde limitation occurred with initial substrate concentrations of 102 mM (10.8 g/l) benzaldehyde and 150 mM (13.2 g/l) pyruvate (Figure 4.10). Benzaldehyde limitation occurred in flasks with 203 mM (21.5 g/l) starting benzaldehyde. At starting benzaldehyde concentrations of 254 mM (26.9 g/l), 304 mM (32.3 g/l), and 406 mM (43.1 g/l) no substrate limitation occurred.

Table 4.3 shows that the yield of *R*-PAC on benzaldehyde consumed was higher at lower substrate levels. The yield of *R*-PAC on pyruvate increased with substrate level. A control with no enzyme and initial substrate levels of 406 mM (43.1 g/l)

benzaldehyde and 600 mM (52.9 g/l) pyruvate lost 101 mM (10.7 g/l) benzaldehyde and 45 mM (4 g/l) pyruvate over 18 hours. Losses of benzaldehyde could possibly be attributed to evaporation and/or losses due to association with the Teflon seal in the reaction vial. Loss of pyruvate could be attributed to degradation. It is well known that pyruvic acid polymerizes unless pure and stored in airtight containers (Merck Index), and its instability has been previously reported in studies of PDC (Ankar 1948). It is apparent from the above control that pyruvate degrades under reaction conditions.

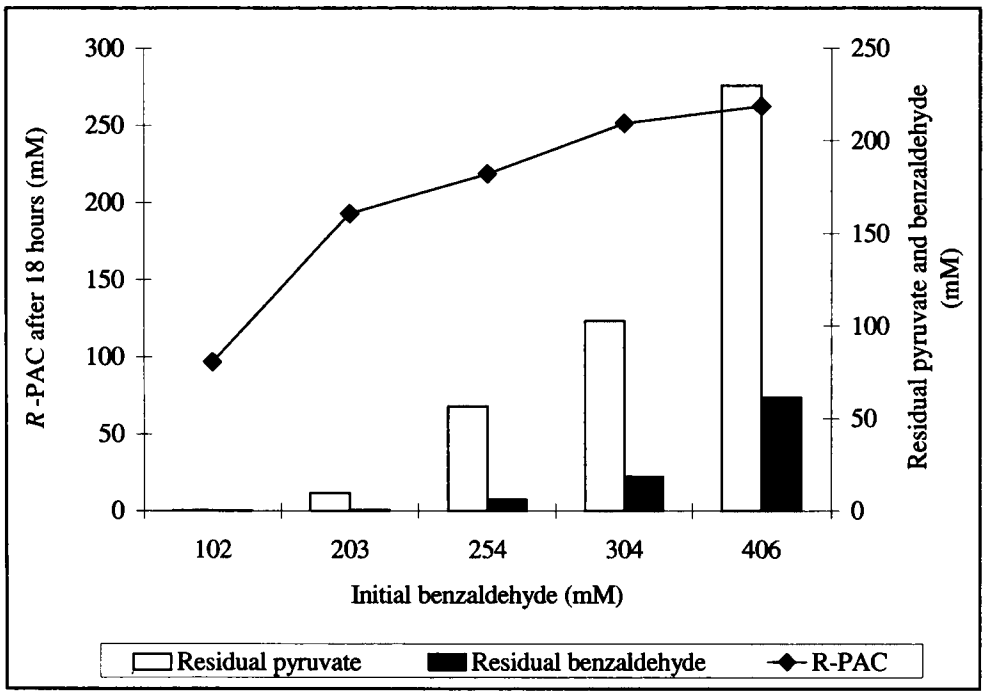


Figure 4.10: *R*-PAC formation as a function of benzaldehyde concentration in the benzaldehyde emulsion biotransformation (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, 7 U/ml carboligase activity, 18 hours).

Table 4.3: Molar yields of *R*-PAC on substrates consumed with various substrate levels in the benzaldehyde emulsion biotransformation (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, 7 U/ml carboligase activity, 18 hours).

Initial benzaldehyde (mM)	Yield $_{(R\text{-PAC/benzaldehyde})}$ (%)	Yield $_{(R\text{-PAC/pyruvate})}$ (%)
102	95.4	64.2
203	95.2	66.0
254	87.7	67.7
304	87.9	71.9
406	76.2	70.4

4.3.4 Effect of Enzyme Level

The effect of enzyme level on final *R*-PAC concentrations, molar yields of *R*-PAC on substrates, and specific product formation per unit of carboligase activity was evaluated. *R*-PAC formation with various enzyme activity levels was measured after 40 hours at 4 °C and pH 7.0 from 406 mM benzaldehyde and 600 mM pyruvate in MOPS buffer (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C). Detailed methodology is described in Section 2.6.8.2.

Figure 4.11 shows that *R*-PAC production increased with increasing enzyme level reaching a maximum at 280 mM (42 g/l) with 9.4 U/ml. Specific production of *R*-PAC (mg *R*-PAC/U) reached a maximum of 9.3 mg *R*-PAC/U at 3.1 U/ml, in comparison to 5.6 mg *R*-PAC/U at 7 U/ml under the same conditions. Yield of *R*-PAC on both pyruvate and benzaldehyde utilized increased with enzyme level. At 7 U/ml the molar yields did not increase further, reaching 75 % for molar yields of both *R*-PAC on pyruvate and benzaldehyde consumed. At low PDC concentrations, small concentrations of *R*-PAC were achieved, with possible degradation of unconsumed substrates leading to low yields. The low levels of pyruvate consumed at low enzyme concentrations suggests that enzyme inactivation was rapid or that substrate utilization

rates were slow. In these cases the unconsumed substrates would be incubated for longer periods of time in reaction buffer facilitating degradation. As enzyme concentrations were increased, the amount of unconsumed substrate decreased, reducing the impact of substrate degradation on yields.

Residual substrate levels indicate that the biotransformation was not substrate limited at any of the carboligase activities examined (Figure 4.12).

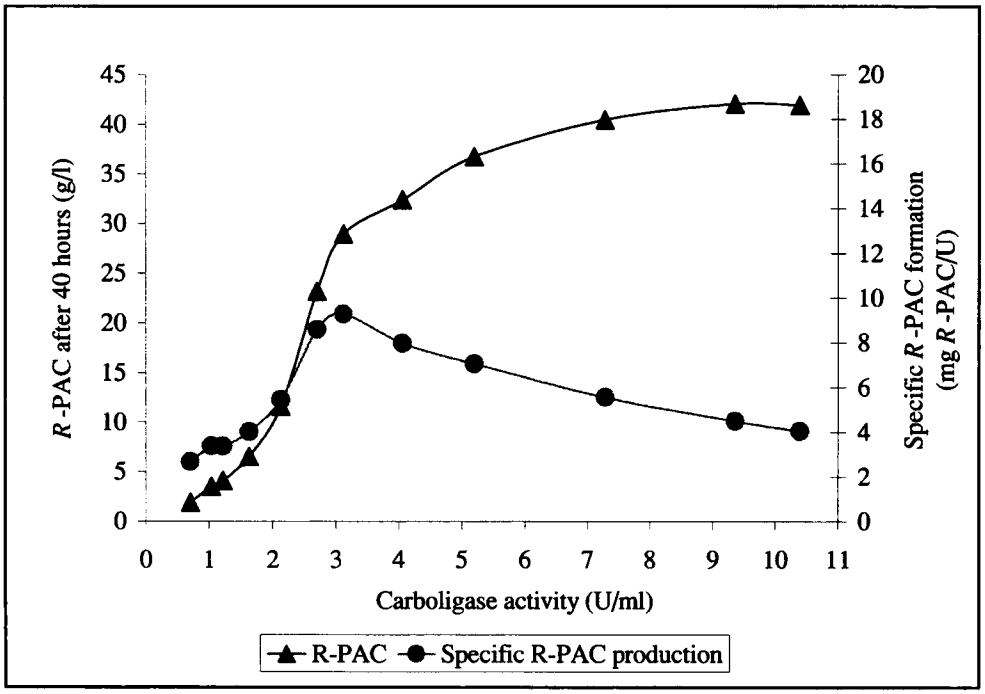


Figure 4.11: *R*-PAC formation as a function of initial carboligase activity in the benzaldehyde emulsion biotransformation (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, 406 mM benzaldehyde, 600 mM pyruvate, 40 hours).

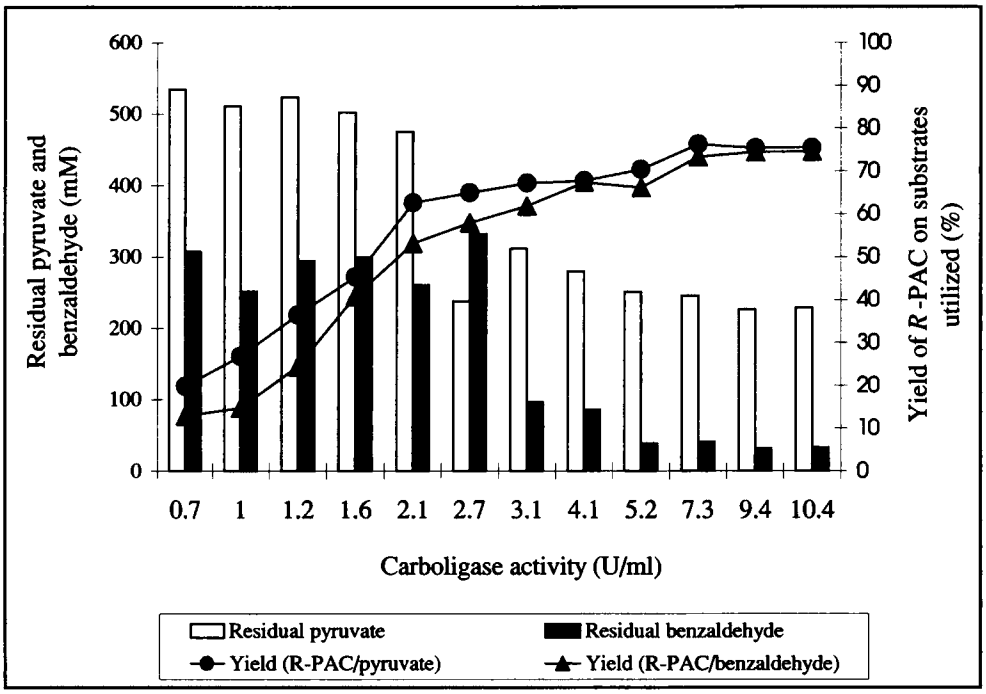


Figure 4.12: Influence of carboligase activity on residual substrate levels and the molar yields of *R*-PAC on substrates utilized in the benzaldehyde emulsion biotransformation (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, 406 mM benzaldehyde, 600 mM pyruvate, 40 hours).

4.4 *R*-PAC Formation and Enzyme Stability Under Reaction Conditions with Benzaldehyde Emulsions

A full profile of the biotransformation was performed detailing substrates, product, by-products and enzyme activity using partially purified PDC (batch #2). Initial substrate levels were 406 mM (43.1 g/l) benzaldehyde and 600 mM (52.9 g/l) pyruvate 4 °C and pH 7.0 in a MOPS buffer (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C). An initial carboligase activity of 7 U/ml was used. Detailed methodology is described in Section 2.6.8.3. The full profile of the benzaldehyde emulsion reaction over 81 hours is shown in Figure 4.13. The molar yields of *R*-PAC, acetoin and acetaldehyde on substrate utilized are shown in Table 4.4.

After 57 hours, *R*-PAC production reached 302 mM (45.3 g/l) *R*-PAC with no further product formation after this time. At this time, acetoin and acetaldehyde concentrations were 39.3 mM (3.5 g/l) and 16.2 mM (0.7 g/l) respectively. The final concentration of *R*-PAC was greater than that achieved in previous experiments described in Section 4.3.3 and 4.3.4. This increase was due to the increased reaction time needed to complete the reaction.

The molar yields of *R*-PAC on benzaldehyde and pyruvate utilized were 82 % and 63 % respectively. The control with no enzyme present lost 165 mM (17.5 g/l) benzaldehyde and 154 mM (13.6 g/l) pyruvate over the 81 hour period. Degradation of pyruvic acid under reaction conditions and loss of benzaldehyde due to association with the glass vial or cap may explain some of the losses that were also experienced in the control with no enzyme present.

The molar yields of *R*-PAC on substrates utilized reflect these losses. The molar yield of *R*-PAC on pyruvate utilized reported in this Section was slightly lower than that achieved in biotransformations with shorter reaction times in Section 4.3.3 and 4.3.4. Possible increased pyruvate degradation occurring with longer reaction times could impact on the yield calculation.

The pH measured after 81 hours was 7.4.

The enzyme was stable over the first two hours of the reaction. After 2 hours the enzyme began to inactivate, with 5 % carboligase activity remaining after 57 hours and complete inactivation after 81 hours.

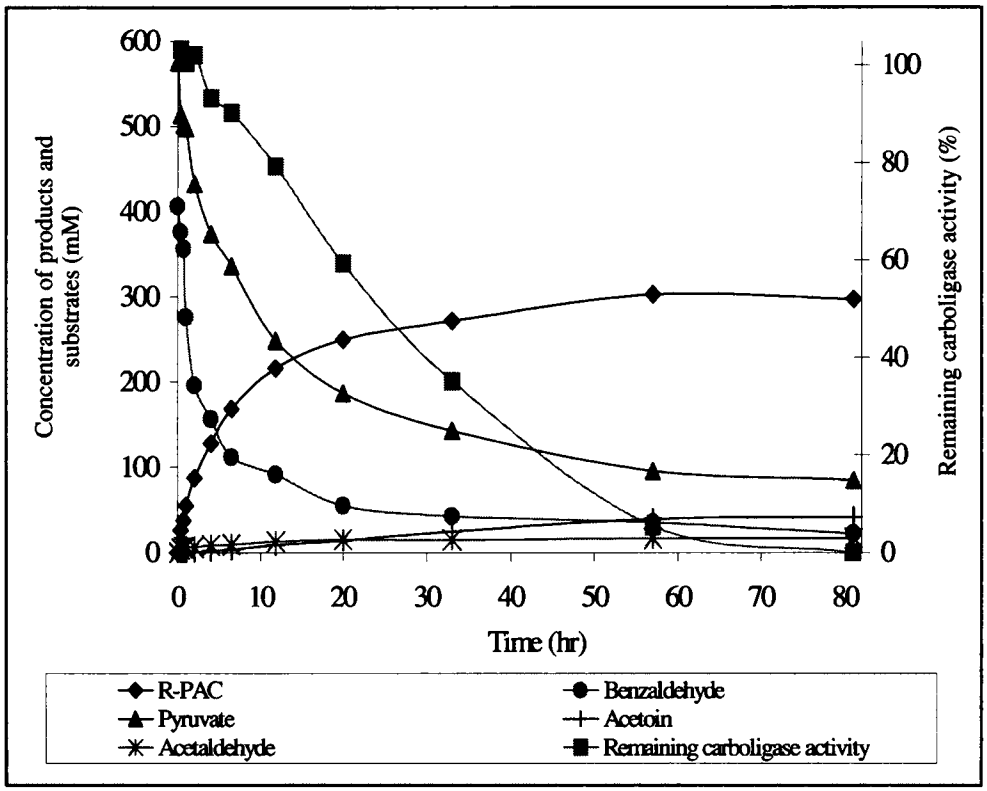


Figure 4.13: Full reaction profile of the benzaldehyde emulsion biotransformation (7 U/ml carboligase activity, 406 mM benzaldehyde, 600 mM pyruvate, 2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, batch #2).

Table 4.4: Molar yields of product and by-products on substrates utilized at 57 hours in the benzaldehyde emulsion biotransformation (7 U/ml carboligase activity, 406 mM benzaldehyde, 600 mM pyruvate, 2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, batch #2).

Yield $_{(R\text{-PAC/benzaldehyde})}$	82 %
Yield $_{(R\text{-PAC/pyruvate})}$	63 %
Yield $_{(\text{acetoin/pyruvate})}$	16 %
Yield $_{(\text{acetaldehyde/pyruvate})}$	3 %

4.5 Discussion and Conclusions

The factors affecting both enzyme stability and *R*-PAC production by partially purified PDC from *Candida utilis* have been investigated in this Chapter. The study of these influences could help identify the limitations of cell-free *R*-PAC production in the benzaldehyde emulsion systems reported by Shin (1994), Chow (1998), and Rosche *et al.* (2002a). Increased knowledge obtained from this study can be used to design a reactor that maximizes *R*-PAC formation per amount of enzyme catalyst.

Factors affecting PDC inactivation

1) Effect of benzaldehyde

The study of pyruvate decarboxylase (PDC) inactivation in this Chapter has identified that benzaldehyde was the most significant inactivating influence. This level of inactivation could be significantly reduced by delivering benzaldehyde as a solution thereby preventing contact of the enzyme with benzaldehyde droplets. The most significant evidence for this conclusion being the significantly reduced inactivation of PDC in the presence of 50 mM (5.3 g/l) benzaldehyde delivered as a solution as compared to an emulsion at 50 mM.

When benzaldehyde was delivered as an emulsion, the enzyme may have associated around the benzaldehyde droplets, resulting in high local concentrations of benzaldehyde in contact with the enzyme. The resulting inactivation may have been due to the same mechanisms as in the solution but occurring at a greater rate due to the higher local concentrations. Alternatively, a change in the mechanism of inactivation may have occurred, possibly due to the formation of a micellular structure of enzyme molecules associated at the interface between benzaldehyde and the aqueous phase. Association of non-polar amino acid side chains with benzaldehyde droplets may disrupt the protein structure. Interfacial inactivation has been reported for several enzyme systems, such as β-hydroxysteroid dehydrogenase and several lipases (Cecil and Louis 1970; Halling 1994; Ghatorae *et al.* 1994b). The large variations encountered with benzaldehyde emulsions may reflect the difficulty in obtaining and maintaining a

relatively constant total interfacial area. Ghatorae *et al.* (1994a) discusses the changes in viscosity, density, interfacial tension and interfacial rheology that occur when an enzyme is inactivated in the presence of an emulsion. These changes are likely to influence the interactive interfacial area.

A previous study of *Candida utilis* PDC also showed similar rapid enzyme inactivation when exposed to benzaldehyde emulsions with reported variation in decarboxylase activity measurements during the time profile of inactivation. It was noted also that significant variation of inactivation rates and *R*-PAC production occurred between different batches of enzyme (Shin 1994; Chow 1998). In this Chapter, standardization of *Candida utilis* PDC characteristics was achieved by using a concentrated 2 M MOPS buffer. Rosche *et al.* (2002a) found that the half-life of *R.javanicus* PDC in the absence of benzaldehyde increased 7 fold with an increase of MOPS concentration from 0.05 M to 2 M. In this study, enzyme stability and *R*-PAC levels achieved per unit of carboligase activity were reproducible in this buffer regardless of the specific decarboxylase activity, specific carboligase activity or activity index of the enzyme batch.

The addition of serine and cysteine protease inhibitors did not affect enzyme activity nor improve enzyme stability in a phosphate buffer. Addition of BSA improved the stability of some batches of enzyme but did not have a beneficial effect for all batches. The use of MOPS to standardize enzyme characteristics is suitable for lab-scale experiments, however the buffer is expensive and is therefore not suitable for an industrial process. Rosche *et al.* (2002a) has found that MOPS buffer may be replaced with more cost effective materials that also improve enzyme stability. It was found that as an alternative to 2 M MOPS, partially purified PDC from *R.javanicus* was stabilized by 2 M glycerol, 0.75 M sorbitol, 10 % w/v polyethylene glycol 6000 or 1 M KCl.

Recent studies by Satianegara (unpublished results) have shown that a crude extract preparation of *C.utilis* PDC was significantly more stable to benzaldehyde emulsions than a partially purified preparation. Crude extract with an initial carboligase activity of 5 U/ml showed a half-life of approximately 4 days when exposed to 400 mM benzaldehyde emulsion at 6°C in MOPS buffer (1.1 M MOPS, 0.11 M citrate, 20 mM Mg^{2+}, 1mM TPP, pH 6.4). Partially purified PDC had a half-life of 6 hours under the

same conditions with an initial carboligase activity of 12 U/ml Satianegara (unpublished results). The crude extract produced significantly more *R*-PAC per unit of enzyme (19.4 mg *R*-PAC/U) in comparison to the maximum achieved using a partially purified preparation (9.3 mg *R*-PAC/U).

The mechanism by which the enzyme is inactivated by benzaldehyde is unknown, however several possible reasons for this inactivation may be:

(a) Reaction of benzaldehyde with NH or SH groups

The reaction of aldehydes such as benzaldehyde with specific groups of proteins is well documented. Aldehydes may react with amine side-chains of amino acids and SH groups such as cysteine. The histidine imidazole NH group and the NH group of tryptophan are also reported to bind aldehydes (Herriott 1947). Schubert (1936) reported that cysteine forms aldehyde condensation products with formaldehyde, butyric aldehyde, chloral, benzaldehyde and furfural. Milun (1957) showed that primary amines react with salicyclicaldehyde to form a yellow Schiff-base.

Studies of *Saccharomyces cerevisiae* PDC have shown that each subunit possesses 4 cysteines, which do not form disulfide bridges. It has been concluded in several studies that the C221 cysteine residue is important for pyruvate decarboxylase activation and activity (Baburina *et al.* 1994; Baburina *et al.* 1998a,b). In these studies (E)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid (CPB) was incubated with the enzyme. The decarboxylation product from CPB was the reactive aldehyde, cinnamaldehyde. It was shown by Baburina *et al.* (1998b) that cinnamaldehyde covalently bonded to the C221 cysteine residue and at least one other cysteine residue, resulting in enzyme inactivation and loss of substrate activation. This could be a possible mechanism for pyruvate decarboxylase inactivation due to benzaldehyde.

(b) Disruption of solvent structure

It was shown by Arjunan *et al.* (1996) that 12 water molecules interact with subunits to crosslink the PDC protein, in addition to 6 water molecules in each active site involved in hydrogen bonding with the cofactors. In addition to the aldehyde reactions discussed

above, hydrophobic benzaldehyde may disturb essential water molecules, which may be important for enzyme stability.

2) Effect of *R*-PAC

In this study *R*-PAC was shown to irreversibly inactivate carboligase activity. The level of enzyme inactivation measured indicates that *R*-PAC formation would have a significant impact on the reaction, although it would contribute less significantly than enzyme inactivation due to benzaldehyde emulsions. Shin (1994) also found that *R*-PAC inactivated *Candida utilis* PDC, with a 20 % loss of decarboxylase activity when exposed to 70 mM *R*-PAC for 1 hour at 4 °C in a phosphate buffer. By comparison, in this study a 20 % loss of carboligase activity occurred in 40 hours when exposed to 70 mM *R*-PAC in a MOPS buffer at 4 °C. The enzyme preparation was therefore more stable under the conditions evaluated in this study. This improvement in stability may arise from differences in the method of enzyme recovery or buffer species used.

3) Effect of acetoin

Prior to the current investigation, enzyme inactivation due to acetoin was unreported in the literature. The inactivation due to acetoin and *R*-PAC may be due to a hydrophobic effect, involving the stripping of essential water molecules from the enzyme, or due to the action of ketone groups, which can react with NH or SH groups of the protein in the same manner as aldehydes (Brown 1982; Harding 1985).

4) Effect of acetaldehyde

Enzyme inactivation due to acetaldehyde was minimal. High levels of irreversible enzyme inactivation due to acetaldehyde have been reported for *Z.mobilis* PDC (Bruhn *et al.* 1995). In this study, partially purified *C.utilis* PDC in 50 mM MES buffer was stable when exposed to 30 mM acetaldehyde for 15 hours at 4°C. Under similar conditions, wild type *Z.moblis* PDC irreversibly inactivated by 50 % when exposed to 87 mM acetaldehyde, in 20 minutes, with nearly 100 % activity loss after 110 minutes (Bruhn *et al.* 1995). Inhibition of yeast PDC decarboxylation and carboligation rates by

acetaldehyde have been reported by Juni (1961) for *Saccharomyces cerevisiae* and by Shin and Rogers (1996b) for *Candida utilis* PDC. Juni (1961) showed that acetaldehyde was inhibitory, but not an inactivating influence. Inhibition of pyruvate decarboxylation was reversible upon removal of acetaldehyde.

Factors influencing *R*-PAC production

1) Inhibition due to *R*-PAC

In this study *R*-PAC was shown to have no inhibitory effect on initial reaction rates or final yields in the presence of up to 153.6 mM (23 g/l) *R*-PAC at the beginning of the reaction. It may be possible that concentrations higher than this could inhibit the reaction, however the combination of high concentrations of *R*-PAC and substrates in this buffer resulted in an emulsion, which could have impacted on the evaluation. For this reason concentrations higher than 153.6 mM were not evaluated. Reaction inhibition due to *R*-PAC was previously documented for *Candida utilis* PDC by Chow (1998). The product inhibition constant (Kp) was calculated to be 48 g/l (320 mM), with this constant determined from a comparison of measured rates of *R*-PAC production with rates determined from a developed model. This study shows however that no inhibition was found with up to 153.6 mM *R*-PAC. These results indicate that the mathematical model of the biotransformation could be further developed.

2) Reproducibility of *R*-PAC production

Enzyme stability evaluated in a phosphate buffer with benzaldehyde emulsion conditions exhibited large variations in stability. *R*-PAC formation in the same buffer exhibited variability as discussed by Shin (1994) and Chow (1998). For the results described in this Chapter, *R*-PAC production in MOPS buffer with benzaldehyde delivered as an emulsion showed reproducible *R*-PAC production for each batch of enzyme. This improvement in reproducibility is a significant advancement of the enzymatic cell-free system using *C.utilis* PDC. These results may suggest that:

(i) the enzyme may be more stable to inactivation by benzaldehyde emulsions in a MOPS buffer in comparison to a phosphate buffer.

(ii) benzaldehyde dispersed in a concentrated MOPS buffer may result in a more stable emulsion and therefore reproducible inactivation kinetics. A more stable emulsion was observed with high benzaldehyde concentrations when measuring *R*-PAC formation in the presence of pyruvate. Further investigations by Satianegara (unpublished results) have confirmed that reproducible inactivation kinetics were obtained in the presence of benzaldehyde emulsions when evaluated in a MOPS buffer (1.1 M MOPS, 0.11 M citrate, 20 mM Mg^{2+}, 1mM TPP, pH 6.4). The level of enzyme inactivation was significant in the presence of a 400 mM benzaldehyde emulsion, with a 50 % loss of carboligase activity after 6 hours at 6 °C with an initial carboligase activity of 12 U/ml.

R-PAC production by partially purified PDC from *Candida utilis* was evaluated previously by Shin (1994) and Shin and Rogers (1996b). It was found that 191 mM (28.6 g/l) *R*-PAC was produced in 8 hours from 7 U/ml decarboxylase activity. In this Chapter it was shown that by using similar biotransformation conditions optimized for *R.javanicus*, the concentration of *R*-PAC could be increased to 302 mM (45.3 g/l) in 57 hours from 7 U/ml carboligase activity. This represented 5.6 mg *R*-PAC produced per unit of carboligase activity. The time profile of this reaction demonstrates that 191 mM (28.6 g/l) was produced also in 8 hours, however additional product formation was achieved in this study by improving the reproducibility of enzyme stability, enhancing enzyme stability and by improving pH control which resulted from addition of 2 M MOPS buffer. The maximum amount of product per unit of enzyme was achieved with 3.1 U/ml producing 192 mM (28.8 g/l) *R*-PAC representing 9.3 mgPAC/U. By comparison partially purified *R.javanicus* PDC produced 50.6 g/l *R*-PAC in a 29 hour reaction period with the same initial substrate concentrations (Rosche *et al.* 2002a). Comparing these results with the similar levels achieved with *Candida utilis* PDC indicates that the improvement in *R*-PAC concentrations was not specific to the PDC source. In this study *Candida utilis* PDC was evaluated under these conditions due to the added benefit of MOPS buffer in standardizing enzyme stability. This provides a comparison of *R*-PAC production with data from other simple batch emulsion systems,

together with a basis for comparison with the new processes described in Chapter 5 and 6.

The full profile of the benzaldehyde emulsion biotransformation indicates that at 57 hours no further product formation occurred despite having residual pyruvate, benzaldehyde and low levels of enzyme activity. It is most likely that rising pH and possible inhibition due to acetaldehyde and acetoin were responsible for the cessation in *R*-PAC formation. On-line pH control may maximize product formation per amount of enzyme in this simple batch reactor. Without removal of by-products, or minimizing their production, however, it is unlikely that further product formation at high reaction rates would be possible.

In summary, in Chapter 4 it was established that:

(1) pyruvate decarboxylase from *Candida utilis* was irreversibly inactivated most significantly by benzaldehyde and acetoin, and less significantly by *R*-PAC. In contrast to *Z.mobilis* PDC, *C.utilis* PDC was very stable in the presence of acetaldehyde.

(2) strong enzyme inactivation occurred in the presence of benzaldehyde emulsions, with dramatic improvements in enzyme stability resulting from supply of benzaldehyde as a solution only.

(3) *R*-PAC did not inhibit the reaction at concentrations up to 153.6 mM.

(4) *R*-PAC production with simultaneous pH control by 2 M MOPS buffer was successfully applied to *Candida utilis* PDC. Reproducible enzyme inactivation kinetics and *R*-PAC production were achieved with all batches of enzyme regardless of specific activity and activity index of the preparations by using a concentrated MOPS buffer.

(5) a possible relationship exists between the activity index of PDC and the level of acetoin formation during the biotransformation. A lower concentration of acetoin was formed using the enzyme batch exhibiting the higher activity index. Further investigation of this possible relationship is needed, including establishing a protocol for controlled alteration of the activity index of the enzyme preparation. Identical stability

characteristics and *R*-PAC production were achieved regardless of this difference in activity index.

(6) a full profile of *R*-PAC production with benzaldehyde supplied as an emulsion showed that the reaction was limited by rising pH and possible inhibition due to by-product formation. Significant enzyme inactivation occurred also during the course of the reaction.

These results suggest that a reaction system delivering low levels of toxic benzaldehyde at a controlled pH of 6.5-7.0, combined with continuous *R*-PAC, acetaldehyde and acetoin extraction could result in higher levels of product per unit of enzyme and increased productivities. In Chapter 5 and 6 a two-phase reaction system is designed and evaluated to achieve these goals.

CHAPTER 5

EVALUATION OF ORGANIC SOLVENTS FOR *R*-PAC PRODUCTION IN THE TWO-PHASE SYSTEM

5.1 Introduction

R-PAC production by partially purified pyruvate decarboxylase (PDC) from *Candida utilis* was shown in Chapter 4 to be affected by a combination of factors including irreversible inactivation by the substrate benzaldehyde, increased inactivation by benzaldehyde emulsions, irreversible inactivation by the product *R*-PAC and irreversible inactivation due to by-product acetoin. These factors limit the amount of *R*-PAC achievable in a benzaldehyde emulsion batch reactor.

These limitations suggest that an ideal reaction system for maximum *R*-PAC production should minimize enzyme inactivation by delivery of soluble low levels of toxic substrate benzaldehyde, while also removing inhibitory and inactivating products and by-products from the enzyme as they are formed.

A two-phase system is proposed consisting of an organic phase containing the bulk of toxic benzaldehyde and produced *R*-PAC, and an aqueous phase containing the enzyme and substrate pyruvate. The most critical factor in such systems is the selection of a suitable organic solvent, which is compatible with the enzyme and is capable of correctly partitioning substrates and products between phases.

R-PAC production in aqueous-organic solvent two-phase systems has been evaluated previously using whole cells of *Saccharomyces cerevisiae*, and resulted in low levels of *R*-PAC formation (Nikolova and Ward 1992a,c; Laurence *et al.* 2001; Smallridge *et al.* 2001b). The use of cell free extracts for *R*-PAC production in two-phase systems has not been previously reported in the literature, and may offer the potential for increased *R*-PAC concentrations and yields.

The objective of this Chapter is to screen various organic solvents for enzyme biocompatibility and to evaluate selected solvents for their effect on *R*-PAC production in a two-phase reaction system. Enzyme from batch #2 was used in Chapter 5.

5.2 Effect of Various Organic Phase Solvents on Enzyme Stability

The effect of organic solvents on enzyme stability was evaluated in a two-phase system. The significance of solvent partition coefficients (log P) and the effect of these solvents on various properties of the enzyme have been discussed in Section 1.2.2.7. This investigation will also determine if a general correlation exists between enzyme stability and the log P of the solvent. A correlation would provide a basis for prediction of enzyme stability in additional solvents not evaluated in the following screen.

Long chain alkanes, alcohols and other solvents having log P values ranging from 0.8 to 6.6 were selected to provide a range of hydrophobicities and chemical properties. The 13 solvents screened and their corresponding log P values are shown in Table 5.1.

The volume ratio of organic phase solvent to the aqueous phase was maintained at 1:1. Organic phase solvent was layered on top of the enzyme at 7 U/ml carboligase activity in MOPS buffer (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C). A control with no solvent added was stirred under the same conditions. The bottom phase was stirred slowly thereby minimizing turbulence at the interface, and maintaining phase separation at all times. The ratio of interfacial surface area to solvent volume was maintained at 1.05:1 (cm^2/cm^3). Carboligase activity was measured directly from the aqueous phase. Detailed methodology is described in Section 2.7.1.

Table 5.1: Log P values of organic solvents evaluated in the two-phase system solvent screen (Lanne *et al.* 1987).

Solvent	Log P
1) Butanol	0.8
2) Pentanol	1.3
3) Octanol	2.9
4) Nonanol	3.4
5) Hexane	3.5
6) Heptane	4.0
7) Octane	4.5
8) Nonane	5.1
9) Dodecane	6.6
10) Toluene	2.5
11) Methylcyclohexane	3.7
12) Benzaldehyde	Not known
13) Methyl-tert-butyl ether (MTBE)	Not known

5.2.1 Effect of Long Chain Alcohols on Enzyme Stability

The effect of long chain alcohols on enzyme stability in the two-phase system is shown in Figure 5.1. Enzyme carboligase activity increased initially for the first 30 hours in the presence of octanol and nonanol in comparison to the control. This increase in enzyme activity was followed by slow sustained inactivation. All other alcohols evaluated resulted in immediate enzyme inactivation with no initial increase in activity. The enzyme was more rapidly inactivated in the presence of butanol in comparison to the other solvents evaluated.

Residual carboligase activities after 264 hours of exposure to the alcohols plotted against log P values are shown in Figure 5.2. There was evidence of increasing stability with increasing log P, however a greater range of alcohols should be evaluated to confirm this observation.

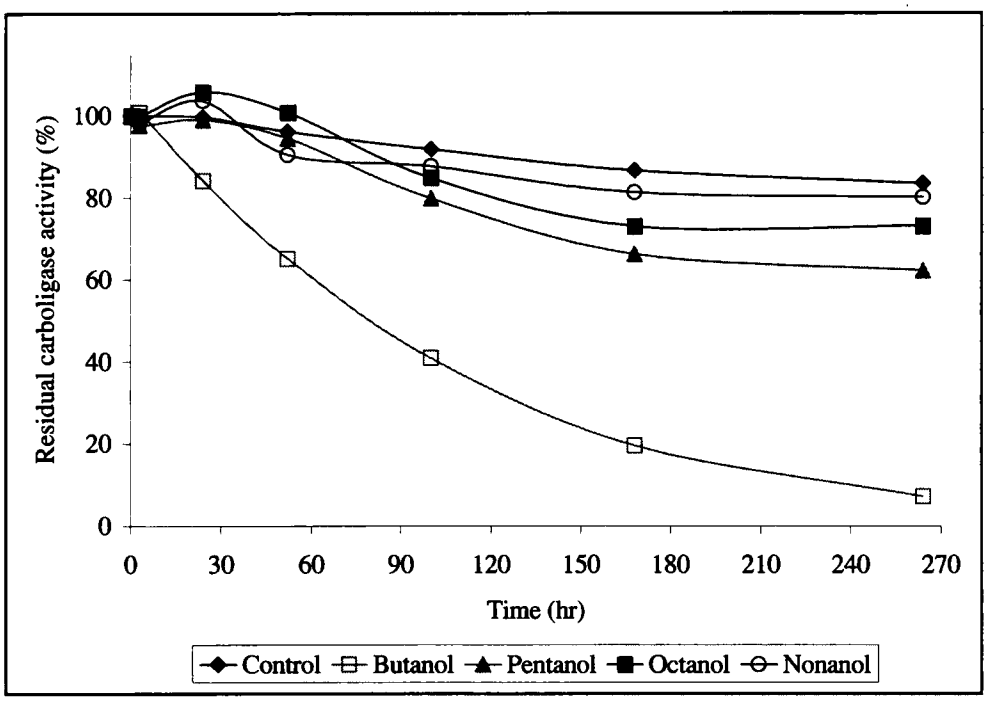


Figure 5.1: Effect of long chain alcohols on enzyme stability in the two-phase system (aqueous phase contained 2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, and an initial carboligase activity of 7 U/ml).

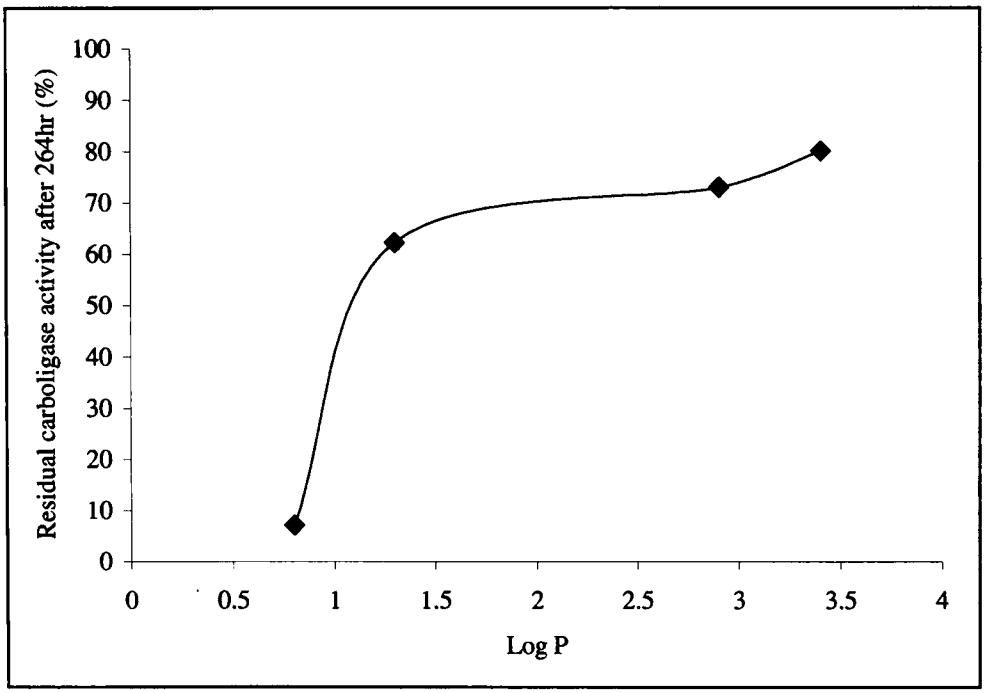


Figure 5.2: Relation between log P of long chain alcohols and enzyme stability in the two-phase system (aqueous phase contained 2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, and an initial carboligase activity of 7 U/ml).

5.2.2 Effect of Long Chain Alkanes on Enzyme Stability

The changes in enzyme stabilities over time, when exposed to alkanes of various chain lengths, are shown in Figure 5.3. There was a general trend found for all the alkanes, that the enzyme activity decreased over an initial three hour period in comparison to the control with no solvent present. After 3 hours the activity recovered from the initial decline and increased over the following 30 hours. After 30 hours the activity steadily declined. This initial rapid decrease in activity was not observed with long chain alcohols.

The relation between the residual carboligase activities after 264 hours of exposure to alkanes and the corresponding log P values of the alkanes is shown in Figure 5.4. There was no correlation of reduced enzyme activity with increasing carbon chain length or log P value.

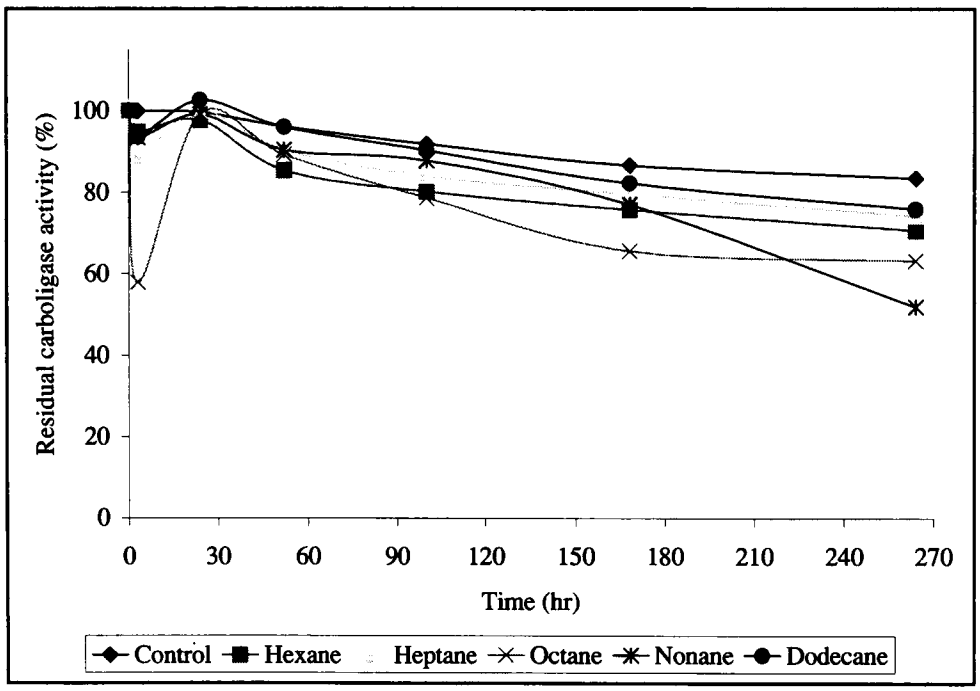


Figure 5.3: Effect of long chain alkanes on enzyme stability in the two-phase system (aqueous phase contained 2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, and an initial carboligase activity of 7 U/ml).

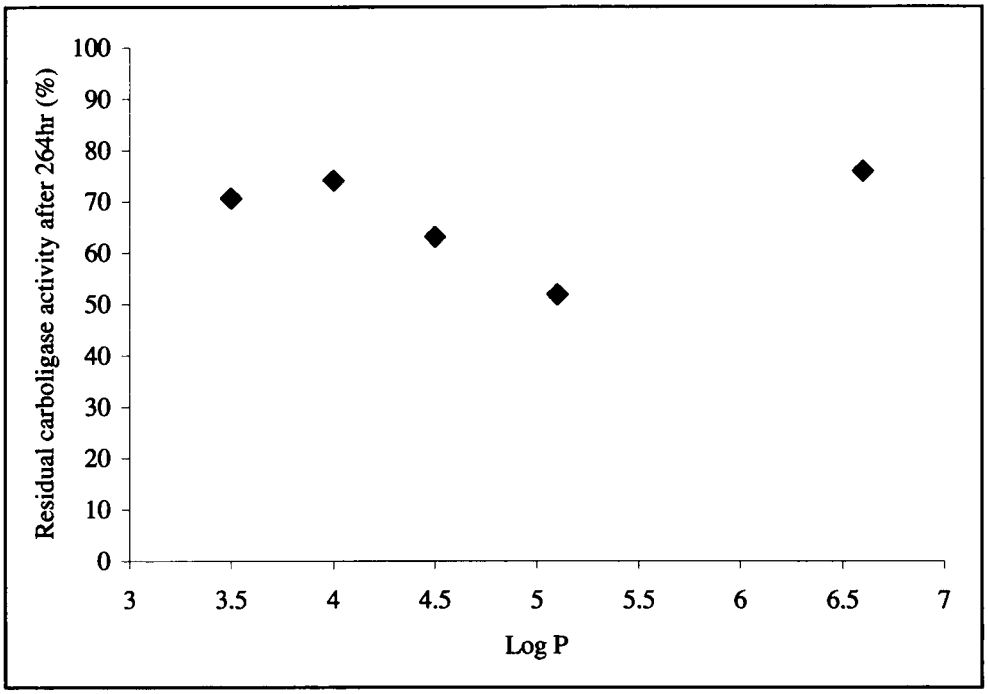


Figure 5.4: Relation between log P of long chain alkanes and residual enzyme activity in the two-phase system (aqueous phase contained 2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, and an initial carboligase activity of 7 U/ml).

5.2.3 Effect of Other Miscellaneous Solvents on Enzyme Stability

Enzyme stability evaluated for a miscellaneous class of solvents including the substrate benzaldehyde is shown in Figure 5.5. Enzyme incubated with MTBE resulted in an initial small increase in activity over 30 hours followed by sustained inactivation, similar to the pattern observed with the alcohols octanol and nonanol. MTBE is the only solvent from all those studied which resulted in a final activity level higher than the control. Benzaldehyde rapidly inactivated the enzyme.

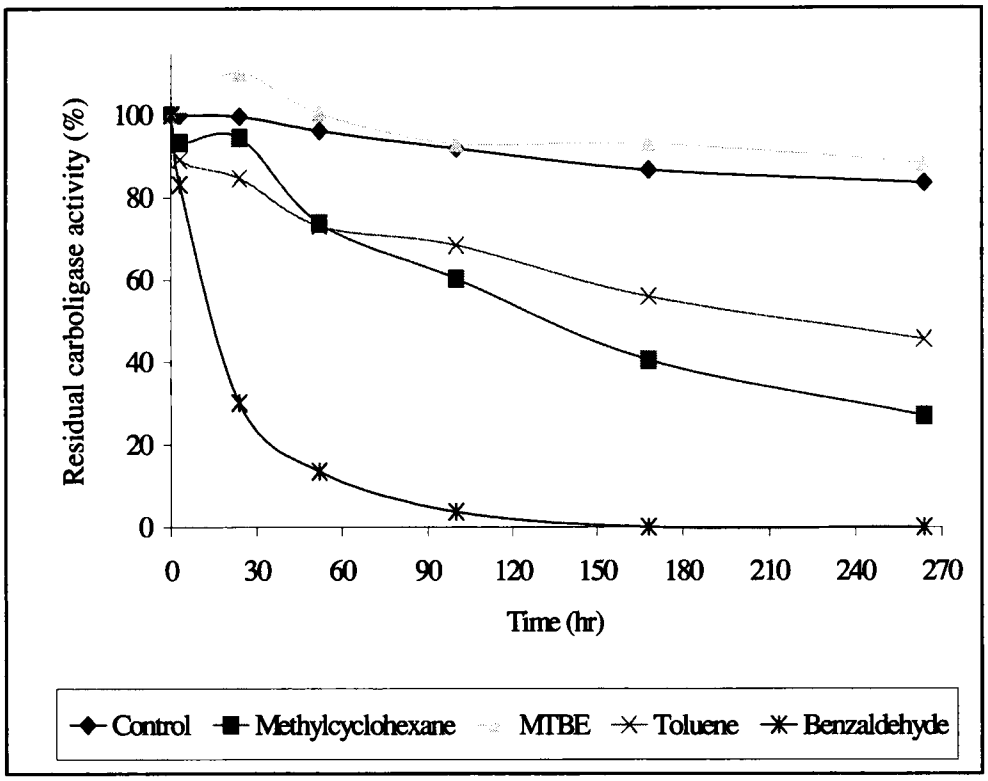


Figure 5.5: Effect of miscellaneous solvents on enzyme stability in the two-phase system (aqueous phase contained 2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, and an initial carboligase activity of 7 U/ml).

5.2.4 Comparison of Enzyme Stabilities

A comparison of residual enzyme activities for all solvents evaluated is shown in Figure 5.6. The carboligase activity after 264 hours of exposure was compared to a control with no solvent. The solvent MTBE had a positive effect on enzyme stability with a lower level of inactivation over 264 hours in comparison to the control. With all other solvents selected, the loss of enzyme carboligase activity over 264 hours was greater than in the control. The error bars represent highest and lowest values for the two vials evaluated per organic solvent.

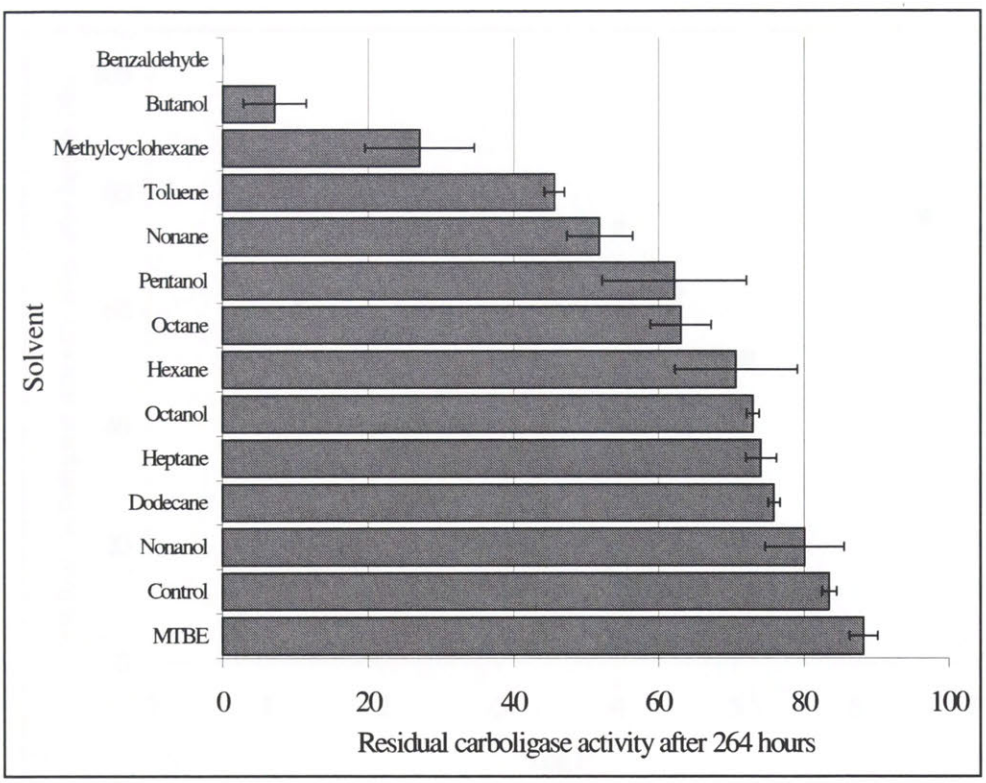


Figure 5.6: Comparison of enzyme stabilities in the presence of various solvents in the two-phase system (aqueous phase contained 2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, slowly stirred with phase separation maintained and an initial carboligase activity of 7 U/ml). The error bars represent highest and lowest values for the two vials evaluated per organic solvent.

5.2.5 Evaluation of Enzyme Stabilities on the Basis of Log P Values of Various Solvents

The residual carboligase activities after 264 hours of exposure to the various solvents is plotted as a function of log P in Figure 5.7. The data shows that there was no overall relationship between enzyme stability and the log P value of the solvent. There may be a relation between enzyme stability and log P for the long chain alcohols as indicated in Figure 5.2. Other classes of solvents however showed no correlation.

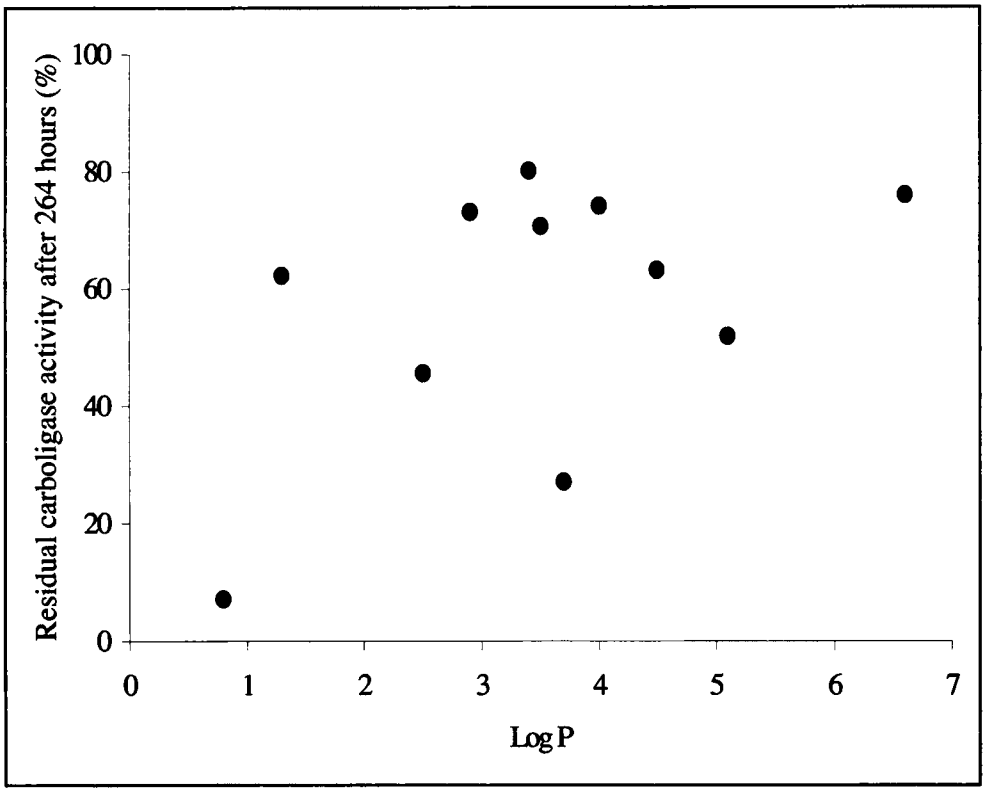


Figure 5.7: Plot of log P values of various solvents and residual enzyme activities in the two-phase system (aqueous phase contained 2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, and an initial carboligase activity of 7 U/ml).

5.3 *R*-PAC Production as a Function of Organic Phase Solvent

The ability of partially purified pyruvate decarboxylase to produce *R*-PAC in a two-phase system was evaluated with each solvent selected above. By evaluating *R*-PAC formation with a range of solvents, it may be possible to identify properties of the solvent which are important for enzyme activity.

R-PAC production was evaluated with 1.8 M benzaldehyde in the organic solvent phase and 1.43 M pyruvate in aqueous MOPS phase (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C). The benzaldehyde was fully miscible with the organic phase at 4°C. The reaction was started with the addition of enzyme to the aqueous phase at 7.3 U/ml

carboligase activity. The volume ratio of organic phase solvent to aqueous phase was maintained at 1:1. Directly after enzyme addition the reaction was stirred rapidly forming an organic phase emulsion to increase reaction rates. After 72 hours a sample was taken from both phases for analysis of pyruvate, benzaldehyde and *R*-PAC. Detailed methodology is described in Section 2.7.2.

The enzyme produced *R*-PAC with each different solvent in the organic phase. The concentrations of *R*-PAC in the organic phase and aqueous phase for each solvent are shown in Figure 5.8. The error bars represent highest and lowest values for the two vials evaluated per organic solvent. The largest amounts of *R*-PAC produced were with octanol and nonanol as organic phase solvents. 670 mM (100.6 g/l) of *R*-PAC was measured in the organic phase with an additional 113 mM (17 g/l) in the aqueous phase for octanol, and 605 mM (90.8 g/l) in the organic phase and 130 mM (19.5 g/l) in the aqueous phase for nonanol.

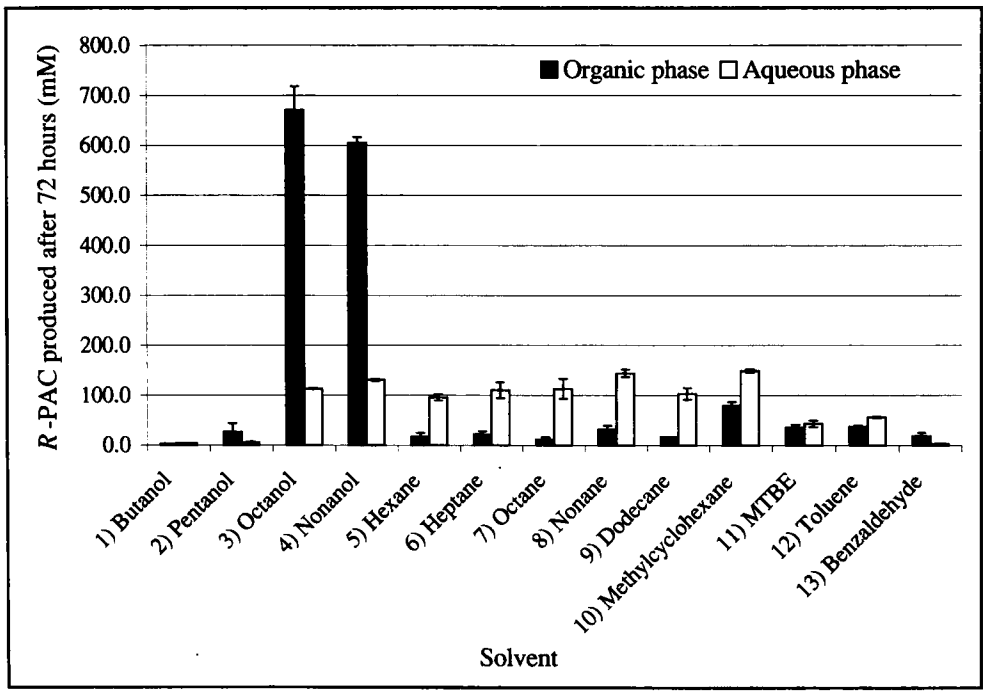


Figure 5.8: Production of *R*-PAC after 72 hours in the two-phase system using various organic phase solvents. The error bars represent highest and lowest values for the two vials evaluated per organic solvent.(organic phase contained 1.8 M benzaldehyde; aqueous phase contained 1.43 M pyruvate, 7.3 U/ml PDC, 2 M MOPS, 1 mM TPP, and 1 mM Mg^{2+}at pH 7.0 and 4 °C, rapidly stirred).

The amount of *R*-PAC produced per unit of carboligase activity as shown in Figure 5.9 reaches 16.1 mg *R*-PAC/U with octanol as organic phase solvent and 15.1 mg *R*-PAC/U for nonanol. In both cases this is a significant improvement over the benzaldehyde emulsion system in the absence of an additional organic solvent phase, where 6.4 mg *R*-PAC /U was achieved with a comparable 7 U/ml enzyme, and 11 mg *R*-PAC/U was achieved with 3.8 U/ml. All other solvents evaluated produced lower amounts of product per amount of enzyme than in the benzaldehyde emulsion system.

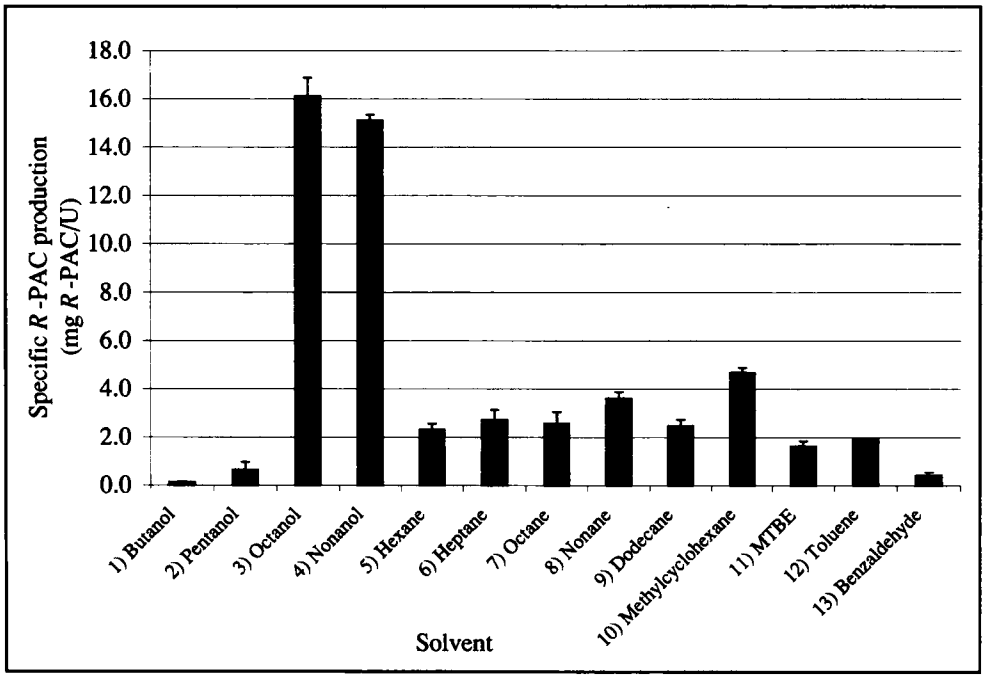


Figure 5.9: Comparison of specific *R*-PAC production for various solvents in the two-phase system. The error bars represent highest and lowest values for the two vials evaluated per organic solvent (organic phase contained 1.8 M benzaldehyde; aqueous phase contained 1.43 M pyruvate, 7.3 U/ml PDC, 2 M MOPS, 1 mM TPP, and 1 mM Mg^{2+}at pH 7.0 and 4 °C, rapidly stirred).

The yields of *R*-PAC on pyruvate and benzaldehyde consumed for each solvent system are shown in Table 5.2. The highest yield of *R*-PAC on pyruvate consumed was obtained with octanol and nonanol, achieving 55 % and 52 % respectively. Nonane and methylcyclohexane have acceptable yields of *R*-PAC on pyruvate consumed, achieving 42 % and 38 % respectively, although the overall level of pyruvate decarboxylated and the amount of *R*-PAC formed was low relative to octanol and nonanol. Reactions using all other solvents resulted in low final molar yields of *R*-PAC on pyruvate consumed and low final concentrations of *R*-PAC. The low levels of pyruvate that were decarboxylated with solvents other than octanol and nonanol indicate that the low concentrations of *R*-PAC produced were due to the low rates of pyruvate decarboxylation. A control consisting of substrates without enzyme showed that loss of both benzaldehyde and pyruvate occurred in this system, with a loss of 405 mM pyruvate and 55 mM benzaldehyde. These losses were included in the calculation of molar yield of *R*-PAC formed from benzaldehyde or pyruvate consumed. For some solvents such as butanol and MTBE, the level of substrate degradation was greater than the amount of substrate utilized for *R*-PAC formation. Therefore low yields were reported for reactions with low overall levels of pyruvate and benzaldehyde utilization.

Residual substrate concentrations and final product concentrations in each phase after a reaction time of 72 hours are shown Table 5.3. Benzaldehyde levels in the aqueous phase (Table 5.3) were sufficiently high to support *R*-PAC formation from the pyruvate that was decarboxylated with all solvents tested. Therefore low product formation with most of the solvents was not due to poor partitioning of benzaldehyde into the aqueous phase for enzymatic conversion.

Yields of *R*-PAC on benzaldehyde consumed for octanol and nonanol were 85 % and 84 % respectively.

The distribution of *R*-PAC between the two phases was different for each solvent. Figure 5.8 shows that *R*-PAC partitioned strongly into the organic phase when octanol and nonanol were used. Pentanol and benzaldehyde also cause preferential partitioning of *R*-PAC away from the enzyme.

Table 5.2: Molar yields of *R*-PAC on substrates utilized for various organic solvents in the two-phase system (organic phase contained 1.8 M benzaldehyde; aqueous phase contained 1.43 M pyruvate, 7.3 U/ml PDC, 2 M MOPS, 1 mM TPP, and 1 mM Mg^{2+}at pH 7.0, 4 °C, 72 hours).

Solvent	Yield $_{(R\text{-PAC/benz})}$ (%)	Yield $_{(R\text{-PAC/Pyr})}$ (%)
1) Butanol	20	3
2) Pentanol	52	24
3) Octanol	85	55
4) Nonanol	84	52
5) Hexane	52	29
6) Heptane	49	24
7) Octane	63	16
8) Nonane	72	42
9) Dodecane	76	24
10) MCH*	64	38
11) MTBE**	48	9
12) Toluene	66	11
13) Benzaldehyde	25	12

* MCH = methylcyclohexane; ** MTBE = methyl-tert-butylether

Note: Yields are based on mole of *R*-PAC formed per mole of benzaldehyde or pyruvate utilized.

Table 5.3: Residual substrate concentrations and final product concentrations in the two-phase system solvent screen (organic phase contained 1.8 M benzaldehyde; aqueous phase contained 1.43 M pyruvate, 7.3 U/ml PDC, 2 M MOPS, 1 mM TPP, and 1 mM Mg^{2+}at pH 7.0, 4 °C, 72 hours).

Solvent	Organic phase (mM)		Aqueous phase (mM)		
	Benz.	*R*-PAC	Pyruvate	Benz.	*R*-PAC
1) Butanol	1730	3	1160	35	4
2) Pentanol	1680	26	1300	60	5
3) Octanol	840	670	0	40	113
4) Nonanol	910	605	5	20	130
5) Hexane	1480	17	1040	105	95
6) Heptane	1430	22	890	100	110
7) Octane	1490	12	630	110	113
8) Nonane	1470	32	1010	85	144
9) Dodecane	1530	17	930	110	104
10) MCH*	1330	79	830	110	150
11) MTBE**	1570	36	590	65	43
12) Toluene	1530	37	615	130	56
13) Benzaldehyde	1550	18	1260	165	3

* MCH = methylcyclohexane; ** MTBE = methyl-tert-butylether

5.4 Discussion and Conclusions

In this Chapter a two-phase system for enhanced *R*-PAC production has been investigated with the aim of minimizing irreversible inactivation of pyruvate decarboxylase due to benzaldehyde, *R*-PAC and acetoin. This investigation involved screening for a suitable solvent that improved *R*-PAC formation and minimized enzyme inactivation.

In Chapter 5 the stability of semi-purified pyruvate decarboxylase from *Candida utilis* was determined in a two-phase system with a range of hydrophobic organic solvents exhibiting varying chemical structures and properties. The enzyme exhibited enhanced stability to methyl-tert-butyl-ether (MTBE) in comparison to a control exposed to no solvent. MTBE may interact favorably with the active center or on the surface of the enzyme to improve stability. Hydrophobic interaction by solvents such as MTBE can reduce the amount of bound water molecules on the enzyme. A loss of bound water can cause the protein to fold to a more stable configuration or it may also reduce water dependent reactions such as proteolysis (Ó Fágáin 1997). Hydrogen bonding could not be responsible for improvement in stability since MTBE has no polarized hydrogen bonds necessary for hydrogen bond formation (Brown 1982). Pyruvate decarboxylase exhibited good stability to solvents other than MTBE, however the enzyme inactivated more than the control in each case.

The log P of the solvents tested could not be correlated to the stability of pyruvate decarboxylase in a two-phase system. There is only a general trend of increasing stability with log P for the long chain alcohols evaluated. Several reports indicate that log P can be used to predict enzyme stability towards solvents in two phase biocatalysis and systems where the reaction buffer is saturated with solvent (Lanne *et al.* 1987; Bruce and Daugulis 1991; Gupta *et al.* 1997). As confirmed in this study of pyruvate decarboxylase, the above method of prediction however, is not suitable for all enzyme systems where stability and activity strongly depend on the solvent rather than the log P (Bauer *et al.* 1999).

R-PAC was produced in a two-phase system with all solvents tested. Large increases in *R*-PAC production were achieved using octanol or nonanol as organic phase solvents. With octanol, 670 mM (100.6 g/l) *R*-PAC was achieved in the organic phase, with a further 113 mM (17 g/l) in the aqueous phase. With nonanol, 605 mM (90.8 g/l) *R*-PAC was achieved in the organic phase with an additional 130 mM (19.5 g/l) *R*-PAC in the aqueous phase. These final concentrations are much higher than the 300 mM (45 g/l) *R*-PAC produced in the benzaldehyde emulsion batch reaction (Chapter 4).

Specific *R*-PAC production has been increased from 6.4 mg *R*-PAC/U with the benzaldehyde emulsion batch reactor to 16.1 mg *R*-PAC/U and 15.1 mg *R*-PAC/U for octanol and nonanol respectively in the two-phase system. Significantly more *R*-PAC was produced per unit of semi-purified enzyme for the two-phase system.

The molar yields of *R*-PAC on pyruvate consumed for octanol and nonanol were 55 % and 52 % respectively, with lower yields reported for the majority of other solvents evaluated. These low yields suggest the formation of by-products acetoin and acetaldehyde which may be influenced by the high initial pyruvate concentrations that were used. Pyruvate that was degraded, possibly by dimerization, was included in the yield calculation and may be an additional factor contributing to low yields. By-product formation will be further investigated in Chapter 6. The molar yields of *R*-PAC on benzaldehyde consumed for octanol and nonanol were 85 % and 84 % respectively.

R-PAC production using other solvents was achievable, but offered no advantage over the benzaldehyde emulsion system, producing less overall *R*-PAC per unit of enzyme.

Low final *R*-PAC concentrations achieved with all solvents other than octanol and nonanol may be due to inhibition of the biotransformation by the solvent. While some solvents affected enzyme stability only moderately, under reaction conditions they may induce a conformational change or prevent access of pyruvate or benzaldehyde to the enzyme active site resulting in low levels of decarboxylation and/or carboligation. Alternatively the presence of substrates may cause a conformation change that could alter the enzymes susceptibility to the solvent. There was no correlation between enzyme stability towards solvents and *R*-PAC formation, which could also be due to a change in mechanism of enzyme inactivation due to increased stirring. Under reaction

conditions the system was rapidly stirred, which may increase contact of the enzyme with the solvent at the interface, resulting in increased enzyme inactivation and therefore lower final *R*-PAC concentrations.

An alternative explanation for the lack of correlation between enzyme stability and *R*-PAC formation, may be that for stability determination, the enzyme from the aqueous phase was diluted before being assayed. This dilution may have lowered the solvent concentration to a level in the carboligase assay where it did not inhibit *R*-PAC formation. This would be the case for solvents which caused reversible inhibition of *R*-PAC formation and the effect therefore reduced or eliminated by dilution.

Good solvent characteristics regarding partitioning and enzyme stability do not necessarily guarantee high *R*-PAC production. A solvent such as pentanol, did not inactivate the enzyme significantly, caused preferential partitioning of *R*-PAC away from the enzyme into the organic phase and allowed suitable delivery of benzaldehyde to the enzyme in the aqueous phase. However, despite these favorable characteristics, very little *R*-PAC was produced, reaching only 0.64 mg *R*-PAC/U. Solvents such as pentanol may significantly and reversibly inhibit enzyme activity.

In Chapter 4 it was shown that *R*-PAC irreversibly inactivated pyruvate decarboxylase and therefore continuous extraction of *R*-PAC would be beneficial for enzyme stability. Besides its partitioning characteristics, the chemical nature of the solvent plays a role in the distribution of *R*-PAC between organic and aqueous phases. Hexane with a log P of 3.5 distributes *R*-PAC preferentially to the aqueous phase, while nonanol with a log P of 3.4 distributes *R*-PAC, which is also an alcohol strongly into the organic phase. It is unlikely that such a small difference in log P could be responsible for such a dramatic difference in distribution. Alcohols may be also more suitable solvents as they facilitate extraction of *R*-PAC from the aqueous phase into the organic phase after production.

The production of *R*-PAC in a two-phase system using partially purified enzyme is an improved process when compared to immobilized whole cell two-phase *R*-PAC production previously evaluated by Nikolova and Ward (1992a,c). They reported a maximum final *R*-PAC concentration of (0.45 g/l) from 50 g/l pyruvate and 6 g/l benzaldehyde in 6 hours with hexane as organic solvent and 10 % (v/v) moisture.

In their experiments *R*-PAC formation was limited by solvent induced cell membrane damage and loss of substrate benzaldehyde to byproduct benzylalcohol by the action of alcohol dehydrogenase. Long chain alcohols were not evaluated in the whole cell study. Smallridge *et al.* (2001b) also produced less than 1 g/l *R*-PAC in a two-phase system utilizing non-fermenting cells of *Saccharomyces cerevisiae*, an aqueous phase consisting of 0.6-1.2 ml buffer per gram of yeast and a petroleum spirit organic solvent phase.

High concentrations of *R*-PAC were specifically achieved with the alcohols octanol and nonanol. It has been reported that solvent soluble in an aqueous phase can impact on enzyme stability in a concentration dependent manner. Enzymes stability or activity may increase until a particular concentration is reached, above which enzyme inactivation begins (Khmelinitsky *et al.* 1991b; Gupta *et al.* 1997). For nonanol and octanol, the partitioning coefficients may be sufficiently high to restrict aqueous phase concentrations of the solvent to a range that is beneficial for enzyme stability or activity in the presence of substrates. Solvents such as pentanol that partition more strongly into the aqueous phase, may expose the enzyme to concentrations of solvent much higher than the ideal concentration, thereby limiting *R*-PAC formation. It may be the case with these alcohols that small concentrations soluble in the aqueous phase may form hydrogen bonds or hydrophobically interact with the enzyme, improving stability and increasing reaction rates. It has been reported by Gupta *et al.* (1997) that water miscible alcohols are often compatible with enzymes. The alcohols form a hydrogen bond with the enzyme, which substitutes for bound water molecules limiting enzyme damage. Alcohols may also improve access of benzaldehyde to the active center. The role of ethanol in enhancing the activity of pyruvate decarboxylase has been also reported by Shin (1994) who reported improvements in both initial rates of *R*-PAC formation and enzyme stability in the presence of up to 3 M ethanol.

In summary, the screening of 13 solvents in a two-phase system identified octanol and nonanol as the most suitable organic phase solvents for *R*-PAC production from benzaldehyde and pyruvate using partially purified PDC. Increases in the final concentration and amount of *R*-PAC per unit of enzyme indicate the potential for a two-phase system mode of production. Further studies reported in Chapter 6 will evaluate the potential of a two-phase system based on octanol. Octanol was selected over

nonanol due to the higher final concentration of *R*-PAC achieved and the higher molar yield of *R*-PAC on pyruvate consumed in comparison to nonanol.

CHAPTER 6

EVALUATION OF *R*-PAC PRODUCTION BY PARTIALLY PURIFIED PDC IN THE TWO-PHASE SYSTEM UTILIZING OCTANOL

6.1 Introduction

A screen for a suitable solvent as the organic phase of a two-phase system identified octanol as the most promising solvent. High final concentrations of *R*-PAC were obtained in comparison to other solvents.

Detailed investigation of *R*-PAC production in the octanol two-phase system is reported in this Chapter with the following aims:

- to investigate the extractive properties of octanol which may contribute to high *R*-PAC production. The partition coefficients for all substrates, product *R*-PAC and by-products will be determined.

- to characterize enzyme inactivation due to benzaldehyde in the presence of octanol, for comparison with the benzaldehyde emulsion batch reaction detailed in Chapter 4.

- to evaluate the effect of substrate concentration, enzyme concentration and degree of agitation on enzyme inactivation and *R*-PAC formation.

- to assess the scalability of *R*-PAC production in the two-phase system.

6.2 Product and Reactant Partitioning

The partition coefficients for substrates and products were determined in the two-phase system with octanol as the organic phase solvent and a MOPS based buffer as the aqueous phase.

Octanol containing *R*-PAC, benzaldehyde, acetoin or acetaldehyde was added to an equal volume of MOPS buffer (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C). The octanol phase was layered above the aqueous phase, maintaining phase separation by slow stirring of the lower aqueous phase. Each flask was equilibrated for 24 hours at 4 °C before sampling from both phases to determine component concentrations. Detailed methodology is described in Section 2.7.3.

Several concentrations of each component were evaluated and an average value of log P was calculated. Table 6.1 shows the concentrations in organic and aqueous phases for all components, and the resultant calculated log P values. Benzaldehyde partitioned strongly into the octanol phase with a log P of 1.4. Acetaldehyde and *R*-PAC also partitioned preferentially into the octanol phase, showing log P values of 0.7 and 0.6 respectively. Acetoin partitioned preferentially into the aqueous phase as indicated by a log P value of – 0.5. Sodium pyruvate was not soluble in octanol, exhibiting a total preference for the aqueous phase.

Table 6.1: Partition coefficients for each reaction component in an octanol/MOPS two-phase system

Component	Concentration in octanol phase $[x]_{octanol}$ (mM)	Concentration in aqueous phase $[x]_{aqueous}$ (mM)	Log P = $Log_{10}([x]_{octanol}/[x]_{aqueous})$
Benzaldehyde	188.0	10.2	1.3
	511.1	21.3	1.4
	1008.7	35.4	1.5
	1738.1	50.3	1.5 **Average: 1.4**
Acetaldehyde	9.8	1.9	0.7
	18.4	4.7	0.6
	38.6	8.7	0.7
	70.2	19.9	0.6 **Average: 0.7**
R-PAC	28.3	7.9	0.6
	63.7	15.3	0.6
	107.7	32.0	0.5
	818.3	133.2	0.8 **Average: 0.6**
Acetoin	2.0	6.9	-0.5
	6.0	17.8	-0.5
	9.2	30.7	-0.5
	16.9	60.4	-0.6 **Average: -0.5**
Pyruvate (sodium salt)	Not soluble	Fully soluble	-

6.3 Enzyme Inactivation due to Benzaldehyde

Enzyme inactivation due to the toxic substrate benzaldehyde was characterized with octanol as organic phase solvent. The enzyme was exposed to various concentrations of benzaldehyde in octanol at 7 U/ml in MOPS buffer (2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5 at 4 °C). The ratio of organic phase to aqueous phase was 1:1. The two phases were stirred forming an emulsion. The large rate of pyruvate decarboxylation

observed in the screen with octanol as organic phase solvent, indicates that the pH is expected to rise significantly due to consumed protons. The buffer pH was therefore reduced from 7 to 6.5, and the MOPS buffer concentration was increased from 2 M to saturation point (2.5 M) to improve buffering of the reaction. The remaining carboligase activity was determined in the aqueous phase after three hours. Enzyme batch #2 was used for experiments in Chapter 6, except for scale-up of the two-phase reactor in Sections 6.5.3 and 6.6.2, where enzyme batch #4 was used. Two controls were included:

a) enzyme exposed to octanol only and
b) enzyme stirred alone without octanol or benzaldehyde.

Detailed methodology is described in Section 2.7.4.

Figure 6.1 shows that the enzyme in the aqueous phase could withstand benzaldehyde levels up to 1800 mM in the organic phase. The error bars represent the highest and lowest values for two vials evaluated for each benzaldehyde concentration. The remaining activity was significantly higher than if the enzyme was exposed to benzaldehyde alone. For example, in the benzaldehyde emulsion system with no second phase, the enzyme was completely inactivated by 100 mM benzaldehyde within 3 hours with variable inactivation rates (see Chapter 4). Delivering benzaldehyde in octanol has reduced the toxic effect of benzaldehyde on the enzyme. The control exposed to octanol retained 98.7 % of its initial activity. The enzyme exposed to no benzaldehyde or octanol retained 95 % of its initial activity. The enzyme with 300 mM benzaldehyde in octanol retained 88.7 % of its initial activity, while at 1800 mM benzaldehyde it retained 65.8 %.

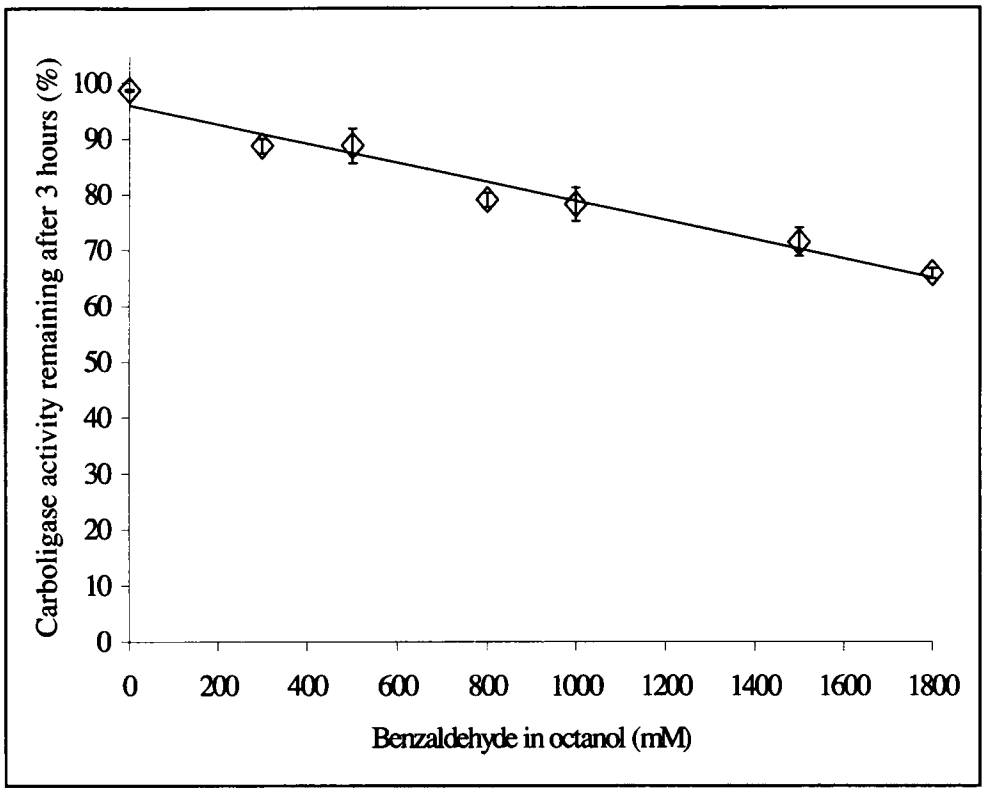


Figure 6.1: Carboligase activity remaining after 3 hours exposure to benzaldehyde in octanol (7 U/ml, 4 °C, organic octanol phase containing benzaldehyde and the aqueous phase containing 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5). The error bars represent the highest and lowest values for two vials evaluated for each benzaldehyde concentration.

6.4 *R*-PAC Formation in MOPS Buffer Saturated with Octanol

In aqueous-organic two-phase systems a portion of the organic phase is sparingly soluble in the aqueous phase. Low concentrations of solvent in the aqueous phase in contact with the enzyme can influence both stability and activity (Halling 1994). In this Section, *R*-PAC production and enzyme stability under reaction conditions were evaluated in an octanol saturated aqueous MOPS buffer with no second octanol phase present.

A MOPS buffer (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C) saturated with octanol was prepared as described in Section 2.7.5. The production of *R*-PAC from

50 mM soluble benzaldehyde, 75 mM pyruvate and 7 U/ml carboligase activity in this octanol saturated buffer is shown in Figure 6.2. A control was included where the reaction was evaluated without octanol present in the buffer. Enzyme activity was measured after 22 hours with gel filtration to remove substrates and products.

Figure 6.2 and Table 6.2 show that the initial rate of *R*-PAC production and the final concentration in buffer saturated with octanol exceeded that of a control without octanol. The initial reaction rate with octanol present was 46 % greater than without octanol. The yield of *R*-PAC on benzaldehyde consumed for the reaction in octanol saturated buffer was 67.2 %, and greater than 61.9 % in the control. Higher levels of pyruvate were converted to acetoin in the control, with a yield of 38.9 % in comparison to 30.4 % in the reaction saturated with octanol. Acetaldehyde concentrations after 22 hours were low in both reactions. No activity was lost after 22 hours in both reactions and all of the supplied pyruvate was consumed.

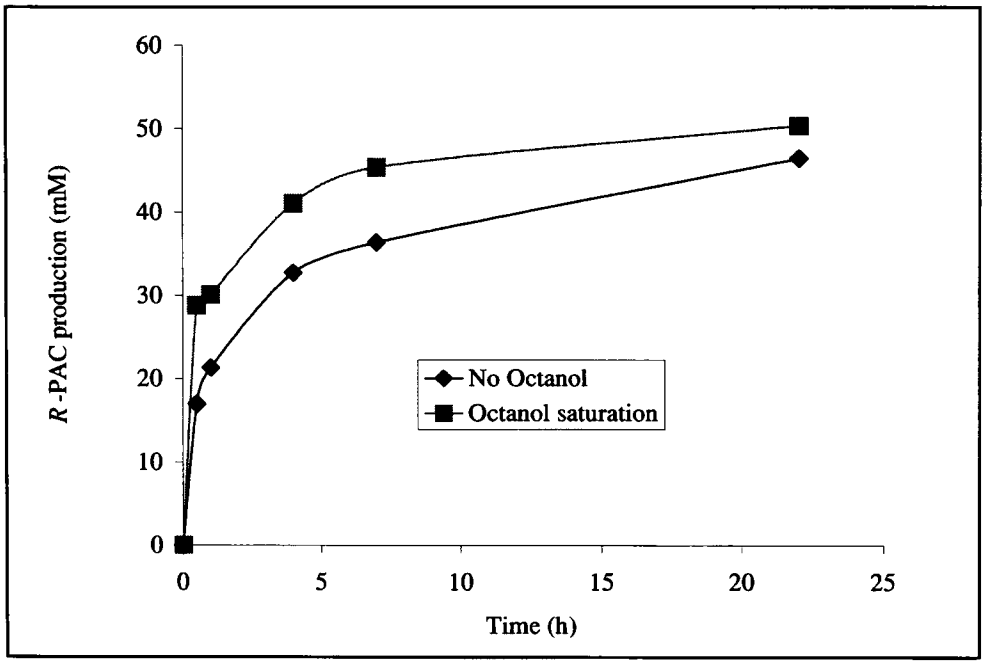


Figure 6.2: Effect of octanol saturation on *R*-PAC production (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, 7 U/ml, 50 mM soluble benzaldehyde, 75 mM pyruvate).

Table 6.2: Effect of octanol saturation on yields of products and by-products (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 7.0 at 4 °C, 7 U/ml, 50 mM soluble benzaldehyde, 75 mM pyruvate, 22 hours)

	No octanol	Octanol saturation
Initial reaction rate (mM *R*-PAC/h)*	33.9	49.6
R-PAC after 22 hours (g/l)	46.4	50.4
R-PAC after 22 hours (mM)	309.3	336
Final benzaldehyde (mM)	2.8	0.1
Final pyruvate (mM)	0	0
Final acetoin (mM)	14.6	11.4
Final acetaldehyde (mM)	<1	<1
Yield $_{(R\text{-PAC/benzaldehyde})}$ %	98.3	100.0
Yield $_{(R\text{-PAC/pyruvate})}$ %	61.9	67.2
Yield $_{(\text{acetoin/pyruvate})}$ %	38.9	30.4
Yield $_{(\text{acetaldehyde/pyruvate})}$ %	<1.3	<1.3

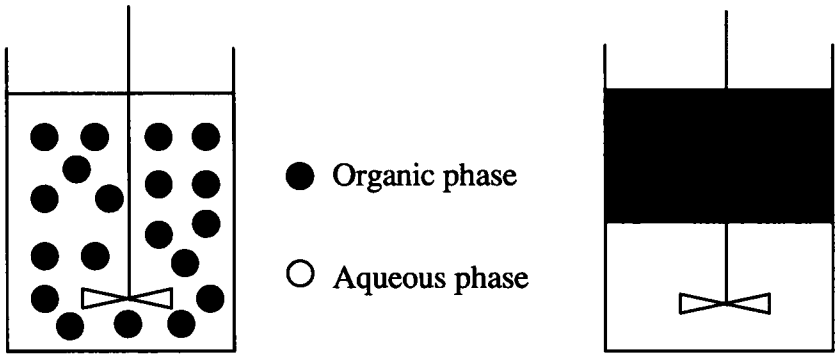
* Initial reaction rate was calculated from the 30 minute timepoint.

Note: Yields are based on moles of product formed per mole of substrate utilized.

6.5 *R*-PAC Production in the Rapidly Stirred Emulsion Two-Phase Reaction System

R-PAC production in the two-phase system was evaluated with two modes of production:

1. Rapidly stirred emulsion.
2. Slowly stirred system with phase separation maintained.



This section evaluates the rapidly stirred emulsion system for *R*-PAC production.

6.5.1 Effect of Benzaldehyde Concentration

The influence of benzaldehyde concentration on *R*-PAC production was investigated to determine if there is optimum balance between enzyme inactivation and *R*-PAC formation.

Various concentrations of benzaldehyde in octanol were layered above an equal volume of MOPS buffer (2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5 at 4 °C) containing 7 U/ml carboligase activity and 1.43 M pyruvate. After 40 hours a sample was taken from the organic and aqueous phases for analysis of pyruvate, benzaldehyde and *R*-PAC. Detailed methodology is described in Section 2.7.6.1.

R-PAC formation as a function of benzaldehyde concentration in the organic phase is shown in Figure 6.3. The maximum amount of *R*-PAC produced was 730 mM (110 g/l) in the organic phase and 70 mM (10.5 g/l) in the aqueous phase. This level of *R*-PAC was achieved with an initial concentration of 1500 mM benzaldehyde. Pyruvate was not limiting in any of the reactions, however the level of benzaldehyde remaining after 40 hours in the flasks containing initial benzaldehyde concentrations of 500 mM and 800 mM indicates that there was insufficient benzaldehyde at the initial concentrations to further increase *R*-PAC levels (Table 6.3). The relatively low molar yield of *R*-PAC on pyruvate utilized with an initial benzaldehyde concentration of 500 mM indicates insufficient benzaldehyde delivery to the aqueous phase with possible conversion of pyruvate to by-products acetoin or acetaldehyde. The maximum amount of product achieved per unit of carboligase activity was 18.2 mg *R*-PAC/U, achieved with an initial benzaldehyde concentration of 1500 mM in the octanol phase (Table 6.3).

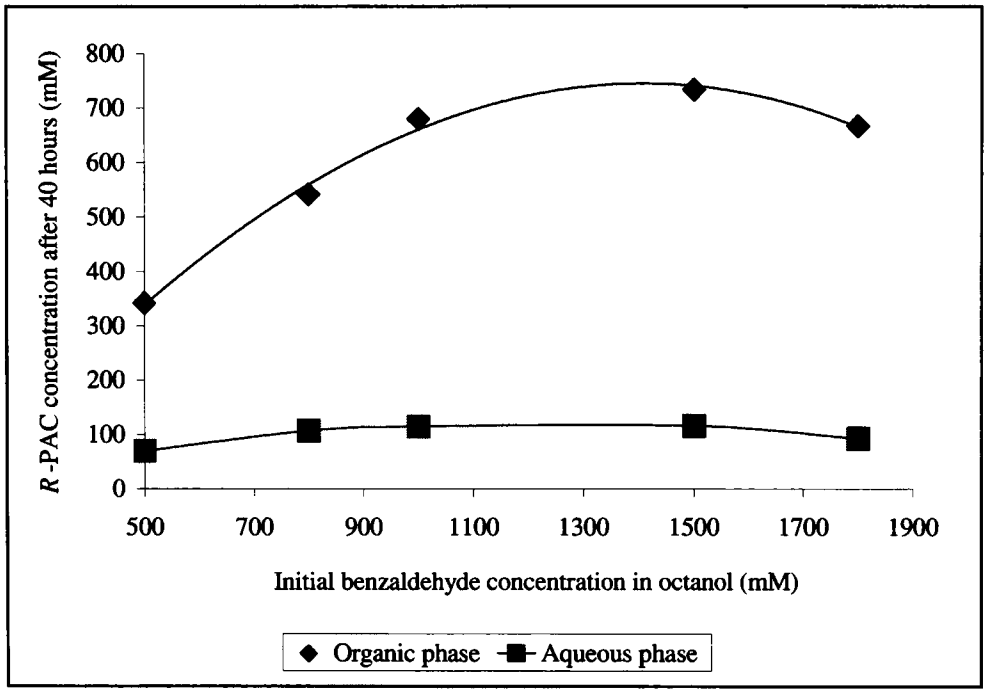


Figure 6.3: Amount of *R*-PAC produced as a function of initial benzaldehyde concentration in the rapidly stirred emulsion two-phase system (4 °C, 40 hours, organic octanol phase contained benzaldehyde at various concentrations, and the aqueous phase contained 7 U/ml carboligase activity, 1.43 M pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

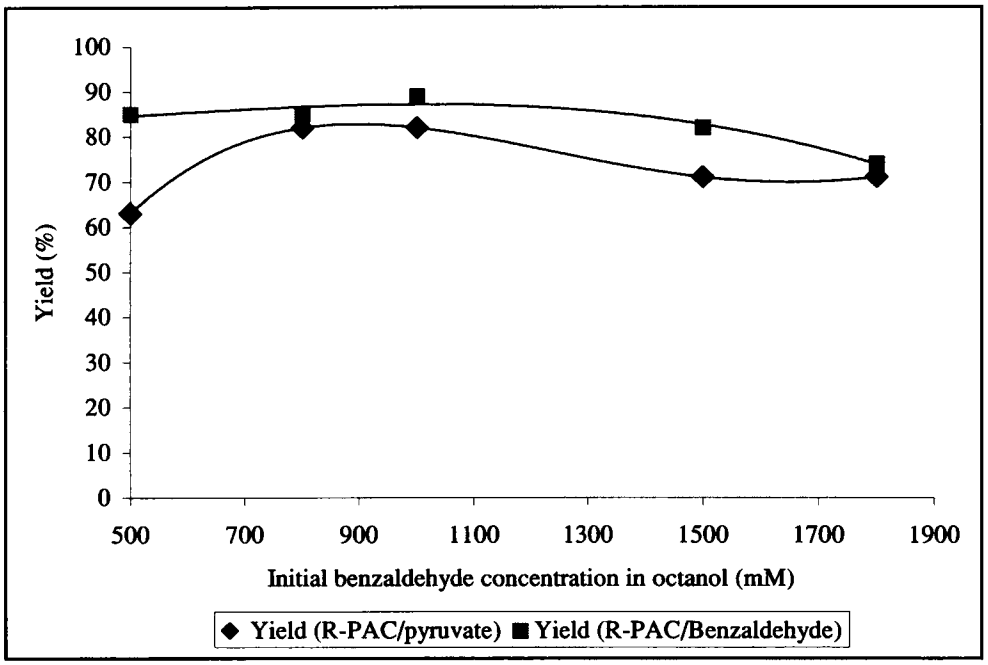


Figure 6.4: Molar yields of *R*-PAC on pyruvate and benzaldehyde utilized in the rapidly stirred emulsion two-phase system (4 °C, 40 hours, organic octanol phase contained benzaldehyde at various concentrations, and the aqueous phase contained 7 U/ml carboligase activity, 1.43 M pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

Table 6.3: Specific *R*-PAC production and remaining substrates in the rapidly stirred emulsion two-phase system with various initial benzaldehyde levels (4 °C, 40 hours, organic octanol phase contained benzaldehyde at various concentrations, and the aqueous phase contained 7 U/ml carboligase activity, 1.43 M pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

Initial benzaldehyde	500 (mM)	800 (mM)	1000 (mM)	1500 (mM)	1800 (mM)
Residual benzaldehyde (mM)					
Organic phase	20	40	112	525	944
Aqueous phase	1	4	13	16	29
Residual pyruvate (mM)	720	585	410	200	330
Specific *R*-PAC production (mg *R*-PAC/U)	8.8	13.9	17.0	18.2	16.2

6.5.2 Effect of Enzyme Concentration

The enzyme activity level was varied in the rapidly stirred emulsion two-phase system to determine if specific production (mg *R*-PAC/U) and yield of *R*-PAC on pyruvate utilized could be increased. Initial substrate levels were 1500 mM benzaldehyde and 1450 mM pyruvate. Detailed methodology is described in Section 2.7.6.2.

R-PAC produced after 40 hours for each carboligase activity evaluated is shown in Figure 6.5. There was a trend of increasing *R*-PAC production with increasing enzyme activity. The maximum amount of *R*-PAC obtained was 946 mM (142 g/l) in the organic phase and 127 mM (19 g/l) in the aqueous phase with 8.5 U/ml carboligase activity. 24 mM pyruvate remained at the end of the reaction and benzaldehyde was not limiting at any enzyme level (see Table 6.4). Higher initial concentrations of sodium pyruvate could not be prepared with batch operation due to its limited solubility. Production at higher PDC activities was not examined, as the biotransformation would have been limited by pyruvate exhaustion.

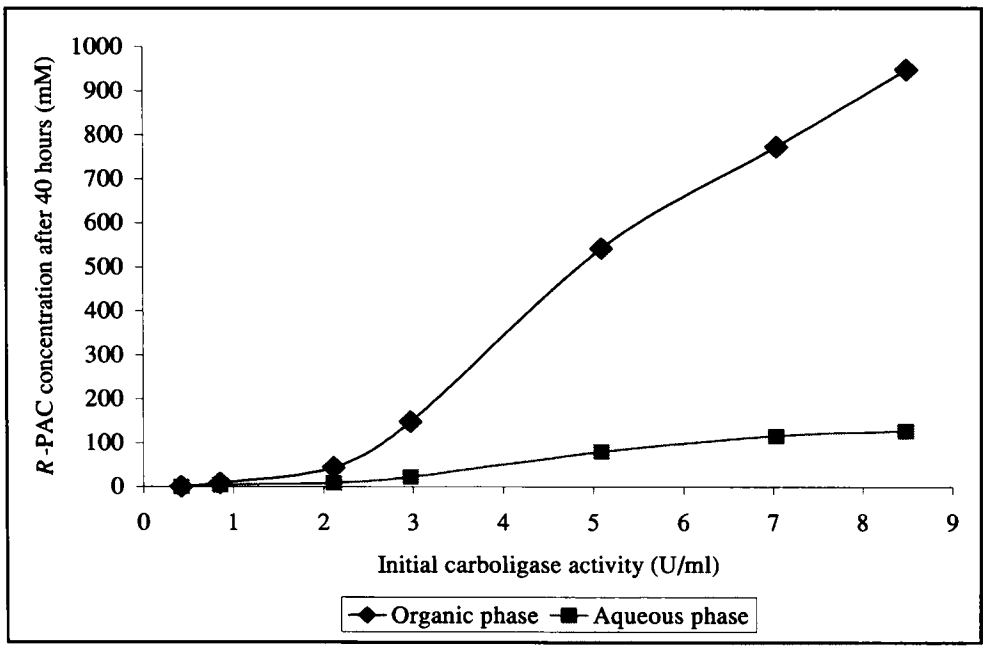


Figure 6.5: *R*-PAC production as a function of carboligase activity in the rapidly stirred emulsion two-phase system (4 °C, 40 hours, organic octanol phase contained 1500 mM benzaldehyde and the aqueous phase containined1430 mM pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

The yield of *R*-PAC on pyruvate utilized increased with enzyme concentration and reached a plateau between 72-75 % with 5 U/ml PDC. The yield of *R*-PAC on benzaldehyde utilized also increased with enzyme concentration and reached a plateau between 91-95 % with 3 U/ml PDC (see Table 6.4). A 57mM loss of benzaldehyde and 393 mM pyruvate occurred in the control with no enzyme present, indicating that substrate loss occurs independently of the biotransformation. The yield calculation was based on the total amount of substrate consumed including losses. At low enzyme levels, with a small amount of *R*-PAC produced and a small amount of substrate consumed, these losses have a large influence on the yield. At enzyme levels less than 3 U/ml, the yields were therefore compromised significantly.

Table 6.4: Substrate and product concentrations, and yields in the rapidly stirred two-phase system as a function of initial carboligase activity (4 °C, 40 hours, organic octanol phase contained 1500 mM benzaldehyde and the aqueous phase contained 1430 mM pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

Initial carboligase activity	0.4 (U/ml)	0.9 (U/ml)	2.1 (U/ml)	3.0 (U/ml)	5.1 (U/ml)	7.0 (U/ml)	8.5 (U/ml)
R-PAC after 40 hours							
Organic phase (g/l)	0	1.2	6.6	22.1	81.3	115.8	142.1
(mM)	0	8	44	147.3	542	772	947.3
Aqueous phase (g/l)	0	0.5	1.3	3.3	11.9	17.4	19
(mM)	0	3.3	8.7	22	79.3	116	126.7
Specific *R*-PAC production (mg *R*-PAC/U)	0	2.0	3.7	8.6	18.3	18.9	19.0
Residual benzaldehyde							
Organic phase (mM)	1245	1316	1302	1271	823	466	312
Aqueous phase (mM)	47	42	40	38	22	11	7
Residual pyruvate (mM)	1344	1326	1252	1132	588	224	24
Yield $_{(R\text{-PAC/pyruvate})}$ %	0	9.1	26.6	53.2	72.1	72.4	75.3
Yield $_{(R\text{-PAC/benzaldehyde})}$ %	0	8.0	33.3	88.6	94.9	86.8	90.9
Yield $_{(\text{acetoin/pyruvate})}$ %	3.5	19.0	10.2	6.7	0.93	1.3	1.5

Specific production of *R*-PAC (mg *R*-PAC/U) increased with enzyme activity and reached a plateau at 5 U/ml with 19 mg *R*-PAC/U as shown in Figure 6.6. In conclusion it was found that increasing the enzyme activity level thereby maintaining high yields and high specific production made an improvement in the final concentration of *R*-PAC.

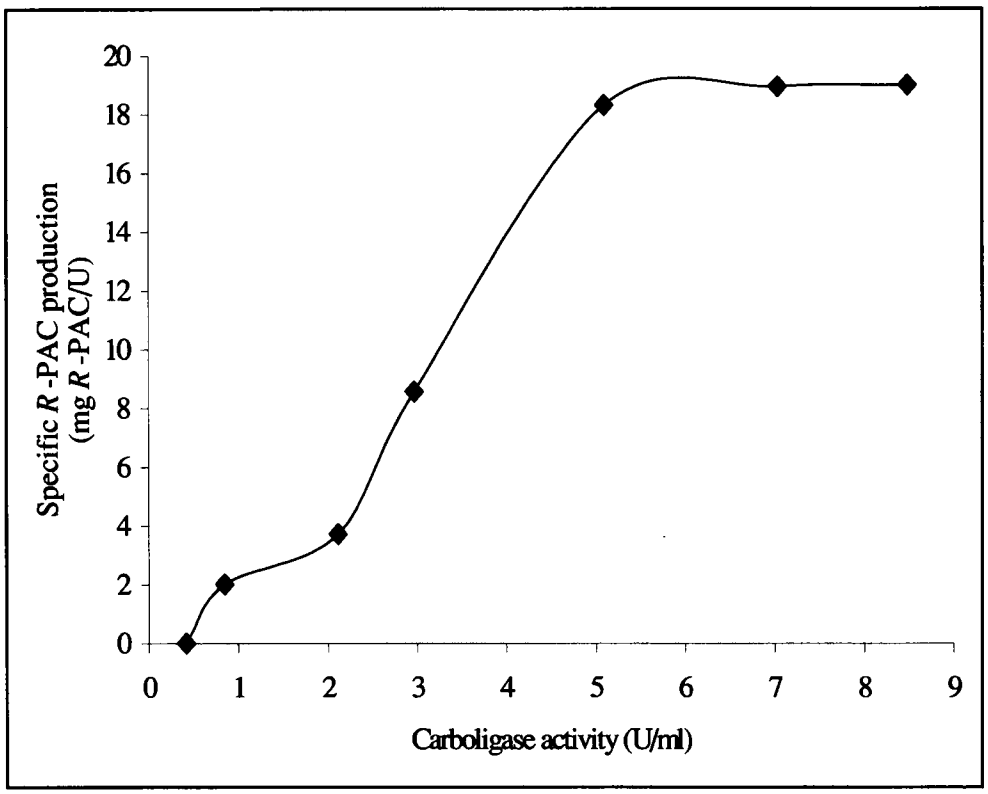


Figure 6.6: Specific production of *R*-PAC as a function of activity level in the rapidly stirred emulsion two-phase reaction (4 °C, 40 hours, organic octanol phase contained 1500 mM benzaldehyde and the aqueous phase contained 1430 mM pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

6.5.3 Kinetic Evaluation of *R*-PAC Formation in the Rapidly Stirred Emulsion system with Scale Up

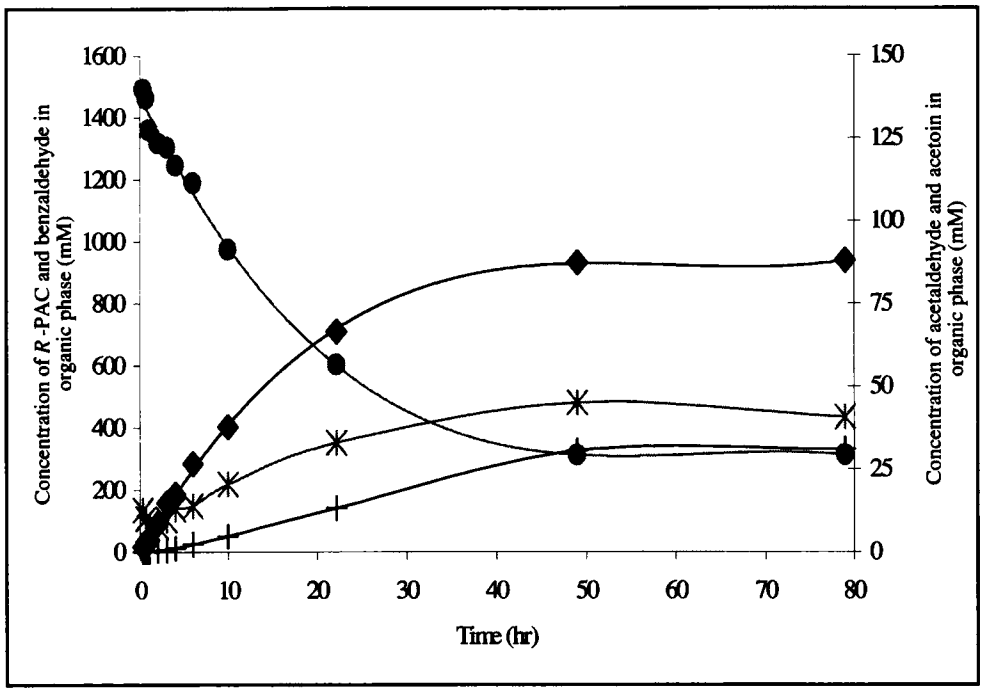
The fully stirred two-phase system was monitored over 80 hours with full profiling of substrates, products, byproducts and enzyme activity to gain an understanding of the system limitations. The total reaction volume was increased from 2.16 ml to 104 ml to assess the viability of scale-up. The reaction was performed at conditions where

maximum final concentration and specific production of *R*-PAC had been achieved previously. These conditions were 1.5 M benzaldehyde in octanol, 1.43 M pyruvate and 8.5 U/ml carboligase activity in MOPS buffer (2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5 at 4 °C). 52 ml of organic phase and 52 ml of aqueous phase were stirred rapidly to form an emulsion. Enzyme batch #4 was used for the experiment described in this Section (6.5.3). Detailed methodology is described in Section 2.7.6.3.

Samples from organic and aqueous phases were analyzed for substrates, product and by-product concentrations. Enzyme activity was monitored from the aqueous phase after gel filtration to remove interfering substances. Figure 6.7(a) profiles organic phase concentrations, and Figure 6.7(b) profiles aqueous phase concentrations of all components in the rapidly stirred two-phase system.

With scaled-up operation, 140.5 g/l (937 mM) *R*-PAC was achieved in the organic phase with an additional 18.5 g/l (123 mM) in the aqueous phase after 49 hours. The yields of *R*-PAC and by-products on substrates consumed are shown in Table 6.5. *R*-PAC production in a scaled-up reaction compared well with the small scale in terms of final *R*-PAC concentration and yields of product on substrates utilized.

(a)



(b)

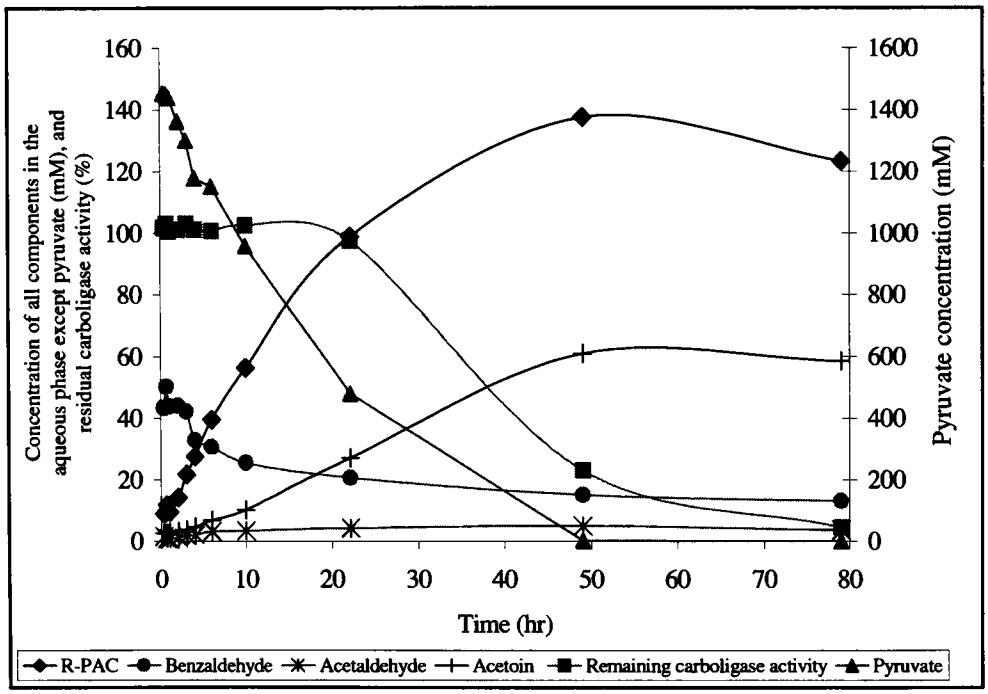


Figure 6.7 (a) and (b): Profile of the organic phase (a) and the aqueous phase (b) of the rapidly stirred emulsion two-phase system (4 °C, organic octanol phase contained 1.5 M benzaldehyde and the aqueous phase contained 8.5 U/ml carboligase activity, 1.43 M pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

By-product acetoin and acetaldehyde production increased over the course of the reaction, but the final levels in both phases were low. The yields of acetoin and acetaldehyde on pyruvate were 12.4 % and 3.1 % respectively. The total amount of pyruvate unaccounted for was 10.4 %. A loss of pyruvate occurred in a control with no enzyme present and was attributed to dimerization. The control with no enzyme present lost 206 mM benzaldehyde and 425 mM pyruvate over the 80 hours. More acetoin was formed in the larger scale system, which could be due to the characteristics of enzyme batch #4. Enzyme batch # 4 was shown in Chapter 4 (Table 4.2) to produce increased concentrations of acetoin in comparison to enzyme batch #2 which was used for the smaller scale reactions.

Table 6.5: Comparison of *R*-PAC production, yields and by-products for small and large-scale production in the rapidly stirred two-phase system (4 °C, organic octanol phase contained 1.5 M benzaldehyde and the aqueous phase contained 8.5 U/ml carboligase activity, 1.43 M pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

	Large scale 52 ml each phase	Small scale 1.08 ml each phase
R-PAC (g/l)		
Organic phase	140.5	142.0
Aqueous phase	18.5	19.0
Reaction time (h)	49	40
Specific *R*-PAC production (mg *R*-PAC/U)	18.7	19.0
Yield $_{(R\text{-PAC/benzaldehyde})}$ (%)	90.2	90.9
Yield $_{(R\text{-PAC/pyruvate})}$ (%)	73.0	75.3
Yield $_{(\text{acetaldehyde/pyruvate})}$ (%)	3.1	not determined
Yield $_{(\text{acetoin/pyruvate})}$ (%)	12.4	1.5

Pyruvate was completely consumed after 49 hours of operation. At this point, 23 % of the initial carboligase activity remained, indicating the potential for further product formation with additional pyruvate feeding. *R*-PAC formation ceased at 49 hours due to pyruvate limitation.

The enzyme did not lose any activity over the first 20 hours of operation. After 20 hours, the enzyme began to inactivate. Benzaldehyde levels in the aqueous phase were low and declined over the course of the reaction. The relatively low concentrations suggest that benzaldehyde was not responsible for the significant level of enzyme inactivation.

Acetaldehyde levels remained very low in the aqueous phase reaching a final concentration of 0.16 g/l (3.6 mM). The low level of acetaldehyde would not attribute significantly to enzyme inactivation, with only 2 % loss of activity over 15 hours at 4 °C when exposed to 30 mM acetaldehyde (Chapter 4). Acetoin levels were also relatively low in the aqueous phase reaching a maximum of 5.15 g/l (58.4 mM). Acetoin contributed to activity loss as indicated in Chapter 4.

R-PAC levels increased in the aqueous phase, reaching 18.5 g/l (123.3 mM), with 80 % of this level produced in the first 20 hours during the time when there is no enzyme inactivation. Previous studies in Section 4.2.2 show that increasing concentrations of *R*-PAC can influence enzyme inactivation, with a loss of 40 % of the activity over 7 days at 4 °C when exposed to 7.5 g/l (50 mM) *R*-PAC. The pH of the aqueous phase increased during the reaction as shown in Table 6.6. It is likely that the rise in pH contributed to enzyme inactivation after 20 hours. Other contributing factors were acetoin and *R*-PAC in the aqueous phase.

Table 6.6: pH profile of the aqueous phase during scaled up operation of the rapidly stirred emulsion two-phase system (4 °C, organic octanol phase contained 1.5 M benzaldehyde and the aqueous phase contained 8.5 U/ml carboligase activity, 1.43 M pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

Time (hour)	pH
0	6.5
1-4	7.0
4-22	7.0-7.5
22-80	7.5-8.0

6.6 *R*-PAC Production in the Phase Separated Two-Phase System

The effect of stirring rate on specific production of *R*-PAC (mg *R*-PAC/U) was determined in this section. Slowly stirred phase separated operation has been evaluated, where two distinct phases exist with a set interfacial area.

6.6.1 Effect of Enzyme Concentration

R-PAC formation in a phase separated two-phase system was evaluated at various enzyme concentrations for direct comparison with the rapidly stirred emulsion operation in Section 6.4.

The ratio of interfacial surface area to volume of organic phase was 1.05:1 (cm^2/cm^3). 1.5 M benzaldehyde in octanol was mixed with an equal volume of MOPS buffer (2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+} pH 6.5 at 4 °C) with varying levels of enzyme and 1.43 M pyruvate. The reactions were stirred slowly with phase separation maintained for the duration of the reaction. The reaction was stopped after 395 hours when available pyruvate was completely consumed. Enzyme batch # 2 was used for the experiment described in this Section (6.6.1). Detailed methodology is described in Section 2.7.7.1.

R-PAC levels achieved after 395 hours of operation are shown in Figure 6.8. *R*-PAC production peaked with 3.8 U/ml of enzyme, achieving 167.3 g/l *R*-PAC (1115 mM) in the organic phase and 27.8 g/l (185 mM) in the aqueous phase. The yield of *R*-PAC on benzaldehyde was 98 % and a yield of *R*-PAC on pyruvate was 92.9 % (Table 6.7). The yields were higher than those achieved in the rapidly stirred system (Table 6.4). A control with no enzyme present lost 55 mM pyruvate and 80 mM benzaldehyde over 395 hours. Substrate losses were lower in comparison to rapidly stirred operation and could lead to improved yields by increasing the availability of substrates for the reaction. The final pH rose to 8 in all flasks.

Residual substrate levels show that the system was limited by available pyruvate in the aqueous phase, with no pyruvate remaining in any flask. Benzaldehyde was not limiting in any flask.

Specific *R*-PAC production reached a maximum at a low enzyme level of 0.9 U/ml, with 142 mg *R*-PAC formed per unit of carboligase activity. There was a general trend of increasing specific production (mg *R*-PAC/U) with lower enzyme levels. This trend differs from the rapidly stirred emulsion two-phase system, where specific production increased with enzyme activity and at much lower levels than the phase separated system (Figure 6.9).

Specific *R*-PAC productivity (mg *R*-PAC/U/h) is compared for the two modes of operation in Figure 6.10. At low enzyme levels, phase separated operation produces more product per amount of enzyme over the same period of time. At enzyme levels higher than 2.8 U/ml a rapidly stirred system produces more product in a shorter time period. At 5 U/ml, the productivity plateaus at a maximum of 0.47 mg *R*-PAC/U/h with rapidly stirred operation.

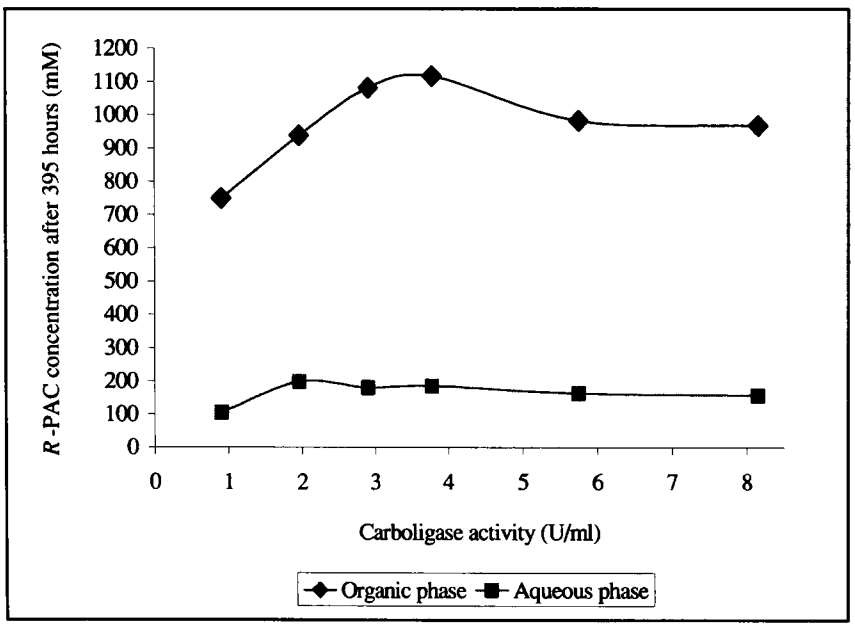


Figure 6.8: Effect of PDC activity on *R*-PAC production in the phase separated two-phase system. (4 °C, 395 hours, organic octanol phase contained 1.5 M benzaldehyde and the aqueous phase contained 1.43 M pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

Table 6.7: Yields, substrate and product concentrations in the phase separated two-phase system as a function of initial carboligase activity (4 °C, 395 hours, organic octanol phase contained 1.5 M benzaldehyde and the aqueous phase contained 1.43 M pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

Initial carboligase activity	0.9 (U/ml)	2.0 (U/ml)	2.9 (U/ml)	3.8 (U/ml)	5.8 (U/ml)	8.2 (U/ml)
R-PAC after 395 hours						
Organic phase (g/l)	112.2	140.6	162.0	167.3	147.2	145
(mM)	748	937.3	1080	1115.3	981.3	966.7
Aqueous phase (g/l)	15.6	29.6	27.0	27.8	24.3	23.5
(mM)	104	197.3	180	185.3	162	156.7
Specific *R*-PAC production (mg *R*-PAC/U)	142	85.1	65.2	51.3	29.6	20.5
Residual benzaldehyde in organic phase (mM)	606	271	151	174	277	293
Residual pyruvate (mM)	0	0	0	0	0	0
Yield $_{(R\text{-PAC/pyruvate})}$ %	60.8	81.0	87.4	92.9	81.6	80.3
Yield $_{(R\text{-PAC/benzaldehyde})}$ %	95.3	92.3	93.3	98.0	93.4	93.0
Yield $_{(\text{acetoin/pyruvate})}$ %	1.5	4.3	7.1	8.2	12.0	14.0

Note: Yields are based on moles of product formed per mole of substrate utilized.

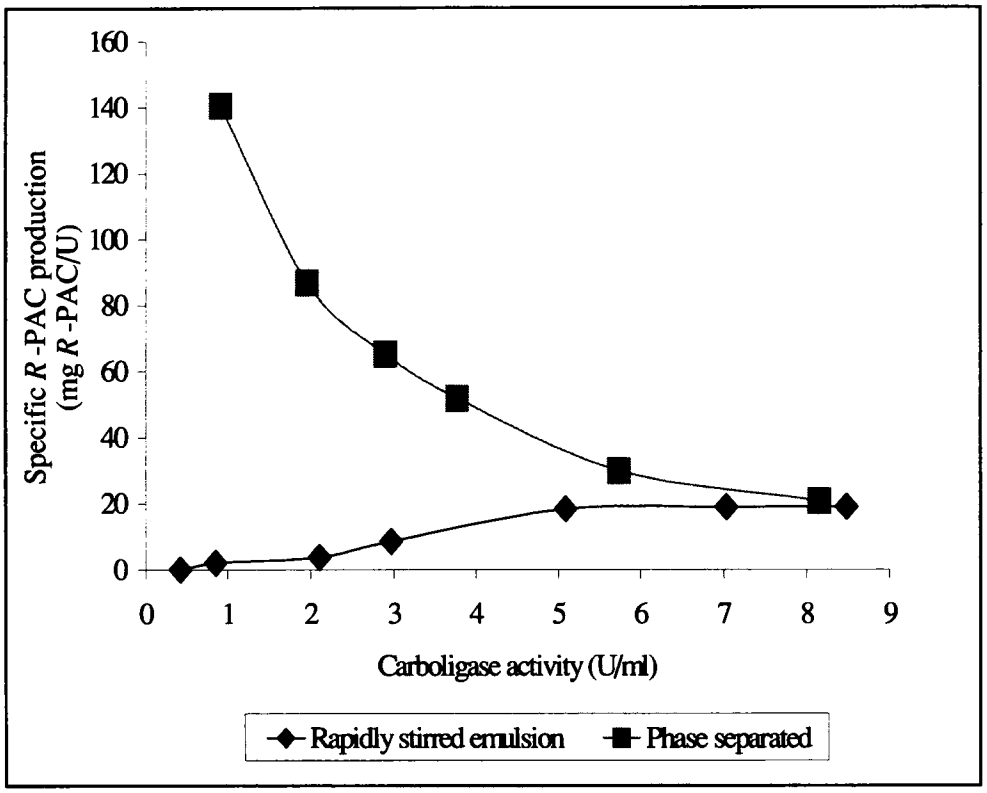


Figure 6.9: Comparison of specific *R*-PAC production for the rapidly stirred emulsion system and the phase separated system.

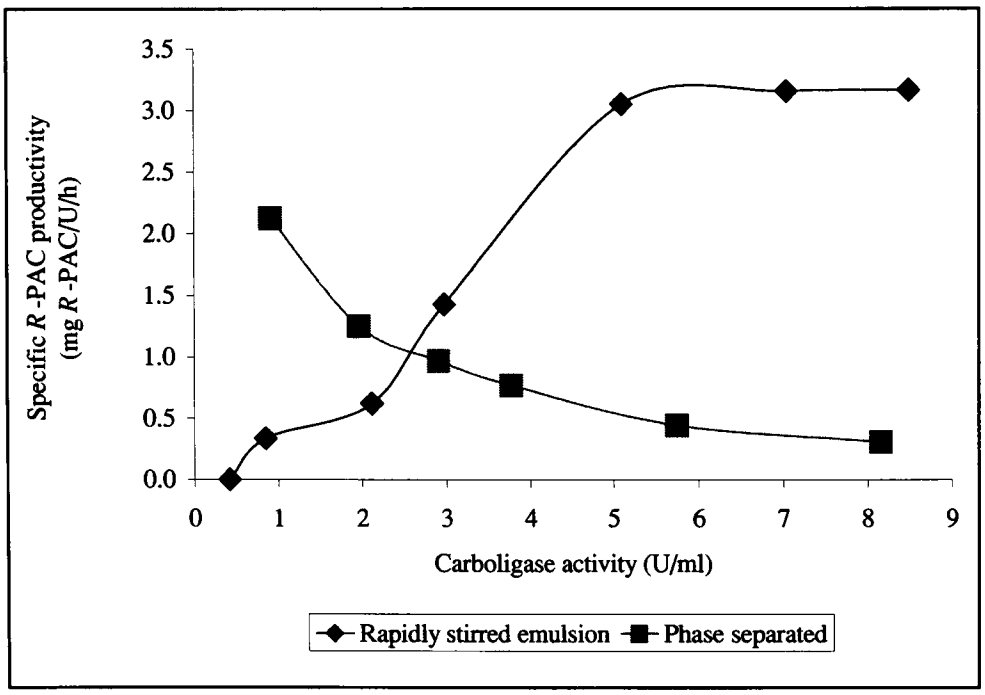


Figure 6.10: Comparison of specific *R*-PAC productivity for the rapidly stirred emulsion system and the phase separated system.

6.6.2 Kinetic Evaluation of *R*-PAC Formation in the Slowly Stirred Phase Separated System with Scale Up

The phase separated two-phase system was monitored over 395 hours with full profiling of substrates, products, by-products and enzyme stability to gain an understanding of the system limitations. A study of *R*-PAC production with varying enzyme levels established that maximum specific *R*-PAC production (mg *R*-PAC/U) occurred with 0.9 U/ml, while the maximum final *R*-PAC level occurred with 3.8 U/ml.

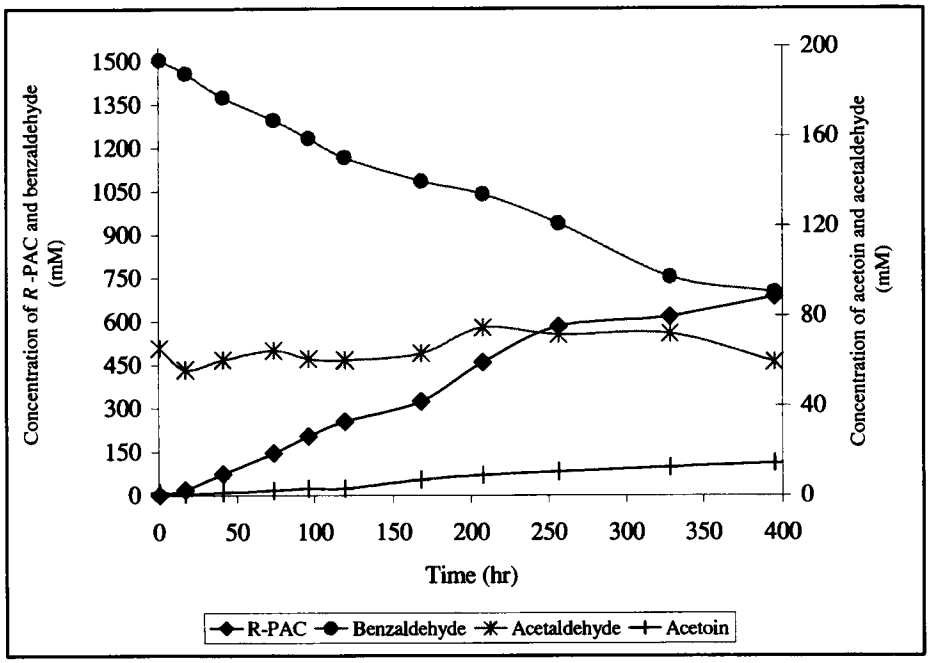
For the larger scale investigations, the following reactions were performed with 1.5 M benzaldehyde in octanol, 1.43 M pyruvate and enzyme at 0.9 U/ml and 3.8 U/ml in MOPS buffer (2 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5 at 4 °C).

Scale-up of a phase separated system should involve maintaining a fixed phase volume to interfacial surface area ratio. The partitioning rate of benzaldehyde, *R*-PAC and by-products between phases would be expected to vary with a change in this ratio. Formation of *R*-PAC and removal of *R*-PAC inhibition would be subsequently affected by this change. However maintaining this ratio would result in reactors with a phase height of only 1 cm. The system was therefore scaled according to conventional height to diameter ratios. The resulting ratio of interfacial surface area to volume of solvent was 0.47:1 (cm^2/cm^3) in comparison to 1.05:1 (cm^2/cm^3) at the small scale. The reaction volume was increased to assess the viability of scale-up. 52 ml of organic phase and 52 ml of aqueous phase were stirred slowly to maintain phase separation. Enzyme batch #4 was used in the experiment described in this Section (6.6.2). Detailed methodology is described in Section 2.7.7.2.

Samples from organic and aqueous phases were analyzed for substrates, product and by-product concentrations. Enzyme activity was monitored from the aqueous phase after gel filtration to remove interfering substances.

Figure 6.11(a) profiles organic phase concentrations, and Figure 6.11(b) profiles aqueous phase concentrations of all components in the phase separated two-phase system operated with 0.9 U/ml carboligase activity.

(a)



(b)

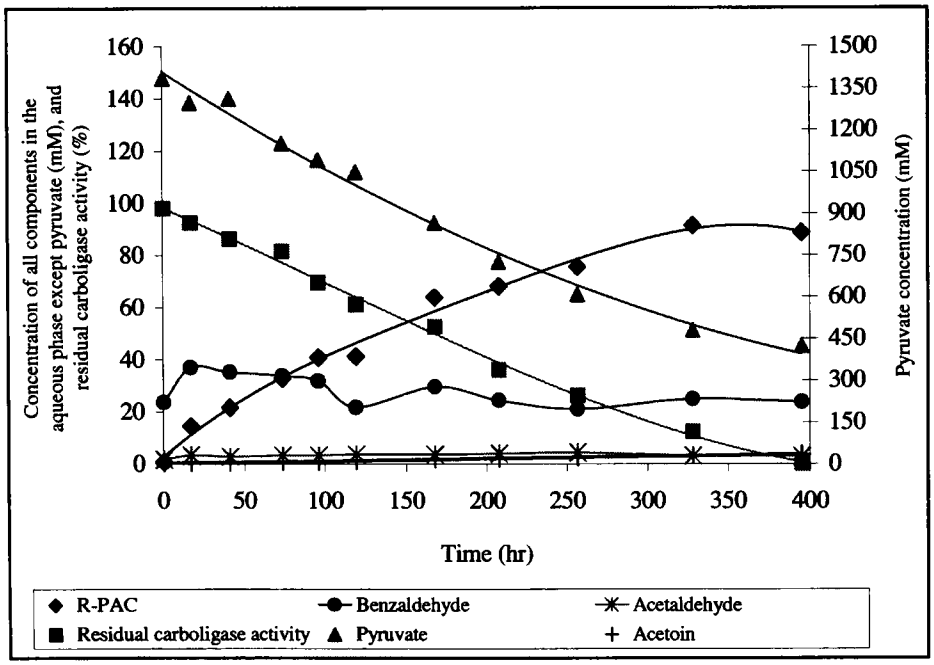


Figure 6.11(a) and (b): Profile of the organic phase (a) and the aqueous phase (b) of the phase separated two-phase system at 0.9 U/ml carboligase activity (4 °C, organic octanol phase contained 1.5 M benzaldehyde and the aqueous phase contained 0.9 U/ml carboligase activity, 1.43 M pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

At this activity level, 90.8 g/l (605.3 mM) *R*-PAC was formed in the organic phase, with an additional 13.3 g/l (88.7 mM) in the aqueous phase. Both final *R*-PAC levels and specific *R*-PAC production (mg *R*-PAC/U) were lower than those achieved at the smaller scale. The available benzaldehyde in the aqueous phase was comparable with the small scale (Table 6.8). The final pH was 7.5.

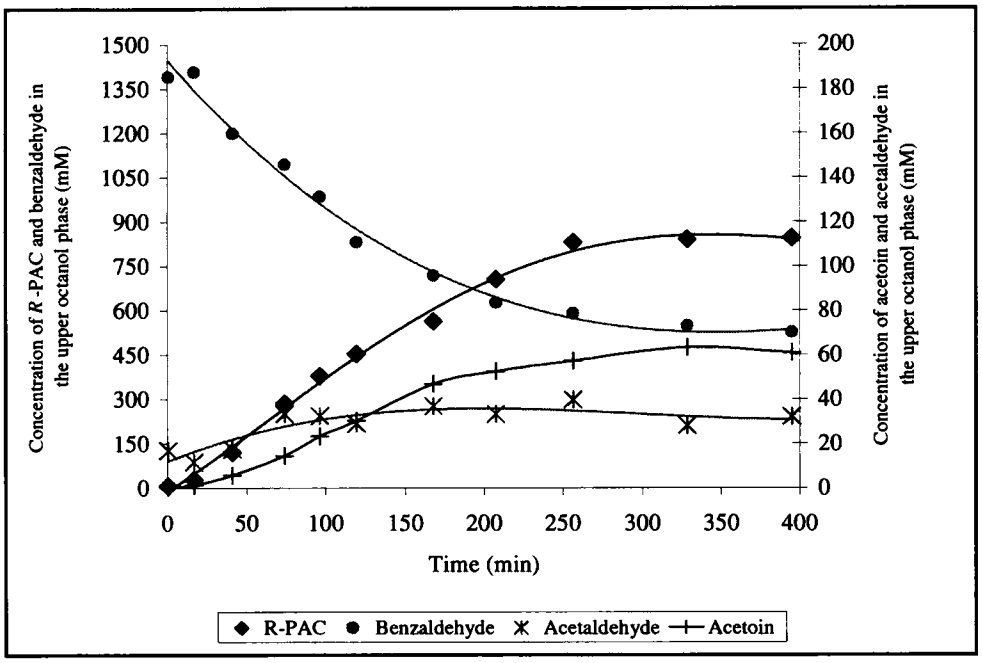
At 0.9 U/ml the amount of decarboxylated pyruvate has been reduced with large scale operation. Significantly more acetoin was produced with scale up which may have impacted on enzyme stability as shown in Chapter 4. The enzyme was completely inactivated by the end of the reaction. The yield of *R*-PAC on benzaldehyde consumed was 98.7 %, which was comparable with the small scale. The yield of *R*-PAC on pyruvate was higher than the small scale at 77.6 %. Pyruvate degradation may have been more significant at the smaller scale due to a higher contact of the aqueous phase at the benzaldehyde/octanol interface. This degradation would lead to a lower yield of *R*-PAC on pyruvate consumed.

Figure 6.12 (a) and (b) show *R*-PAC formation in phase separated conditions with an activity of 3.8 U/ml. Also for 3.8 U/ml, a lower amount of *R*-PAC was formed than at the smaller scale, with 126.0 g/l (840 mM) *R*-PAC formed in the organic phase and 18.7 g/l (124.5 mM) in the aqueous phase. Pyruvate limitation prevented higher levels of *R*-PAC formation. There was a much lower yield of *R*-PAC on pyruvate than at the lower scale. Acetoin levels were much higher at the larger scale. More acetoin was formed in the larger scale system, which could be due to the characteristics of enzyme batch #4. Enzyme batch # 4 was shown in Chapter 4 (Table 4.2) to produce increased concentrations of acetoin in comparison to enzyme batch #2 which was used for the smaller scale reactions.

The final pH was 7.7.

The initial timepoint for both 0.9 U/ml and 3.8 U/ml biotransformations were taken after 0.5 hour. The graphs show that acetaldehyde production was already significant at this time. A comparison of the effect of scale-up for the enzyme concentrations is shown in Table 6.8.

(a)



(b)

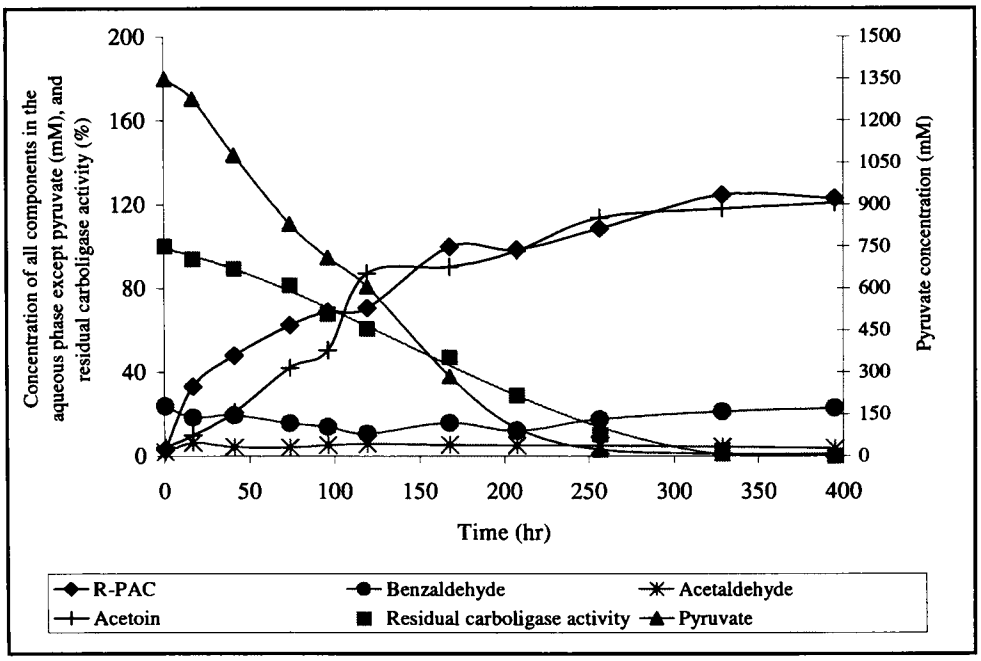


Figure 6.12 (a) and (b): Profile of the organic phase (a) and the aqueous phase (b) of the phase separated two-phase system at 3.8 U/ml carboligase activity (4 °C, organic octanol phase contained 1.5 M benzaldehyde and the aqueous phase contained 3.8 U/ml carboligase activity, 1.43 M pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

Table 6.8: Comparison of *R*-PAC production with small and large scale operation in the phase separated two-phase system (4 °C, organic octanol phase contained 1.5 M benzaldehyde and the aqueous phase contained 1.43 M pyruvate, 2.5 M MOPS, 1 mM TPP, 1 mM Mg^{2+}, pH 6.5).

	0.9 U/ml large scale	0.9 U/ml small scale	3.8 U/ml large scale	3.8 U/ml small scale
R-PAC (g/l)				
Organic phase (g/l)	102.8	112.2	126.0	167.3
(mM)	685.0	748.0	840.0	1115.3
Aqueous phase (g/l)	13.3	15.6	18.7	27.8
(mM)	84.5	104.0	124.5	185.3
Reaction time (h)	395	395	329	395
Specific *R*-PAC production (mg *R*-PAC/U)	128.9	142.0	38.1	51.3
Yield $_{(R\text{-PAC/benzaldehyde})}$ (%)	98.7	95.3	99	98
Yield $_{(R\text{-PAC/pyruvate})}$ (%)	77.6	60.8	67.8	92.9
Yield $_{(\text{acetaldehyde/pyruvate})}$ (%)	6.2	ND	2.3	ND
Yield $_{(\text{acetoin/pyruvate})}$ (%)	9.4	0.7	25.1	4.1
Remaining pyruvate (mM)	422	0	7.0	0

* ND: Not determined

6.7 Enantiomeric Excess of *R*-PAC Produced in the Two Phase System

The Enantiomeric Excess (EE) was determined for *R*-PAC produced in the two-phase system from four batches of semi-purified enzyme. In all cases, the EE for *R*-PAC was > 99 %. This EE value is higher than the 96 % obtained with *R*-PAC produced in the benzaldehyde emulsion batch reactions.

6.8 Gas Chromatography-Mass Spectrophotometry of *R*-PAC Produced in the Two-Phase System

A sample of *R*-PAC produced in the two-phase system was analyzed by Gas Chromatography Mass Spectrophotometry (GC-MS) to confirm its identity. A sample of *R*-PAC from the octanol phase of a fully stirred reaction that produced 142 g/l *R*-PAC in the upper octanol phase from 8.5 U/ml over 40 hours was analyzed.

The following samples were analyzed:

- 200 μg/ml octanol in ethyl acetate.
- 200 μg/ml octanol and 35 μg/ml *R*-PAC standard in ethyl acetate.
- 200 μg/ml octanol and 35 μg/ml *R*-PAC produced in the two phase system in ethyl acetate.

Trace ionization chromatograms (TIC) for *R*-PAC standard and sample are shown in Figure 6.13 and Figure 6.14. The 5.03 min peak in these chromatograms was identified as octanol by comparing the mass spectrum of the peak to a database containing a recorded spectrum for that compound. The 6.23 min peak corresponds to PAC and the mass spectrum diagrams for both the standard and sample are shown in Figure 6.15 and Figure 6.16. The mass spectrum for *R*-PAC produced at high levels in the two-phase system exhibited an identical mass spectrum to that of the standard.

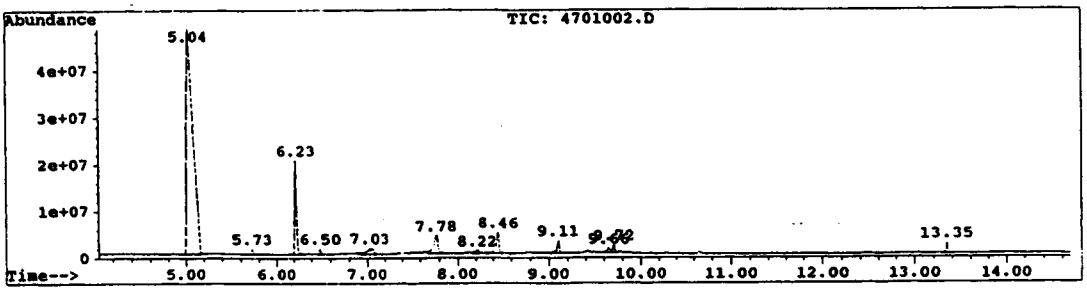


Figure 6.13: Trace ionization diagram for *R*-PAC standard in octanol (200 µg/ml octanol and 35 µg/ml *R*-PAC standard in ethyl acetate).

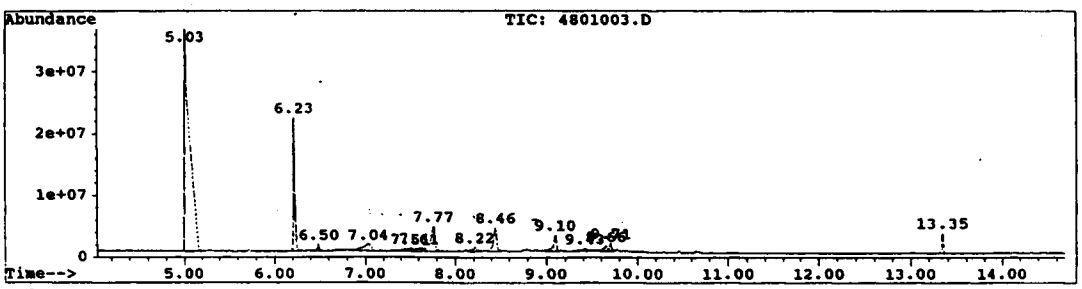


Figure 6.14: Trace ionization diagram for *R*-PAC sample in octanol (200 µg/ml octanol and 35 µg/ml *R*-PAC sample in ethyl acetate).

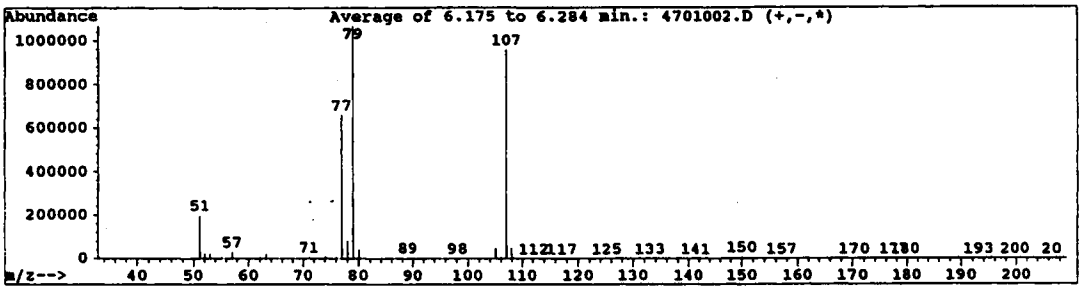


Figure 6.15: Mass spectrum for *R*-PAC standard in octanol (200 μg/ml octanol and 35 μg/ml *R*-PAC standard in ethyl acetate).

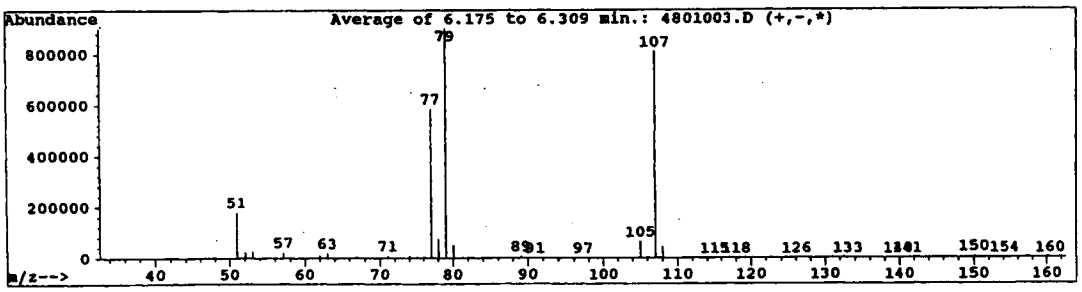


Figure 6.16: Mass spectrum for *R*-PAC sample in octanol (200 μg/ml octanol and 35 μg/ml *R*-PAC sample in ethyl acetate).

6.9 Discussion and Conclusions

Studies reported in Chapter 5 identified octanol as a potential organic phase solvent for use in a two-phase system for *R*-PAC production using partially purified PDC from *Candida utilis*. In Chapter 6 the octanol two-phase system was further investigated to maximize *R*-PAC formation and to identify system limitations and challenges.

Partition coefficients (log P) determined for all substrates and products of the two-phase system indicated that the following partitioning characteristics might be of benefit for minimizing enzyme inactivation and improving *R*-PAC production.

(a) strong partitioning of toxic benzaldehyde into the octanol phase away from the enzyme contained in the aqueous phase. Low soluble levels of benzaldehyde were delivered continuously from the octanol phase to the aqueous phase. With a benzaldehyde level of 1.8 M in the octanol phase there was approximately 50 mM benzaldehyde in the aqueous phase in contact with the enzyme. Results from Chapter 4 indicate that soluble low levels of benzaldehyde ($\leq$ 50 mM) minimize enzyme inactivation.

(b) *R*-PAC preferentially partitioned into the organic phase, with continuous extraction during the biotransformation. In Chapter 4 it was shown that exposure of pyruvate decarboxylase to increasing concentrations of *R*-PAC resulted in irreversible enzyme inactivation. Therefore continuous extraction of *R*-PAC into the octanol phase away from the enzyme in the aqueous phase would minimize loss of PDC activity during the biotransformation.

(c) by-products acetaldehyde and acetoin (to some extent) partitioned into the octanol phase away from the enzyme. Acetaldehyde partitioned strongly, whereas acetoin exhibited a preference for the aqueous phase. In both instances, however, some removal of by-products away from the enzyme may alleviate product inhibition due to acetaldehyde and inactivation due to acetoin. Shin and Rogers (1996b) reported decreased initial reaction rates and final concentrations of *R*-PAC in the presence of increasing concentrations of free acetaldehyde for partially purified PDC from *C.utilis*.

The initial reaction rates were influenced significantly, with an 85 % reduction of initial reaction rate in the presence of 50 mM acetaldehyde. Continuous extraction of acetaldehyde may increase initial reaction rates and is one possible reason for high levels of *R*-PAC formation in the octanol two-phase system.

In the two-phase system, enzyme is exposed to low concentrations of benzaldehyde in the aqueous phase and also to high concentrations of benzaldehyde at the octanol/MOPS buffer interface. Enzyme inactivation in the two phase system suggests that although benzaldehyde is partitioned into the octanol phase, there may be inactivation of the enzyme at the interface. The enzyme lost 34 % of its initial activity over 3 hours when exposed to 1.8 M benzaldehyde in octanol with approximately 50 mM benzaldehyde in the aqueous phase. Results from Chapter 4 show that the enzyme exposed to 50 mM benzaldehyde alone, with no second phase present retained 100 % of its activity. Also octanol alone did not affect the enzyme stability over this time period (see Figure 6.1).

Enzyme inactivation at interfaces has been documented for several enzymes, showing that proteins can absorb either reversibly or irreversibly, affecting activity and native structure (Cecil and Louis 1970; Graham and Phillips 1979a,b; Halling 1994). In general inactivation occurs when the hydrophobic core of the enzyme is attracted to the solvent, thereby flattening the enzyme across the interface. As the enzyme denatures and precipitates away from the surface, it is replaced by new enzyme from the bulk aqueous phase (Ghatorae *et al.* 1994a;b).

In benzaldehyde emulsion reactions described in Chapter 4, the initial concentration of benzaldehyde was limited by enzyme inactivation to 400 mM (42.4 g/l) at 7 U/ml. Delivery of benzaldehyde in octanol allowed higher initial starting concentrations of benzaldehyde, with continuous delivery of low concentrations of benzaldehyde to the enzyme in the aqueous phase, with the simplicity of a batch operated system. Enzyme membrane reactors and enzyme immobilization would also allow delivery of more substrate at low levels, however both of these systems involve the use of expensive membranes and immobilization matrices, which make scale up of the process more complicated. The rapidly stirred two-phase system was shown to scale up with reproducible *R*-PAC formation and yields.

The octanol that was soluble in the aqueous MOPS phase was shown to have a beneficial impact on initial reaction rates and final yields, while also lowering acetoin by-product formation. This result provides additional explanation for the increased production of *R*-PAC in the octanol two-phase system. Low concentrations of alcohols such as octanol and nonanol in contact with the enzyme may improve access of benzaldehyde to the active center. Shin (1994) found that up to 3 M ethanol in phosphate buffer improved initial reaction rates for partially purified PDC from *Candida utilis*. With 3 M ethanol, a 40 % improvement *R*-PAC formation was achieved over a 30 minute reaction period. When not undergoing a reaction, up to 2 M ethanol improved enzyme stability at 4 °C in phosphate buffer.

Studies reported in this chapter have established that a balance between enzyme stability and activity ensures high levels of *R*-PAC production. This balance can be influenced by the concentration of benzaldehyde in the organic phase and the ratio of phase volume to interfacial area. A rapidly stirred system with a large surface area of contact between phases, resulted in high rates of *R*-PAC production for the lifetime of the enzyme. With this mode of operation 140.5 g/l (936.7 mM) *R*-PAC was produced in the organic phase with an additional 18.5 g/l (123.3 g/l) in the aqueous phase after 50 hours. The phase separated system maintaining minimal contact between aqueous and organic phases, showed slower reaction times, allowed the enzyme to maintain activity longer and produce more *R*-PAC per amount of enzyme. With this mode of operation 167.3 g/l (1115.3 mM) *R*-PAC was produced in the organic phase with an additional 27.8 g/l (185.3 g/l) in the aqueous phase after 395 hours. Scale up of the phase separated system emphasized the importance of mass transfer between phases as it was shown that a change in the phase volume to interfacial area ratio influenced *R*-PAC production. This was attributed to changes in rates of transfer of benzaldehyde to the enzyme and *R*-PAC and by-products away from the enzyme. In such a system the benzaldehyde level in the organic phase may be increased sufficiently to influence transfer of benzaldehyde to the enzyme in the aqueous phase, allowing *R*-PAC formation instead of pyruvate decarboxylation to by-products when the benzaldehyde concentration is too low in comparison to the pyruvate concentration. Alternatively the enzyme level could be further reduced. A phase separated system would allow easier fed batch operation, and continuous product extraction. However these scale up issues need to be addressed to ensure high *R*-PAC production.

Increased acetoin production at the larger scale for both rapidly stirred and phase separated operation may be due to the enzyme batch used. Enzyme batch #4 was shown in Chapter 4 (Table 4.2) to produce more acetoin than batch #2 which was used for the smaller scale experiments. For the rapidly stirred system, identical *R*-PAC concentrations were achieved at both small and larger scale, indicating that *R*-PAC production was reproducible for the two batches of enzyme.

Under optimized conditions large increases in the amount of product per unit of enzyme activity were achieved in the octanol two-phase system. In the rapidly stirred and phase separated systems, 18.7 mg *R*-PAC/U and 142 mg *R*-PAC/U were achieved respectively, which is a significant improvement over the maximum of 9.3 mg *R*-PAC/U achieved in the benzaldehyde emulsion system described in Chapter 4. A more detailed comparison of *R*-PAC production is discussed in Chapter 7.

The full potential of the two-phase system was restricted by pyruvate availability and buffer exhaustion. Both limitations are easily overcome by additional substrate feeding and pH control. Significant loss of enzyme activity also occurred in the two-phase system and it is likely that rising pH influenced the loss of activity. As the pH rises from 6.5 to 8.0, it has been shown that the enzyme begins to dissociate into dimers with a resultant loss of activity (Gounaris *et al.* 1971; Hopmann 1980). Shin and Rogers (1996b) demonstrated that *R*-PAC formation rates by partially purified PDC from *Candida utilis* significantly declined at pH values above 7.

Substrate losses in controls with no enzyme present were attributed to pyruvate degradation and possible association of benzaldehyde with glass vials and caps. These losses were greater under rapidly stirred conditions. Contact of pyruvate with solvents octanol and benzaldehyde may increase its rate of degradation. The degradation has a significant influence on the yield calculations. Utilizing a fed batch system may reduce losses of pyruvate, and improving bioreactor design may reduce losses of benzaldehyde.

In summary, large increases in the amount of product per unit of enzyme, final *R*-PAC concentrations and productivity have been made with the two-phase system utilizing octanol. The success of this system is attributed to partitioning of benzaldehyde, *R*-PAC, acetoin and acetaldehyde away from the enzyme, with the additional increase in

reaction rates due to residual octanol in the aqueous phase. Knowledge of system limitations indicates that the system has potential for additional product formation and improved yields.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 Introduction

This thesis has focused on the development of a cell-free enzymatic process for enhanced production of *R*-PAC for use in ephedrine and pseudoephedrine synthesis. Previous investigations by Shin (1994), Chow (1998) and Rosche *et al.* (2002b) demonstrated the potential of partially purified *Candida utilis* PDC in *R*-PAC production. *R*-PAC concentrations were improved through pH control and enzyme stabilization of partially purified *Rhizopus javanicus* PDC in a benzaldehyde emulsion process (Rosche *et al.* 2002a). Further investigations in this thesis addressed the issues of reproducibility of enzyme stability and subsequent *R*-PAC formation, involving standardizing bioreactor operation, enzyme recovery procedures and investigating the factors affecting enzyme stability and activity.

A detailed study of the most significant factors affecting enzyme stability and activity not only solved reproducibility problems but highlighted the most important characteristics of *C.utilis* PDC that prevented further increases in *R*-PAC formation. Identification of these characteristics provided the basis for the design of an improved aqueous-organic two-phase enzymatic bioreactor that maximized product formation, thus making the cell-free process more competitive with the traditional whole-cell system involving benzaldehyde feeding to baker's yeast in molasses-based medium.

7.2 Production and Recovery of PDC

The aim of this section was to induce pyruvate decarboxylase activity under controlled conditions that were based on the recommended findings by Shin (1994) and Chow (1998). The emphasis was to ensure reproducible bioreactor operation for PDC induction and to recover suitable levels of partially purified PDC from the bioreactor to support further investigation.

Pyruvate decarboxylase (PDC) was successfully induced in *Candida utilis* using a two-step respiratory quotient (RQ) control strategy. A respiratory phase to maximize cell growth was achieved by controlling the RQ at a value of 1. After sufficient biomass production, fermentative conditions were induced in the second stage by increasing the RQ to a value of approximately 4. In this second stage, PDC activity increased rapidly, reaching an average enzyme yield of 278 units of decarboxylase activity and 77 units of carboligase activity per gram dry weight of cells (U/g). During the fermentation the ratio of carboligase activity to decarboxylase activity (activity index) was 0.25-0.33. Small variations in the level of decarboxylase and carboligase activity per gram of cells between different bioreactors could have been the result of uncontrolled variations in RQ in this system. RQ control could be further improved by incorporating on-line calculation of proportional/integral control constants and by reducing the airflow to the bioreactor after respiratory growth.

Crude extracts containing pyruvate decarboxylase were prepared successfully from *Candida utilis* cells by a combination of freeze thawing and blending with glass beads. The enzyme could be recovered from the crude extract with high yield by acetone precipitation between concentrations of 40-50 % [v/v] solvent. The resultant precipitate was freeze-dried and ground to a fine powder. The preparation was found to be stable following long-term storage for at least three years at –20 °C. The preparation containing pyruvate decarboxylase was dissolvable in any media and was a convenient preparation for investigation of enzyme properties.

Batches of enzyme prepared in the same manner from four separate experiments under controlled conditions exhibited different levels of stability in phosphate buffer incubated at 4 °C and when exposed to benzaldehyde solutions at the same temperature. To achieve reproducible characteristics in terms of stability and product formation, it was necessary to perform all reactions and evaluations in concentrated MOPS buffer. High concentrations of MOPS were shown to stabilize *Rhizopus javanicus* PDC, with a 7 fold increase in half-life when the MOPS concentration was increased from 0.05 M to 2 M (Rosche *et al.* 2002a). The half-life of partially purified *C.utilis* PDC was estimated to be 500 hours in 2 M MOPS buffer, and 138 hours in the presence of a solution of 50 mM benzaldehyde in the same buffer at 4 °C and pH 7.0. Partially purified *C.utilis* PDC at 7 U/ml was significantly more stable in comparison to *R. javanicus* PDC at 10 U/ml which showed a half-life was 14.5 hours in 2 M MOPS under the same conditions (Rosche *et al.* 2002a). Leksawasdi (unpublished results) showed that the half-life of *R.javanicus* PDC was 5 hours when exposed to 50 mM benzaldehyde in 0.6 M MOPS at 4 °C and pH 7.0.

Previous studies of enzyme stability and *R*-PAC formation in a phosphate buffer indicated that enzyme inactivation due to exposure to benzaldehyde and final *R*-PAC yields varied between batches of enzyme (Chow 1998). The studies in this thesis indicate that variable enzyme inactivation was due to interaction with benzaldehyde emulsions and also to variation in the long-term enzyme stability between batches of enzyme exposed to benzaldehyde solutions in phosphate buffer incubated at 4 °C. Replacement of phosphate buffer (40 mM) with concentrated MOPS buffer overcomes this problem of variability when exposed to benzaldehyde solutions.

The activity index (AI) is defined as the ratio of carboligase activity to decarboxylase activity and allowed comparison of results based on carboligase activity in this thesis to those results reported in the literature and in previous studies (Shin 1994; Chow 1998), based on decarboxylase activity. It is interesting to note that batches of partially purified *C.utilis* PDC were shown to exhibit a larger variation in AI between 0.19 to 2.07, in comparison to the crude extract (0.25-0.33). This change in AI occurred during acetone precipitation and/or freeze drying steps in the recovery of PDC from the crude extract. This

variation in AI did not have an impact on the level of *R*-PAC formation, the enantiomeric excess or enzyme stability when experiments were standardized to the same carboligase activity (U/ml). There was some indication that the AI may influence the level of by-product acetoin formation, with more acetoin formed in the enzyme preparation with the lower activity index. Increased acetoin production was observed for batch #4 (lower activity index) in comparison to batch #2 (higher activity index) in both the benzaldehyde emulsion reactor and the scale-up of the rapidly stirred emulsion and phase separated two-phase systems. A change in the AI induced by site-directed mutagenesis techniques was previously reported for *Z.mobilis* PDC (Bruhn *et al.* 1995), however there is no reported variation in activity index due to PDC recovery procedures. A more detailed investigation of the conditions needed to control and increase the AI of PDC could possibly improve the characteristics of the enzyme preparation. More detailed studies are required into the significance of changes into the activity index of the PDC preparation.

7.3 Factors Affecting Enzyme Stability and Activity

The influences of product *R*-PAC, by-products acetoin and acetaldehyde, and substrate benzaldehyde on enzyme stability were investigated. The most significant finding was the dramatically increased enzyme inactivation which occurred when benzaldehyde was delivered as an emulsion. It was found that the carboligase activity was maintained for a significantly longer period of time when exposed to benzaldehyde solutions only. A further significant factor contributing to enzyme inactivation was the by-product acetoin (5-60 mM), and of less significance the product *R*-PAC (concentrations up to 274 mM). Acetaldehyde at a concentration of 30 mM did not have an impact on enzyme stability. It was established further that *R*-PAC concentrations up to 153 mM do not inhibit initial reaction rates or final yields of *R*-PAC.

R-PAC formation was evaluated in a simple batch reactor developed by Rosche *et al.* (2002a), with benzaldehyde delivered as an emulsion to evaluate enzyme stability under harsh reaction conditions. It was found that a maximum of 45 g/l *R*-PAC with *C.utilis* PDC

could be achieved in 57 hours. The concentration of *R*-PAC achieved was significantly higher than the maximum of 28.6 g/l that was achieved with *C.utilis* PDC by Shin (1994), although lower than the 50.6 g/l *R*-PAC produced in 29 hours with *R.javanicus* PDC (Rosche *et al.* 2002a). This increase in product formation in comparison to the results of Shin (1994) can be attributed to enhanced enzyme stability of the enzyme preparation in concentrated MOPS buffer, improved buffering of the biotransformation and possibly to the removal of ethanol from the reaction media. *R.javanicus* PDC produced higher final concentrations of *R*-PAC in a shorter reaction time, despite exhibiting a shorter half-life than *C.utilis* PDC when evaluated in MOPS buffer and when exposed to a 50 mM benzaldehyde solution (Leksawasdi, unpublished results; Rosche *et al.* 2002a). The higher final concentration of *R*-PAC achieved with *R.javanicus* PDC could be due to the use of a more concentrated MOPS buffer (2.5 M) and a lower initial pH of 6.5 in comparison to *C.utilis* (2 M MOPS and pH 7.0).

Despite advances in the level of product achieved in this benzaldehyde emulsion batch reactor, the biotransformation was restricted by irreversible enzyme inactivation due to benzaldehyde, *R*-PAC and acetoin. A pH rise over the biotransformation from 7.0 to 7.4 may have additionally limited the reaction.

7.4 Two-Phase System

From these findings an aqueous-organic two-phase reactor was developed that contained high concentrations of benzaldehyde in an octanol phase which partitioned benzaldehyde at low soluble concentrations to the aqueous MOPS phase, with enzyme and pyruvate contained in this latter phase. Formed *R*-PAC, acetaldehyde and acetoin were extracted away from the enzyme into the organic phase. This system allowed delivery of higher concentrations of benzaldehyde (1.5 M) in a batch-operated system by partitioning the bulk benzaldehyde in the organic phase away from the enzyme in the aqueous phase. Benzaldehyde at these high concentrations (1.5 M) in an emulsion reactor with no second organic phase would have resulted in rapid rates of enzyme inactivation and low final

concentrations of *R*-PAC. In addition, the continuous extraction of *R*-PAC and acetoin into the organic phase would further reduce enzyme inactivation.

Solvent screening

A screen of various solvents for an appropriate organic phase in the two-phase system identified nonanol and octanol as the most suitable solvents for *R*-PAC formation. Several other solvents limited *R*-PAC formation, possibly due to inhibition of carboligase activity, enzyme inactivation or insufficient partitioning of product *R*-PAC and/or by-product acetoin away from the enzyme into the organic phase. Subsequent evaluation of *Z.mobilis* PDC in the two-phase reaction system also identified alcohols as the most suitable organic phase solvents for *R*-PAC production from benzaldehyde and acetaldehyde (Rosche, unpublished results). From the solvent screen described in Chapter 5, octanol was selected for further studies of *R*-PAC formation due to the higher molar yield of *R*-PAC/pyruvate utilized and the higher final concentration of *R*-PAC in comparison to nonanol. The reactor was evaluated both with rapidly stirred emulsion operation and with slow stirring to maintain the organic and aqueous phases as phase-separated.

Previous studies in a two-phase system using whole cells of baker's yeast produced only 0.45 mg/ml *R*-PAC in a 6 hour process (Nikolova and Ward 1992a,c). In this system the most suitable solvent was found to be hexadecane due to cell damage experienced with other solvents ethylacetate, butylacetate, chloroform, toluene, dodecane and hexadecane. Alcohols were not evaluated in this screen and it is possible that they may have been more suitable solvents for minimizing cell damage and therefore maximizing *R*-PAC formation.

R-PAC production in the two-phase system

In the present investigation, in the rapidly stirred system, high levels of *R*-PAC were achieved in 49 hours, with 140 g/l produced in the organic octanol phase and an additional 21 g/l in the aqueous MOPS phase. A specific production of 18.9 mg *R*-PAC per unit of carboligase activity was determined. The system achieved reproducible product formation

when scaled up from 2.16 ml to 104 ml bioreactor working volume. This reaction was limited by complete utilization of pyruvate and rising pH. Significant carboligase activity remained after 49 hours, showing that further product formation should be possible with additional pyruvate feeding and pH control.

In the phase-separated system, high levels of *R*-PAC were achieved with 167.3 g/l and 27.8 g/l in the organic and aqueous phases respectively for 3.8 U/ml, and 102.8 g/l and 13.3 g/l in the organic and aqueous phases respectively for 0.9 U/ml. Significantly more *R*-PAC was formed per unit of carboligase activity (51.3 mg *R*-PAC/U at 3.8 U/ml and 142 mg *R*-PAC/U at 0.9 U/ml) than that achieved in the rapidly stirred emulsion system. However, a much longer time period was needed to achieve these levels, most probably due to mass transfer limitation resulting from a combination of slow stirring rate and a low ratio of interfacial area to volume.

The ratio of the interfacial area to volume of solvent was larger in the rapidly stirred system resulting in higher reaction rates than in the phase-separated system. However, the enzyme inactivation rate was greater in the rapidly stirred system due probably to increased contact of the enzyme with benzaldehyde, acetoin and *R*-PAC at the aqueous-organic interface. In addition shear effects may contribute to increased enzyme inactivation with a higher rate of stirring. The half-life of PDC in the rapidly stirred reactor was 37 hours, while longer half-lives of 170 hours at 0.9 U/ml and 150 hours at 3.8 U/ml were determined in the phase separated reactor. Optimization of the two-phase system will most likely involve balancing the rate of benzaldehyde delivery and enzyme concentration against the rate of enzyme inactivation.

Comparison of processes for *R*-PAC production

A comparison of the operational characteristics of the various enzymatic processes for *R*-PAC formation are shown in Figures 7.1-7.7 and compared with the traditional process. For the yeast-based batch fermentation process maximum literature values in the range 10-22 g/l *R*-PAC produced in 10-14 hours from 13-28 g/l biomass were estimated from

literature (Mahmoud *et al.* 1990a; Rogers *et al.* 1997; Tripathi *et al.* 1997; Oliver *et al.* 1999). Therefore the average values calculated from this data for comparisons in Figures 7.1-7.7 were16 g/l *R*-PAC in 12 hours from approximately 20 g/l dry weight biomass. Two-phase data in Figures 7.1-7.7 were derived from the small-scale reactions.

A number of criteria can be used to compare the various modes of bioreactor operation for the enzymatic production of *R*-PAC, and for the further comparison with the traditional yeast based biotransformation.

Possible criteria are:

1) final *R*-PAC concentrations (g/l)
2) specific *R*-PAC production (mg *R*-PAC / U carboligase activity)
3) specific productivity (mg *R*-PAC / U carboligase activity/ day)
4) volumetric productivity (g *R*-PAC / l / day)
5) molar yields based on substrates utilized (*R*-PAC / benzaldehyde and *R*-PAC / pyruvate)

For a direct comparison with the traditional process, the specific production (g *R*-PAC/ g biomass) and productivity (g *R*-PAC / biomass/ day) can be used. Both of these criteria are significant in the economic evaluation of the various processes as it brings the comparison to a common basis by taking into account the PDC which can be produced by yeast (U/g biomass).

Figure 7.1 shows that the final concentrations of *R*-PAC achieved in the cell-free enzymatic biotransformations were significantly higher in comparison to the whole cell fermentation process, with the highest levels achieved by *Candida utilis* PDC in the two-phase process developed in Chapter 5 and 6. These high levels of *R*-PAC would possibly be of advantage particularly in downstream processing for *R*-PAC recovery.

Figure 7.2 shows that the highest amount of *R*-PAC produced per unit of carboligase activity was achieved in the two-phase system at low enzyme level (0.9 U/ml) with phase separation maintained. However, the time to achieve this specific production was 395 hours in comparison to 40 hours in the rapidly stirred system. This is reflected in the specific productivities shown in Figure 7.3, which illustrate that the highest specific productivity (mg *R*-PAC/U/day) could be achieved with the two-phase rapidly stirred system. Figure 7.4 demonstrates that the productivity (g *R*-PAC/l/day) was comparable between the benzaldehyde emulsion reactor with *R.javanicus* PDC and with the two-phase rapidly stirred system. The lower productivity of the phase separated two-phase system in comparison to other approaches is a result of the slower reaction rate.

A comparison of yields of moles of *R*-PAC achieved per mole of substrates consumed is shown in Figure 7.5. In all cases the yields of *R*-PAC based on benzaldehyde utilized were much greater than for the whole-cell process where the yield was lower due to the loss of benzaldehyde to benzyl alcohol resulting from the action of oxidoreductases such as alcohol dehydrogenase. Some loss of benzaldehyde possibly due to association with the glass vial or cap (5-10 %) was experienced in the enzymatic system and may be overcome by improved design of the reactor in scale-up. The molar yields of *R*-PAC based on pyruvate utilized varied from 60 % to 93 % theoretical for the different enzymatic processes. There was evidence of both pyruvate degradation and by-product acetoin and acetaldehyde formation in these processes which compromised the yields. Programmed feeding of pyruvate may lower its degradation by dimerization and reduce by-product formation.

Determination of the amount of *R*-PAC achieved per gram of cells (Figure 7.6) for all processes provides additional information for comparison of the different biotransformation processes, and demonstrates the superiority of the two-phase enzyme process when based on this criterion, however Figure 7.7 shows that the whole cell system produced more *R*-PAC/g/day in comparison to the enzymatic process due to the rapid biotransformation process (10 hours) when benzaldehyde is added to relatively concentrated cells of *S.cerevisiae* in a molasses-based medium.

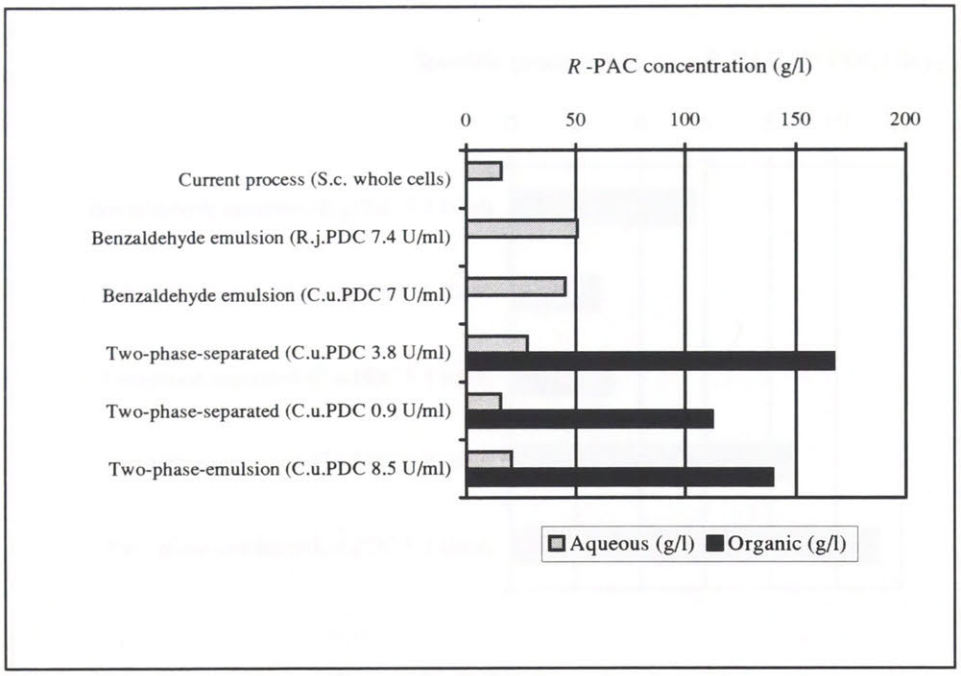


Figure 7.1: Comparison of final concentrations of *R*-PAC achieved in various processes (S.c : *Saccharomyces cerevisiae*; R.j: *Rhizopus javanicus*; C.u: *Candida utilis*).

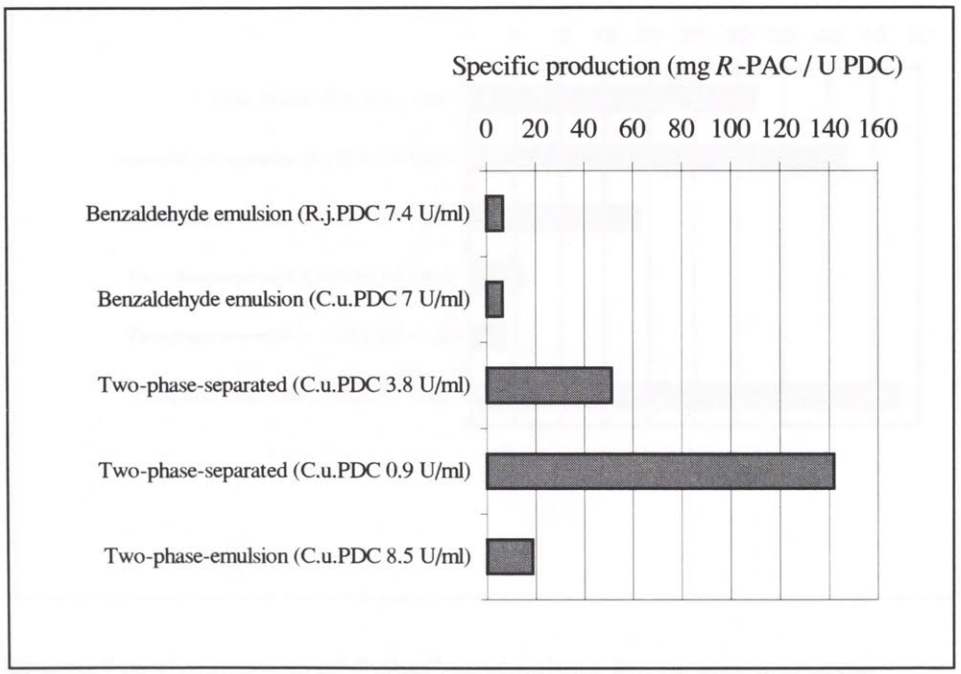


Figure 7.2: Comparison of specific *R*-PAC production for various processes (S.c : *Saccharomyces cerevisiae*; R.j: *Rhizopus javanicus*; C.u: *Candida utilis*).

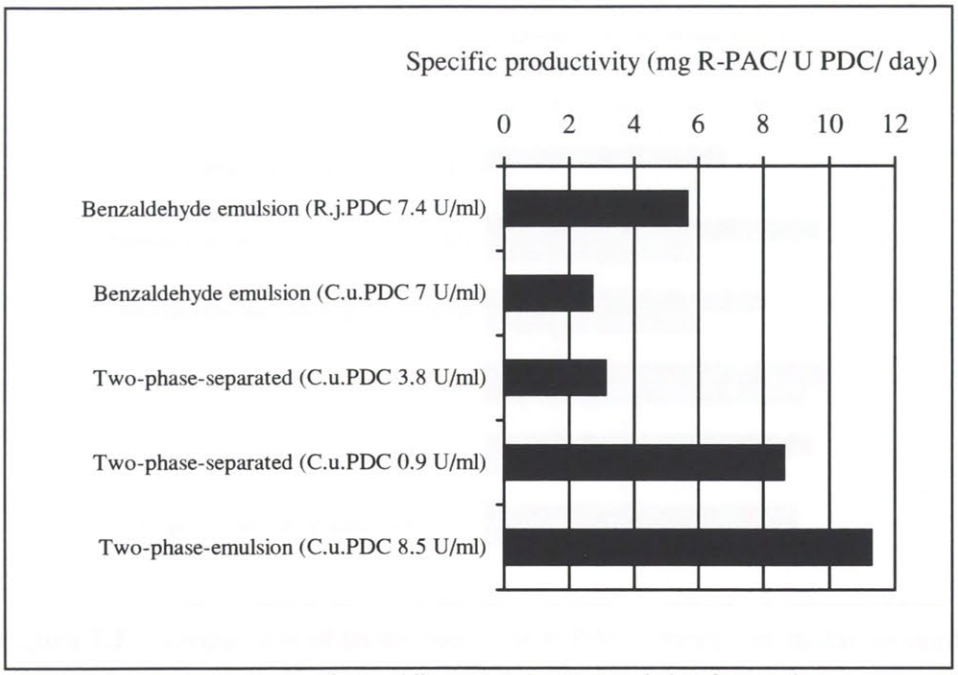


Figure 7.3: Comparison of specific *R*-PAC productivity for various processes (S.c : *Saccharomyces cerevisiae*; R.j: *Rhizopus javanicus*; C.u: *Candida utilis*).

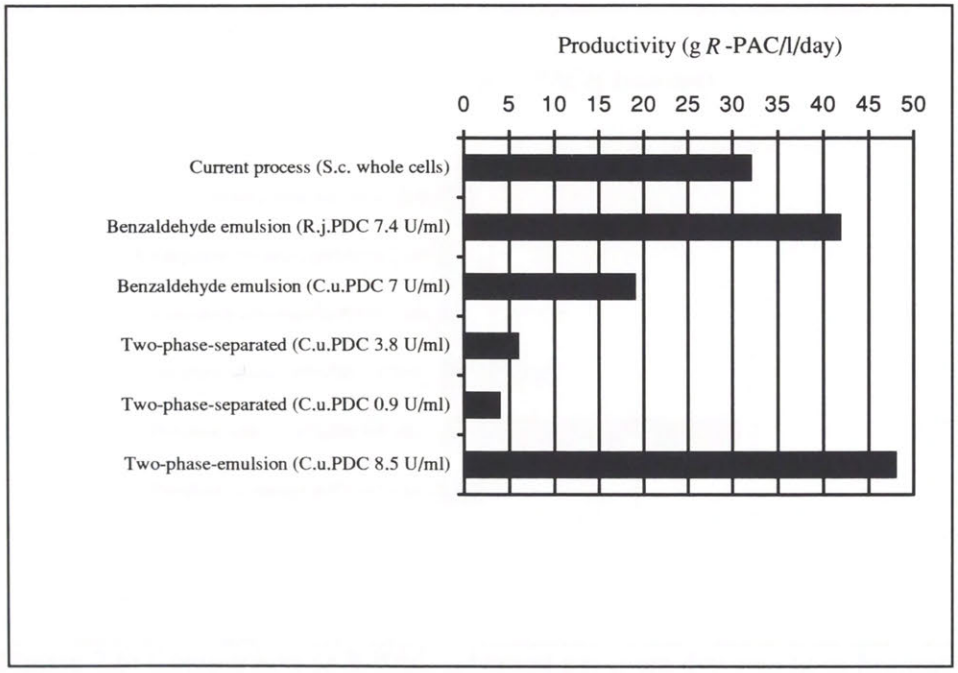


Figure 7.4: Comparison of *R*-PAC productivity for various processes (S.c : *Saccharomyces cerevisiae*; R.j: *Rhizopus javanicus*; C.u: *Candida utilis*).

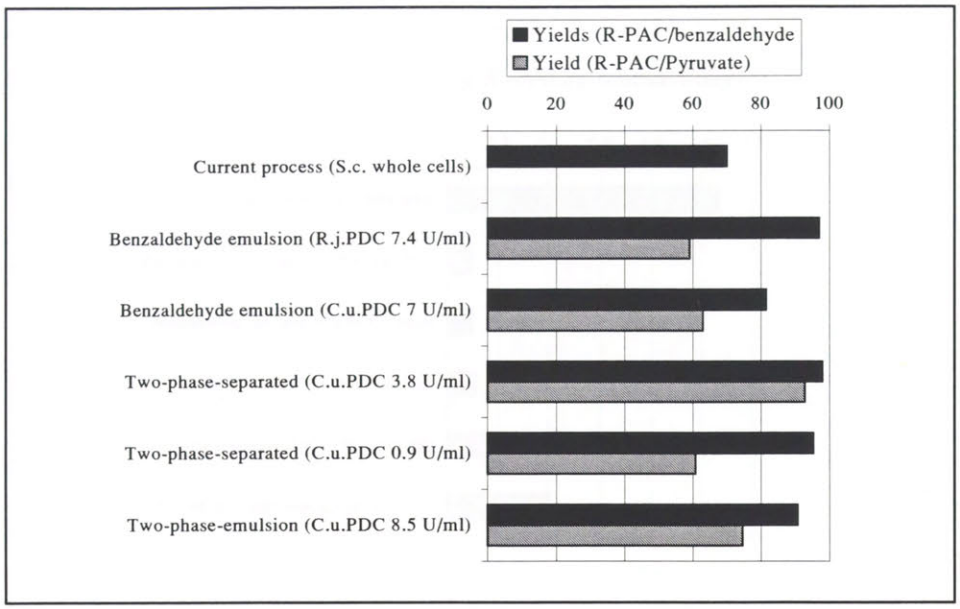


Figure 7.5: Comparison of molar yields of *R*-PAC formed per molar amount of substrates consumed for various processes (S.c : *Saccharomyces cerevisiae*; R.j: *Rhizopus javanicus*; C.u: *Candida utilis*).

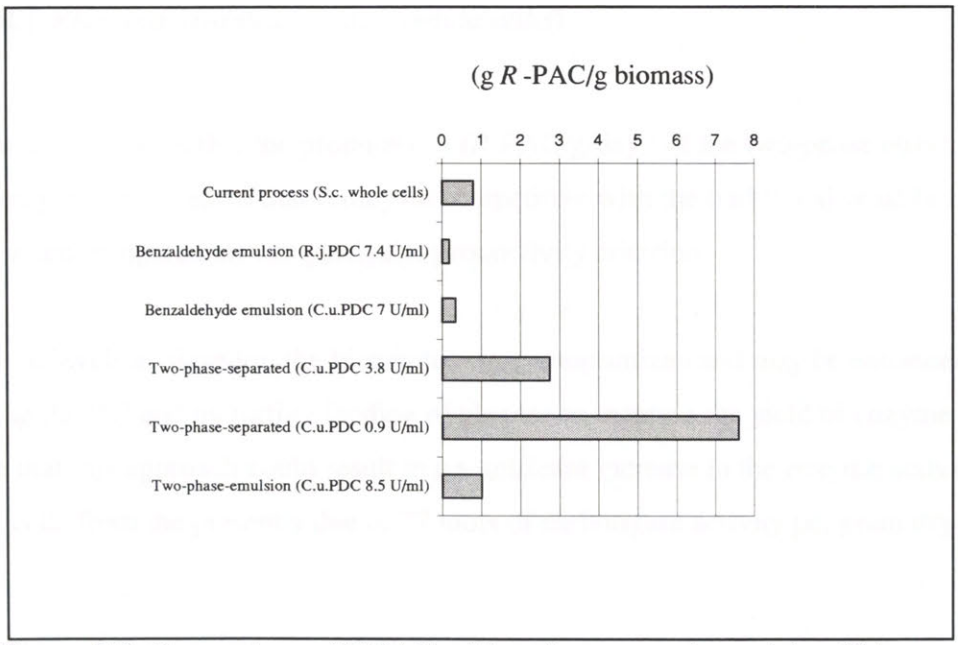


Figure 7.6: Comparison of *R*-PAC achieved per gram dry weight of biomass per day. The calculation was based on the level of enzyme recovered from the fermentation and recovery protocol (S.c : *Saccharomyces cerevisiae*; R.j: *Rhizopus javanicus*; C.u: *Candida utilis*).

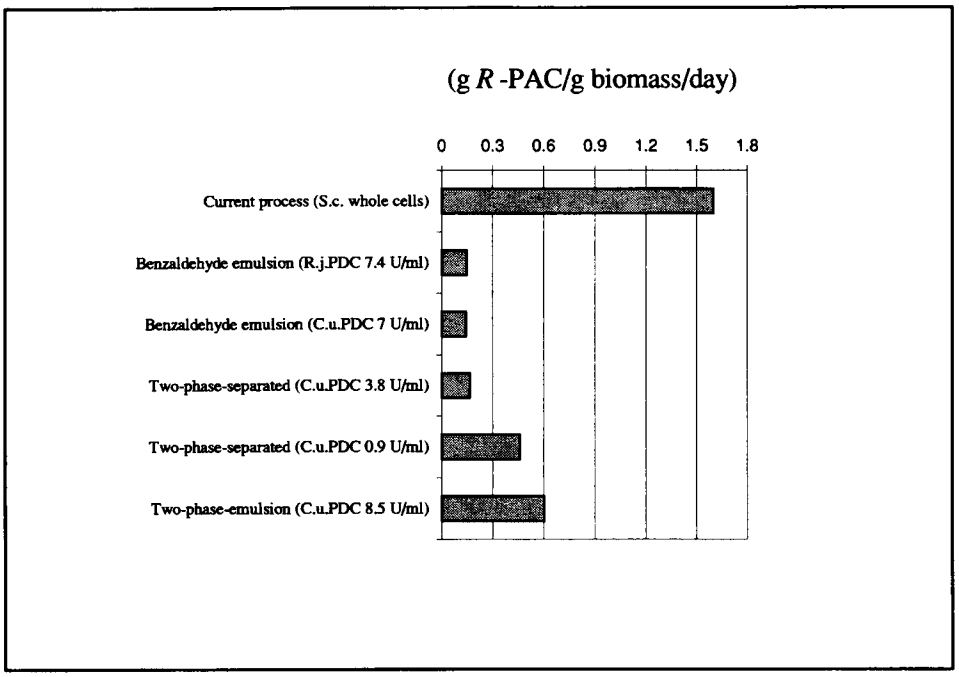


Figure 7.7: Comparison of *R*-PAC achieved per gram dry weight of biomass per day. The calculation was based on the level of enzyme recovered from the fermentation and recovery protocol (S.c : *Saccharomyces cerevisiae*; R.j: *Rhizopus javanicus*; C.u: *Candida utilis*).

There are several ways that the productivity (*R*-PAC/g/day) of the two-phase enzymatic system may be improved so that it may be competitive with the traditional yeast based process when compared on this particular productivity criterion:

(a) enzyme levels achieved in the bioreactor were unoptimized and may be enhanced by increasing the RQ and by further feeding of glucose to increase the yield of enzyme. It is possible that this approach could result in a significant increase in the enzyme activity per gram of cells from the present value of 77 units of carboligase activity per gram dry weight of cells.

(b) the use of crude extract or whole cells which would avoid a loss of some 40 % of PDC activity that occurred during acetone precipitation.

(c) a reduction in the reaction time which may be achieved by increasing the temperature of the biotransformation, although higher temperatures (e.g. 25 °C) could result in more acetaldehyde or acetoin formation.

Other groups have reported that a continuously operated enzyme membrane reactor (EMR) using *Z.mobilis* PDC, stabilized by site-directed mutagenesis, achieved a high space-time yield of 81 g *R*-PAC/l/day (Goetz *et al.* 2001; Iwan *et al.* 2001). However the final concentrations of *R*-PAC achieved were low at 3.4 g/l with a conversion of 22.5 % of the 50 mM substrates fed initially. This yield was increased to 45 % by incorporating a cascade of EMR's. In comparison to results in our evaluation, this enzymatic process has a cost advantage from using acetaldehyde rather than the more expensive pyruvate as a substrate. However its lower *R*-PAC concentrations and yields along with enzyme production and recovery from recombinant *E.coli*, are significant limitations. In addition, the cost of pyruvate may be reduced significantly in the future by controlled fermentation processes with yeast or recombinant *E.coli*.

In conclusion, a study of the factors influencing *R*-PAC formation and enzyme stability in the biotransformation of pyruvate and benzaldehyde to *R*-PAC by *Candida utilis* PDC has provided the basis for the development of an enzymatic two-phase reactor. This reaction system demonstrated increased final concentrations of *R*-PAC, improved the amount of product achieved per amount of enzyme and increased the specific productivity in comparison to the benzaldehyde emulsion reactor. The system also competes well with other reported processes using *Z.mobilis* PDC.

Additional improvements in the amount of enzyme produced during the fermentation and increases in the productivity may further enhance the commercial potential of a two-phase process in comparison to the current industrial process. As discussed in Section 7.4, there are a number of possible ways to achieve these additional improvements. Any decision to replace a traditional yeast-based biotransformation with an enzyme-based process will ultimately depend on the results of a detailed economic evaluation and the significance of the various component cost factors.

Comparison with other two-phase studies

R-PAC formation in the two-phase system described in Chapters 5 and 6 highlighted the importance of the selection of a suitable solvent for enzyme compatibility and partitioning of substrates, by-products and product. A balance between interphase mass transfer, enzyme concentration and enzyme stability was found to be critical for achieving high rates of *R*-PAC production.

Botes *et al.* (2001) and Lotter *et al.* (2001) demonstrated from an extensive solvent screen, that alcohols were the most suitable solvents for the enantioselective hydrolysis of (2,3-epoxypropyl)benzene by whole-cells of *Rhodotorula* sp. Octanol was found to be one of the most suitable solvents for maintaining epoxide hydrolase activity in the cells, with up to 20% [v/v] solvent concentration. A study of partially purified epoxide hydrolase activity from *Agrobacterium radiobacter* by Baldascini *et al.* (1999) showed that octanol contributed significantly to enzyme inactivation in comparison to octane or hexane. Enzyme inactivation was attributed to octanol dissolved in the aqueous phase and due to inactivation at the octanol/aqueous interface. These studies highlight that although octanol has been suitable in other studies (Botes *et al.* 2001; Lotter *et al.* 2001), the application of octanol in two-phase reactors is dependent on the specific reaction system, for example whole-cell or isolated enzyme catalysts. This conclusion supports literature and the results found in the solvent screen in Chapter 5, where it was found that no overall correlation of solvent biocompatibility parameters (for example, log P) can be used for all enzymes or processes.

In this thesis the extraction of *R*-PAC into the octanol phase is beneficial for reducing enzyme inactivation and could be important for reducing possible end-product inhibition at high *R*-PAC concentrations. Continuous extraction of *R*-PAC could also be important for recovery of *R*-PAC in downstream processing. Octanol was shown by Husken *et al.* (2001) to be a suitable solvent for the production and extraction of 3-methylcatechol in a two-phase system. Octanol was selected in this system for its compatibility with whole-cells of *P.putida* and also due to the preferential partitioning and continuous extraction of

3-methylcatechol into octanol after formation. Continuous extraction of 3-methylcatechol was an important consideration for increasing the overall product concentration beneficial for downstream processing (Husken *et al.* 2001).

An important advantage of the two-phase bioreactor for *R*-PAC production was the partitioning of toxic substrates benzaldehyde into the organic phase away from the enzyme thereby reducing enzyme inactivation. Other two-phase systems have also attributed reduced enzyme inactivation due to the partitioning of toxic substrates or products away from the enzyme (Biselli *et al.* 1995; Baldascini *et al.* 1999; Xin *et al.* 2000; Husken *et al.* 2001).

Mass transfer as affected by stirring rate was highlighted as an important consideration for *R*-PAC formation in the two-phase system. Baldascini *et al.* (1999) has discussed the implications of mass transfer limitation on the enzymatic resolution of epoxides in a two-phase system. In these studies a model simulating the reaction showed that mass transfer limitations have a large impact on the yield of epoxide. An increased rate of enzyme inactivation occurred with an increase in the interfacial area of contact (Baldascini *et al.* 1999) as also demonstrated for pyruvate decarboxylase in the two-phase system in this thesis.

7.5 Possible Future Studies

In this study, significant improvements in *R*-PAC production using partially purified pyruvate decarboxylase from *Candida utilis* have been achieved. Future studies should focus on improving the critical parameters of g *R*-PAC/g biomass and g *R*-PAC/g biomass/day and directed at the following:

1.Optimum enzyme production and stabilization

- Optimization of PDC production in a bioreactor with additional glucose feeding. In this study a maximum of 77 units of carboligase activity/g biomass was achieved in a controlled bioreactor. Recently and 162 U of carboligase activity/g biomass was achieved in shake flask experiments with the identical medium (Rosche *et al.* 2002b), and it is possible that additional glucose feeding may further improve this yield of enzyme. The objective then should be to establish conditions in a controlled bioreactor to maximize the amount of PDC per gram of cells, which would increase therefore the amount of *R*-PAC formed per amount of cells per day.

- Chemical crosslinking of the enzyme for improved stability and activity under reaction conditions. Currently the half life of PDC in the rapidly stirred two-phase system is 37 hours. Crosslinking has been shown to improve the stability of enzymes in reaction conditions and it may be possible to improve the half life of *Candida utilis* PDC under two-phase reaction conditions. Further stabilization of the enzyme may improve the amount of *R*-PAC produced per gram of biomass from which the enzyme is recovered.

- Enhancing PDC activity and stability by sequencing the gene encoding for PDC from *Candida utilis* and comparing the DNA sequence and amino acid composition of the protein to those other sources that have favourable *R*-PAC production, stability and substrate range characteristics. From this comparison a site-directed mutagenesis strategy could be developed to improve the enzyme. In addition, the gene encoding for PDC from *Candida utilis* could be expressed in a suitable host organism to be further improved by directed evolution techniques. Directed evolution techniques could aim to improve the half life of PDC under reaction conditions or to allow the enzyme to directly convert acetaldehyde and benzaldehyde to *R*-PAC.

- The use of permeabilized whole cells which may provide a protective compartment for the enzyme thereby reducing enzyme inactivation due to benzaldehyde.

- Further investigation of enzyme recovery procedures to produce an enzyme batch of reproducible high stability and activity without the use of additives or expensive buffers, with possible use of crude extract for *R*-PAC formation.

- Further investigations into methods to control and manipulate the activity index (AI) of the enzyme preparation. In addition, the significance of AI on by-product acetoin formation should be determined.

2.Further development of the two-phase system

- Further evaluation of the two-phase system with pH control and additional substrate feeding, with the possibility of using pyruvic acid for combined pyruvate feeding and pH control. The effect of pH control on enzyme stability should be evaluated to confirm that the decline in enzyme activity is attributed to pH rise over the reaction. In addition the two-phase reactor should be evaluated at increased temperature up to 25 °C to maximize productivity without overproduction of by-products.

- Development of a two-phase system incorporating a membrane at the interface which prevents direct interaction between the enzyme and the organic phase containing toxic benzaldehyde. This design may minimize interfacial inactivation and allow more product formation from the enzyme catalyst.

- Investigation and subsequent minimization of pyruvate degradation in the two-phase reactor. Prevention of degradation may improve molar yields of *R*-PAC on pyruvate utilized and reduce the cost associated with substrate loss.

- Investigation of the effect of high concentrations of pyruvate on initial reaction rates and final *R*-PAC concentrations in the two-phase system. If pyruvate is found to limit the reaction, then a programmed feeding profile could further improve reaction rates and decrease the operation time.

- Further evaluation of *R*-PAC production in the two-phase system from the substrates acetaldehyde and benzaldehyde utilizing pyruvate decarboxylase from wild type *Z.mobilis* PDC and from the more stable mutant PDC's.

In summary, the most relevant further work would be to complete the development of a cost competitive enzymatic two-phase system for *R*-PAC production. Future improvements in enzyme stability by site-directed mutagenesis or directed evolution techniques would additionally enhance the cost advantages of this enzymatic process.

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Appendix A

Sample Chromatograms and Calibration Curves

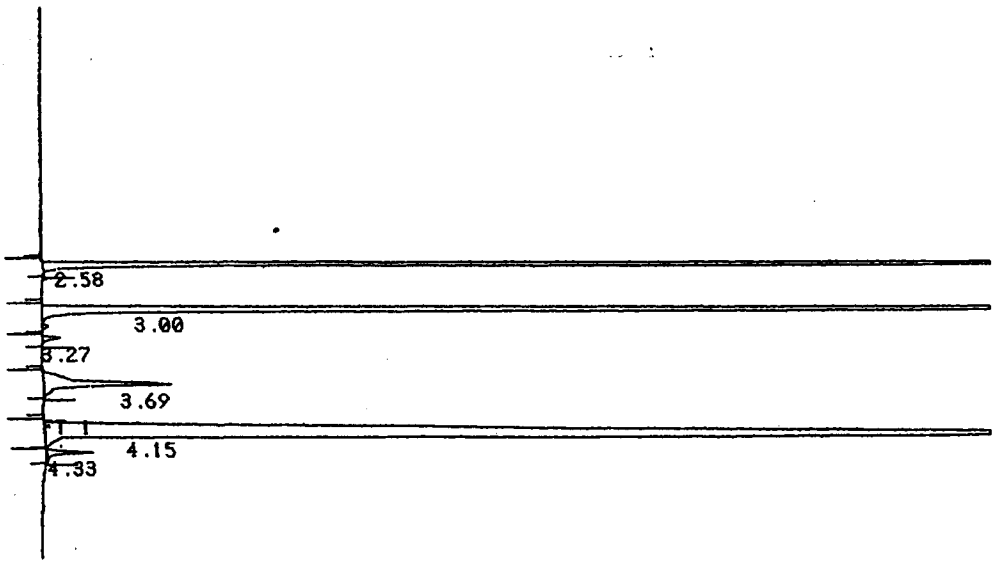

Figure A1 (a): Sample chromatogram for quantification of *R*-PAC (4.15 min) and benzaldehyde (2.58 min) in octanol (3.00 min) analyzed by capillary gas chromatography.

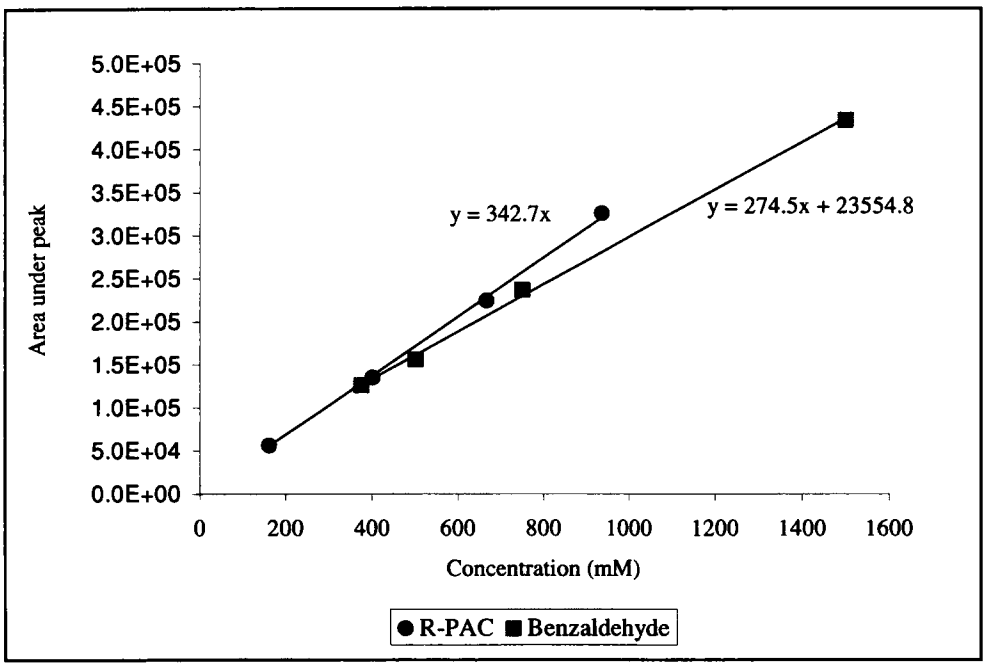

Figure A1 (b): Calibration curves for quantification of *R*-PAC and benzaldehyde in octanol analyzed by capillary gas chromatography.

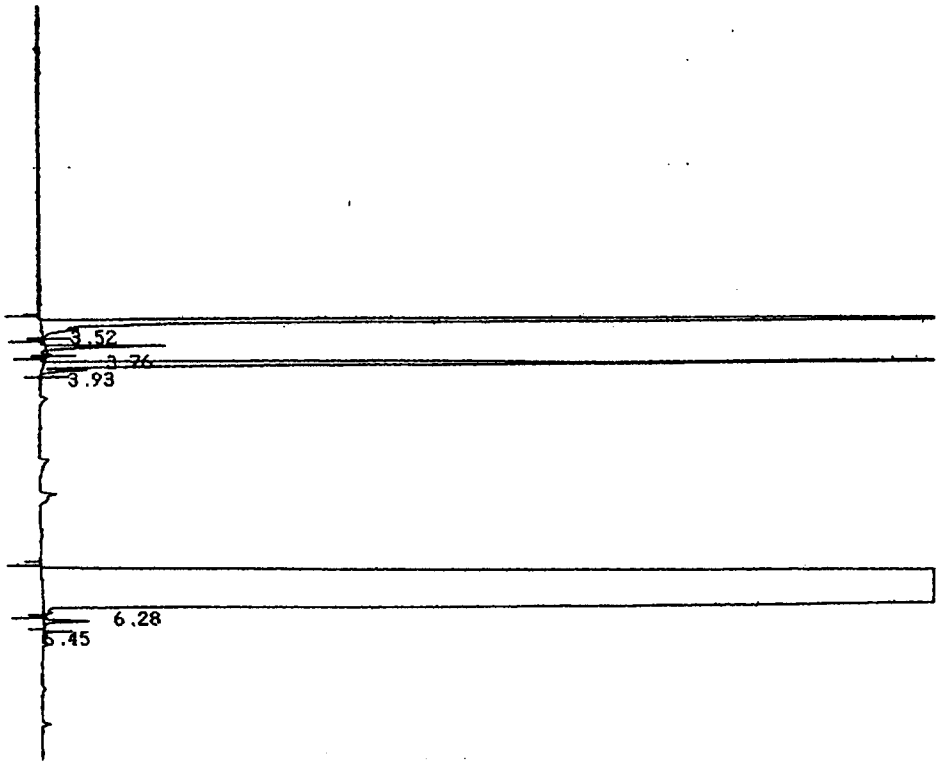

Figure A2 (a): Sample chromatogram for quantification of acetoin (3.76 min) and acetaldehyde (3.52 min) in octanol (6.28 min) analyzed by capillary gas chromatography.

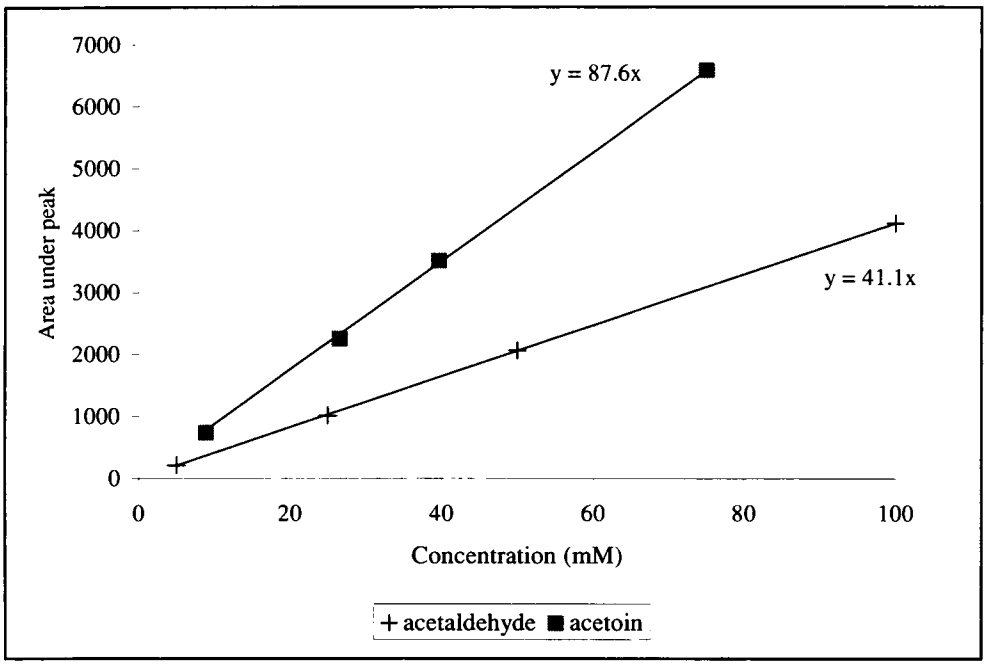

Figure A2 (b): Calibration curve for quantification of acetoin and acetaldehyde in octanol analyzed by capillary gas chromatography.

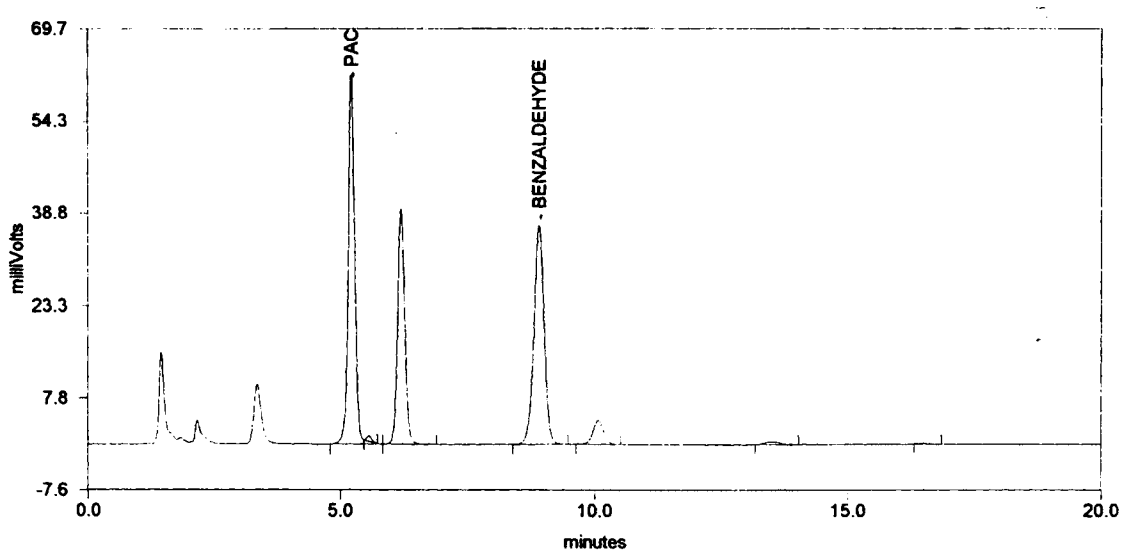

Figure A3 (a): Sample chromatogram for aqueous phase *R*-PAC (5.24 min) and benzaldehyde (8.91 min) analyzed by HPLC.

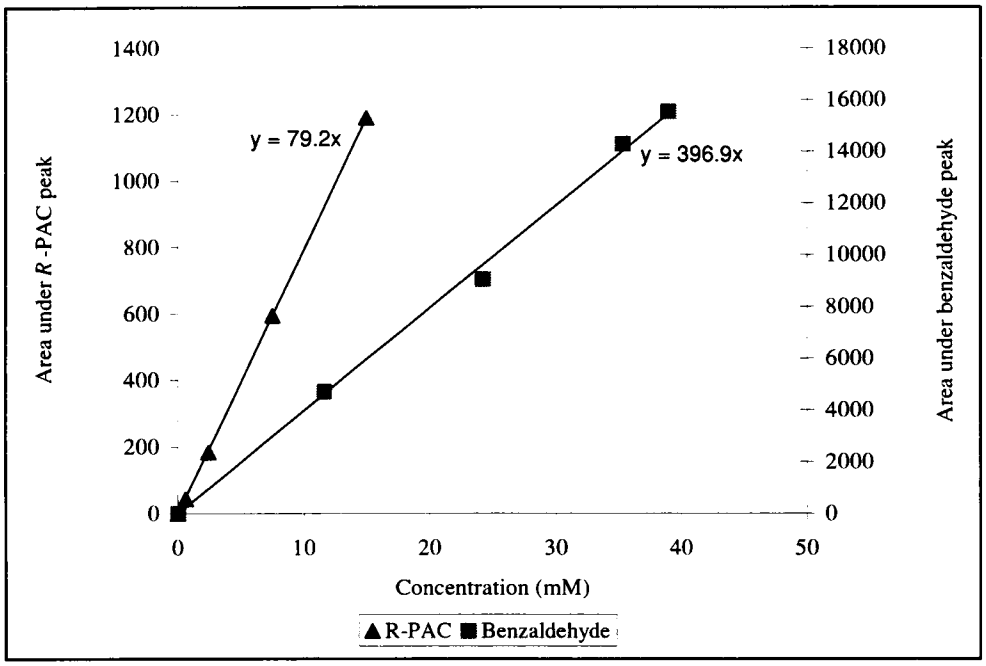

Figure A3 (b): Calibration curves for quantification of aqueous phase *R*-PAC and benzaldehyde analyzed by HPLC.

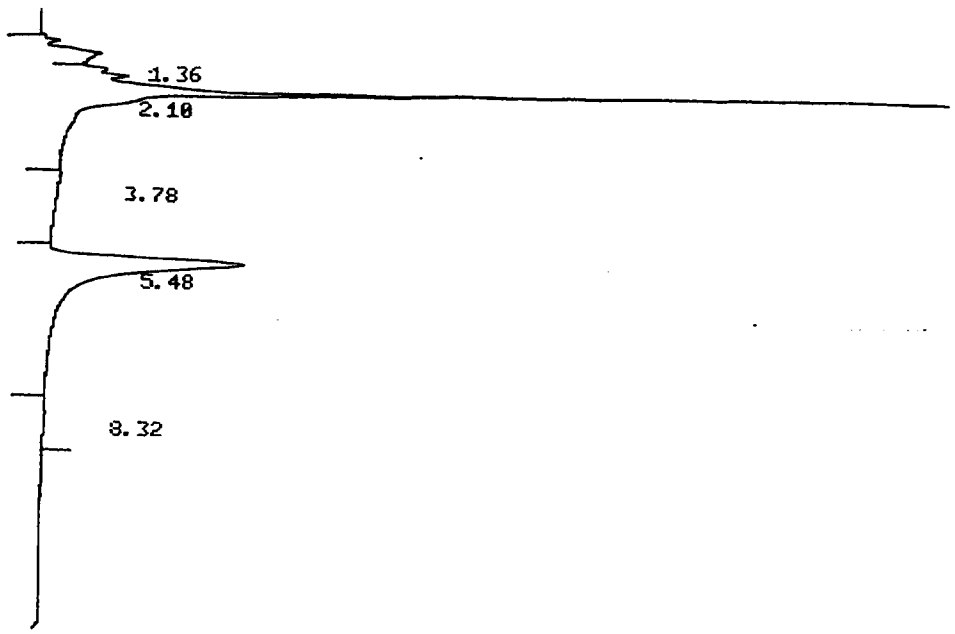

Figure A4 (a): Sample chromatogram for aqueous phase acetoin (5.48 min) by gas chromatography.

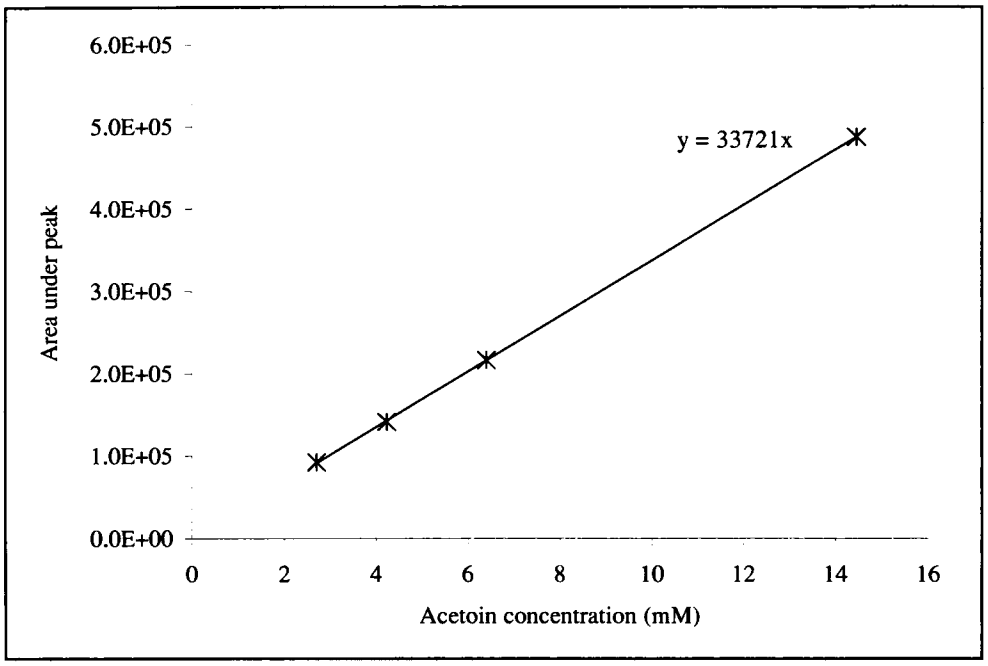

Figure A4 (b): Calibration curve for quantification of aqueous phase acetoin analyzed by gas chromatography.

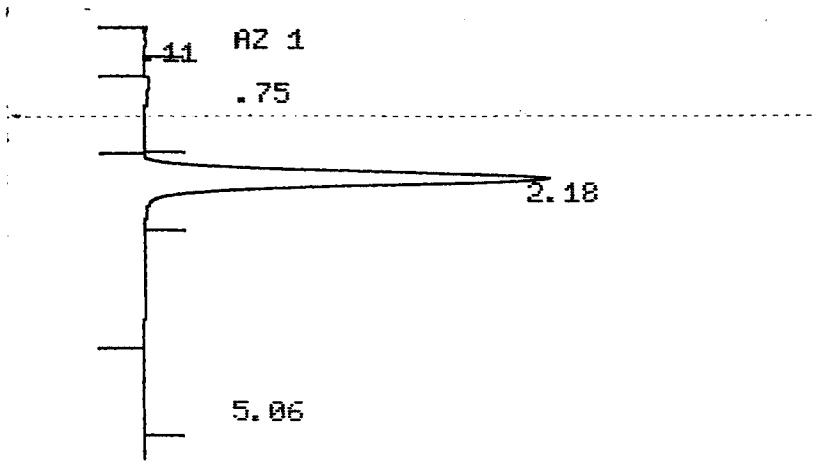

Figure A5 (a): Sample chromatogram for aqueous phase ethanol (2.18 min) analyzed by gas chromatography.

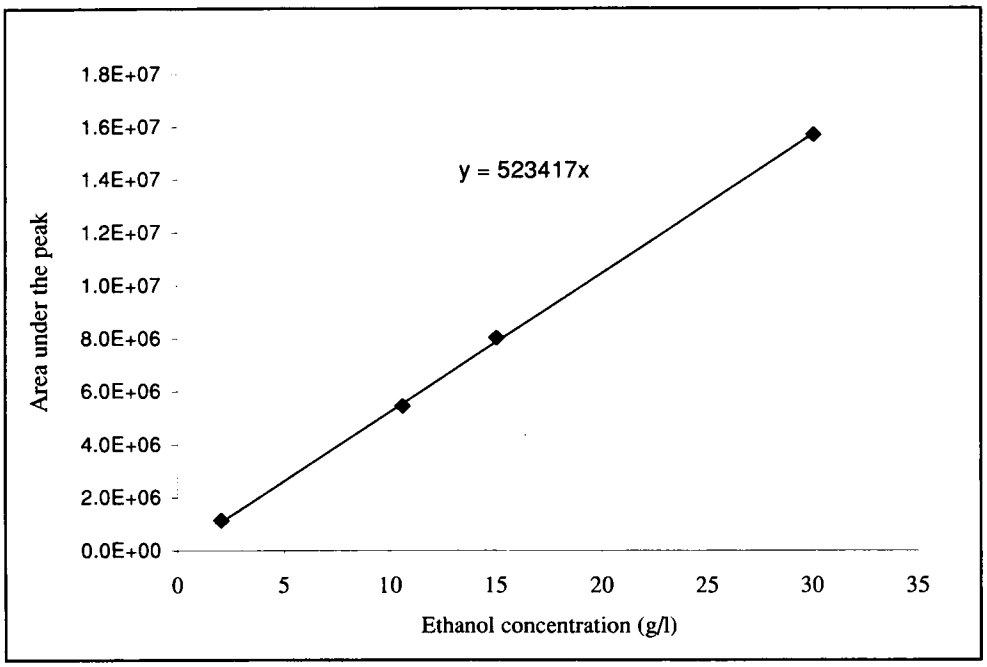

Figure A5 (b): Calibration curve for quantification of aqueous phase ethanol analyzed by gas chromatography.

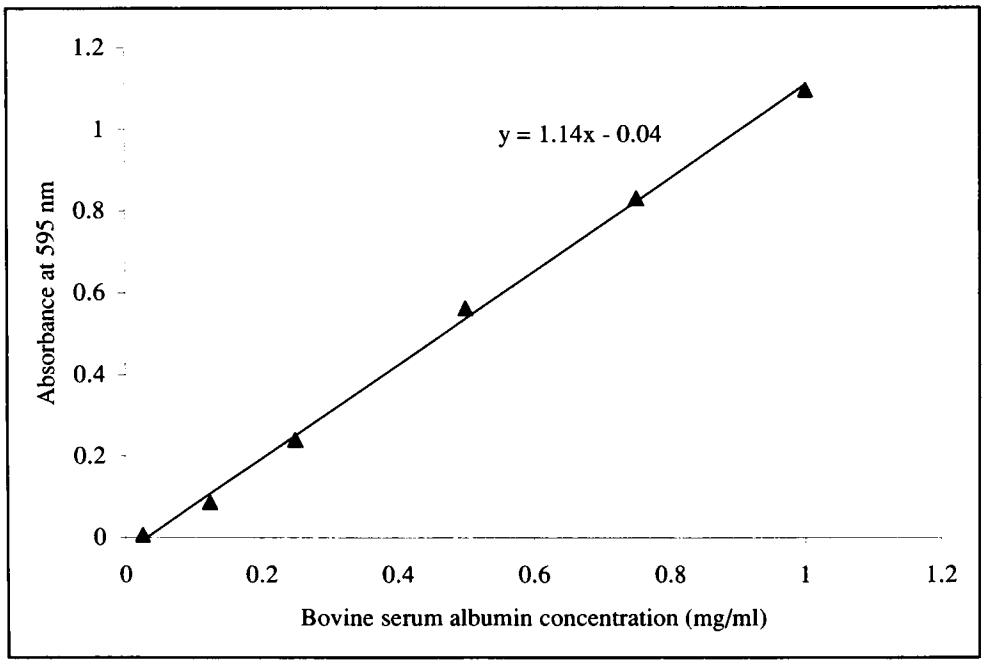

Figure A6: Calibration curve for bovine serum albumin (BSA) using the Coomassie® plus protein assay reagent.

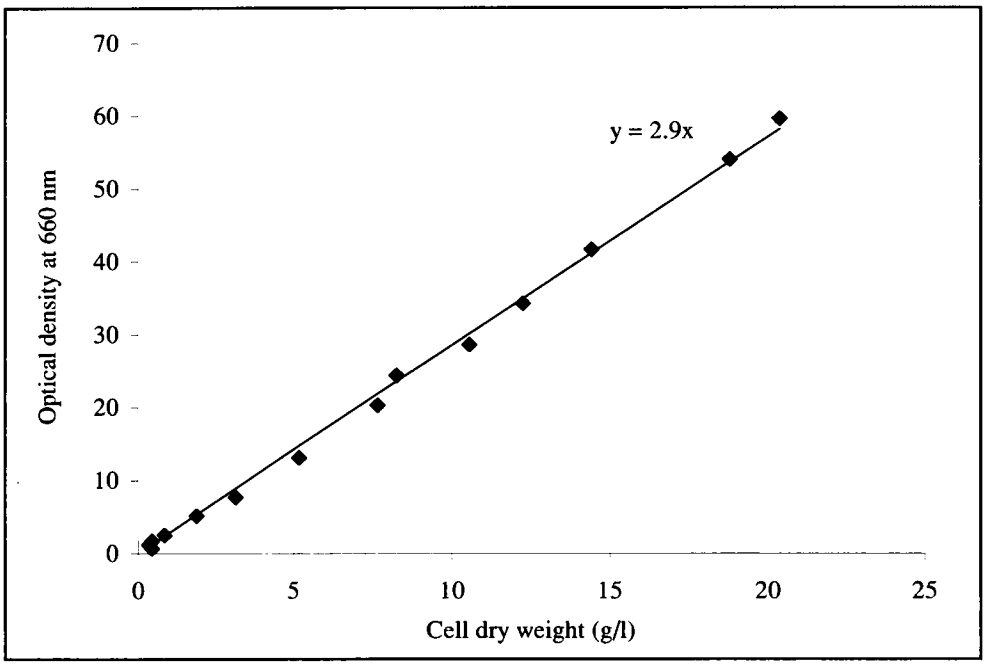

Figure A7: Correlation between optical density of fermentation samples and the cell dry weight for *C.utilis*.

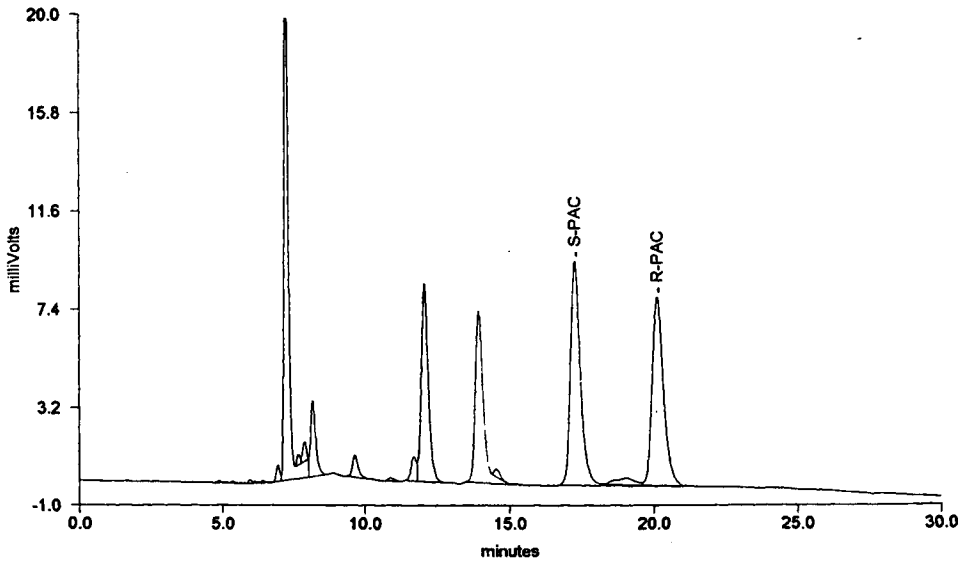

Figure A8: Sample Chromatogram for *R* (20.5 min) and *S* (17 min) enantiomers of PAC as analyzed by HPLC.

Appendix B

Abbreviations And Nomenclature

R-PAC	*R*-phenylacetylcarbinol
PDC	Pyruvate decarboxylase
RO	Reverse osmosis
USD	United States dollar
TPP	Thiamine pyrophosphate
MOPS	3-[N-morpholino]propanesulfonic acid
MES	2-[N-morpholino]ethanesulfonic acid
MTBE	Methyl-tert-butylether
MCH	Methylcyclohexane
Benz.	Benzaldehyde
EE	Enantiomeric excess
P	Partition coefficient
ND	Not determined
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NAD	Nicotinamide adenine dinucleotide (oxidized form)
ADH	Alcohol dehydrogenase
EMR	Enzyme membrane reactor
EDTA	Ethylenediaminetetraacetate
OUR	Oxygen uptake rate
CER	Carbon dioxide evolution rate
RQ	Respiratory quotient
AI	Activity index
BSA	Bovine serum albumin
HPLC	High performance liquid chromatography
GC	Gas chromatography

MS	Mass spectrophotometry
amu	Atomic mass units
U.V.	Ultra violet
NMR	Nuclear magnetic resonance
rpm	Revolutions per minute
RCF	Relative centrifugal force
FID	Flame ionization detector
Pty.	Proprietry
Ltd.	Limited
V or v	Volume
vvm	volume per volume per minute
w/v	weight per volume
v/v	volume per volume
µM	Micromolar
mM	Millimolar
M	Molar
A	Absorbance
w	Weight
g	Gram
mg	Milligram
^{o}C	Degrees celcius
kDa	Kilodalton
Da	Dalton
U	Unit
%	Percentage
$	Dollar
µm	Micromole
mmol	Millimole
mol	Mole
µl	Microlitre
ml	Millimeter
l	Litre

t	Time
min	Minute
h	Hour
nm	Nanometer
mm	Millimeter
cm	Centermeter
m	Meter
µm	Micrometer
psi	Pounds per square inch
Ø	Diameter
W	Watt