To My Family
and particularly Mum and Dad
without whose continued love and support
I may never have made it this far

THANKS

EVALUATION AND COMPARISON OF TWO-LIQUID PHASE HYDROLYTIC REACTION IN A STIRRED TANK AND MEMBRANE BIOCATALYTIC REACTOR

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by

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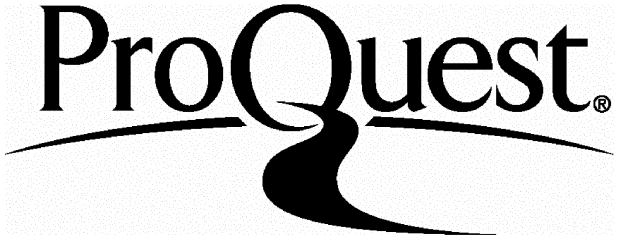

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Abstract

The introduction of a second liquid phase as a means to improving the productivity of bioreactions involving poorly water soluble compounds is now a fairly well established technique. It is particularly applicable for the generation of chiral intermediates via enzyme catalysed hydrolytic resolution. The work presented in this thesis attempts to address issues which will aid in the understanding of elements involved with the commercial development of such processes.

Hydrolytic reactions can be facilitated by both esterases and lipases. In this study a purified pig liver esterase and a crude porcine pancreatic lipase were found to catalyse hydrolysis of a model substrate benzyl acetate, in a two liquid phase medium, in a stirred tank reactor. In previous study pig liver esterase was found to catalyse hydrolysis solely in the aqueous phase, in contrast porcine pancreatic lipase was found to catalyse reaction at the interface.

In the stirred tank reactor, the activity of the esterase was dependent on the mass transfer conditions in the reactor as influenced by operating variables of phase ratio and agitation rate. Using data from previous studies and that obtained in this study mass transfer conditions were evaluated and optimum conditions of operation in the stirred tank reactor were identified. The optimal mass transfer conditions in the reactor did not support large aqueous phase concentrations of the pig liver esterase. The lipase activity was influenced by the amount of interface in the reactor. The optimal interfacial operating conditions supported large aqueous phase concentrations fo the lipase preparation at maximum activity.

The esterase was prone to interfacial effects and product inhibition and thus had limited stability. Resultingly overall conversion of substrate was low. In contrast the lipase was comparatively more stable and the overall conversion of substrate using lipase as the catalyst was much greater.

Although reaction could be carried out in the stirred tank reactor, difficulties were forseen in the downstream separation of reactor contents for product recovery and catalyst reuse. Conditions in the stirred tank reactor, necessary to maintain optimal enzyme activity, led to the irreversible destruction of the enzyme. To overcome some of these problems a further reaction device was studied, a membrane bioreactor. Fundamentally the two reactors differed in the way in which contact of the two phases was facilitated, in the stirred tank reactor this was by dispersion, in the membrane reactor by contact across a membrane surface, and the enzyme location, in the stirred tank reactor solubilised in the aqueous phase and in the membrane bioreactor immobilised onto the membrane surface.

The lipase catalysed hydrolysis was compared in the membrane reactor with its hydrolysis in the stirred tank reactor. The activity of the enzyme in the membrane reactor was severely limited in comparison to activity in the stirred tank reactor. Limitations were greatest at high enzyme loads. Several ideas were proposed as to why this might be. Although the activity of the enzyme was limited, the stability was much improved. The time required to achieve the same degree of conversion in the membrane reactor as achieved in the stirred tank reactor was greater. The membrane reactor facilitated phase separation throughout the course of the reaction and enzyme was reused in several subsequent reactions with some loss of activity between runs.

The results and methods used to obtain them provide useful tools and guidelines and a scientific basis which can be applied to the evaluation of similar reaction systems in order to identify the most effective process option.

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1. Introduction

1.1. Biocatalysis

Catalysis is the process by which compounds can be transformed to generate compounds having different propeties. The transformation is brought about by the presence of a compound which participates in the reaction but is not itself changed. The processes which define and sustain organic life are catalytic processes. Many of these processes employ proteinaceous structures known as enzymes as catalyst.

Although the catalyst has a specific function in the life process and acts on specific substrates, structural similarities of other compounds enable catalyst action on a variety of compounds which are not natural substrates for the catalyst. The exploitation of this property allows the access to a wide range of potentially useful compounds and the transformation of potentially harmful compounds to less harmful compounds. Many transformations can be achieved chemically. However a number of properties of these "life catalysts" or biocatalysts make them potentially more desirable than their chemical counterparts for transformation processes. They can be highly specific, giving rise to reduced by-products and optically pure products, are able to operate under relatively mild conditions of pH, temperature and pressure, have high catalytic activity and often the number of process steps to generate the final compound is reduced.

To facilitate transformations the biocatalyst may be utilised in its normal working environment, a whole cell biocatalyst. Alternatively the biocatalyst may be removed from this environment, either artificially or cellularly excreted, and used in this form, an enzymatic biocatalyst. The advantages of whole cell biocatalysts may be apparent where the transformation process as a whole requires the presence of more than one catalyst for completion or further energy in the form of co-factors. The use of a whole cell conveniently brings together the enzymes and cofactors in a convenient package. It also eliminates the need for further preparative operations. In contrast enzymes might provide advantages in terms of limiting alternative transformation processes, providing

higher catalytic activity with less limits to substrate access. Enzymes may be used in their crude form, in a purified state and attached to inert supports, immobilised. Similarly a whole cell biocatalyst may be used either in its native state or immobilised to an inert support.

Although previously enzymes were thought of as expensive catalysts, advancements in areas of biotechnology now provide a wide range of enzymes at comparatively low cost. Further genetic engineering has paved the way for the preparation of altered enzymes with improved physical and chemical properties. Enzymes are now applied to the generation of a wide range of products in the pharmaceutical, food, biotechnology and agricultural industries. One area where enzymes provide particular oppurtunities is in the synthesis of organic compounds and the exploitation of their specificities to the production of chiral compounds (Whitesides and Wong 1985, Akiyama *et al* 1988, Sih and Wu 1989).

1.2. Biotransformation of Poorly Water-soluble Organic Compounds

Some organic compounds are soluble in aqueous media and thus their transformation can be accomplished in conventional aqueous phase biocatalytic processes. However many potentially transformable organic compounds are poorly soluble in an aqueous environment. This poor solubility gives rise to poor volumetric productivities and limits cost effective production. A number of ways have been envisioned by which the biotransformation of poorly water-soluble organic compounds can be achieved more productively.

1.2.1. Cosolvent Systems

In aqueous-organic solvent mixtures, a water soluble solvent can be used resulting in increased solubility of poorly soluble substrates and reduced water activity. Water miscible organic solvents have been utilised to facilitate steroid transformations, (Fukui *et al* 1977, Freeman and Lilly 1987). Reduced water activity is useful in

carrying out synthesis reactions such as the synthesis of peptides, (Reslow *et al* 1988). However inclusion of water-miscible solvents only raises the solubilities to a limited extent. High cosolvent concentration will lead to loss of activity. It has been observed for an enzyme catalysed reaction that there is a dependence of activity on the cosolvent concentration, (Mozhaev *et al* 1989). Methods to stabilise enzyme against the denaturing effects of cosolvents have been reviewed (Mozhaev *et al* 1990a, Mozhaev *et al* 1990b).

1.2.2. Anhydrous Systems

Although an amount of water is necessary for enzyme activity it has been identified to be much less than is needed to form a monolayer on the enzyme surface, (Zaks and Klibanov 1988a and 1988b). In anhydrous organic solvent systems water content is reduced to an amount where no discrete aqueous phase is present. The enzyme is not soluble in this system and is present as suspended catalyst particles. The chemical modification of enzymes to facilitate solubility in anhydrous organic solvents has been suggested, (Inada *et al* 1986). Enzymes utilised in an anhydrous media may have a very rigid structure which may enhance its stability. Inactivation of enzymes generally requires water. The thermal stability of enzymes in anhydrous organic media has been recognised, (Zaks and Klibanov 1984).

1.2.3. Two-Liquid Phase Systems

A third alternative was recognised in which a reaction system consisting of two discrete phases; an aqueous phase and an organic phase can be employed, (Lilly 1982). The existence of two phase aqueous systems is also recognised for different water soluble polymers, (Andersson and Hahn-Hagerdal 1990), however these will not be considered. Two major types of two phase system might be distinguished, (Halling 1990). In one the amount of aqueous phase is relatively small and restricted to the

microenvironment of the biocatalyst, this is considered to be a virtually anhydrous system.

The second system is one in which the aqueous and organic phases are both present in relatively large amounts, for the purposes of definition this is considered to be a truly two-liquid phase system and the system of interest in this study. Figure 1.1 shows a schematic representation of a two-liquid phase reaction medium. The organic phase may be the reactant itself or act as a reservoir for poorly soluble reactants. The biocatalyst is contained within the aqueous phase and transfer of reactants and products between the two can take place

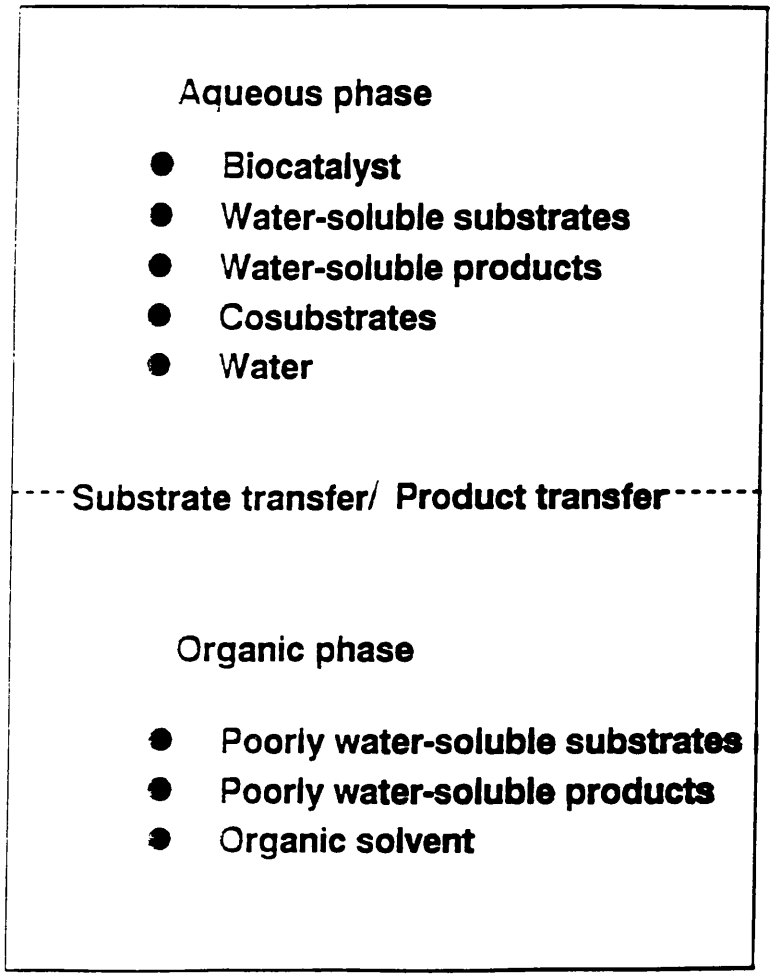


Figure 1.1 Schematic representation of two-liquid phase biocatalytic reaction medium

The arguments for the use of a two-liquid phase system are better solubility of substrates and/or products, the biotransformation of steroids serves as an early example (Carrea and Cremonesi 1987). In some cases product inhibition might be reduced by removal of product into the organic phase and thus away from the biocatalyst. Esterification and transesterification reactions , not normally feasible in aqueous media, are more feasible, (Borzeix 1992). Shifts in the reaction equilibrium can be facilitated, (Eggers *et al* 1989). Further recovery of products may be made easier by their concentration in the organic phase, (Woodley and Lilly 1990). Arguments against the use of organic solvent two phase systems are inhibition and catalyst deactivation and the potential for formation of difficult to separate emulsions.

1.3. Two-Liquid Phase System Classification

Figure 1.2, (Lilly *et al* 1987) shows a classification system based on the nature of the aqueous phase, continuous (A1 and B1) or discontinuous (A2, B2 and C3), and the catalyst form, soluble or insoluble. Further classification has been based on the number and distribution of reaction components, table 1.1, (Lilly and Woodley 1985). Whether the aqueous phase is continuous or discontinuos will be a consequence of the relative phase volumes. A two-phase system might also consist of enzyme in small water droplets as in A2, figure 1.2, stabilised by surfactants, (Martinek 1989).

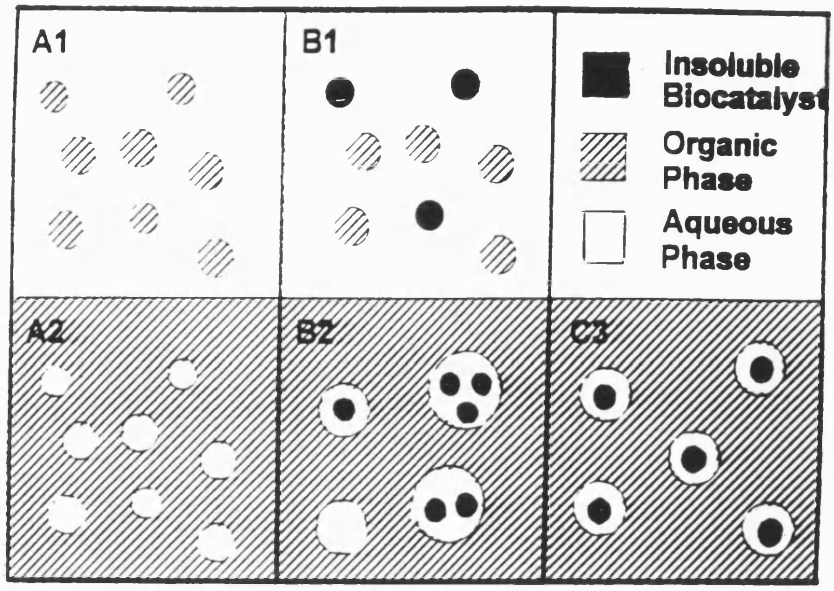


Figure 1.2 Diagramatic representation of two-liquid phase biocatalytic reaction systems. (From Lilly *et al* 1987).

Number of Components	Phase	
	Aqueous	Organic
TWO	P	S
	-	SP
THREE	S	P1,P2
	S1,S2	P
	S1	S2,P
	S1,P	S2
	P1	S,P2
FOUR	S1,P1	S2,P2
	-	S1,S2,P1,P2

Table 1.1 Product and Substrate distributions for two-liquid phase biocatalytic reactions. (From Lilly and Woodley 1985).

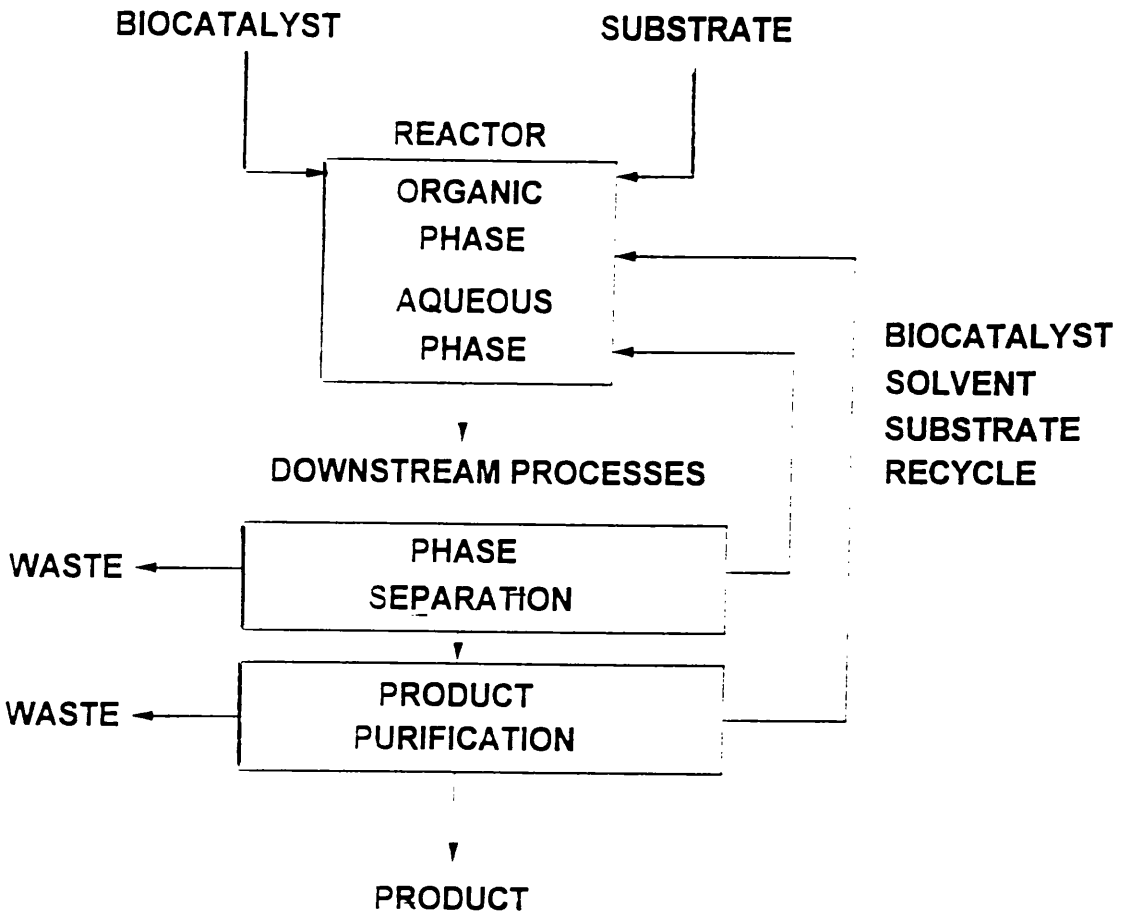


Figure 1.3 Two-liquid phase biocatalytic process schematic.

1.4. Two-liquid Phase Biotransformation: Process Considerations

The two-liquid phase process as a whole can be represented as shown in figure 1.3. Within this process a number of elements require consideration. These include elements of biocatalyst selection, solvent selection, elements of downstream processing and reactor options.

1.4.1. Biocatalyst Selection

The selection of suitable biocatalysts for application to two-phase biotransformation will depend on efficient screening programs to identify suitable biocatalysts able to carry out the transformation of interest at a high rate whilst retaining operational stability in the two liquid-phase reaction environment. Further to

this the efficient production of identified catalysts will be required. The production criteria will influence the performance characteristics. The biocatalyst will have implications in the design of the two-liquid phase biocatalytic process, (Woodley *et al* 1990). A number of issues for the operation of the biocatalyst in the two-phase system require consideration.

1.4.2. Solvent Selection

The choice of a suitable organic solvent with which to run the biotransformation is a prime concern. For a reaction where the substrate is the second liquid phase there is no choice of a second phase. Where the substrate is to be dissolved then a number of solvent types can be applied. such as alkanes, alkenes, esters, alcohols, ethers perfluorocarbons, ect. Solvent choice will be dependent on the use to which it is being put and will involve the consideration of substrate and product solubilties and partitioning between the phases. (Martinek *et al* 1981 and Eggers *et al* 1989). Further to this is the consideration of solvent on the basis of how it will affect the activity and stability of the enzyme. Measures of solvent toxicity have been proposed, (Laane *et al* 1987). Log P, which is the logarithm of the partition coefficient has been recognised as a suitable indicator. More recently a three dimensional approach to the prediction of solvent effects on enzymes has been proposed which takes into account polar, dispersive and hydrogen bonding interactions, (Schneider 1991). Another recent approach has been to consider selection based on the denaturation capacity, (Khmelnitsky *et al* 1991). As well as the application of single solvent, mixtures of solvents may lead to the generation of a more suitable second phase, (Bruce and Daugulis 1991).

1.4.3. Downstream Processing

Product recovery, substrate, solvent and biocatalyst reuse are all important aspects to be considered in the operation of a two liquid phase system for process

efficiency. Extractive biocatalysis in two-liquid phase systems is a means to improving product recovery, (Woodley and Lilly 1990). Phase separation upon reaction completion is necessary to remove product and reuse non-converted substrates and solvents. Separation might be facilitated by one of several methods, hydroclone, electrostatic coalescence, bed filtration, membrane filtration and gravity decanting are examples, (Woodley, thesis). Biocatalyst reuse might be facilitated by immobilisation. Other elements of the process will be influential on the downstream operations including elements of biocatatalyst selection, solvent selection and reactor options, (Lilly *et al* 1987).

1.4.4. Two liquid phase bioreactors

A key consideration in the two liquid phase bioprocess is selection of a suitable reactor. Attention is focused on the development of product specific, high productivity bioreactors. Prime concern for a two-liquid phase bioreactor is the contact of the two phases to allow transfer of substrates and products. A number of reactor types have been employed for the biotransformation of poorly soluble compounds by two-liquid phase technology.

1.5. Stirred Tank Reactors (STRs)

The most conventional approach has been the use of Stirred Tank Reactors (STRs). STRs are used in a variety of processes in both the biological and chemical industries. It is a suitable vessel for the operation of two-liquid phase biotransformations since agitation of the reactor contents results in the dispersion of one phase in the other and the necessary area of contact is generated.

Both bacterial and enzymic catalysts have been used in two-liquid phase systems operated in STRs. The stereospecific hydrolysis of *d,l*-menthyl acetate has been carried out in a STR using *Bacillus subtilis*. Mass transfer is an important criteria and influences the reaction rates. (Brookes *et al* 1986). Menthyl acetate hydrolysis has also

been carried out using the enzyme pig liver esterase, (PLE), as a catalyst in a STR, (Williams *et al* 1987). Mass transfer has also been implicated as influential on the kinetics of the enzyme cholesterol oxidase in a two-liquid phase STR, (Ramelmeier and Blanch 1989). Other biocatalytic reactions in two-liquid phase STRs have been studied, (Carrea *et al* 1988, Umemura and Hirohara 1989 and Mukataka *et al* 1985).

In STRs the volume of the two phases has a marked effect on mass transfer of substrates and products between the two phases, (Lilly *et al* 1990, Williams *et al* 1990). Rate of agitation is also influential on the transfer process, (Williams *et al* 1990). Phase ratio and rate of agitation have also been shown to influence the stability of biocatalysts, both bacterial and enzymic, in the presence of toxic solvents in STRs, (Williams *et al* 1987, Harrop *et al* 1992). Scale up of two-liquid phase biotransformations in STRs based on the maintenance of power input per unit volume has been discussed and illustrated using the hydrolysis of menthyl acetate by PLE, (Woodley 1990b).

Although STRs have proved useful for carrying out two-liquid phase biocatalytic processes, problems associated with substrate and product separation, continuous operation, and restricted access to the biocatalyst can be forseen. Some of the associated drawbacks might be overcome by operating in less conventional reactors. Some of those include packed bed reactors (Brady *et al* 1988, Yang and Rhee 1992), fluidised bed reactors (Kosugi *et al* 1990)), mixer settler cascades (Funada *et al* 1992) and liquid impelled loop reactors (Tramper *et al* 1987). However a more recent innovation in the area of biotechnology has been in the use of membranes bioreactors, (MBRs). The ability to combine separation with catalytic activity and biocatalyst retention makes them particularly interstesting in terms of overcoming some of the drawbacks of two-liquid phase bioprocesses in STRs.

1.6. Membrane Bioreactors (MBRs)

In the past membrane technologies have been applied to a wide range of applications in bioprocesses including air cleaning and sterilisation, separation duties

and functional membranes, biosensors. Membrane bioreactors utilise the ability of membrane material to separate liquid and/or solid phases while at the same time maintaining contact and selectively allowing specific components to transfer across the membrane.

1.6.1. Classification

Ultrafiltration as well as microfiltration and dialysis membranes have been used. Various materials have been used in the construction of the membranes. They can be operatated in a convective mode where solution is passed through the membrane under pressure. Alternatively, in diffusive mode, solutions pass either side of the membrane and diffusion of substrates and products can take place through the membrane. They can be operated in single pass operation in which medium enters at the inlet and exits at the outlet, essentially representing a plug flow reactor. Alternatively outlet solutions can be recirculated through the reactor and the reactor can be said to represent a well mixed reactor. Membranes can be either flat, spiral, tubular or hollow fibre and for commercial application can be packaged into modules to support high surface area to volume ratio. They will have a defined molecular cut off and thus exclude molecules of a certain size while allowing passage of smaller molecules. With respect to biocatalyst, two major groups can be distinguished based on the catalyst form. In one the biocatalyst is in solution and not attached to the membrane and in the other the biocatalyst is immobilised onto the membrane.

Biocatalyst solution systems have been applied in continuous stirred tank ultrafiltration reactors. A simple form of this is the stirred ultrafiltration cell in which a single flat membrane serves to retain biocatalyst in the reaction solution, figure 1.4a. A more suitable system for large scale operation is the use of a STR linked to an ultrafiltration module, figure 1.4b. The products of the reaction are separated in the ultrafiltration step while the unreacted substrate can either pass into the permeate or be retained. The biocatalyst is retained by the membrane and recirculates. Dialysis membrane systems have also been applied as flat membranes in STRs and as a separate

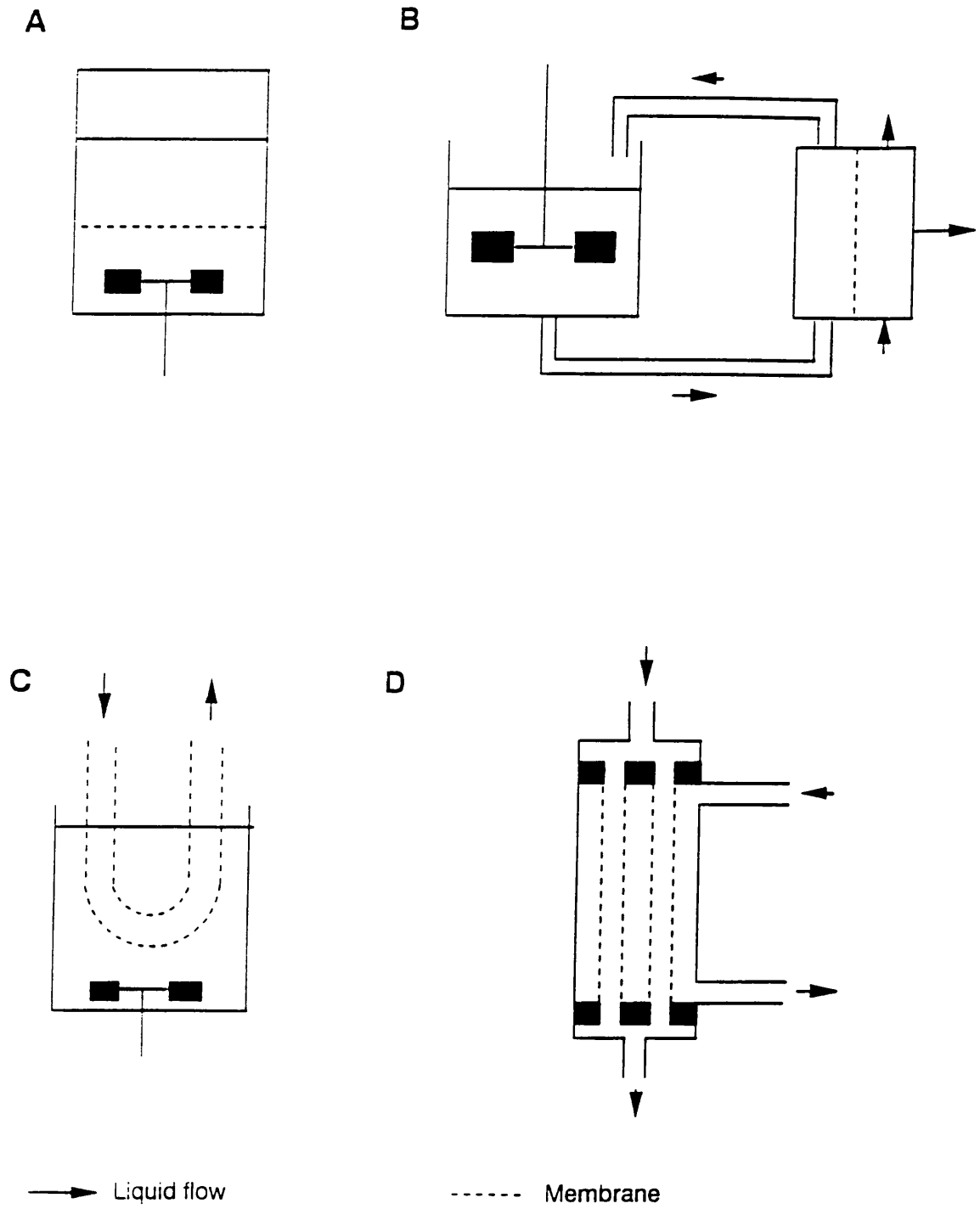


Figure 1.4 Membrane bioreactor types: Stirred ultrafiltration cell (a), Continuous stirred tank linked to ultrafiltration cell (b), Hollow fiber beaker dialyser (c), Hollow fibre module (d).

dialysis membrane unit linked to a STR. Dialysis membranes have also been applied in hollow-fibre beaker dialysers, figure 1.4c The biocatalyst solution is either in the beaker and substrate in the fibre or biocatalyst solution in the hollow fibre and substrate in the beaker. Hollow fibre reactors can also be used where substrate may circulate through the lumen of the membrane and biocatalyst solution through the shell side of the hollow fibre figure 1.4d..

The second group is one in which the membrane also functions as a support for the biocatalyst. This group can be divided based on the method of immobilistaion. The biocatalyst may be physically retained in the macroporous area of the membrane by adsorption. The biocatalyst may be immobilised by either filling the reactor with the biocatalyst solution for a period of time over which the biocatalyst adsorbs or by backflushing of the biocatalyst solution through the membrane, as the biocatalyst cannot pass the membrane it is retained in the macroporous area. Biocatalyst may also adsorb to the surface of a ultrafiltration membrane forming a gel layer. This method makes use of the concentration polarisation phenomenom. Adsorbed biocatalysts can be removed from the membrane and thus the membrane can be reused for further loadings.

Alternatively membranes may be prepared in which the biocatalyst is added to the casting solution when the enzyme is being prepared and thus the biocatalyst is irreversibly entrapped in the membrane. Biocatalyst can also be irreversibly immobilised to a membrane utilising the natural functional groups of the membrane for covalent linkage of the biocatalyst. Chemical treatment of previously adsorbed biocatalyst can generate covalent bonds without the necessity of reactive groups on the membrane.

1.6.2. Membrane Bioreactors in Biotechnology

Use of membrane bioreactors in the field of whole cell bioprocesses include their use in cell culture to remove inhibitive products away from the reaction environment. One of the first reactors to be designated a membrane bioreactor was applied to the culture of *Serratia marcescens* (Gerhardt and Gallup 1963) A 12 fold increase in cell

mass was achieved as a result of using dialysis culture in a STR. More recently an extractive bioprocessing of ethanol has been performed by immobilised yeast cells in a multimembrane unit (Steinmeyer *et al* 1988). Both anaerobic and aerobic cell culture have been undertaken in membrane devices. The anearobic bacterium *Lactobacillus delbreukii* has been immobilised in hollow fibre modules for the production of lactic acid. (Vick Roy *et al* 1982). Single hollow fibre systems are thought to be unsuitable for aerobic culture due to the problems of oxygen supply and cell leakage, (Inloes *et al* 1983). Means to overcome the problem of oxygen supply have been proposed in the form of dual hollow fibre systems. (Chang *et al* 1986). Membranes have been applied to the retention of cells in membrane recycle reactors for increased cell biomass and resulting productivity, (Vick Roy *et al* 1983, Schlote and Gottschalk 1986).

As well as the culture of bacterial cells membranes have also found application in the culture of both plant and animal cells. As early as 1972 human choriocarcnoma cells were grown on bundles of membrane fibres. A number of other workers have since developed hollow fibre systems for mammalian cell culture, (Tharakan and Chau 1986, Altshuler *et al* 1986). A hollow fibre reactor for the production of phenolics by plant cells was developed, (Jose *et al* 1983)

Membrane bioreactors utilising enzymes first emerged with the hydrolysis of starch carried out by Butterworth and co-workers, (Butterworth *et al* 1970). A number of applications of single phase enzyme membrane bioreactors can be recognised including hydrolysis reactions of high molecular weight substrates such as starch, (Butterworth *et al* 1970), cellulose (Hong *et al* 1981) and protein, (Cheryan and Deeslie 1982). Simple bioconversion reactions can be facilitated such as the hydrolysis of urea, (Furusaki *et al* 1990) and amino acid production, (Leuchtenberger and Plocker 1983). Cofactor requiring reactions can be carried out using membrane bioreactors, (Wandrey *et al* 1984), and are made more economical by the retention of cofactors. Complex multi enzymic reactions can be carried out using membrane immobilised enzyme reactors, (Matson and Quinn 1986). In reactions which require oxygen, membrane mediated transfer can be utilised limiting the direct contact of air with the enzyme protein which can often lead to its denaturation.

1.6.3. Membrane Bioreactors in Two Liquid Phase Bioprocesses

This ability for simultaneous separation and catalytic abilty makes enzyme membrane reactors potential candidates for use in the biotransformation of poorly soluble organic substrates by two-liquid phase reaction. The developments of membrane reactors for two liquid phase bioprocesses has been mainly in the food industry to carry out the conversions of fats and oils using lipases.

Continuous stirred tank (CST) membrane reactors have been investigated. A simple CST dialysis membrane reactor has been used for the hydrolysis of olive oil by lipase in a AOT-iso-octane reverse micellar system, (Chiang and Tsai 1992). The membrane served to retain the reversed micelles in the reactor while removing inhibitive product without removing surfactants. A CST ultrafiltration membrane bioreactor has similarly been investigated with respect to this application, (Prazeres *et al* 1993)

More common is the use of flat plate and hollow fibre enzyme membrane bioreactors. In an early study the synthesis of glycerides in a microporous flat sheet membrane reactor with hydrophobic character was investigated, (Hoq *et al* 1984). The enzyme was present in a glycerol water solution and separated from the fatty acid solution by the membrane. The glycerol-water-lipase solution was continually recycled through the reactor and exess water generated in the reaction removed from the recycle stream so as to limit the occurence of the hydrolytic reaction. Contact of the fatty acid feed stream with the gycerol-lipase water solution at the membrane surface facilitated the reaction and glycerides were collected in the feed stream. The fatty acid feed stream was not recycled. A number of parameters were investigated including the effects of water content, enzyme concentration, fatty acid flow rate and operational stability of the lipase. A 90 % conversion of the fatty acid to equimolar quantities of mono and diglycerides could intially be achieved in this reactor with a conversion of 70 % when the reactor was operated for one month. Further to this study Hoq and co-workers, (Hoq *et al* 1985a), also examined the glyceride synthesis by immobilised lipases from several bacterial strains using the same system. It was commented that the strength of

adsorption of the enzyme depended on the hydrophobicity of the enzyme itself as well as the nature of the interface. Some of the lipase is adsorbed directly on the interface in the pores and some is also adsorbed on the non-porous part of the membrane.

The hydrolysis reaction of olive oil has also been investigated in the hydrophobic microporous flat sheet membrane reactor, (Hoq *et al* 1985b). Liquid fat made up one process stream separated by the membrane from the other process stream, the aqueous phase buffer. In contrast to the synthesis studies, neither process stream was recycled. Glycerol was collected in the aqueous stream and fatty acid in the fat stream. Lipase was immobilised onto the membrane at the liquid interface and it was suggested that the adsorption was partially specific. Hydrolysis percent depended on the interfacial enzyme concentration. Significant differences between hydrolysis percentage was observed between process streams operated cocurrently and countercurrently. Flow rate, (mean residence time), of the buffer solution was again observed to influence the concentration of glycerol which accumulated in the buffer phase. Above a certain interfacial enzyme concentration no further increase in the percentage of hydrolysis was observed and it was suggested that this might be due to diffusional limitations. A stabilising effect of initial concentrations of glycerol in the buffer phase was in evidence although above a certain level the esterification reaction was favoured resulting in lower conversion.

The hydrolysis of olive oil by lipase was also investigated in a hollow fiber hydrophobic membrane bioreactor, (Hoq *et al* 1985c). Enzyme was absorbed onto oil impregnated hollow fibers at 2.5 units cm^{-1} in contrast to 12.4 units cm^{-1} for a flat sheet membrane reactor. Further comparison of the flat sheet reactor with the hollow fibre reactor was undertaken. (Yamane *et al* 1986a). The dependence of hydrolysis on the flow rate relative to membrane area was investigated and modelled. In the hollow fibre reactor rates of hydrolysis agreed with the developed model at comparatively high values of flow rate relative to membrane area. At lower flow rates it was suggested non uniform flow of oil through the hollow fibres was rate limiting. The hollow fiber had higher performance in terms of area/volume criteria than the flat sheet membrane reactor.

The glycerolysis of fat by lipase in solution has been investigated in a single flat sheet microporous hydrophobic membrane, (Yamane *et al* 1986b), and a modular system of flat sheet membranes, (Koizumi *et al* 1987). Effects of enzyme concentration. flow rates, flow directions and operational stability were assesed along with the effect of water content on the conversion of fat to glycerides. The scale up of the reactor from single flat sheet to the modular system could be achieved on the basis of oil flow rate to total membrane area. Types of membrane material for the operation of this system were also investigated and found to affect the reactor performance, (Yamane *et al* 1987).

More recently the hydrolysis of butterfat by a lipase from *Aspergillus niger* was investigated. The free fatty acids produced are important in flavour characterisation of dairy products. Intial study used a polypropylene microporous flat sheet membrane operated in a plug flow mode, (Malcata 1991a). Characterisation of adsorption of the crude lipase preparation revealed the specificity of adsorption for lipase proteins in the preparation. Half life of the immobilised lipase was 19.9 days at 35 ^{o}C. Overall degree of conversion was a function of reactor space time, dictated by the flow rate. Further study was carried out using a polypropylene microporous hollow fiber reactor. The physical adsorption of lipase onto the membrane was temperature dependent, (Malcata *et al* 1991b). The reactor was operated in a plug flow mode with buffer pumped through the lumen and melted butterfat pumped cocurrently through the shell side. The overall release of free fatty acid from the butterfat was modeled as a function of space time and time elapsed after immobilisation using rate expressions derived from kinetic analysis of the reaction, (Malcata *et al* 1992a). This model was further applied to the prediction of the free fatty acid profile of the product dependent on the flow rate (Malcata *et al* 1992b). Investigation of temperature effscts revealed desorption of the enzyme at temperatures above 35 ^{o}C and resultantly decreased conversion. Long term compensation for the loss of lipase activity should be obtained at the expense of larger residence times rather than by changing the operating temperature, (Malcata *et al* 1992c).

Advantages over the conventional batch process operated by the addition of free lipase to immobilised butterfat were: (1) no need for addition of emulsifiers and vigorous stirring, (2) the tangential flow pattern of oil and buffer leads to low pressure drop along the reactor. (3) natural feedstocks of butterfat can be processed without extensive plugging of the membrane, (4) retention of the lipase within the pores of the membrane decreases the potential for contamination of the product via residual lipase and avoids the need for thermal treatment of the efffluent stream, (5) the useful operational life of the lipase is extended. (6) the reactor can be easily regenerated following the release of adsorped lipase with ethanol, (7) crude enzyme preparations can be used as the source of lipase since the adsorption process is extremely selective towards lipase, (8) the space time of the reactor can be increased in order to maintain a constant level of hydrolysis as lipase activity declines.

More recently a spiral wound membrane reactor has been used to effect the hydrolysis of milkfat using an immobilised *Candida rugosa* lipase, (Garcia *et al* 1992). In contrast to the hollow fibre and flat sheet membranes the feedstock is not fed as separate phases but the oil/aqueous mixture is fed as an emulsion at one end of the reactor, the hydrolytic reaction takes place on passage through the reactor and product leaves the reactor ar the other end. The advantages to be gained by operating this type of reactor, in contrast to a hollow fibre reactor for the same system, were a greater amount of enzyme adsorbed per unit of membrane area and a high ratio of exposed area to volume of the emulsion which helps to avoid diffusional limitations on conversion. A disadvantage is that product still leaves the reactor in an emulsion.

The production of fatty acids has also been investigated employing the hydrolysis of tallow by a thermostable lipase from *Thermomyces lanuginosus*. The reactor is a single hollow fiber with one end of the lumen and one end of the shell blocked off. The enzyme was immobilised onto the membrane by microfiltration. Two modes of operation were performed in which tallow entered the lumen or the shell, passed through the membrane and exited with the aqueous phase into a separator, (Taylor *et al* 1986). Immobilisation onto the membrane might lead to clogging and impaired filtration of tallow. however this phenomenon was not in evidence. It was reported that

the stability of the lipase was much improved by the application of this reactor and maintenance of pH, the reactor was operated for up to 4 months. The conversion of the tallow varied between a few percent to 35 % depending on the activity and the tallow feed rate. The activity of the immobilised enzyme was found to be limited at higher enzyme loadings. Comparison of flat sheet membrane reactors of acrylic and glass fiber for the hydrolysis of tallow by its passage through the lipase activated membrane and collection in the aqueous phase has been carried out, (Taylor and Craig 1991). Activity of the enzyme supported by glass fiber was a little higher than the acrylic support. Scale up to a unit of ten sheets of the glass fiber membrane showed a reduction in the activity upon scale up at similar enzyme loads and similar oil fluxes, expressed on the basis of membrane area. It was suggested that the reduced activity on scale up resulted from uneven flow distribution amoung the layers.

As well as hydrophobic membrane materials, hydrophilic membranes have also been investigated. A hollow fiber cellulose membrane reactor has been investigated to determine its suitability to carry out the hydrolysis of triglycerides, (Pronk *et al* 1988). Enzyme immobilisation was by ultrafiltration of enzyme solution from the lumen to the shell of the fibers. Concentration polarisation of lipase resulted in the formation of a "gel layer" on the lumen side surface of the membrane. In this study lipid and aqueous phases were recirculated, lipid through the lumen of the reactor, aqueous phase through the shell, it was considered a perfectly mixed reactor. Inactivation of the lipase during the reaction is a result of enzyme denaturation and not enzyme washout. Good enzyme stability was achieved with an enzyme half life of 43 days at a temperature of 30 ^{o}C. Replacement of the aqueous phase, each day, stopped the build up of inhibitive glycerol concentrations in that phase. The reactor was operated in batch mode and continuous mode. In continuous mode a predetermined flow of lipid was added to a lipid reservoir vessel within the lumen circuit and an equal flow of product removed from the vessel.

A lewis cell with defined interfacial area was compared with a hydrophilic membrane reactor for the hydrolysis reaction of *Candida rugosa* lipase to provide insight into the effects of enzyme concentration and interfacial behaviour, (Pronk and

van't Riet 1991). Interfacial activity of the lipase is the same in the membrane reactor as in the lewis cell at below saturation concentration. At above saturation concentration the interfacial activity in the STR is higher than the lewis cell. The specificity of lipase adsorption was experimentally evidenced in this study by its preferential adsorption in a solution containing BSA. The effect of temperature and pH on the free and membrane immobilised enzyme were also compared, (Pronk *et al* 1992). The pH optima of the enzyme were identical for both free and immobilised enzyme. The enzyme immobilised to the membrane was thermally more stable thans its free counterpart. Effecters of lipase which give rise to increased activity such as NaCl resulted in decreased stability of the lipase in the membrane bioreactor when the effector was present at high concentration. This was a result of destruction of the ionic interactions which help hold the lipase onto the membrane.

The hydrolysis of triacetin has been carried out using a hydrophilic hollow fibre membrane bioreactor in which the fibres were made of polyacrylic nitrile and one in which the fibres were made of cellulose, (Guit *et al* 1991). The oil was fed to the lumen of the reactors either as the neat oil or in various concentrations dissolved in toluene. Both substrate and aqueous phase were recirculated through the reactor and the reactor system was described as a perfectly mixed reactor operated in batch mode. The *Candida cylindracea* lipase employed in the study immobilised equally well onto both supports and its activity and stability were not influenced by the choice of support. The half life of the enzyme in both membrane bioreactors was 6 days. Comparison of the activity of the enzyme immobilised in the polyacrylic nitrile membrane reactor with the enzyme in its native state in an emulsion reactor showed a greater activity of the enzyme immobilised in the membrane bioreactor.

Applications of membrane bioreactors to the hydrolysis of poorly soluble organic substrates also exist. The main application has been recognised in the generation of chiral intermediates for the pharmaceuticals industry (Bratzler 1987 and Young and Bratzler 1990). The enzymatic resolution of N-benzoyl tyrosine ethyl ester (D.L.-BTEE) has been achieved using chymotrypsin immobilised in an assymetric hydrophilic hollow fibre membrane unit. (Matson and Lopez 1989). The substrate was

dissolved in octanol and circulated through the membrane lumen while the aqueous phase buffer was circulated through the shell side. The reactor was operated in a batch mode with reactions running to completion. The operation of the reactor was compared at low and high enzyme loads with respect to effects on the stereoselectivity of the enzyme. The use of high enzyme loads, to maintain high productivity in the reactor and minimise the required membrane area, led to reduced stereospecificity of the reaction. This was a result of diffusional limitations.

Using an assymetric hollow fiber membrane unit the resolution of Ibuprofen trifluoroethyl ester using a *Candida* lipase has been achieved (McConville *et al* 1990). Organic phase was circulated through the lumen and aqueous phase through the shell. The reactor was operated in batch mode. In the membrane bioreactor there was a low ratio of enzyme activity expressed to activity loaded as result of diffusional limitations. The membrane bioreactor in this study was coupled to a membrane extractor for product removal and the process operated continually for over 3-4 months with an enzyme half life of about 1 month.

1.7. Objectives

Two-liquid phase processes using biocatalysts to convert poorly soluble compounds demand commercial application in a cost effective manner. Methods which help to establish an understanding of the reactions benefit commercial application of the process. These methods need to address the collection of data which aids in the establishment of general scientific principles and rules governing the operation of two-liquid phase processs thus enabling optimisation. One key element in the design of these processes is the reactor engineering. Reactor engineering must primarily provide a rationale for reactor choice and process optimisation. In order to provide these elements model systems which have general "commonalities" with the many other systems to which two-liquid phase process can be applied must be studied.

1.7.1. Model Systems

Enzymes are useful to convert organic compounds. As a model catalyst to investigate aspects of two liquid phase reaction they are more suitable than a bacterial catalyst. A bacterial catalyst requires first to be grown, the growth conditions will influence the associated biocatalytic activity, variations may occur between different batches. The surfactant properties of cells, their behaviour at the liquid-liquid interface and the influence of cell concentration on liquid viscocity adds to the number of factors which need to be considered and further complicates interpretation of the results.

One group of enzymes proving to be useful in the bioconversion of organic compounds is the hydrolytic enzymes. Of this group esterases and lipases are particularly attractive. They are able carry out the hydrolysis, esterification and interesterification of a wide variety of esters, acids and alcohols. Hydrolysis involves an attack on the ester bond in the presence of water molecules to produce both an alcohol and a carboxylic acid. Esterification reactions between alcohol and free acid are the reverse of the hydrolysis reaction. Transesterification refers to the exchange of acyl radicals between an ester and an acid, (acidolysis), an ester and an alcohol, (alcoholysis) or an ester and another ester, (transesterification). There is no requirement for cofactors/coenzymes and their specificities provide ideal oppurtunities for assymetric synthesis and resolution. A large number are available from different sources, both animal and bacterial.

1.7.1.1. Pig Liver Esterase (PLE) Catalysed Hydrolysis

Pig liver esterase, (PLE E.C.3.1.1.1), is an esterase with considerable potential in organic synthesis. Its application in assymetric synthesis is particularly well characterised. A number of studies have been carried out with a wide range of symmetric diesters and monoesters, (Zhu and Tedford 1990). It has been particularly well characterised with respect to its application as a catalyst in a two-liquid phase system.

The hydrolysis of menthyl acetate to menthol and acetic acid has been carried out in a two-liquid phase STR. The activity and stability of the catalyst were affected by the acetic acid product of the reaction and the presence of gas-liquid and liquid-liquid interfacial area, (Williams *et al* 1987). The operating parameters of phase ratio, stirrer speed and aqueous phase enzyme concentration also affect the activity associated with the enzyme. For phase ratio changes amount of product formed in 1 hour per gram of enzyme was constant up to a phase ratio of 0.5 but fell at higher values. At a rate of agitation of 2500 rpm product formation rate was comparatively lower than at a rate of agitation of 1500 rpm at fixed phase ratio. Product formed by PLE in 1 hour was independent of its aqueous phase enzyme concentration up to about 0.1 g l^{-1} but decreased at higher concentrations, (Williams *et al* 1990).

Although hydrolysis of menthyl acetate by PLE has recognised the effect of various parameters in the two liquid phase STR a more thorough study has been undertaken for the hydrolysis of benzyl acetate. This reaction is illustrated in figure 1.5.

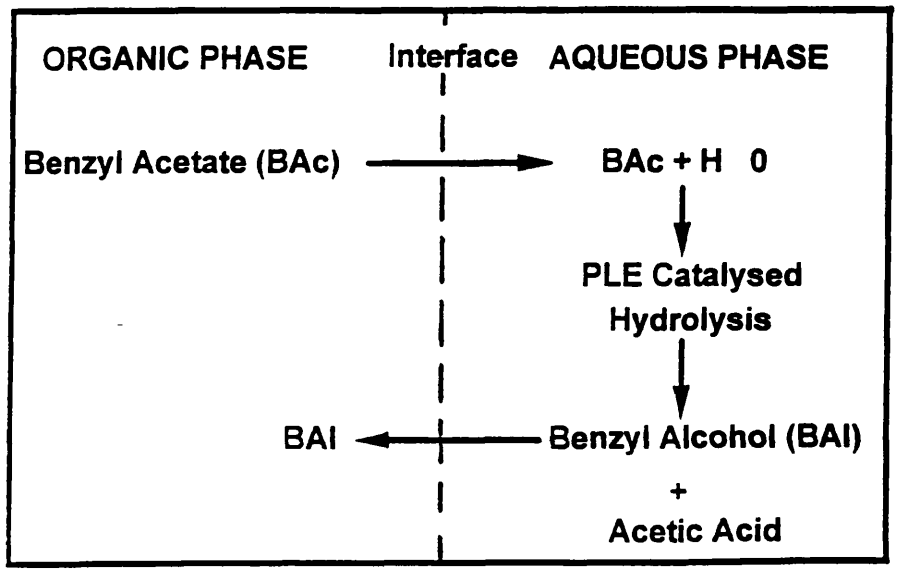


Figure 1.5 Schematic of the hydrolysis of benzyl acetate by pig liver esterase (PLE) in a two-liquid phase biocatalytic reaction system.

There are several advantages to the use of benzyl acetate as a model system in order to elucidate kinetic mechanisms and develop models for an organic-aqueous two-liquid phase system and these are listed below, (Woodley Thesis).

1. There is no requirement for any gas phase components which would add another level of complexity.

2. The kinetic analysis is simplified since the reaction only involves two substrates, one being water in exess and the other the poorly water- soluble benzyl acetate, the rate limiting substrate thus pseudo-one substrate kinetics can be assumed.

3 Benzyl acetate, although poorly water soluble, can be measured in an aqueous phase by HPLC thus making intrinsic kinetic determinations possible. Its saturation concentration in the aqueous phase is lower than the product, benzyl alcohol. This is essential in the measurement of intrinsic kinetics since the benzyl alcohol will not form a second liqid phase and partition the substrate away from the reaction environment.

4. Benzyl acetate itself will form the second liquid phase. It is not necessary to solubilise in a second water immiscible organic solvent with the added complications of selection that will incur. Use of the substrate as organic phase solvent is advantageous in terms of maintaining the equilibrium between organic phase interfacial substrate concentration and aqueous phase interfacial substrate concentration.

Physical properties and chemical structures of the phase components are given in table 1.2.

An analysis of the interaction between substrate transfer and biocatalytic reaction in a two-liquid phase STR has been performed for the PLE catalysed hydrolysis of benzyl acetate and this has proved a valued tool to elucidate the reaction location, (Woodley *et al* 1991).

The intrinsic kinetics of PLE were elucidated in aqueous solutions over a range of substrate concentrations up to saturation concentration, the results of which are depicted in figure 1.6. A maximum reaction rate of 36.5 mmol min^{-1} g^{-1} was observed at substrate concentrations much less than the aqueous phase saturation concentration of 13.5 mM. First order kinetics were observed up to a concentration of 4mM, above which zero order kinetics are observed.

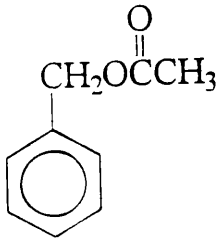
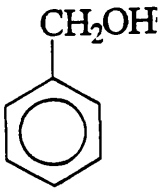
	Reaction Components			
Property	**Substrates**		**Products**	
	Benzyl Acetate	**Phosphate Buffer**	**Benzyl Alcohol**	**Acetic Acid**
MW (g)	150.17	18.016	108.13	60.05
Density ($Kg\ m^{-3}$)	1055.17 at 17°C	998.2 at 20°C	1043 at 20°C	1049 at 20°C
B.Pt (°C)	213.5	100	204.7	118.1
F.Pt (°C)	-51.5	0.0	-15.3	16.7
Log P	1.96	-	1.1	-
Surface tension ($Dynes\ cm^{-1}$)	-	73.05 at 18°C	39.0 at 20°C	27.8 at 20°C
Structure		-		-

Table 1.2. Properties of the model system (From Woodley Thesis)

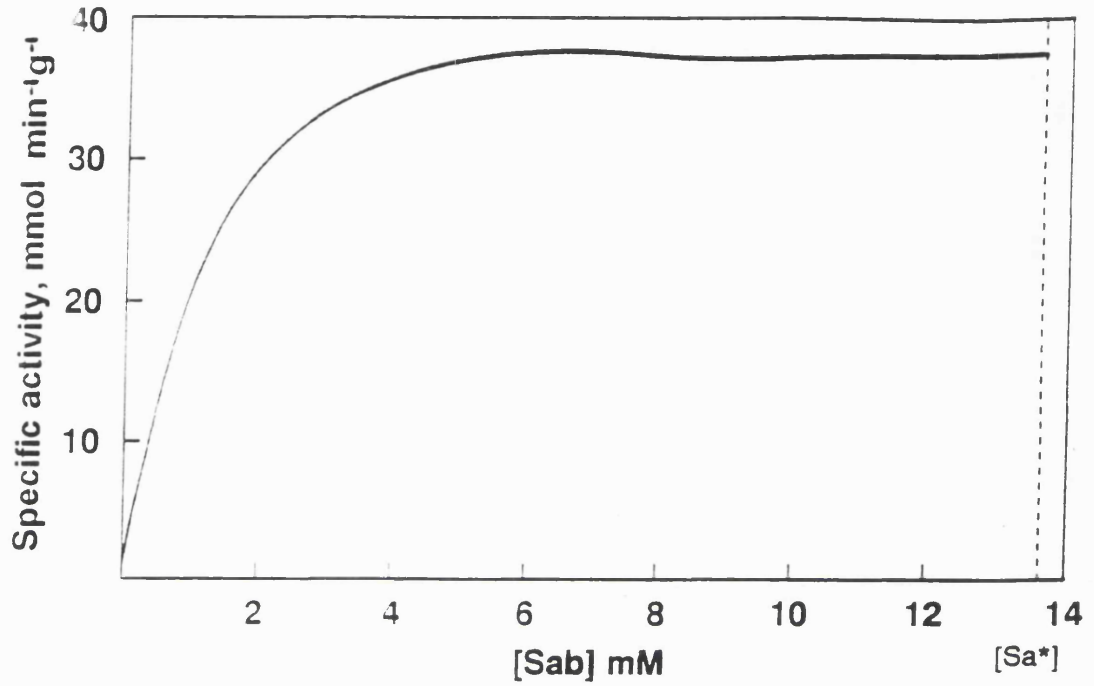


Figure 1.6 Aqueous phase kinetics for hydrolysis of benzyl acetate by PLE. (From Woodley *et al* 1991a); Bulk aqueous phase substrate concentration [Sab], Aqueous phase saturation concentration [Sa*]

Substrate transfer rates were determined in a modified Lewis cell in which the substrate is placed alongside aqueous phase, (excluding catalyst), and both are gently stirred without disturbing the interface. In such apparatus the phase boundary is defined and thus measurable. The rate of transfer of the substrate was determined by measuring the concentration of substrate in the aqueous phase as a function of time. Integration of these values gives a value for the mass transfer coefficient. This value gives a gradient of a linear plot of the transfer rate as a function of aqueous phase substrate concentration, figure 1.7.

The superimposition of the plot in figure 1.7 onto the plot shown in figure 1.6 gave the plot of figure 1.8. The point of intersection of the two plots is the steady state condition where the rates of mass transfer and reaction are equal. At aqueous phase substrate concentrations above this intersection the biocatalytic reaction rate is in exess of the subtrate transfer rate leading to a decrease in aqueous phase substrate concentration to the steady state point of intersection. Conversely, at aqueous phase substrate concentrations below this intersection, the substrate transfer rate is in exess of the biocatalytic reaction rate leading to a rise in aqueous phase substrate concentration to the steady state point of intersection. The point of intersection of the two lines is characteristic of a bulk aqueous phase catalyst.

The aqueous phase bulk reaction model for PLE was also futher evidenced in lewis cell studies. A plot of observed lewis cell reaction rate results, for different sizes of interface and catalyst concentration, against results predicted using the aqueous phase bulk reaction model showed good agreement of the two, (Woodley *et al* 1991)

Since the hydrolysis by PLE can be described by the bulk aqueous phase model then reaction rate will be dependent on bulk aqueous phase substrate concentration and biocatalyst concentration. The bulk aqueous phase concentration of substrate will be dependent on the substrate K_LA. The overall reaction rate will be governed by substrate K_LA and biocatalyst concentration. Optimal operation will occur when the ratio of K_LA to catalyst concentration is such that both potential mass transfer duty and catalytic activity are fully used. For the PLE catalysed hydrolysis of benzyl acetate this relationship has been elucidated, figure 1.9. The line running diagonally through the

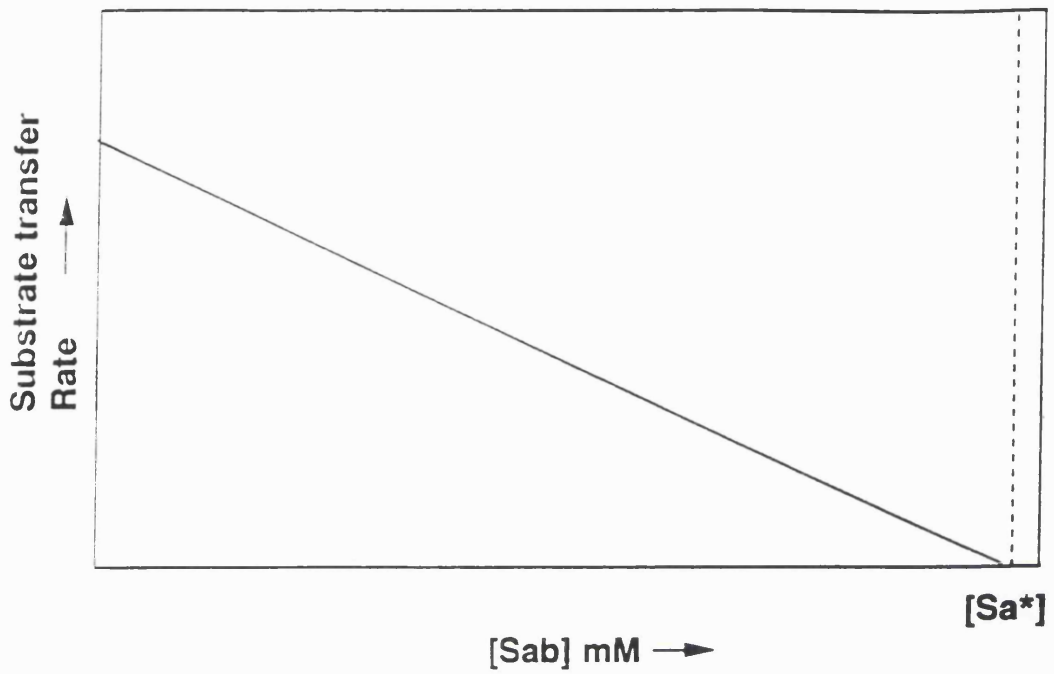


Figure 1.7 Schematic of substrate transfer rate as a function of aqueous phase substrate concentration. Transfer rate = $K_L A$ ([Sa*] - [Sab]). (From Woodley 1990a).

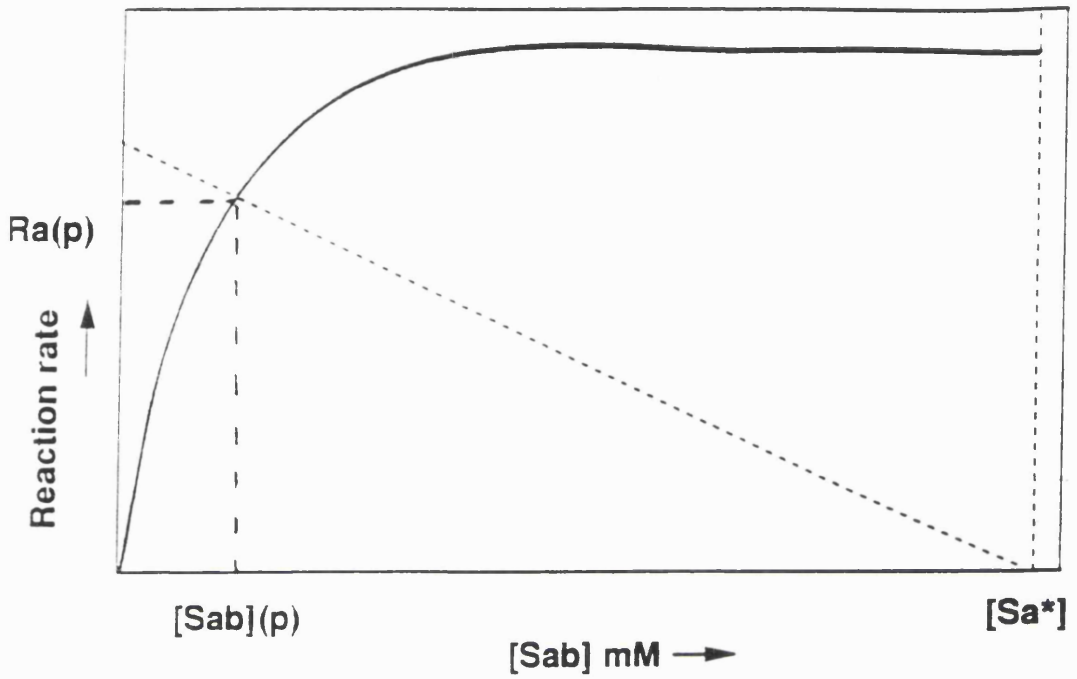


Figure 1.8 Schematic plot of substrate mass transfer rate, K_LA, (------) and biocatalytic reaction rate, (------), as a function of aqueous phase substrate concentration showing the steady state bulk reaction model predictions of rate Ra(p) and aqueous phase substrate concentration [Sab](p). (From Woodley 1990a).

centre of the chart represents optimal operating conditions where enzyme concentration is matched with the minimal $K_L A$ to achieve maximal rate for the PLE of 36.5 mmol min^{-1} g^{-1}.

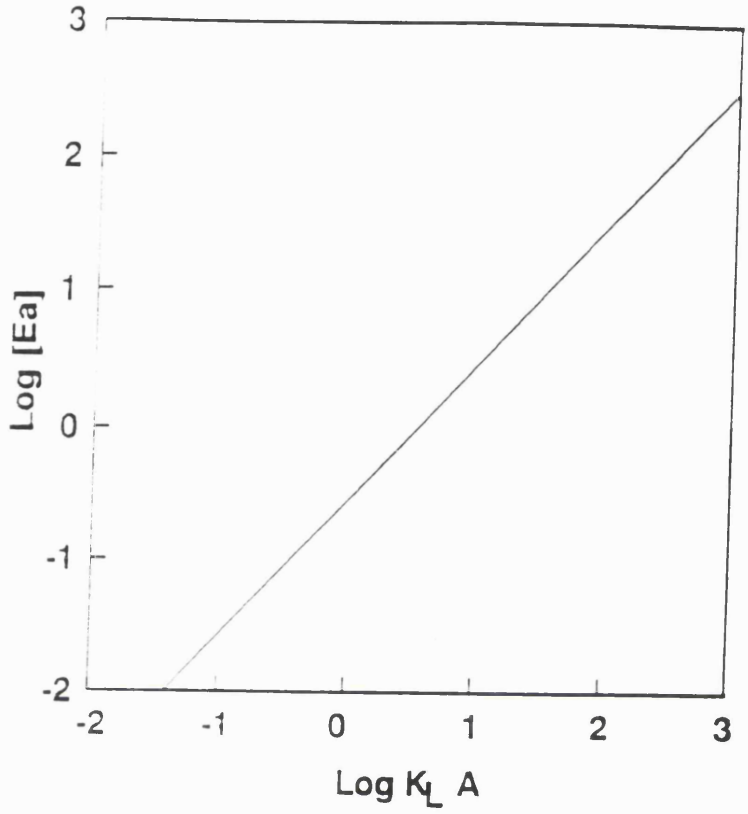


Figure 1.9 Design chart showing optimal conditions of mass transfer and aqueous phase enzyme concentration for benzyl acetate hydrolysis by PLE. (From Woodley 1990a).

Product transfer models for the hydrolysis of benzyl acetate by PLE have also been presented. Lewis cell studies were used to model product transfer in the absence of reaction. Experimental data presented from lewis cell studies in the presence of reaction demonstrated the applicability of the model for predicting phase product concentrations in the two-liquid phase reaction medium as a function of product $K_L A$. This has important implications particularly with regard to product inhibited reactions and its removal from the aqueous reaction environment. Product transfer is however not a problem for the hydrolysis of benzyl acetate with PLE, (Woodley and Lilly 1990).

1.7.1.2. Lipase Catalysed Hydrolysis

An alternative to using an esterase to carry out an hydrolytic reaction is to use a lipase. In contrast to esterases, which act primarily on dissolved substrates in the aqueous phase, lipases are most active at interfaces, (Verger and Haas 1974). At an interface the lipase molecule undergoes a comformational change that exposes the interface binding site, (O'Connor and Bailey 1988). Further comformational change as a result of binding makes the active site accessible to the substrate, (Brady *et al* 1990, Winkler *et al* 1990). This activity associated with an interface makes them particularly interesting for application in a biphasic system in which a liquid-liquid interface is present.

Previous studies have been carried out using lipases in two-liquid phase systems. The hydrolysis of 2-Naphtyl esters has been investigated in a lewis cell to determine kinetics of the enzyme in a two-liquid phase system and compare them with the kinetics in an aqueous system. Activities were greater in the two-liquid phase system, (Miyake *et al* 1991). The nature of the interface in the biphasic system may influence the lipase kinetics. The adsorption of a candidal lipase to both a tributyrin-water interface and a heptane-water interface has been studied. The lipase had a greater affinity for the tributyrin-water interface than the heptane-water interface, (Ekiz *et al* 1988)

Lipases in two-phase STR systems have been applied to the modification of fats and oils, (Halling 1990). An investigation into the hydrolysis of tributyrin by a *Candida cylindracea* lipase has been carried out in a STR, (Martinez *et al* 1992). In this study the dependence of the reaction rate on the phase ratio and speed of agitation was elucidated and modeled with respect to kinetics. At phase ratios below 0.1 increased rate of agitation is necessary to achieve high conversion. Above phase ratio of 0.1 an increase in the speed of agitation had no significant influence.

A wide variety of lipases remain catalytically active in systems containing high proportions of water-immiscible organic liquids. At low water levels in the reaction medium esterification and transesterification reactions are promoted. At higher levels of water the hydrolytic reaction is promoted. The lipase-mediated synthesis of

carbohydrate esters has been investigated in a two phase system using 2-pyrrolidone as a cosolvent for sorbitol, (Janssen *et al* 1991). The water concentration was seen to influence enzyme activity. At high concentrations of water the equilibrium was driven towards hydrolysis. The suitability of a two-liquid phase system for the synthesis of butyl butyrate by esterification using a lipase has been demonstrated. The rate of agitation of the two-liquid phase medium influenced the activity of the lipase, (Borzeix *et al* 1992).

A kinetic analysis of the hydrolysis of olive oil in a two-liquid phase organic-aqueous STR revealed an influence of the phase ratio on the reaction rate and equilibrium conversion. With increasing rate of agitation the rate of reaction initially increased rapidly but was found to increase more slowly at higher rates of agitation, (Tsai *et al* 1991). The hydrolysis of palm oil and beef tallow have also been investigated in two-liquid phase isooctane-aqueous system using high concentrations of substrate in a STR. The degree of hydrolysis was affected by the volume percent of isooctane in the reaction mixture. A 20 % isooctane system brought about the most rapid reaction and highest percentage of hydrolysis, (Mukataka *et al* 1987).

Lipase catalysed hydrolysis has been applied to the resolution of enantiomers in two-liquid phase organic-aqueous systems. Charcteristics of the hydrolysis of an acetic ester in a two-liquid phase system showed a dependence of the activity on the rate of agitation, (Mitsuda *et al* 1989). The enantioselective hydrolysis of Cyano-3-phenoxy benzyl acetate has been studied using an *Arthrobacter* lipase in an organic-aqueous system to elucidate the kinetics of both the free and immobilised lipase, (Mitsuda *et al* 1990). Several different lipases have been used for the hydrolysis of diacetate esters in both the native and immobilised form, (Patel *et al* 1990 and 1992). Lipases have found application in the generation of chiral intermediates for the preparation of β blockers, (Kloosterman *et al* 1988). R,S glycidyl ester has been resolved using porcine pancreatic lipase (PPL). This is a poorly water soluble ester and thus its conversion for commercial production is best achieved in a two-liquid phase system, (Kierkels *et al* 1990). PPL is also able to catalyse the hydrolysis of benzyl acetate and will serve as the model lipase for comparison with the esterase. The reaction is illustrated in figure 1.10..

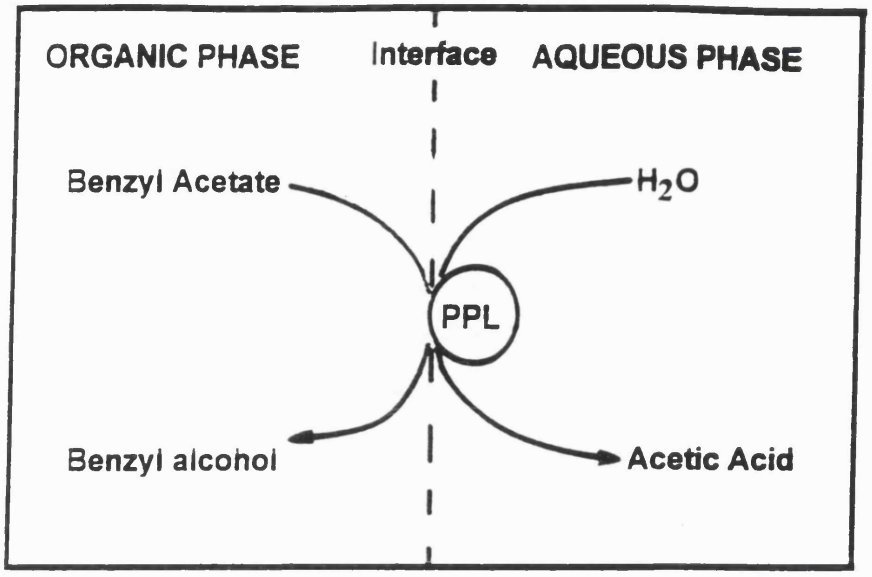


Figure 1.10 Schematic of the hydrolysis of benzyl acetate by porcine pancreactic lipase (PPL) in a two liquid phase biocatalytic reaction system.

1.7.2. Study Outline

The fundamental difference between the two reactors is in the area available for phase contact and how this is defined. In the STR interfacial area depends on the dispersion of one phase within the other, and this will be a function of droplet size. The droplet size will be a function of the STR operating variables. In the MBR the interface is a fixed, absolute area, for a particular module. In this study a commercially available hollow fibre MBR system is to be used. It is made up of a number of individual fibres contained in a module, this allows a large area to phase volume ratio and a relatively large amount of enzyme can be immobilised. The two reactor types are shematically illustrated in figure 1.11 for the STR and 1.12 for the MBR.

In this study the kinetics of the esterase and lipase catalysed hydrolysis will be studied in the STR. Catalyst will be used in its soluble form. In the STR mass transfer-reaction interaction will be studied. Influences of reactor operating variables of enzyme concentration, phase ratio and rate of agitation upon this interaction and the stability of the enzymes will be investigated. It is the intention of these studies to identify the key

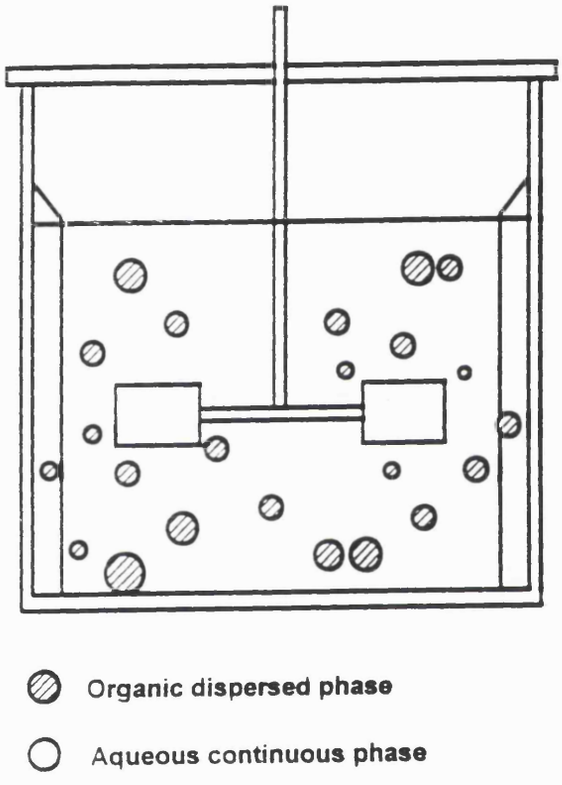


Figure 1.11 Schematic representation of the STR

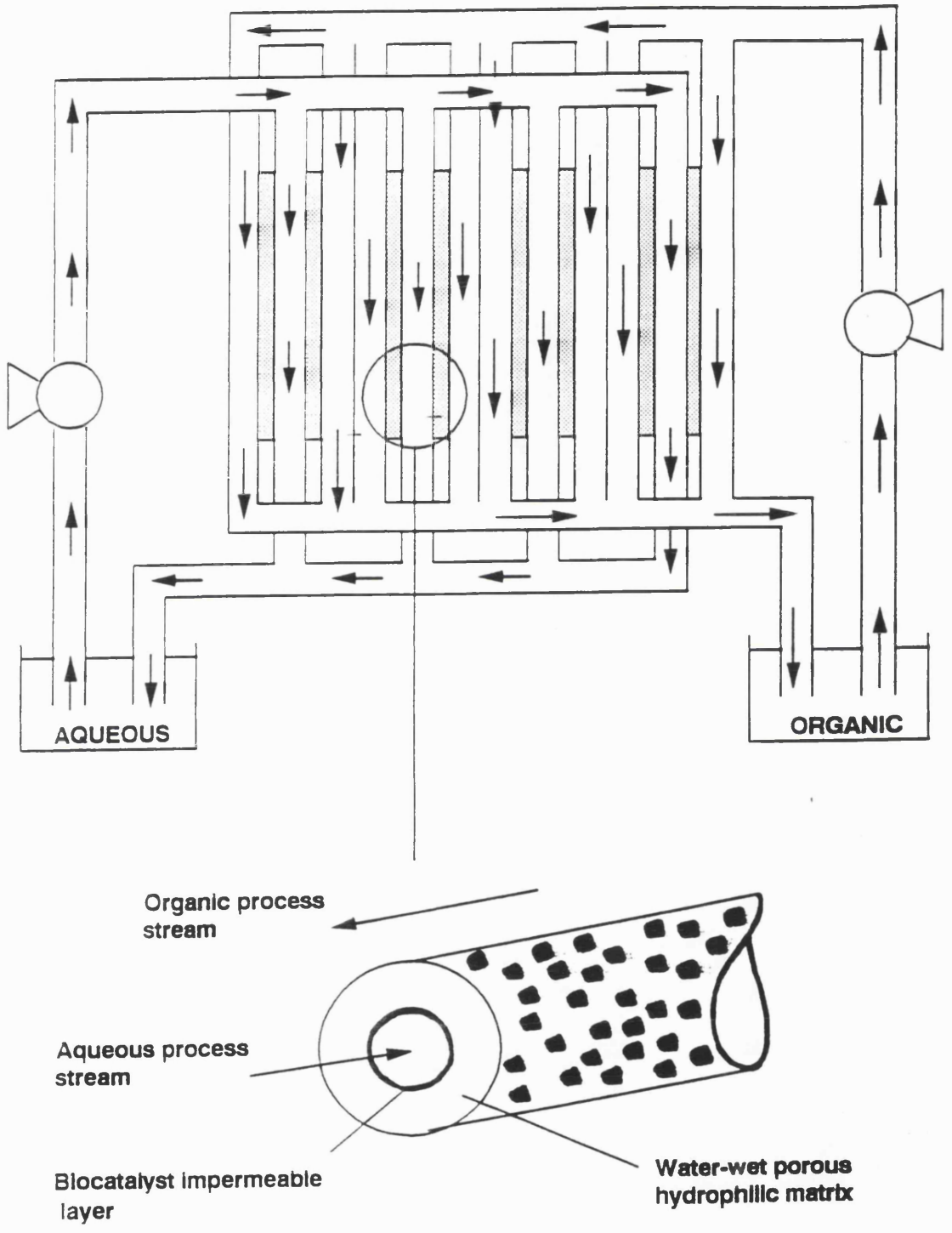


Figure 1.12. Schematic representation of the MBR

interactions as they affect a bulk aqueous phase biocatalyst, the esterase, and biocatlyst exhibiting activity at the interface, the lipase. It is the further intention to model these interactions on the basis of kinetic data previously obtained in Lewis cell studies for the hydrolysis of benzyl acetate by PLE. This will provide widely applicable insight into the optimal operation of STRs using catalysts active within the bulk of the aqueous phase and at the interface. More specifically the study will help to recognise the most suitable catalyst for this particular aplication i.e. the non specific hydrolysis of an ester.

Having identified the more suitable catalyst this will be used in reaction in the MBR to make comparisons with reaction in the STR. The enzyme will be immobilised to the membrane. Ideally to make a valid comparison conditions in the MBR should as much as possible resemble those in the STR. Ideally the same interfacial area in the STR and the MBR should be attained. However this is difficult since no accurate method of determining interfacial area for a two-liquid phase dispersion, (as opposed to an emulsion), has been found at present time. In order to make this comparison the productivity of the enzyme will be accounted for. The reactor will be run with phase recycle and so is representative of conditions in a continuous STR. This study will enable a preliminary choice choice to be made between the use of a STR and a MBR for a given reaction. This will be beneficial in providing a focal point for the overall process design strategy.

2. Materials and Methods

2.1. Materials

2.1.1. Reagents

Benzyl acetate (99+%), benzyl alcohol (99+%, Gold label) and 2-propanol (HPLC grade) were obtained from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, England). Potassium *di*-hydrogen orphophosphate (AnalaR), *di*-potassium hydrogen phosphate (AnalaR), acetonitrile 'Far' UV (Hipersolv) and sodium sulphate (GPR) were obtained from BDH Chemicals Ltd. (Poole, Dorset, England). Sodium azide was obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, England). 'Ultrasil 11' was obtained from Henkel Hygiene GmbH. (Dusseldorf, Germany).

2.1.2. Biocatalysts

Carboxylic-ester hydrolase, EC 3.1.1.1. (Esterase from pig liver as a suspension in ammonium sulphate 10 g l^{-1}) was obtained from the Boehringer Corparation (London) Ltd. (Lewes, East Sussex, England). Lipase, EC 3.1.1.3. (Type II from porcine pancreas approximately 25 % protein containing amylase and protease activity) was obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, England).

2.1.3. The Stirred Tank Reactors (STRs)

STR 1 consisted of a baffled glass vessel (volume: 100 ml, internal diameter: 48 ml) in which was contained 80 ml of reaction medium. This was stirred by a turbine impeller (diameter 24 mm) powered by a Heidolph type 50.113 motor. Temperature was maintained by immersion of the vessel in a constant temperature water bath (Grant Instruments (Cambridge) Ltd., Barrington, Cambs., England).

STR 2 consisted of a baffled and jacketed glass vessel (volume: 150 ml, internal diameter: 48 mm) in which was placed 100 ml of reaction medium. This was stirred by a turbine impeller (diameter 48mm) which was powered by a Heidolph type 50. 113 motor (Germany). Temperature was maintained by water circulation through the jacket from a water bath (Grant Instruments [Cambridge] Ltd., Barrington, Cambs., England). A Radiometer pH-stat (Copenhagen, Denmark) was used to regulate pH of the reactor contents for experiments in which pH was maintained.

2.1.4. The Membrane Bioreactor (MBR)

All reactions carried out for MBR studies were performed in a commercially available membrane bioreactor system, the Sepracor MBR-500 (Marlborough, MA 01752, USA) The membrane module is made of hydrophilic microporous hollow fibre membranes. The membrane has an asymmetric structure with an ultrafiltration skin on one side (lumen) and a porous matrix on the other (shell). The molecular weight cut-off of the ultrafiltration skin, as assayed with dextran, is 95% retention of molecules of molecular weight greater than 50,000 Daltons. Fibre specifications as given are internal diameter 210 +/- 10 microns, external diameter 310 +/- 10 microns, number of fibres in module 8500 +/- 500, fibre length 13.3 cm, nominal membrane area 0.75 m^2. Fluid flow through this module is controlled by the Sepracor model 10 fluid management system. Lumen and shell reservoirs were contained in 500 ml jacketed vessels through which water was circulated from a water bath at the desired reaction temperature. A Radiometer pH-Stat was used to maintain the pH of the aqueous phase.

2.2. Analytical Techniques

2.2.1. Sampling and Preparation

0.4 ml samples were removed from the reactor and placed in 0.8 ml of ice-cold propan-2-ol to stop the reaction and create an homogeneous sample for HPLC analysis.

This was then centrifuged to remove enzyme debris and the supernatant transferred to HPLC vials for analysis.

2.2.2. HPLC Analysis

Benzyl acetate and benzyl alcohol were assayed by HPLC using a *u*-Bondapak C_{18} reverse phase column (0.39 x 15 cm) (Waters Chromatography, Milford, Massachusetts, U.S.A.) and a mobile solvent phase composed of a 50 % (v/v) solution of acetonitrile in de-ionised water. Detection was by UV absorption at a wavelength of 257.5 nm. The solvent flowrate was maintained at 0.5 ml min^{-1}. Retention times for benzyl alcohol and benzyl acetate were 4.8-5.1 min and 7.9-8.5 min, respectively. Known standards were used to produce a standard curve against which the unknowns were compared.

2.2.3. Titration

In experiments where a pH stat was used to maintain the reaction pH the quantity of hydroxide necessary to neutralise the acid by-product in the reaction could be used as an alternative means to measure product concentration. The stoichiometry of the reaction reveals that the acid by-product of the reaction is produced in equimolar ratios to the product alcohol. This is only valid if all the acetic acid generated can be fully accounted for in pH measurements of the aqueous phase by the pH-Stat and thus titrated against i.e. the acetic acid must fully partition into the aqueous phase of the two-liquid phase reaction medium. In order to verify this, results for product concentration measurements obtained by HPLC analysis were compared with the results of the same experiment for product concentrations obtained using measurements calculated from titrant volumes using the pH-Stat. The results for these measurements (see section 2.3.3 for reaction details) are depicted in figure 2.1 and show good correlation between the two methods thus validating the use of titration for reaction kinetic analysis.

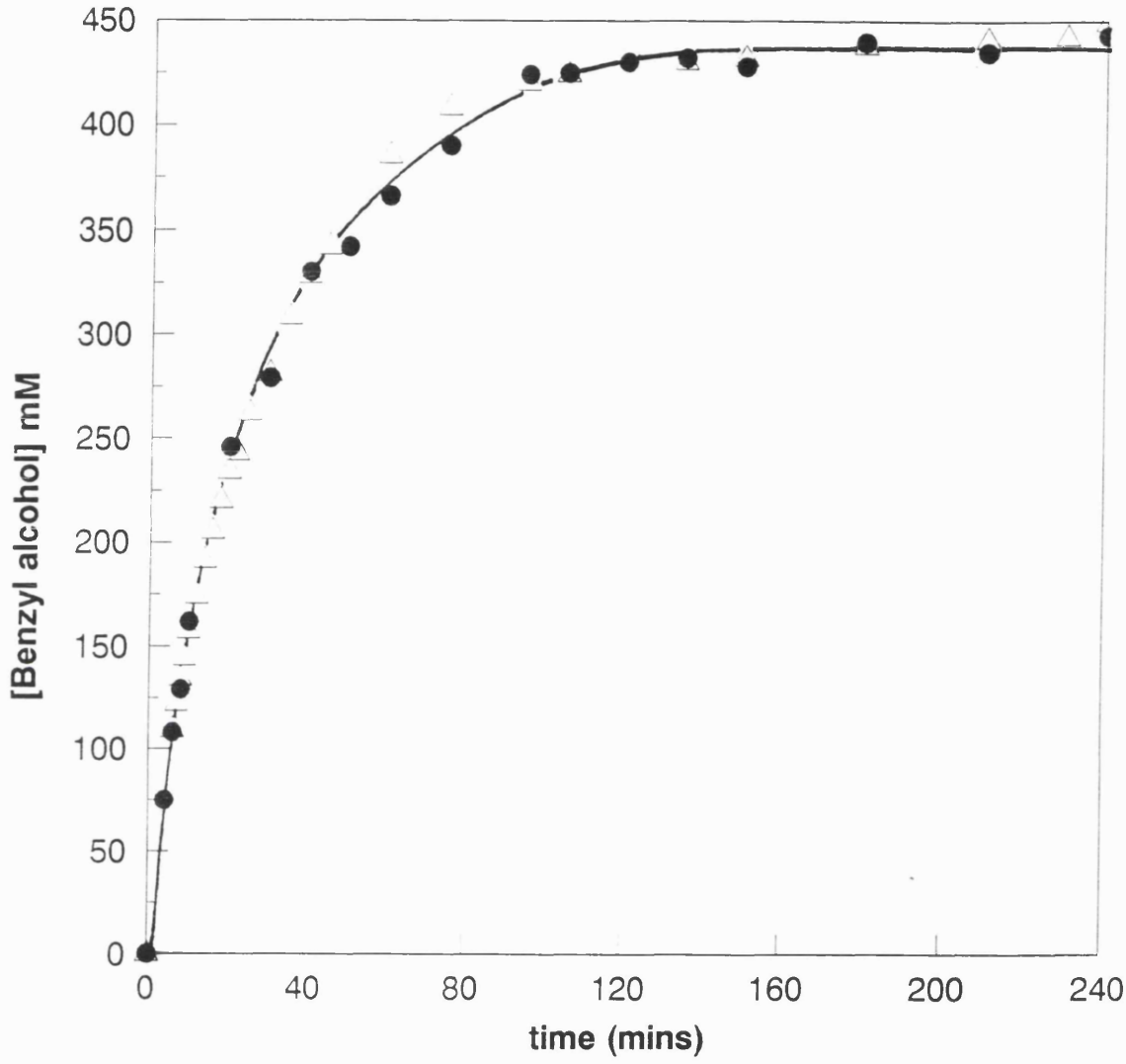


Figure 2.1 Product concentration in the STR with time for the esterase hydrolysis. Reactor conditions: ∅ = 0.4, [Ea] = 0.5 g l^{-1}, n = 1000 rpm; concentration measurement resulting from HPLC analysis (●), Concentration measurement based on titre volume (Δ).

2.2.4. Titrant Selection

In the reactor the acid will be neutralised and thus pH maintained by the addition of hydroxyl ions to the reactor. The first experiments carried out under pH controlled conditions used KOH as a source of hydroxyl ions the resulting salt generated being the potassium salt of acetic acid. However NaOH can also be used as a titrant. It is thus interesting to discover if there is any difference in the effect upon the reaction between the two types of titrant. Two comparative reactions were carried out under the same conditions of phase ratio, 0.4, stirrer speed, 1000 rpm and using the lipase at an aqueous phase enzyme concentration of 4 g l^{-1}. Two reactions were repeated under pH controlled condition using in one experiment 10 M KOH as the titre and in the corresponding experiment 10 M NaOH as the titre. Figure 2.2 shows the progression of the two reactions on the basis of the concentration of product generated The two reactions initially have identical rates of product formation but after 4 hours of operation the activity in the reactor using KOH as the titrant shows a greater rate of decay in comparison to that in the reactor using NaOH as the titrant. Since there seems to be a comparatively negative effect on the stability when using KOH as the titrant all reaction pH 's were maintained using NaOH as the titrant.

2.2.5. Sampling Intervals

For analysis by HPLC samples were initially removed at 1 minute intervals for the first 10 minutes and then at subsequent intervals of from 5 to 10 minutes. For experiments which measured product based on titre volume a continuous real time pen recording of titre volume was obtained. Values for calculation of product concentration could be obtained from this.

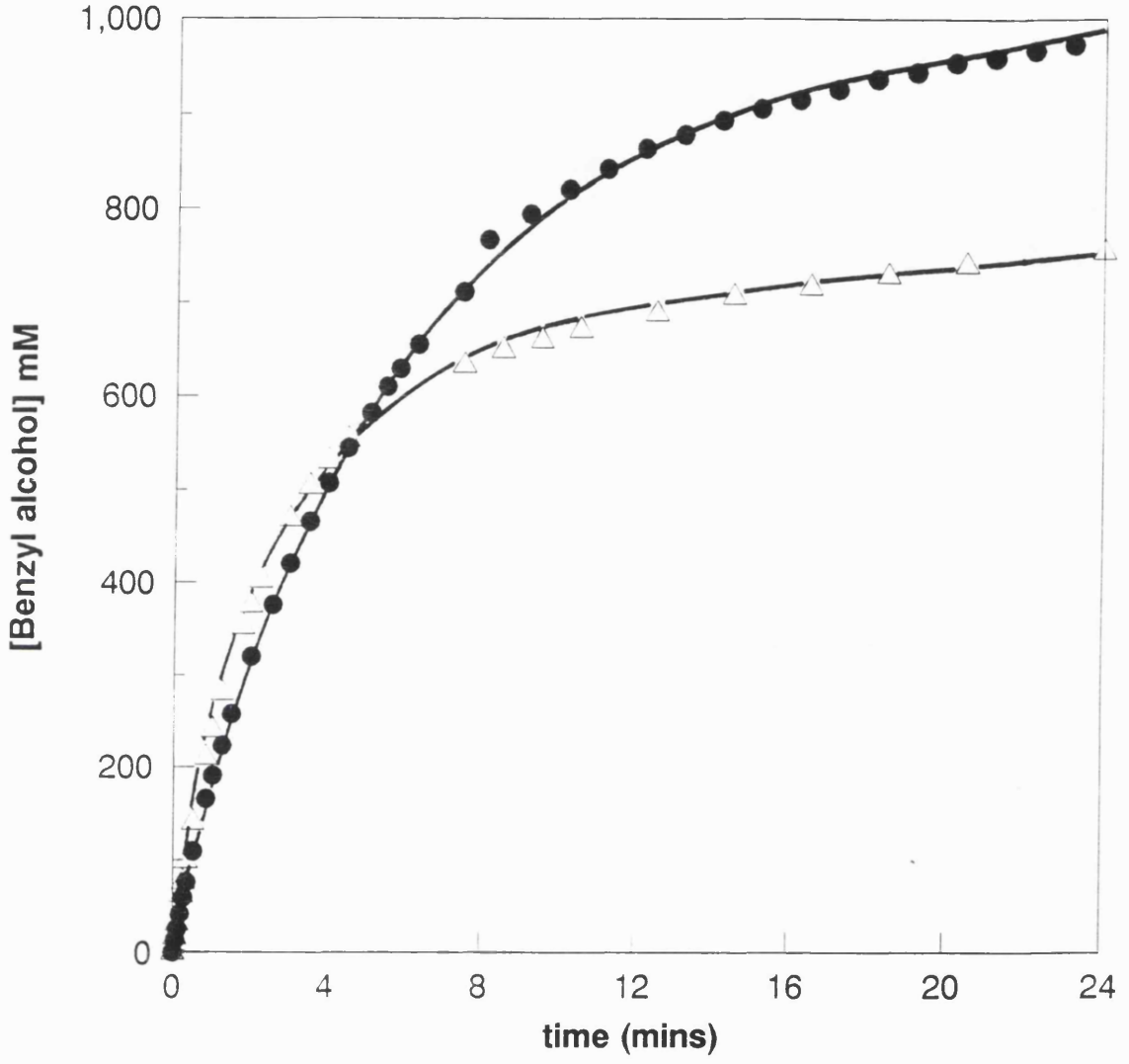


Figure 2.2 Product concentration in the STR with time for the esterase hydrolysis. Reactor conditions: ∅ = 0.4, [Ea] = 4 g l^{-1}, n = 1000 rpm; titre 10 M KOH (Δ), 10 M NaOH (●).

2.3. STR Evaluation with Esterase

2.3.1. Reactor Start Up

For a two liquid phase system phase ratio is a definition of the volume fraction in the reactor occupied by the organic phase and is defined by the following equation.

$$Phase\ Ratio = \frac{Organic\ Phase Volume}{Total\ Liquor\ Volume}$$

Phase ratio thus dictates the volumes of organic and aqueous phases employed upon reactor start up. The following procedure for reactor start up was employed for all esterase catalysed reactions in both STR vessels 1 and 2. A volume of aqueous phase appropriate to the phase ratio being investigated was added to the reactor vessel. Into this was dissolved an amount of enzyme to give the appropriate enzyme concentration under investigation. Stirring was then commenced at the appropriate speed and the appropriate volume of organic phase as dictated by the phase ratio was added to the reactor. The addition of the organic phase represented time zero, the commencement of the reaction. Employing this method of reactor start up the initial bulk aqueous phase substrate concentration, [Sab], is equal to zero, the [Sab] rising from time zero to steady-state [Sab].

2.3.2. Determination of the Effects at Different Aqueous Phase Enzyme Concentrations

These reactions were all carried out in STR vessel 1. A phase ratio (Ø) of 0.75 was employed in carrying out these reactions. In the 80 ml rection medium volume this equated to a 20 ml aqueous phase volume with a 60 ml organic phase volume. Stock solution of esterase was at a concentration of 10 mg ml^{-1}, correspondingly in separate experiments 1, 2, 4, 6 and 8 ml of the stock solution were added to 19, 18, 16, 14 and 12

ml of phosphate buffer to give the appropriate 20 ml volumes of aqueous phase having aqueous phase enzyme concentrations 0.5, 1, 2, 3 and 4 g l^{-1} respectively. Reactor start up was as previously described (sec. 2.3.1). Based on previously reported methodology a sampling interval of 1 minute was deemed adequate to enable the initial linear reaction rate to be determined. Samples were removed and analysed by HPLC, the methodology being as previously described in sections 2.2.1 and 2.2.2.

2.3.3. Determination of pH Effects

The by-product of the hydrolysis of benzyl acetate is acetic acid which partitions into the aqueous phase resulting in a lowering of the pH of this phase as reaction proceeds. At lower pH the enzyme will be affected, (Barker and Jencks 1969) Experiments were performed in STR vessel 1 under non pH controlled conditions. Phase ratios of 0.2 and 0.4 were investigated. At a phase ratio of 0.2 the 80 ml of reaction liquor corresponded to 64 ml of aqueous phase, made up of 51.2 ml of phosphate buffer with 12.8 ml of the stock enzyme solution giving a corresponding aqueous phase enzyme concentration of 2 g l^{-1}, and a 16 ml volume of organic phase. At a phase ratio of 0.4 the 80 ml volume of reactor medium corresponded to 48 ml of aqueous phase, made up of 38.4 ml of phosphate buffer with 9.6 ml of the stock enzyme solution giving a corresponding aqueous phase enzyme concentration of 2 g l^{-1}, and a 32 ml volume of organic phase. Reactor start up was as previously described (sec 2.3.1). Samples were initially removed at 1 min intervals for the first 10 minutes and then at subsequent intervals of 10 minutes. Samples were prepared and analysed by HPLC employing the methodology as previously described (sec 2.2.1 and 2.2.2)

To asses effect of pH the results from the above experiments were compared with corresponding experiments carried out in STR vessel 2 in which the pH was maintained at a constant value of 7 by addition of 5M KOH. A 80 ml volume of reaction medium was used and the reactions carried out as in STR vessel 1 at phase ratios of 0.2 and 0.4 resulting in the use of corresponding aqueous, stock enzyme solutions and organic volumes. Reactor start up was as described (sec 2.3.1) at which

time pH-stat operation was begun. Samples were removed at periodic intervals, prepared, and analysed by HPLC employing the previously described methodology (sec 2.2.1 and 2.2.2). The volume of KOH added to the reactor was monitored and this was equated to values for product concentrations for comparison with the concentrations as measured by HPLC this data being relevant to section 2.2.2 as described therein.

The results of this investigation are illustrated in figure 2.3. Comparison of the reactions carried out under a regime of no pH maintenance with those under a regime where the pH was maintained at 7 by titration with NaOH show a definite effect upon the stability of the esterase. The reactions carried out under non pH maintained conditions showed no activity after only 10 minutes of operation (□ and ● of fig 2.3) whereas the reactions carried out under pH maintenance (■ and O of fig 2.3) showed a degree of activity for a further 80 to 120 minutes resulting in product concentrations 4 times in exess of those observed in the non pH maintained regime. Initial rates were the same in both experiments. Following the results of this investigation all subsequent experiments were carried out under a regime of pH maintenance.

2.3.4. Determination of the Effects of Agitation Rate

The reactions were all carried out in STR vessel 2 . The pH was maintained at 7 by the addition of NaOH using the pH stat. A phase ratio of 0.4 was employed in carrying out these reactions In the 80 ml reaction medium volume this equated to a 48 ml aqueous phase volume with a 32 ml organic phase volume. To make up the enzyme containing aqueous phase 9.6 ml of the stock enzyme solution was added to 38.4 ml of phosphate buffer to give an aqueous phase enzyme concentration of 2 g l^{-1} Reactor start up was as previously described (sec 2.3.1). Concentrations of product were determined based on titre volume. Experiments were repeated at agitation rates, (n) of 750, 1000 and 1800 rpm.

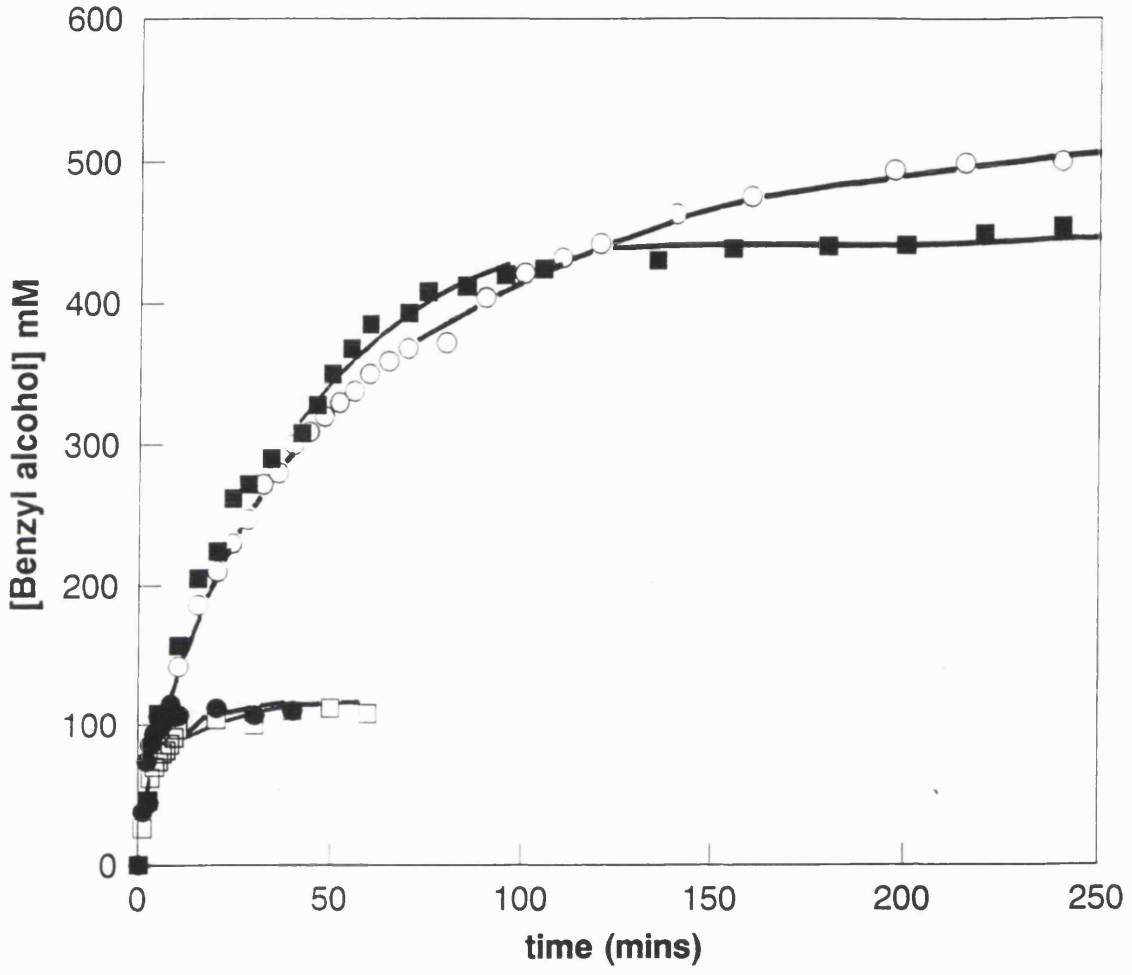


Figure 2.3 Product concentration in the STR with time for the esterase hydrolysis. Reactor conditions: ∅ = 0.4, [Ea] = 0.5 g l^{-1}, n = 1000 rpm; pH maintained (■); ∅ = 0.4 [Ea] = 2 g l^{-1}, n = 1000 rpm, non pH maintained (●); ∅ = 0.2, [Ea] = 0.5 g l^{-1}, n = 1000 rpm, pH maintained (O); ∅ = 0.2, [Ea] = 2 g l^{-1}, n = 1000 rpm, non pH maintained (□).

2.3.5. Determination of the Effects of Phase Ratio

The reactions were all carried out in STR vessel 2. The pH was maintained at 7 by the addition of 10 M NaOH using the pH stat. In separate experiments phase ratios corresponding to 0.2, 0.4, 0.5 and 0.6 were investigated. In the 80 ml reaction medium volume this equated to aqueous phase volumes of 64, 48, 40 and 32 ml respectively with corresponding organic phase volumes of 16, 32, 40 and 48 ml. The aqueous phase was made up of 12.8, 9.6, 8 and 6.4 ml of stock enzyme solution added to 51.2, 38.4, 32 and 25.6 ml of phosphate buffer to give an aqueous phase enzyme concentration of 2 g l^{-1} at all phase ratios. Reactor start up was as previously described (sec 2.3.1). Experiments were carried out at an agitation rate of 1000 rpm and at the selected phase ratios of 0.2, 0.4, and 0.6 at 1800 rpm. Concentrations of product were determined based on titre volume.

In experiments carried out at phase ratio of 0.2 and 0.4 and an aqueous enzyme concentration of 0.5 g l^{-1}, the aqueous phase was made up of 3.2 and 2.4 ml of stock enzyme solution added to 60.8 and 45.6 ml of phosphate buffer with organic volumes of 16 and 32 ml

2.3.6. Determination of the Potential Inhibitory Effect of Product

Reactions were all carried out in STR vessel 2 and pH maintained by the addition of 10 M NaOH with the pH-stat A phase ratio of 0.4 was employed for these experiments equating as previously to a 48 ml volume of aqueous phase and a 32 ml volume in an 80 ml volume of reaction liquor. Sets of experiments were repeated at aqueous phase enzyme concentrations of 0.5 and 2 g l^{-1}, (see sec 2.3.5 for stock and buffer volumes). All experiments were carried out at a stirrer speed of 1000 rpm. Two sets of experiments were performed at each enzyme concentration. The aqueous phase was made up as in previous experiments. In one set of experiments the 32 ml volume of organic phase consisted initially of purely the substrate benzyl acetate as in all previous experiments. In the comparative set of experiments at the two aqueous phase enzyme

concentrations an initial product concentration of 400 mM was applied to the reactor. The organic phase was made up of 27.12 ml of the substrate benzyl acetate and 3.46 ml of the product benzyl alcohol thus representing the time in the reactor at which this concentration of product was generated. Reactor start up was as previously described. Product concentrations were determined from titre volumes.

2.4. STR Evaluation with Porcine Pancreatic Lipase.

2.4.1. Assessment of Aqueous Activity.

In general lipases have been found to be most active at an interfaces, (Verger and Haas 1974). The crude preparation used here may have some hydrolytic activity not associated with the lipase and might show some activity in the bulk aqueous phase environment. In order to investigate this a reaction was carried out in which a purely aqueous phase was presaturated with benzyl acetate to a concentration of 13.5 mM. The reaction was carried out in STR vessel 1 in a 80 ml volume of presaturated aqueous phase. Stirring was commenced at 750 rpm and 0.8 g of the crude enzyme preparation added to the reactor thus giving a reactor protein concentration, (termed enzyme concentration for subsequent experiments - although this is crude), of 2 g l^{-1} Samples were removed and analysed by HPLC as described in section 2.2.1 and 2.2.2.

The product concentration time profile for this experiment is depicted in figure 2.4. The results indicate that the activity in a purely aqueous environment is negligible, the initial activity being evaluated at 0.043 mmol min^{-1} g^{-1} compared to that observed for the esterase in a substrate saturated solely aqueous reaction medium of 37 mmol min^{-1} g^{-1} We can thus assume that any activity measured above the measured value is associated with an interface in the two-liquid phase reaction system.

2.4.2. Reactor Start Up

Phase ratios were as defined previously and determine the respective volumes of aqueous phase and organic phase employed in the reactor. The following procedure was employed for reactor start up for all lipase catalysed reactions in STR vessel 2. Appropriate volumes of organic and aqueous phases were initially added to the reactor vessel. Agitation was commenced at the selected rate. A quantity of the crude enzyme preparation appropriate to the concentration was then added to the reactor This addition of the enzyme was deemed time zero.

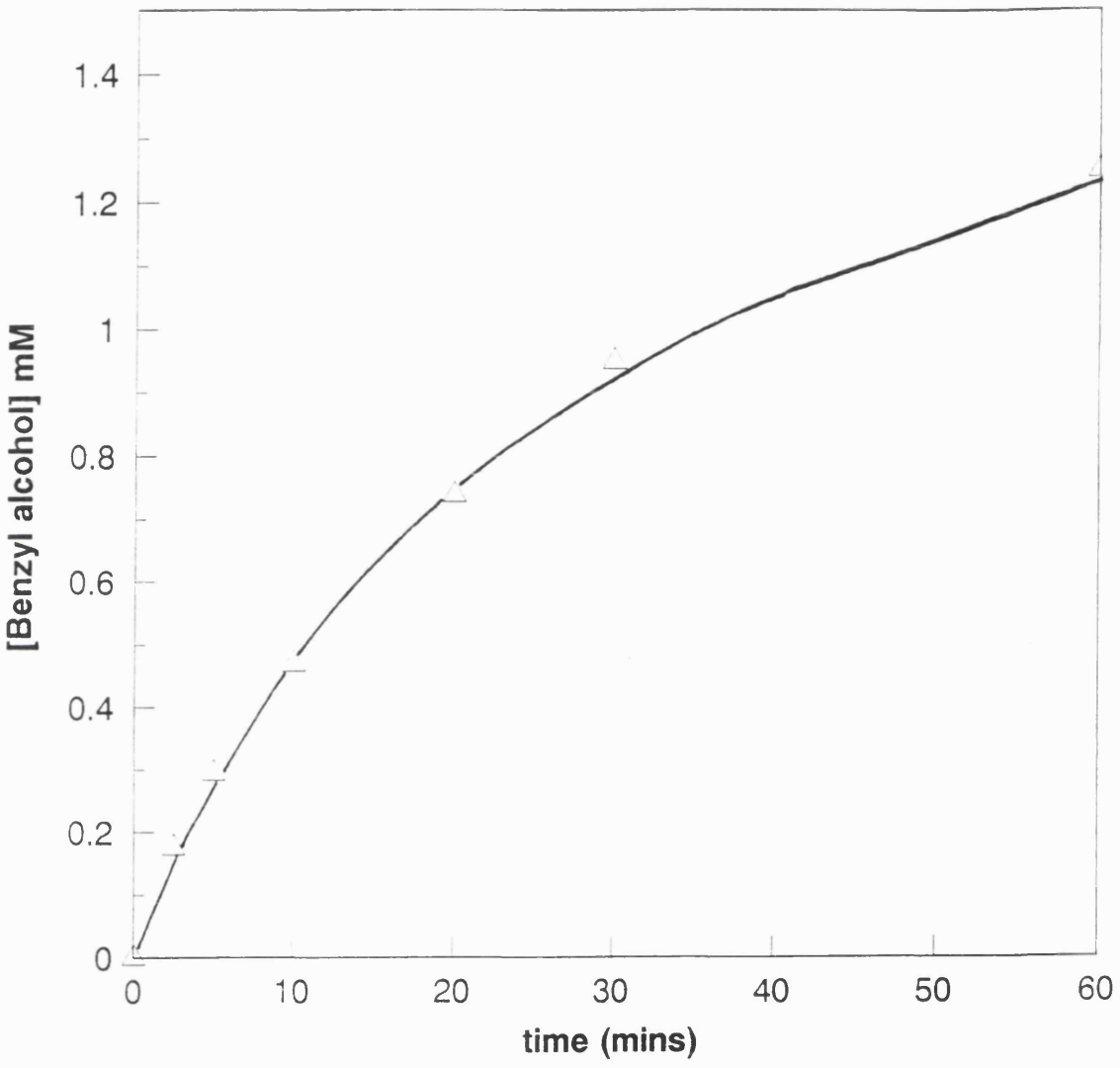


Figure 2.4 Product concentration in the STR with time for lipase hydrolysis in a solely aqueous phase saturated initially with substrate at 13.5 mM; [Ea] = 2 g l^{-1}, n = 750 rpm.

2.4.3. Determination of the Effects at Differrent Aqueous Phase Enzyme Concentrations.

The reactions were all carried out in STR vessel 2 and pH maintained at 7 by the addition of 10 M NaOH with a pH-stat. A phase ratio of 0.4 was employed for these experiments. Medium volume for these experiments was 100 ml equating to a 60 ml volume of aqueous phase and a 40 ml volume of organic phase . Experiments were repeated with the addition of 0.6, 1.2, 2.4, and 4.8 g of the crude enzyme preparation giving enzyme concentrations of 2, 4, 8 and 16 g l^{-1} respectively. Reactor start-up was as previously described. Product concentrations were determined based on titre volume.

2.4.4. Determination of the Effects of Agitation

The reactions were all carried out in STR vessel 2. The pH was maintained at 7 by the addition of 10 M NaOH with the pH-stat. A phase ratio of 0.4 was employed for these experiments. Medium volume for these experiments was 100 ml equating to a 60 ml volume of aqueous phase and a 40 ml volume of organic phase. 1.2 g of the crude enzyme preparation giving an enzyme concentration of 4 g l^{-1} was added to the reactor. Experiments were repeated at stirrer speeds of 1000 and 1800 rpm. Reactor start-up was as previously described. Product concentrations were determined based on titre volume.

2.4.5. Determination of the Effects of Phase Ratio

The reactions were all carried out in STR vessel 2 and pH maintained at 7 by the addition of 10 M NaOH using the pH stat. A 100 ml medium volume was used in the reactor. In separate experiments phase ratios corresponding to 0.2 and 0.4 were investigated. In the 100 ml liquor volume this equated to aqueous phase volumes of 80 and 60 ml respectively with corresponding organic phase volumes of 20 and 40 ml. Crude enzymes in amounts of 1.6 and 1.2 g were used to give an aqueous phase enzyme

concentration of 4 g l^{-1} at all phase ratios. Reactor start up was as previously described. Concentrations of product were determined based on titre volume

2.4.6. Determination of the Potential Inhibitory Effect of Product

The reactions were all carried out in STR vessel 2. The pH was maintained at 7 by the addition of 10 M NaOH with the pH-stat A phase ratio of 0.4 was employed for these experiments equating as previously to a 60 ml volume of aqueous phase and a 40 ml volume in an 100 ml working volume. 1.2 g of crude enzyme preparation was used giving an aqueous phase enzyme concentration of 4 g l^{-1} Experiments were carried out at a stirrer speed of 1000 rpm. Two sets of experiments were performed. In one set of experiments the 40 ml volume of organic phase consisted initially of purely the substrate benzyl acetate as in previous experiments. In the comparative experiment an initial product concentration of 1 M was applied to the reactor the organic phase now being made up of 29 ml of the substrate benzyl acetate and 10.8 ml of the product benzyl alcohol thus representing the time in the reactor at which this concentration of product was generated. Reactor start up was as previously described. Product concentrations were determined from titre volumes.

2.4.7. Substrate Conversion.

All the reactions (biotransformations) described in section 2.4.3 were monitored to completion.

2.5. Membrane Bioreactor Studies

2.5.1. Catalyst Loading

A general procedure for enzyme loading was followed for loading of all specified concentrations of enzyme used in these studies. In separate experiments 10 g and 100 g of PPL was added to 0.1M potassium phosphate (1L) buffer and stirred for 2 hours on a magnetic stirrer so as to fully hydrate the enzyme. This was then centrifuged to remove particulate insoluble material. 50 ml of the remaining supernatant was then removed for determination of activity and STR studies for comparison with MBR. The remaining 950 ml having enzyme activity was used to loaded onto the membrane. The loading procedure was as set out in the protocol received with the system (Sepracor MBR-500 Operations Manual, Sepracor Inc) and was carried out by ulrafiltration of the supernatant from the shell, through the membrane and into the lumen where the filtrate was collected, 50 ml of which was taken for further activity determination in a STR. The size of the enzyme facilitates its containment in the matrix of the membrane by the ultrafiltration skin. The percentage of activity i.e. the amount of enzyme which is associated with the membrane is determined by comparing the activity of the permeate in the STR with the activity of the filtrate in the STR as outlined in the following section.

2.5.2. Activity Determination of Permeate and Filtrate

Activity determination for comparison of the permeates and filtrates was carried out in a STR. The actual reactor used in these determinations was STR vessel 2 and all determinations were carried out at 30^0C. pH was maintained as in previous experiments using the pH-Stat with 10 M NaOH. 50 ml of the enzyme solution (permeate or filtrate) was added to 50 ml of the substrate, benzyl acetate giving a phase ratio in the reactor of 0.5. Agitation was commenced at a rate of 1000 rpm Product concentrations were determined from titre volumes.

Figure 2.5 illustrates the concentrations of product generated as a reaction proceeded in the STR for the both the 10 and 100 g l^{-1} permeates (● and Δ respectively of figure 2.5) and the 10 and 100 g l^{-1} filtrates (O and Δ of figure 2.5). This comparison indicates that there is no activity associated with the filtrate compared with the activity of the original permeate. Thus we can assume that all activity available was associated with the membrane.

2.5.3. Reaction in the Membrane Bioreactor

Having determined the amount of enzyme activity associated with the membrane module a reaction was then carried out in the MBR. 400 ml of 0.1 M phosphate buffer was placed in a jacketed vessel as the lumen reservoir and 400 ml of the substrate benzyl acetate was set up as the shell reservoir. Temperature was maintained by circulation of water at 30^{0}C through the jacketed vessels. A pH-Stat was used to maintain the reaction pH of the phosphate buffer. Both aqueous and organic phases were recirculated and operated initially in batch mode. The reservoirs were continually mixed using magnetic stirrers to avoid concentration gradients. Circulation of the lumen reservoir was begun at 500 ml min^{-1} through the membrane module. To initiate the reaction when the lumen (aqueous) reservoir was fully circulating through the module, circulation of the organic reactant through the membrane module was begun at 500 ml min^{-1}. Once the organic phase was fully circulating the transmembrane pressure was adjusted to give an outlet reading on the shell side of 7 psi. Reaction monitoring was by pH-Stat.

2.5.4. Enzyme reuse in the MBR or Semi-continuous Operation in The MBR

For experiments where the enzyme was reused in the MBR the system was drained of reactant and buffer and air introduced into the system to blow out any excess remaining in the module and piping. Fresh buffer and reactant were reintroduced to their respective reservoirs and reaction was initiated as previously described.

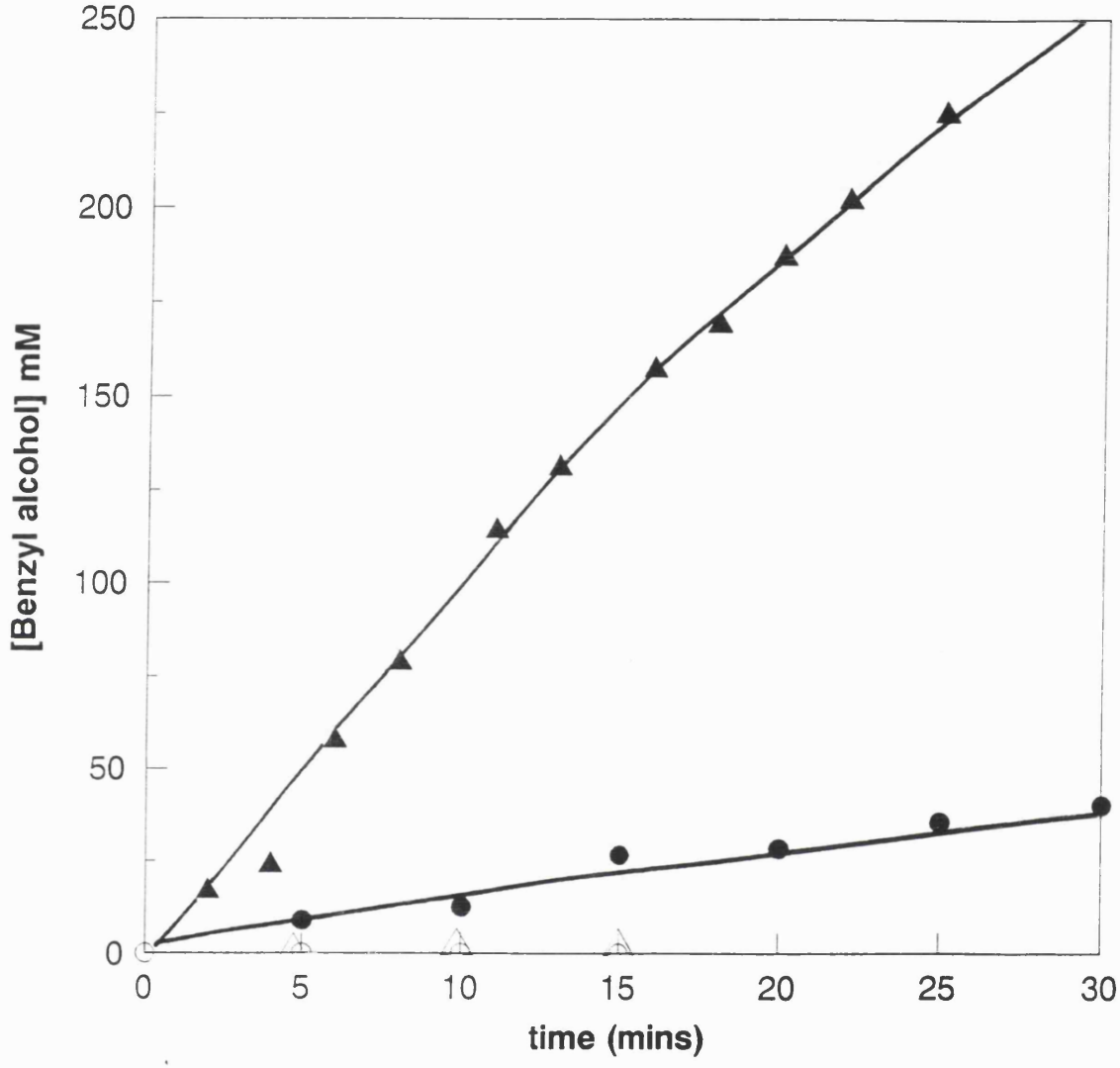


Figure 2.5 Product concentration in the STR with time for lipase hydrolysis. Reactor conditions: ∅ = 0.5, n = 1000 rpm; 10 g l^{-1} permeate (●), 100 g l^{-1} permeate (▲), 10 g l^{-1} filtrate (O and 100 g l^{-1} filtrate (Δ).

2.5.5. Enzyme Removal

Enzyme removal was by a process of backflushing as outlined in the protocol received with the system, (Sepracor MBR-500 Operations Manual, Sepracor Inc). The membrane was cleaned using first a 0.1M NaOH and 1M Na_2SO_4 followed by an industrial membrane cleaning agent Ultrasil-11 (Henkel, Germany) at 0.5 wt % as suggested by Guit *et al* (1991). This procedure leads to complete removal of enzyme from the membrane ready for regeneration with fresh enzyme.

2.5.6. STR Evaluation for MBR Comparison

All reactions were carried out in STR vessel 2 and pH was maintained at 7 by the addition of 10 M NaOH using a pH-Stat. The 50 ml permeate sample, removed after centrifugation in the preparative step for enzyme loading, was used as the enzyme containing aqueous phase for the STR evaluation. The procedure for the evaluation in the STR was as carried out for the activity determinations of the permeate and the filtrate at phase ratio of 0.5, a stirrer speed of 1000 rpm and a temperature of 30°C (see section 2.5.2).

3. RESULTS

3.1. Esterase Catalysed Hydrolysis in the Stirred Tank Reactor.

3.1.1. Enzyme Concentration

The initial product concentration time profiles depicted in figures 3.1 and 3.2 illustrate the results for reactions carried out at a constant phase ratio of 0.75 and agitation rates of 750 and 1000 rpm respectively. Experiments were repeated at the two agitation rates using different aqueous phase enzyme concentrations these being at 750 rpm 0.1, 0.5, 1, 2, and 4 g l^{-1} and at 1000 rpm 0.1, 1, 2 and 4 g l^{-1}. Phase ratio dictates the relative volumes of organic and aqueous phases in the reactor. The concentration of enzyme in the reactor is expressed on an aqueous phase basis only since the enzyme is active in the bulk of the aqueous phase (Woodley *et al* 1991). As phase ratio increases then the relative volume of aqueous phase in the reactor decreases, assuming aqueous phase enzyme concentration remains constant then amount of enzyme in the reactor decreases. Comparisons of specific activity at different phase ratios must make allowances for this fact. Therefore all product is assumed to emanate from the aqueous phase and thus product concentrations are expressed on a solely aqueous phase volume basis for the purposes of evaluating specific activity.

Figures 3.1 and 3.2 were used to plot figure 3.3 which shows the specific activity expressed as a function of [Ea] for reaction carried out in the STR at a phase ratio of 0.75 and agitation rates of 750 and 1000 rpm. The linearity of the data plotted in figures 3.1 and 3.2 show that a steady state between the rate of biocatalytic activity and that of substrate transfer is achieved rapidly and therefore measurements made accurately reflect steady state measurements.

In previous experiments to determine the specific activity in solely aqueous medium, (Woodley thesis), the maximum achievable rate, limited by the kinetic performance of the biocatalyst, was shown to be approximately 37 mmol min^{-1} g^{-1}.

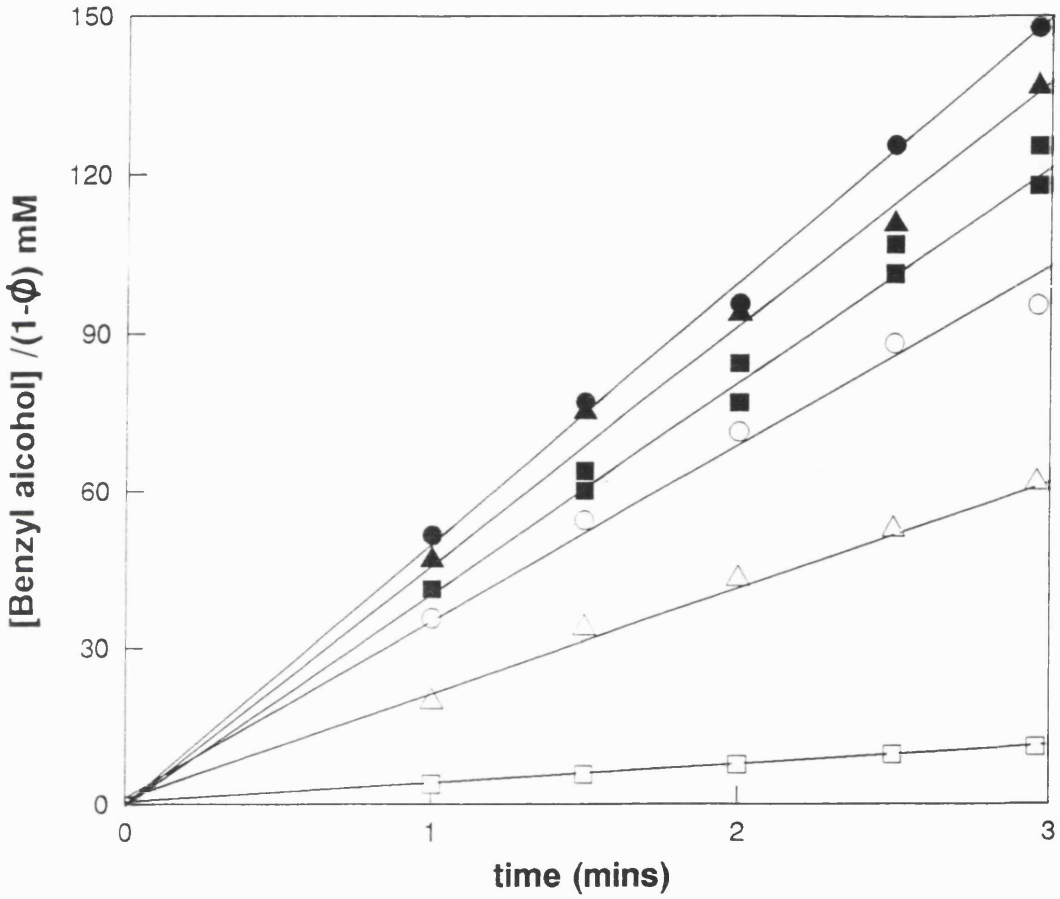


Figure 3.1 Initial two-liquid phase reaction kinetics for esterase hydrolysis in the STR. Reactor conditions: ∅ = 0.75; n = 750 rpm; [Ea] = 0.1 g l^{-1} (□), 0.5 g l^{-1} (Δ), 1 g l^{-1} (O), 2 g l^{-1} (■), 3 g l^{-1} (▲), 4 g l^{-1} (●).

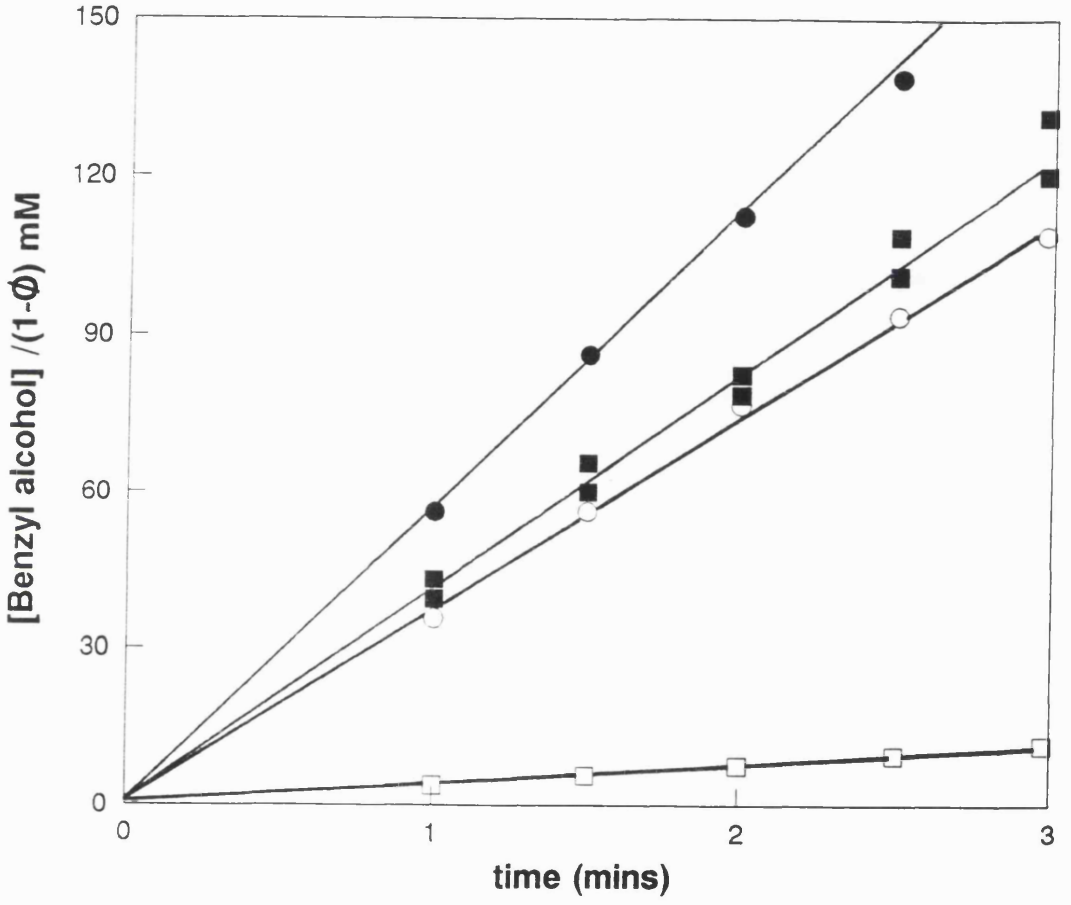


Figure 3.2 Initial two liquid phase reaction kinetics for esterase hydrolysis in the STR. Reactor conditions: ∅ = 0.75, n = 1000 rpm. [Ea] = 0.1 g l^{-1} (□), 1 g l^{-1} (O), 2 g l^{-1} (■), 4 g l^{-1} (●).

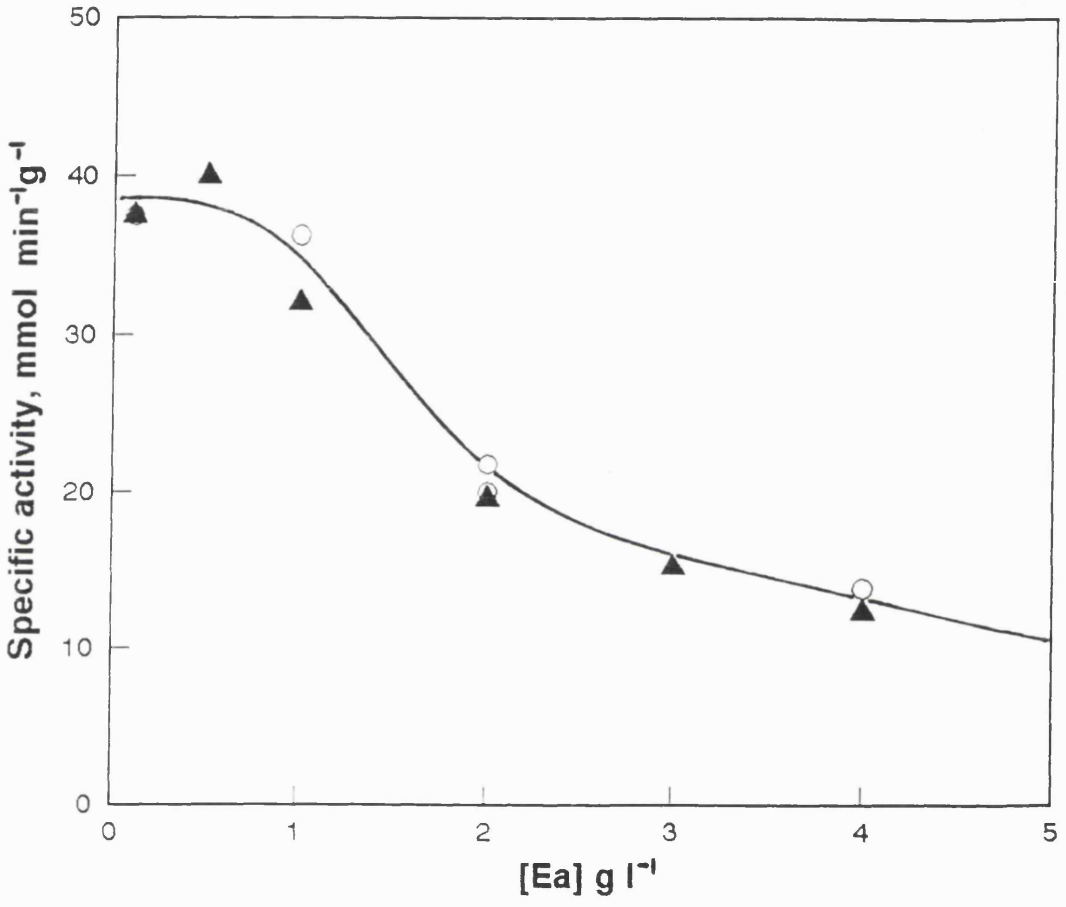


Figure 3.3 Specific activity as a function of aqueous phase enzyme concentration for esterase hydrolysis in the STR. Reactor conditions: ∅ = 0.75; n = 750 rpm (Δ), 1000 rpm (O).

Under the specific reactor conditions for these experiments aqueous phase enzyme concentrations of greater than 1 g l^{-1} resulted in specific activities lower than the previously determined maximum. As [Ea] increased from 1 to 4 g l^{-1} the specific activity was seen to respond inversely. The conditions in the reactor fail to facilitate the optimal kinetic performance of the enzyme.

Specific activity is limited due to an inadequate supply of substrate to the aqueous phase to support maximal activity of the enzyme, resulting in sub-optimal kinetic performance. The results define reactor operating conditions under which substrate mass transfer is the limiting rate in the reaction, the [Ea] at which this transition occurs (1 g l^{-1}) is only valid at the particular substrate mass transfer coefficient in the system under the defined reactor operating conditions of phase ratio of 0.75 and agitation rates of 750 and 1000 rpm.

3.1.2. Agitation Rate

The initial product concentration time profiles depicted in figure 3.4 show the results for reactions carried out at a constant phase ratio of 0.4 and an [Ea] of 2 g l^{-1}. Experiments were repeated at different rates of agitation, 750, 1000 and 1800 rpm respectively. All product concentrations were expressed on an aqueous phase volume basis. From previous work examining reactor homogeneity at agitation rates of 250 to 2500 rpm, agitation rates below 750 rpm resulted in a non-homogeneous reaction liquor in which the phase ratio is not uniform throughout the reactor volume, (Woodley thesis). Measurements were therefore made at stirrer speeds of 750 rpm and above.

Figure 3.4 was used to plot figure 3.5 which shows the specific activity expressed as a function of agitation rate. Results of figure 3.4 accurately reflect steady state measurements. From the previous experiment which examined the effect of differing [Ea] in the reactor we know that at the chosen [Ea] of 2 g l^{-1} the reactor is potentially being operated in a mass transfer limited regime. This assumption is confirmed, in none of these experiments the previously determined maximum specific

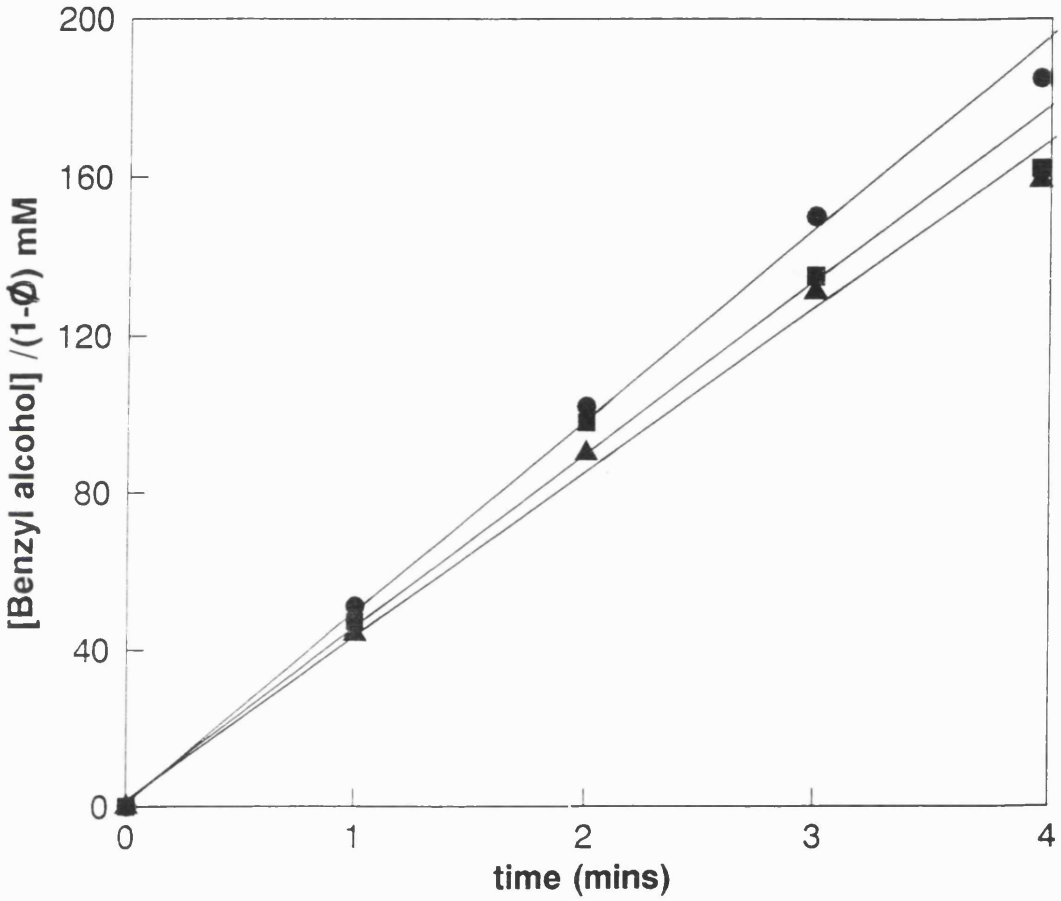


Figure 3.4 Initial two liquid phase reaction kinetics for esterase hydrolysis in the STR. Reactor conditions: ∅ = 0.4, [Ea] = 2 g l^{-1}; n = 750 rpm (▲), 1000 rpm (■), 1800 rpm (●).

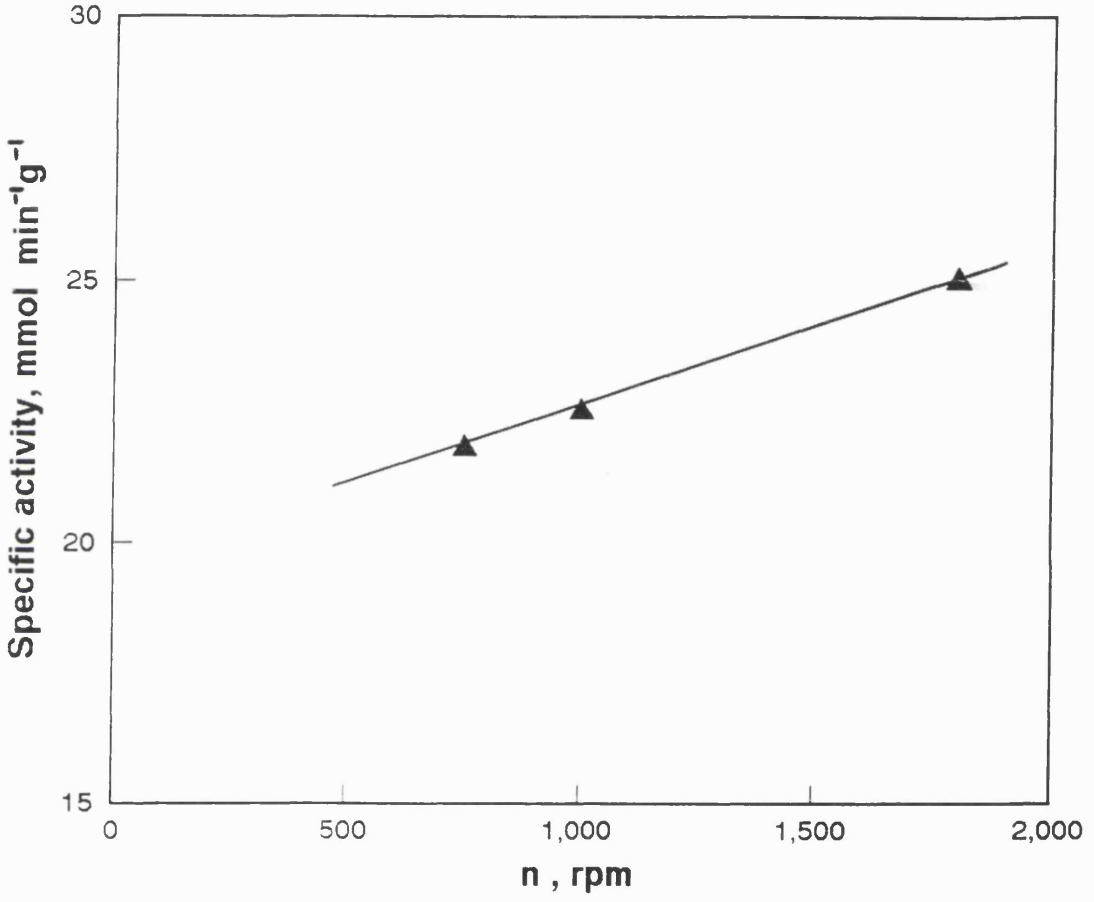


Figure 3.5 Specific activity as a function of agitation rate for esterase hydrolysis in the STR. Reactor conditions: $\varnothing = 0.4$, [Ea] = 2 g l^{-1}.

activity as limited by the kinetics of the enzyme was achieved. The results do however show that as rate of agitation is increased from 750 through 1000 finally to 1800 rpm there is a concomitant increase in specific activity. Since all other variables are constant then this reflects an effect of agitation rate.

The mass transfer coefficient (K_LA) defines the rate at which substrate transfer takes place. The amount of interface over which this transfer takes place is a determinant in this rate, the greater the area of interface the higher the rate. In a two-liquid phase system, in a STR, as the rate of agitation is increased the degree of droplet breakage, relative to coalescence, increases resulting in the generation of smaller dispersed phase droplets in the reactor leading to a greater area of interface over which substrate transfer can take place.

In the two-liquid phase system, the supply of substrate to the enzyme determines the kinetic performance. In order to achieve optimal kinetic performance substrate must be made available in high enough concentration in the aqueous phase to support optimal enzyme activity. The amount of substrate available to the enzyme in the aqueous phase will be determined by its rate of transfer i.e. the K_LA.

In the mass transfer limited environment, as defined under these operating conditions, stirrer speed increase results in increased rate of substrate transfer reflected in increased specific activity of the enzyme.

3.1.3. Stability (Agitation)

The product concentration-time profiles depicted in figure 3.6 show results for reactions carried out at a phase ratio of 0.4 and an [Ea] of 2 g l^{-1}, experiments were repeated at agitation rates of 1000 and 1800 rpm respectively. In order to compare the effect on the stability of the enzyme at these two rates of agitation the reaction was monitored, under pH controlled conditions, over a period of time, (250 mins), where eventually very little enzyme activity remained, evidenced by no further increase in the product concentration in the reactor. Product concentrations, as given here, represent the overall product concentration based on medium volume since the phase ratio was the

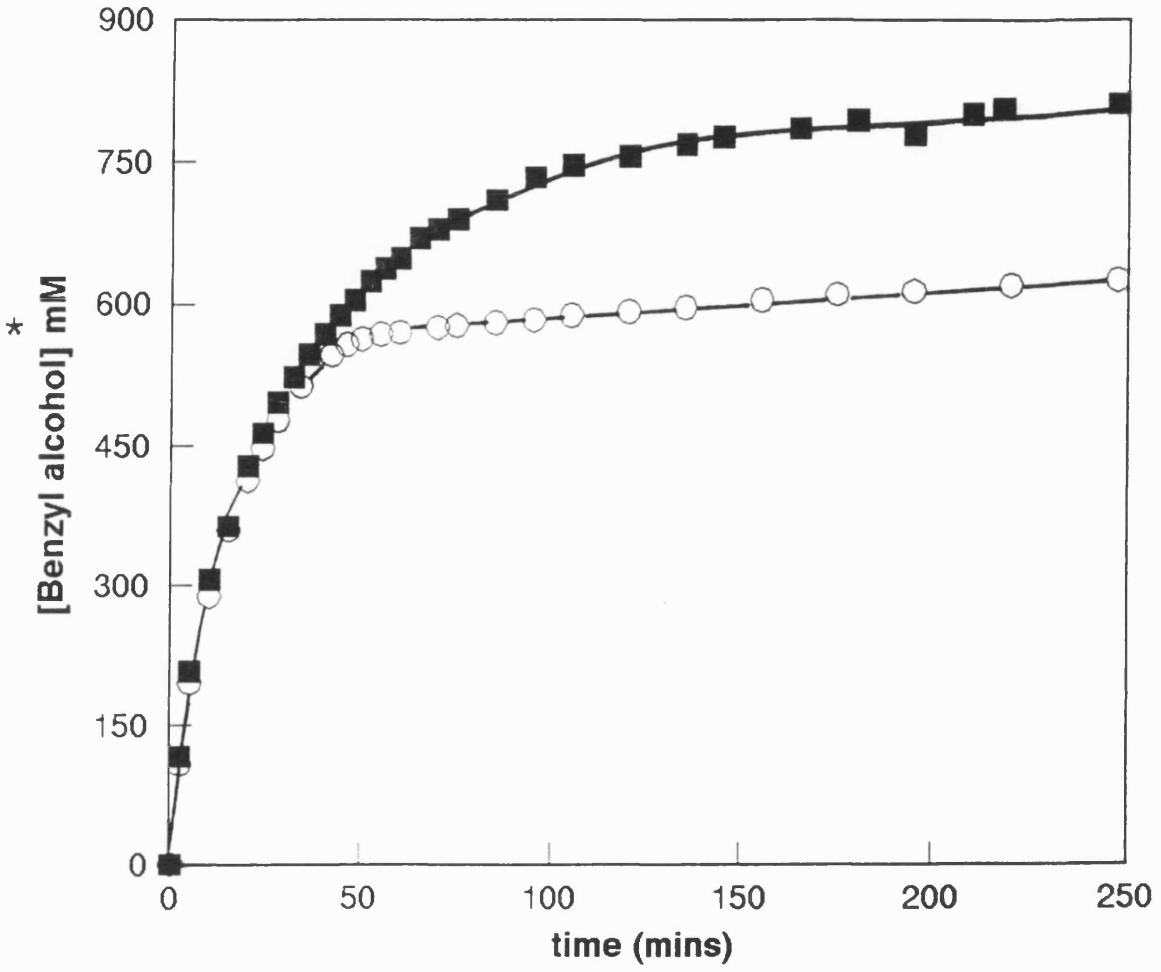


Figure 3.6 Product concentration in the STR with time for the esterase hydrolysis. Reactor conditions: ∅ = 0.4, [Ea] = 2 g l^{-1}; n = 1000 (■), n = 1800 (O).

same and thus the true amount of enzyme in the reactor identical. The slope of the line represents the rate at which product formation is occurring. Previous measurements based on the initial rate of the reaction, i.e. the first 3 minutes of reaction, indicated slightly higher rates at the stirrer speed of 1800 rpm. As reaction proceeds beyond the initial 3 minute period a reduction in the rate of product formation at 1800 rpm compared to 1000 rpm is observed, the comparative reduction becoming more marked as reaction proceeds. At 46 minutes, for the reaction at 1800 rpm, enzyme has ceased to be active in contrast to the activity for the enzyme at 1000 rpm which at a decreasing rate for a further 50 minutes up until 100 minutes at which very little further activity is observed. An effect on enzyme stability can thus be attributed to the rate of agitation, higher rates resulting in reduced stability.

From the previous experiments it was established, by examining the specific reaction rate and relating this to substrate mass transfer in the reactor, an increase interfacial area due to the creation of smaller organic droplets at higher agitation rates. Exposure of the enzyme to such increased organic/aqueous interface has a comparative detrimental effect upon the enzyme stability. We also observed that at the higher stirrer speed air was drawn into the reactor as a separate gaseous phase and thus having its own distinct interface thus this may help to magnify the instability of the enzyme.

Due to the difference in stability a further 200 mM of product was generated for the same amount of enzyme in the reactor operated at 1000 rpm compared with that operated at 1800 rpm. This required a further 50 minutes of operation time. The final yield of product from reactors operated under these conditions based on the percentage of total substrate available in the reactor, which at a phase ratio of 0.4 is 2.6 M, was at 1000 rpm 30 % and at 1800 rpm 23 %.

3.1.4. Phase Ratio

The initial product concentration time profiles depicted in figure 3.7 show results of reactions carried out an agitation rate of 1000 rpm and [Ea] of 2 g l^{-1}, experiments being repeated at different phase ratios of 0.2, 0.4, 0.5, 0.6, and 0.75. With

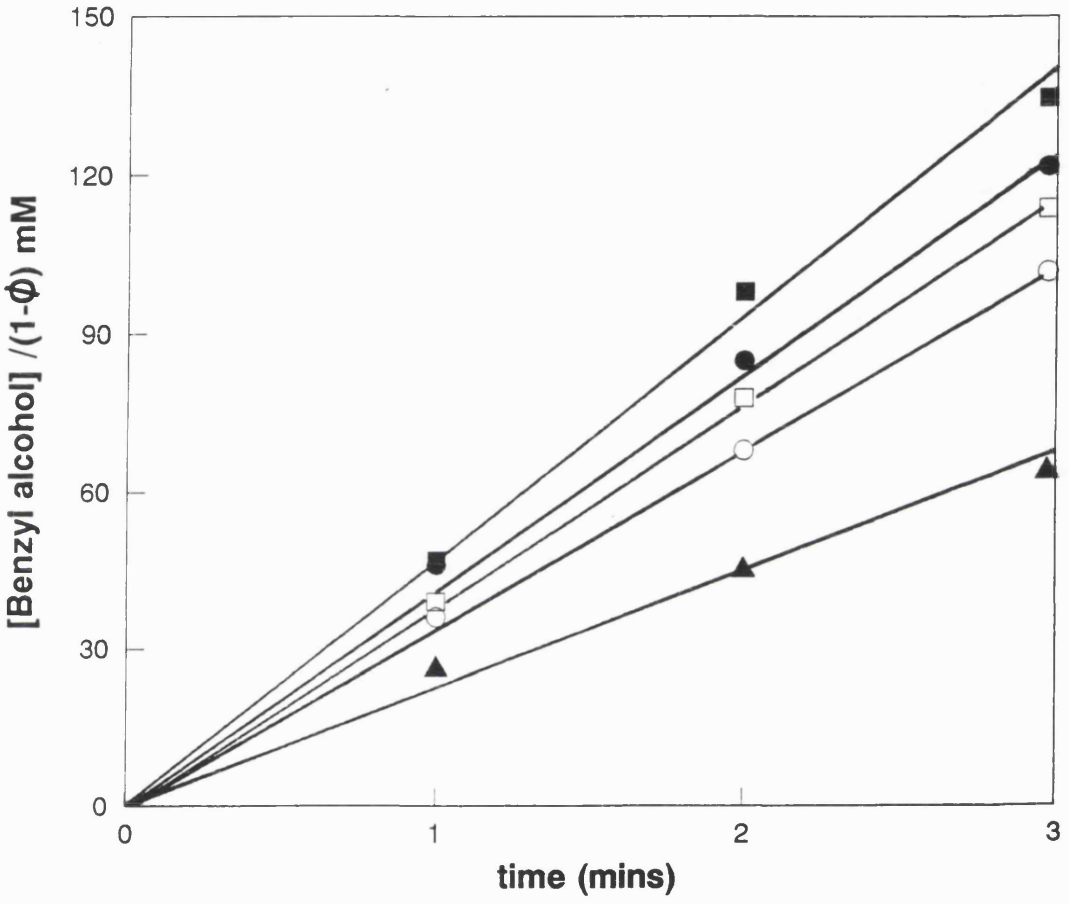


Figure 3.7 Initial two liquid phase reaction kinetics for esterase hydrolysis in the STR. Reactor conditions: n =1000 rpm, [Ea] = 2 g l^{-1}; ∅ = 0.2 (▲), 0.4 (■), 0.5 (●), 0.6 (□), 0.75 (O).

the exception of the phase ratio of 0.75 the reactions were carried out under a regime of pH maintenance, at high phase ratios the degree of inversion makes pH measurement impractical. However as previously discussed in section 2.3.3 measurements of initial activities under pH and non pH maintained regimes correlate well and thus the comparison is valid. The [Ea] was selected so that the reactor was operated in a mass transfer limited regime as for the experiments at different agitation rates. The results reflect steady state measurements. The product concentrations are expressed on an aqueous phase volume basis, particularly important under changing conditions of phase ratio as previously discussed.

Figure 3.7 was used to plot figure 3.8 which shows the specific activity expressed as a function of phase ratio at an agitation rate of 1000 rpm. The maximum specfic activity observed is lower than that previously determined in solely aqueous medium confirming the reactor was operated in a mass transfer limited regime. Therefore any changes in the specific activity are a result of increased availabilty of substrate. Highest activity is at a phase ratio of 0.4. Substrate mass transfer in the reactor is best facilitated at a phase ratio of 0.4 when agitated at 1000 rpm.

Initially at low phase ratios only a small amount of organic volume is present. The interfacial area is small resulting in low substrate K_LA resulting in low enzyme activity. As the phase ratio is increased then the volume of organic phase relative to the volume of aqueous phase is increased. The organic/aqueous interfacial area increases providing greater area and a higher substrate K_LA resulting in higher enzyme activity. As we increase the phase ratio beyond 0.4 a resultant decline in activity is observed due to a lowering of K_LA.

3.1.5. Stability (Phase Ratio)

The product concentration time profiles depicted in figure 3.9 show the results of reactions carried out at an agitation rate of 1000 rpm and [Ea] of 0.5 g l^{-1}, experiments were repeated at phase ratios of 0.2 and 0.4 respectively. In order to

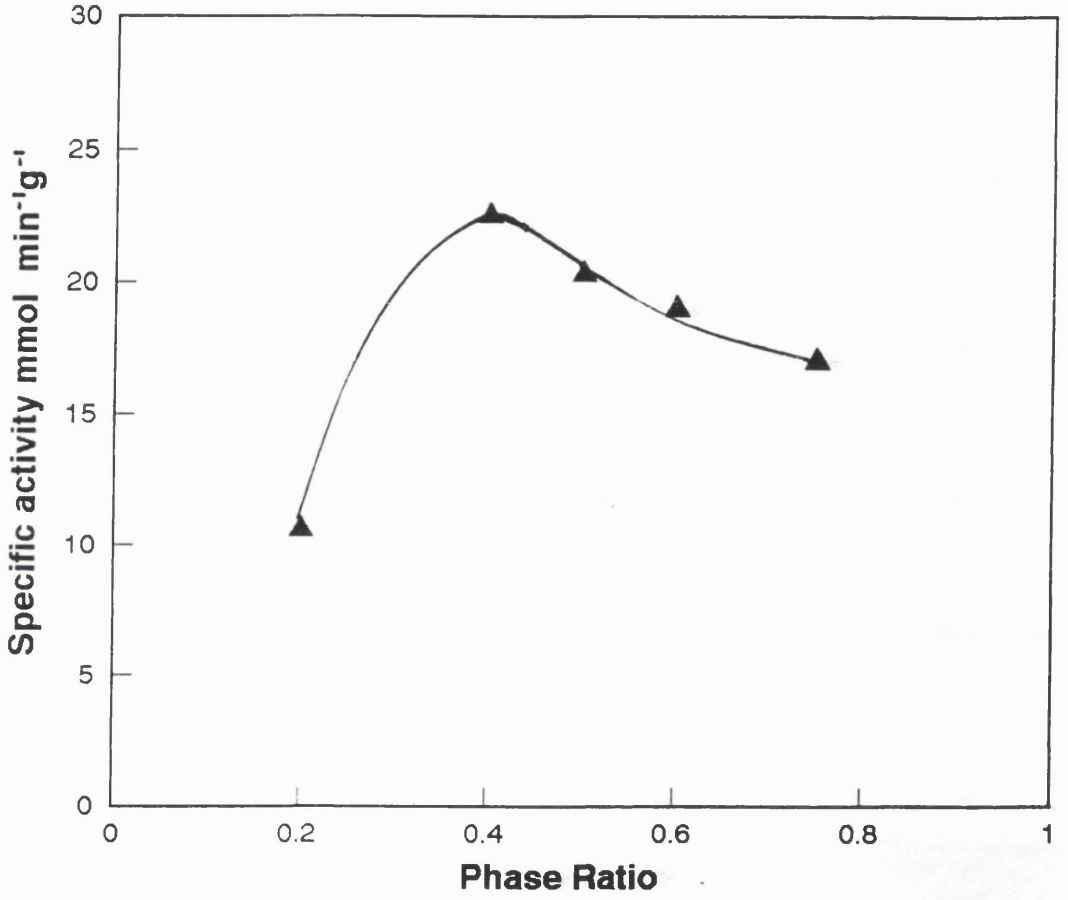


Figure 3.8 Specific activity as a function of phase ratio for esterase hydrolysis in the STR. Reactor conditions: n = 1000 rpm, [Ea] 2 g l^{-1}.

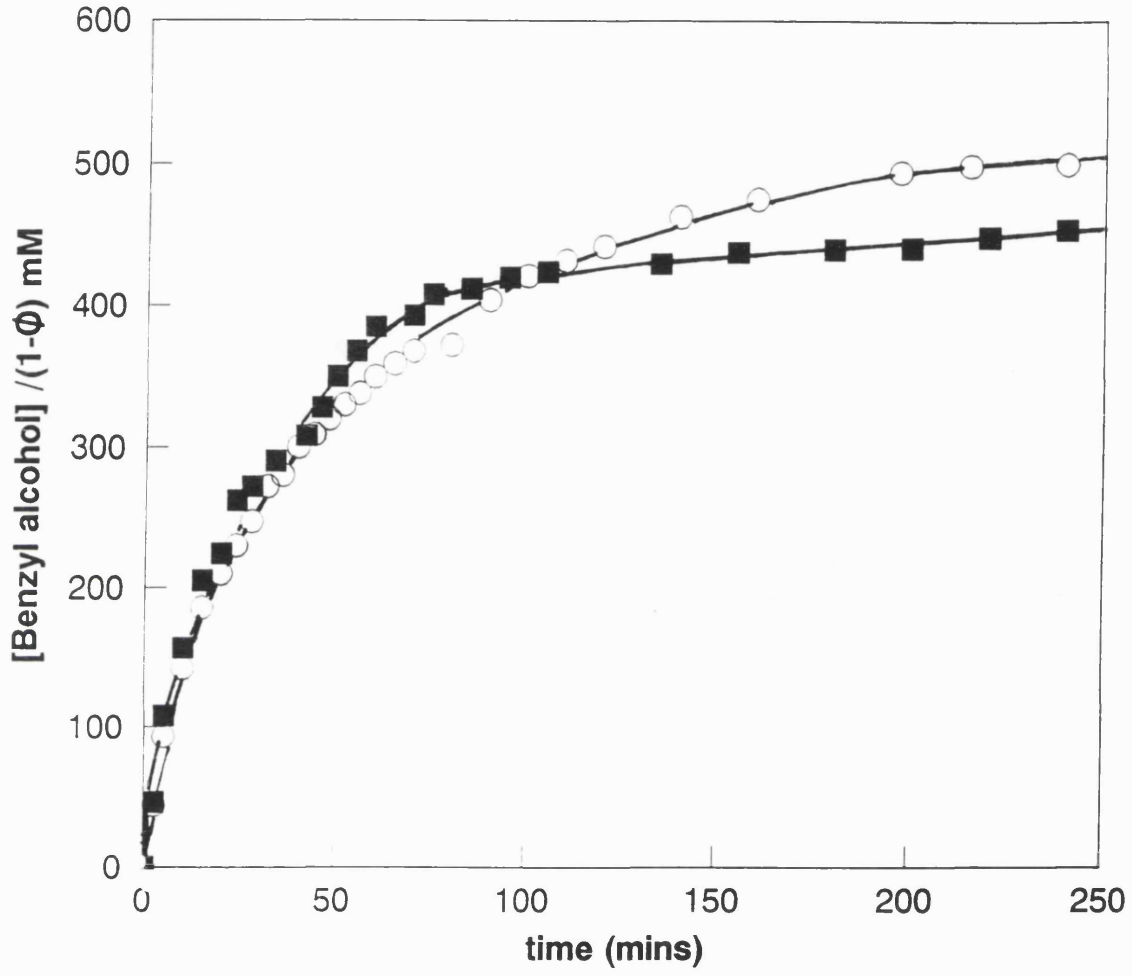


Figure 3.9 Product concentration in the STR with time for the esterase hydrolysis. Reactor conditions: n = 1000, [Ea] = 0.5 g l^{-1}; ∅ = 0.2 (O), 0.4 (■)

compare the effect on stability of the enzyme of the phase ratio the reactions were monitored, under pH controlled conditions, over a period of time, (250 mins), where eventually very little enzyme activity remained, evidenced by no further increase in the product concentration in the reactor. The product concentrations are expressed on an aqueous phase volume. Using an [Ea] of 0.5 g l^{-1} the enzyme activity is no longer limited by substrate mass transfer in the reactor under these reactor operating conditions, (section 3.1.1), as evidenced by the similarity of the initial rates of 37 mmol min^{-1} g^{-1} in both cases.

The slope of the lines represents the rate of product formation. At both phase ratios, for the first 80 minutes, there is a steady equivalent decay in enzyme activity. After 80 minutes of operation, at a phase ratio of 0.4, there is a cessation of the enzyme activity In contrast the enzyme activity at a phase ratio of 0.2 continues, albeit at a much lower rate, for another 100 minutes. A greater area of organic/aqueous interface exists at a phase ratio of 0.4 compared with that at 0.2. (sec 3.1.4). Previous comparison of enzyme stability at two different agitation rates indicated an effect attributable to the creation of greater interface at the higher agitation rate. It is apparrent from this result that an effect akin to that is being observed although this effect is not as pronounced as that observed for a higher rate of agitation, (section 3.1.3).

3.1.6. Phase Ratio and Agitation

The initial product concentration time profiles depicted in figure 3.10 show the results of reactions carried out at an agitation rate of 1800 rpm and an [Ea] of 2 g l^{-1}, experiments were repeated at different phase ratios of 0.2, 0.4 and 0.6 respectively. The [Ea] of 2 g l^{-1} was used so as to be operating in a mass transfer limited regime (section 3.1.1) and all product concentrations were expressed on an aqueous phase volume basis.

The results of figure 3.10 and the previous results of figure 3.7 were used to plot figure 3.11 which shows the specific activity expressed as a function of phase ratio at

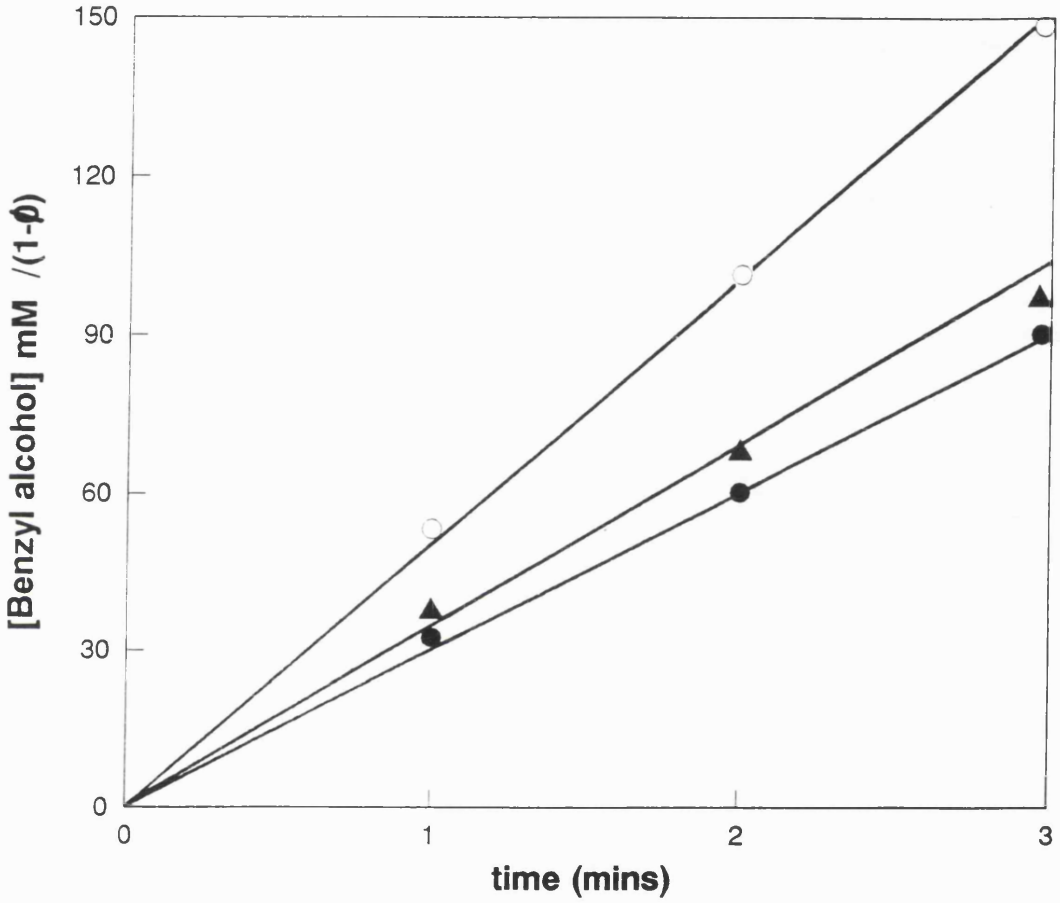


Figure 3.10 Initial two liquid phase reaction kinetics for esterase hydrolysis in the STR. Reactor conditions: n =1800 rpm, [Ea] = 2 g l^{-1}; ∅ = 0.2 (▲), 0.4 (O), 0.6 (●).

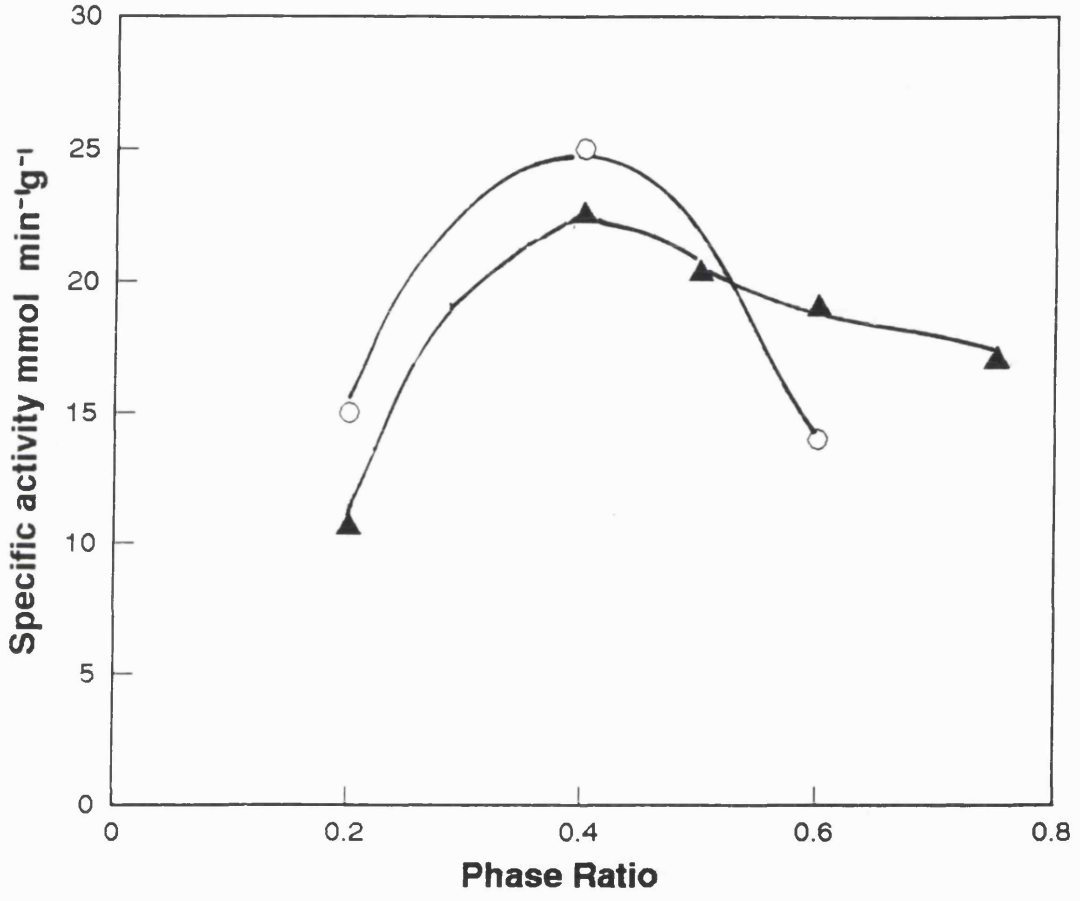


Figure 3.11 Specific activity as a function of phase ratio for esterase hydrolysis in the STR. Reactor conditions: [Ea] 2 g l^{-1}; n = 1000 rpm (▲), n = 1800 rpm (O).

agitation rates of 1000 rpm and 1800 rpm. The plot at 1000 rpm was as previously shown in fig 3.8. Comparing the two plots at phase ratios of 0.2 and 0.4 at increased agitation rate there is a corresponding increase in the specific activity which due to an increase in the substrate K_LA as a result of increased interfacial area (section 3.1.2). Comparing the specific reaction rate at these two agitation rates at a phase ratio of 0.6 shows a decrease in the specific reaction rate at 1800 rpm relative to that at 1000 rpm. This is in contrast to the effect observed at phase ratios of 0.2 and 0.4 implying a decrease in substrate K_LA.

3.1.7. Product Inhibition

The product concentration time profiles depicted in figure 3.12 show the results of reactions carried out at a phase ratio of 0.4 and an agitation rate of 1000 rpm, experiments were repeated at aqueous enzyme concentrations of 0.5 and 2 these were further broken down into two separate experiments at each enzyme concentration. Experiment carried out where no initial quantity of benzyl alcohol was added to the reactor and a comparative experiment where an initial quantity of benzyl alcohol at a reactor concentration of 400 mM was added to the reactor.

Observing the effect of this addition at 0.5 g l^{-1} shows that upon reaction start up an initial amount of benzyl alcohol is produced for both reactions As reaction progresses the rate in the reactor containing an initial quantity of benzyl alcohol rapidly declines compared to that in the reactor in which no initial quantity of benzyl alcohol was present. Figure 3.13 shows the first 3 minutes of this reaction and demonstrates more clearly that the effect of the initial quantity of benzyl alcohol is almost immediate at [Ea] of 0.5 g l^{-1}. In further comparison of these two experiments, in the reactor in which no initial quantity of benzyl alcohol is present, activity continues for a 100 minutes. In contrast the activity in the reactor with the initial quantity of benzyl alcohol present continues only for a 40 minute period of operation.

Observing the reactions carried out at 2 g l^{-1} , similarly upon reaction start up an initial amount of benzyl alcohol is produced in both reactors. For the first 3 minutes of

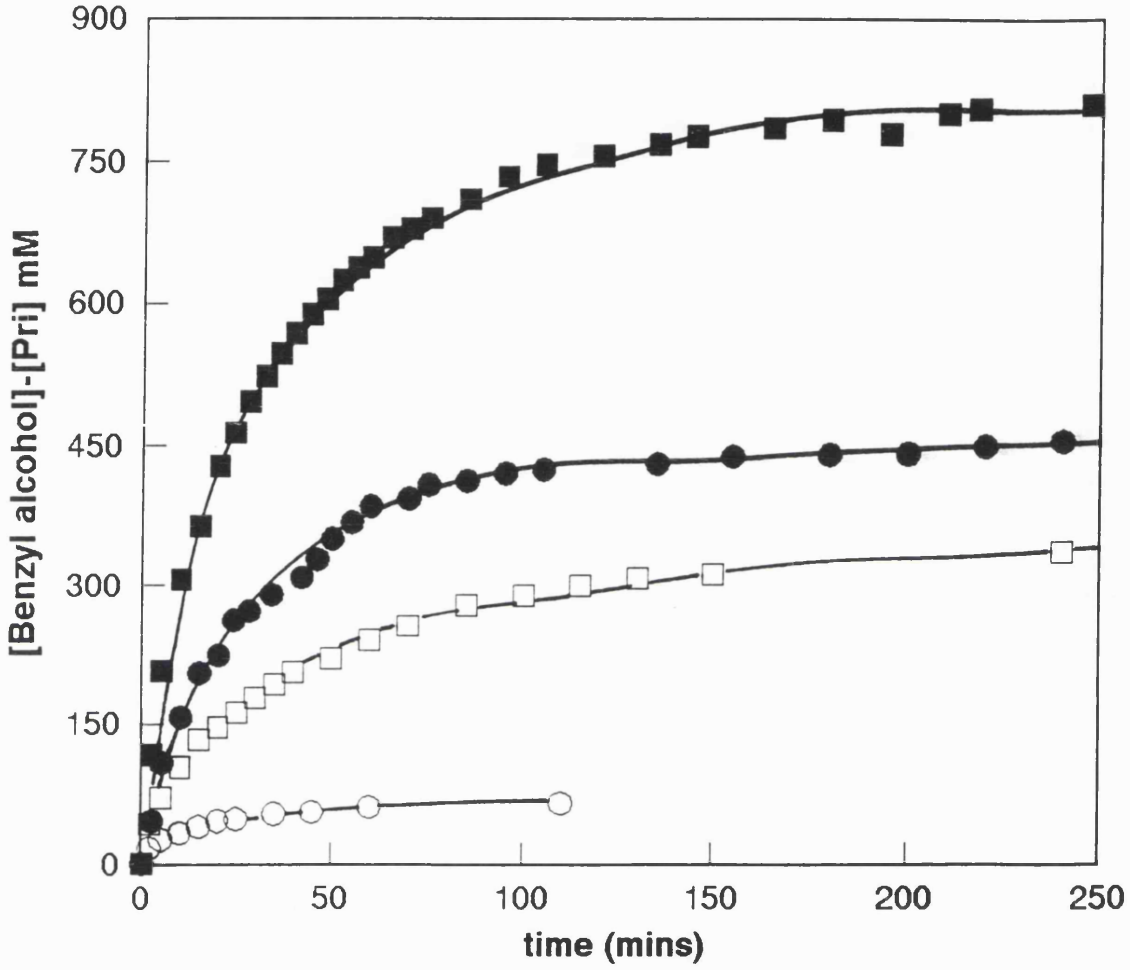


Figure 3.12 Product concentration in the STR with time for the esterase hydrolysis. Reactor conditions: n = 1000 rpm, ∅ = 0.4; [Ea] = 2 g l^{-1}, [Pri] = 0 (■), [Pri] = 400 mM (□); [Ea] = 0.5 g l^{-1}; [Pri] = 0 (●), [Pri] = 400 mM (O)

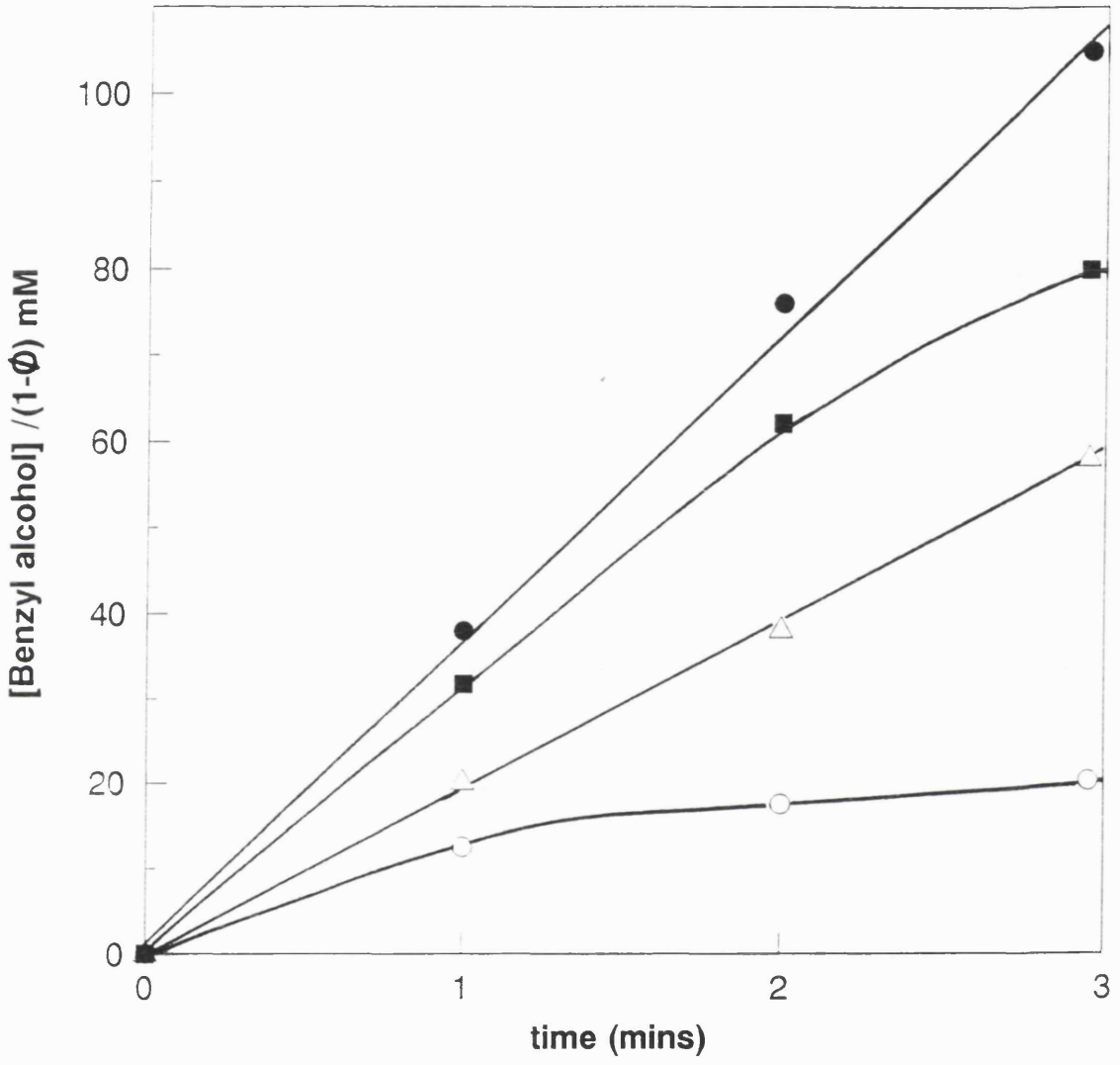


Figure 3.13 Initial two liquid phase reaction kinetics for esterase hydrolysis in the STR. Reactor conditions: ∅ = 0.4, n = 1000 rpm; [Ea] = 0.5 g l^{-1}; [Pri] = 0 (●), [Pri] = 400 mM (O); [Ea] = 2 g l^{-1}, [Pri] = 0 (■), [Pri] = 400 mM (□).

operation, figure 3.13 the rate in both reactors corresponds. After the first few minutes the rate in the reactor containing an initial quantity of benzyl alcohol declines, compared with that observed in the reactor which contained no initial quantity of benzyl alcohol and continues to comparitively decline as reaction progresses. In contrast to the observations made at the enzyme concentration of 0.5 g l^{-1}, activity remains for 100 minutes in both reactors.

The initial product concentration of 400 mM represents, for reactions carried out at 0.5 g l^{-1}, the concentration of product normally generated in the reactor under these conditions upon reaction completion. At an enzyme concentration of 2 g l^{-1} 400 mM repesents a concentration which is half that achieved in the reactor under these conditions upon reaction completion. The effect of benzyl alcohol is thus concentration dependent relative to the enzyme concentration in the reactor hence the more pronounced and immediate effect of the initial quantity of benzyl alcohol at [Ea] of 0.5 g l^{-1}. At 2 g l^{-1} an effect is observed but since a total inhibitory concentration is not present in the reactor, reaction continues until a further 400 mM of product are generated, at which time activity ceases, the additive total of product in the reactor at this point is equal to 800 mM, the concentration of benzyl alcohol in the reactor at which activity ceases in the reactor in which no initial quantity of benzyl alcohol is present.

3.2. Lipase Catalysed Hydrolysis in the STR

3.2.1. Enzyme Concentration

The initial product concentration time profiles depicted in figure 3.14 show the results for reactions carried out at a phase ratio of 0.4 and an agitation rate of 1000 rpm. Experiments were repeated using different aqueous phase enzyme concentrations of 2, 4, 8 and 16 g l^{-1} respectively. Based on previous results with the esterase a phase ratio of 0.4 provides optimal interfacial area in a two-phase system and at 1000 rpm greater stability was observed than operating at 1800 rpm thus these two parameters were chosen as the starting point for studies with the lipase. Since lipases are active at the organic/aqueous interface, (Verger and Haas 1974) then product does not emanate from the aqueous phase as with the esterase and the kinetics of the lipase will thus depend on the interface available All product concentrations , since we are using the same phase ratio, are expressed on the basis of concentration in the total reactor medium volume.

The results of figure 3.14 were used to plot figure 3.15 which shows the specific reaction rate as a function of [Ea] for reaction carried out in the STR at a phase ratio of 0.4 and an agitation rate of 1000 rpm. Observing this plot shows that at all enzyme concentrations investigated the specific reaction rate is comparable. The rates measured are lower than the rates observed for the esterase primarily due to impurity of the lipase preparation.

The interface in the STR for a lipase catalysed reaction serves as the site of activity, if not enough interface is present for all lipase to have access to it then the specific reaction rate for the lipase will alter depending on the availabilty of interfacial area. It would be expected that for a particular interfacial area there is a concentration of enzyme at which the interface is fully saturated. Any further increase in concentration will not be supported by the interface available thus excess enzyme will remain redundant in the aqueous phase, it cannot catalyse the reaction and thus the specific reaction rate will show a decrease. The specific reaction rates in this set of experiments at all enzyme concentrations investigated were the same. Even at the relatively high

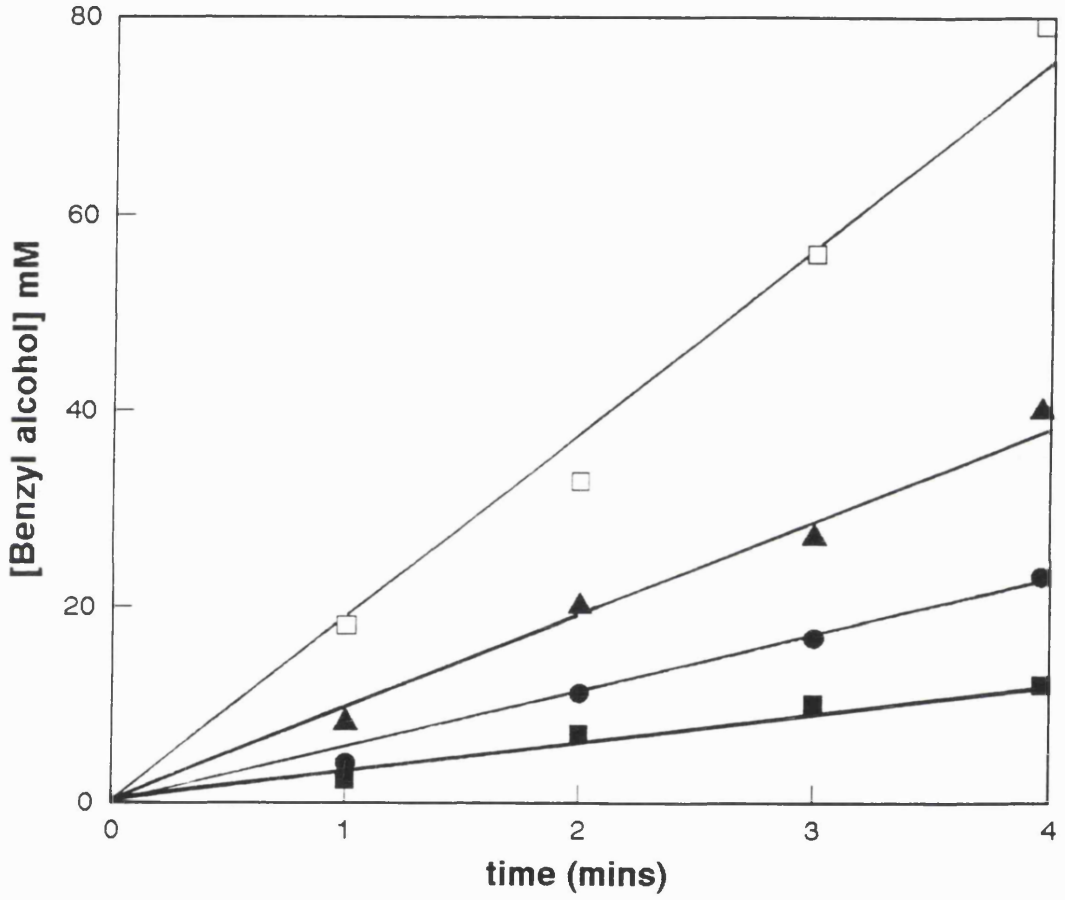


Figure 3.14 Initial two-liquid phase reaction kinetics for lipase hydrolysis in the STR.Reactor conditions: ∅ = 0.4, n = 1000; [Ea] = 2 g l^{-1} (■), 4 g l^{-1} (●), 8 g l^{-1} (Δ), 16 g l^{-1} (□).

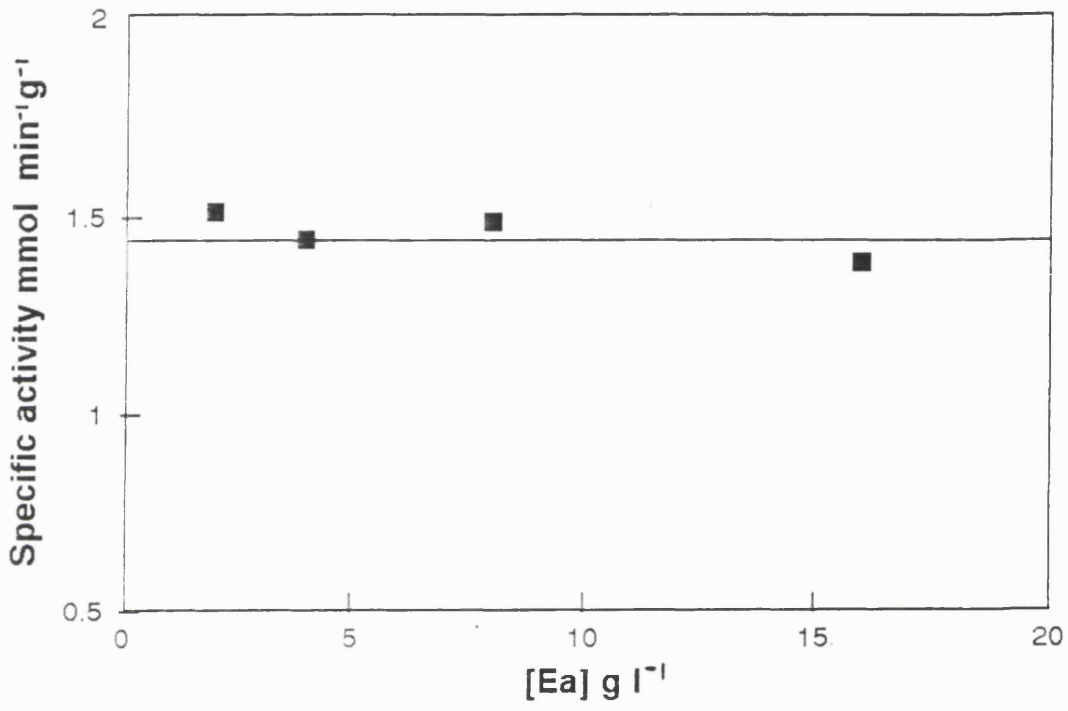


Figure 3.15 Specific activity as a function of aqueous phase enzyme for lipase hydrolysis in the STR. Reactor conditions; $\varnothing = 0.4$, n = 1000 rpm.

concentration of 16 g l^{-1} the interface is not yet fully saturated. This may reflect the impurity of the preparation, even at these high concentrations only a fraction of the concentration has lipase activity, the remaining non lipase proteins not competing with the lipase proteins for access to the interface

3.2.2. Agitation Rate

The product concentration time profiles depicted in figure 3.16 show the results for reactions carried out at a phase ratio of 0.4 and an [Ea] of 4 g l^{-1}. Experiments were repeated at agitation rates of 1000 and 1800 rpm. In order to compare the effect on the stability of the enzyme at these two rates of agitation, the reaction was monitored, under pH controlled conditions, over a period of time, 24 hours, where very little further activity remained, evidenced by little further increase in product concentration in the reactor. Initially rates were identical at both agitation rates indicating that although the interfacial area at 1800 rpm is potentially greater than that at 1000 rpm the enzyme concentration we are operating at does not reflect this difference since the interface is not fully saturated. Continuing to observe the two reactions as they progress, the profiles of product concentration and thus the rates are identical throughout, there is no difference in the stabilty of the enzyme at these two stirrer speeds. Implicit in this is the fact that previously for the esterase, which acts in the bulk of the aqueous phase, a detrimental effect on enzyme stabilty at higher agitation rates was attributed to an interfacial effect (section 3.1.3). In contrast the lipase is active at an interface and it is not expected that by increasing the interfacial area enzyme stability would be significantly affected at below interfacial saturation concentrations.

3.2.3. Phase Ratio

The product concentration time profiles depicted in figure 3.17 show the results for reactions carried out at an [Ea] of 4 g l^{-1} and an agitation rate of 1000 rpm. Experiments were repeated at phase ratios of 0.4 and 0.2 . In order to assess the impact

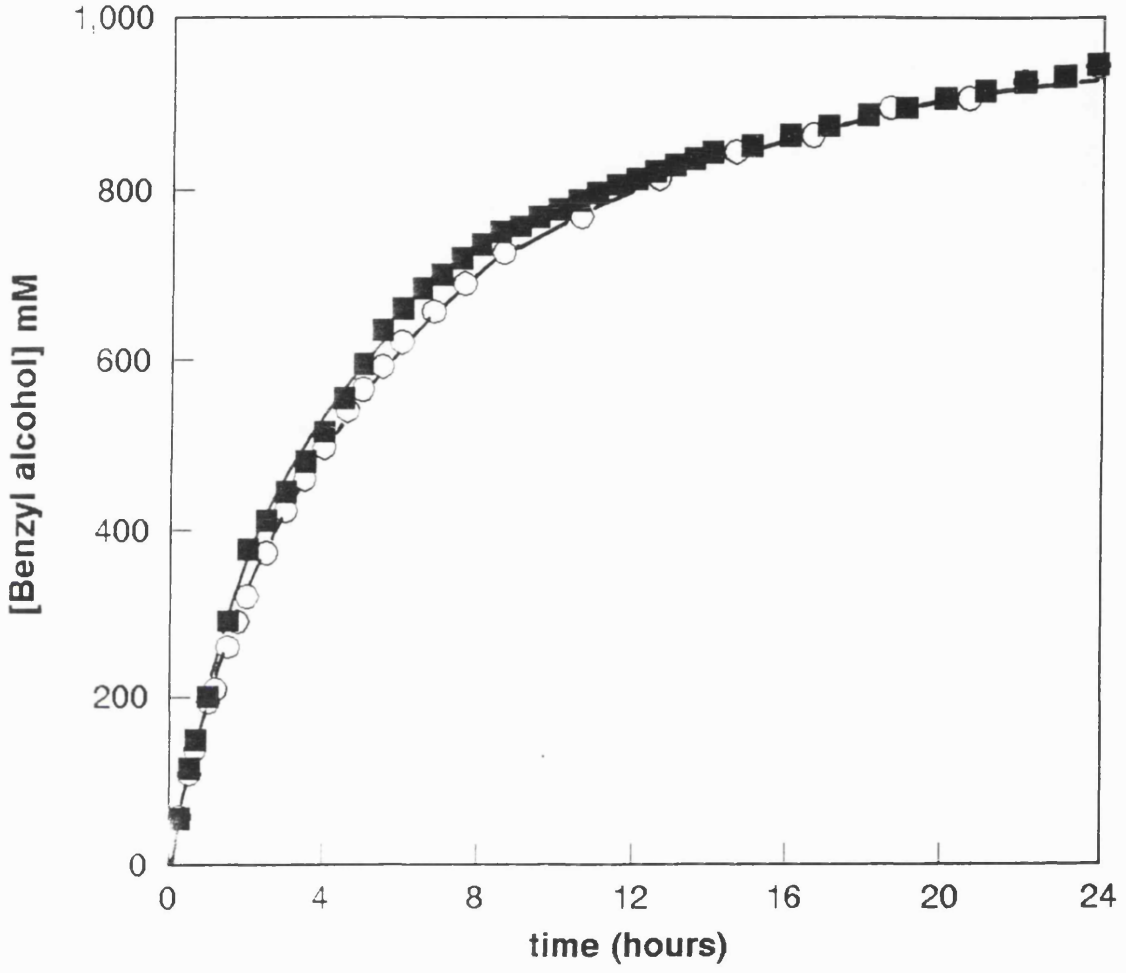


Figure 3.16 Product concentration in the STR with time for the lipase hydrolysis. Reactor conditions. ∅ = 0.4, [Ea] = 4 g l^{-1}; n = 1000 rpm (O), 1800 rpm (■).

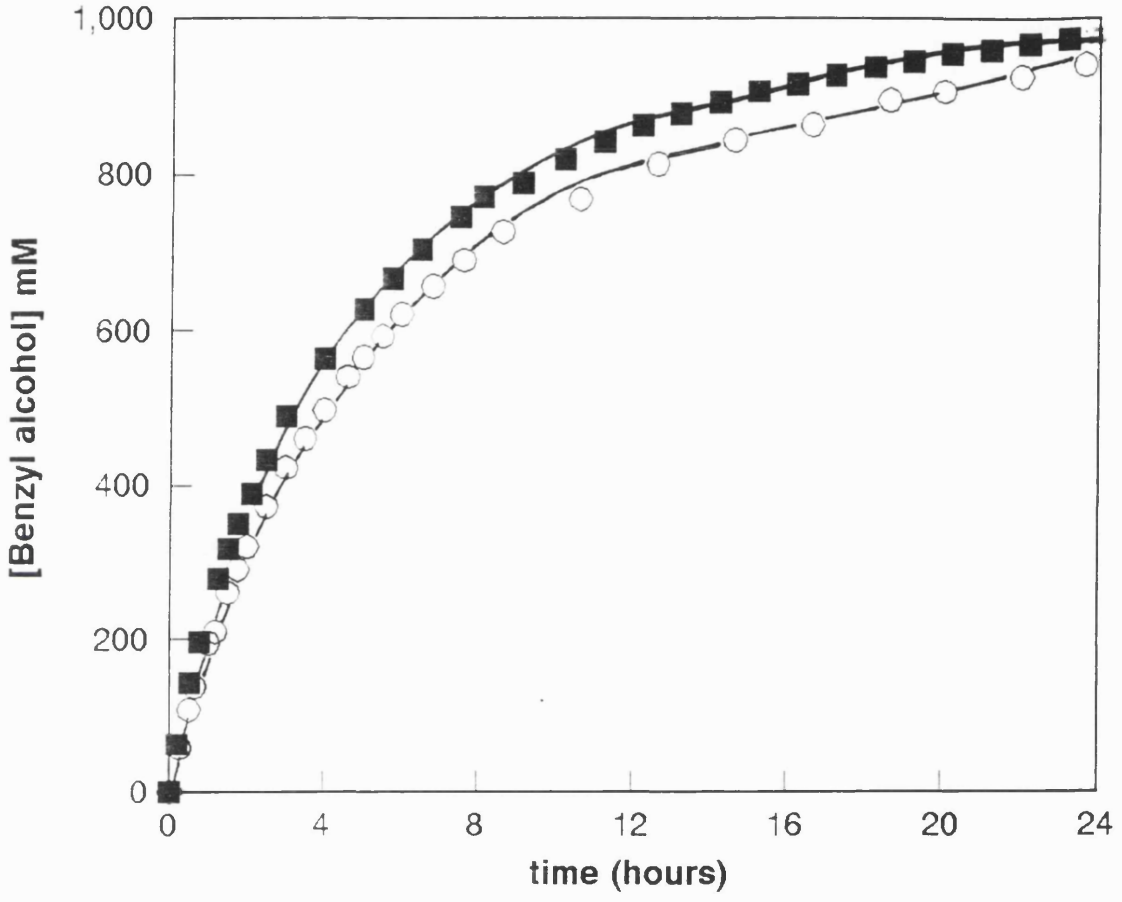


Figure 3.17 Product concentration in the STR with time for the lipase hydrolysis. Reactor conditions. n = 1000 rpm, [Ea] = 4 g l^{-1}; ∅ = 0.2 (■), 0.4 (O).

of phase ratio on stability the reactions were monitored, under pH controlled conditions, over a period of time, 24 hours, where eventually very little activity remained as evidenced by no further significant increase in the concentration of benzyl alcohol.

The profiles are identical. It could be predicted, assuming specific reaction rates to be identical, an increased amount of product at phase ratio of 0.2 since a greater amount of aqueous phase containing enzyme is present compared to that at a phase ratio of 0.4 and product concentration are expressed on a reactor volume basis. This however is not observed. The same amount of product being generated in both reactors implies that at a phase ratio of 0.2 the interface is limiting the enzyme activity since the area of interface cannot support all enzyme present, the interface becomes fully saturated and excess enzyme lies redundant in the aqueous phase where it cannot exhibit activity. The two profiles being significantly identical with cessation of activity at the same time, there is no effect of phase ratio on stabilty.

3.2.4. Product Inhibition

The product concentration time profiles depicted in figure 3.18 show the results for reactions carried out at a phase ratio of 0.4, an agitation rate of 1000 rpm and an [Ea] of 4 g l^{-1}. Experiments were repeated, one with no initial quantity of benzyl alcohol present and a comparative experiment in which an initial quantity of benzyl alcohol was added resulting in a reactor concentration of 1M.

Observing the profiles, initially the two rates are the same. As the reaction progresses beyond 1 hour the rate in the reactor, in which an initial quantity of benzyl alcohol was contained, declines slightly more rapidly and ends up slightly lower than that observed in the reactor in which no initial quantity of benzyl alcohol was present. The final product concentration achieved in the reactor in which an initial amount of benzyl alcohol was added is resultingly slightly lower but from these results the inhibitory effect of benzyl alcohol upon the lipase is only marginal under these conditions.

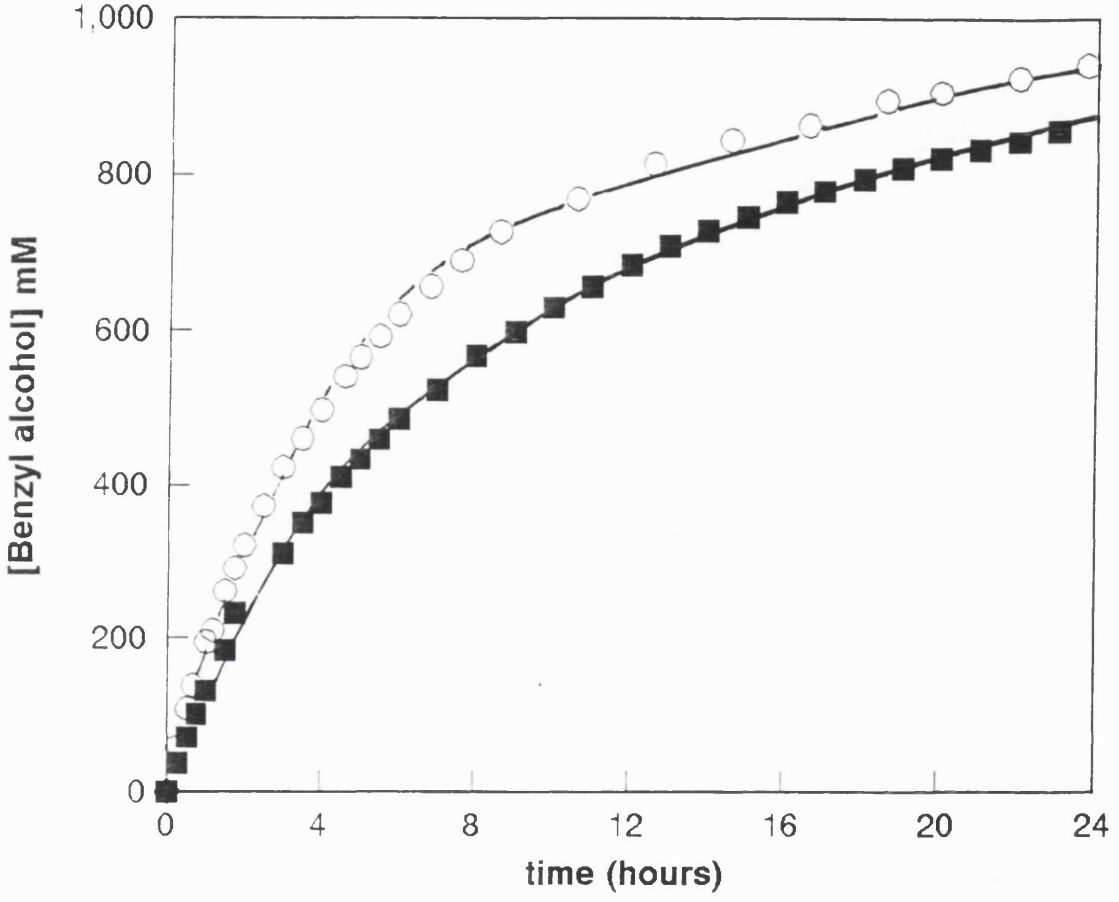


Figure 3.18 Product concentration in the STR with time for the lipase hydrolysis. Reactor conditions: ∅ = 0.4, n = 1000 rpm, [Ea] = 4 g l^{-1}; [Pri] = 0 (O), [Pri] = 1M (■).

3.2.5. Substrate Conversion

The product concentration time profiles depicted in figure 3.19 show the results for reactions carried out at a phase ratio of 0.4 and an agitation rate of 1000 rpm. Experiments were repeated at different aqueous phase enzyme concentrations of 2, 4, 8 and 16 g l^{-1} respectively. The reactions were monitored, under pH controlled conditions, over a period of time, 24 hours, where eventually very little activity remained as evidenced by no further significant increase in the concentration of benzyl alcohol.

Observing the plots at all four enzyme concentrations reveals similar trends in all four plots. For the first four hours of operation rate declines steadily, a further accelerated decline is then observed over the next 8 hour period, followed by a more rapid decline over the next 12 hour period. Rate in the reactors initially for the first 8 hours are significantly similar, however identifying the specific activity after 12 hour of operation time the rates at 2, 4 and 8 g l^{-1} are all identical at 5 mmol hr^{-1} g^{-1}. The specific reaction rate identified at 12 hours for the reactor operated with an enzyme concentration of 16 g l^{-1} was 3.13 mmol hr^{-1} g^{-1}, significantly lower than that at 2, 4 and 8 g l^{-1}. This lowering of activity may reflect one of two possibilties due to the high concentration of product in the reactor. One might assume that it reflects inhibitory levels of product although one would expect this to be in relation to enzyme concentration and might expect to observe similar effects at the other enzyme concentrations. An alternative explaination may be elucidated if we consider the availabilty of substrate at the interface. At enzyme concentrations of 2, 4 and 8 g l^{-1} the substrate converted after 12 hours of operating time represents 50 % and less of the quantity available for conversion, whereas that at 12 hours for an enzyme concentration of 16 g l^{-1} represents 76 % of the quantity of substrate available in the reactor. Knowing that benzyl alcohol partitions primarily into the organic phase, then at 50 % conversion and less the benzyl alcohol will most probably be dissolved in the substrate benzyl acetate the interface primarily being a substrate aqueous phase interface. The enzyme at the interface still has pimarily access to relatively high concentrations of substrate. At

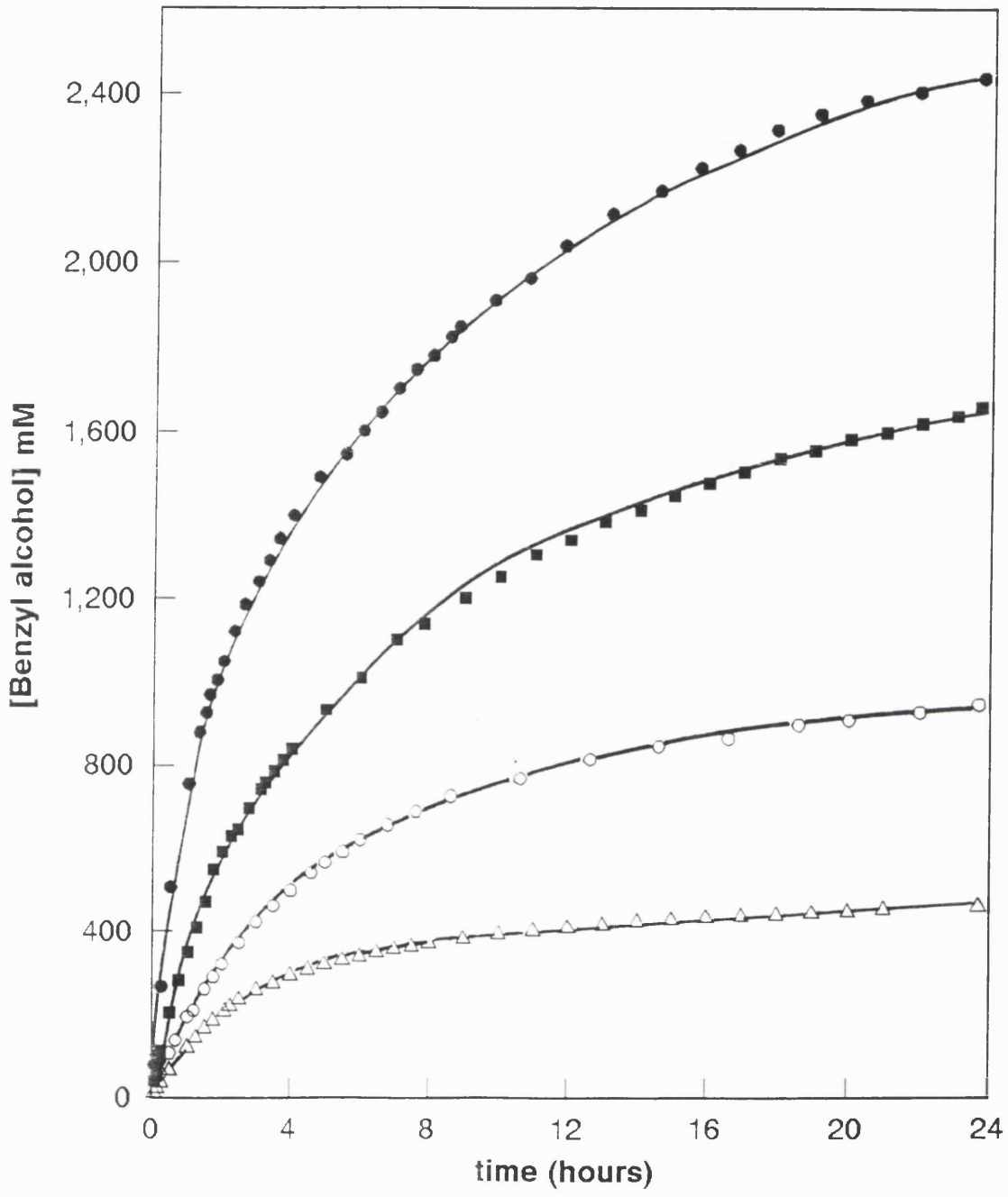


Figure 3.19 Product concentration in the STR with time for the lipase hydrolysis. Reactor conditions: $\varnothing$ = 0.4, n = 1000 rpm; [Ea] = 2 g l^{-1} (Δ), 4 g l^{-1} (O), 8 g l^{-1} (■), 16 g l^{-1} (●)

conversions of greater than 50 % and particularly at 76 % the quantity of product most probably forms the second liquid phase in which the remaining substrate becomes dissolved and thus the interface will now primarily be a product / aqueous interface and the volumetric concentration of substrate is reduced. The enzyme at such an interface will be limited in primary access to substrate, its availability will be hindered by the presence of product and hence a relative decrease in the specific reaction rate will result. The low concentrations of substrate, forces governing substrate migration to the interface and subsequent contact with the enzyme will become rate limiting.

The conversion at 2, 4 and 8 g l^{-1} were 16, 32 and 62 % respectively accurately reflecting the constancy of the specific reaction rates at each doubling of enzyme concentration and the effects within the STR. The conversion returned at 16 g l^{-1} was 92 % at 24 hours.

3.3. Comparison of Lipase Catalysed Hydrolysis in the STR and the MBR

3.3.1. Low Enzyme Load

The quantity of crude enzyme material used for these experiments was 10 g l^{-1}, only 25 % of this material was protein, not all of which accounted for lipase activty and a further amount of activity was lost in the preparation process for enzyme loading (section 2.5.1). The amount of lipase active protein was therefore small in comparison to the potential carrying capacity of the membrane module operated and thus experiments carried out using this initial quantity of enzyme were designated as low enzyme load experiments.

3.3.1.1. In the STR

To make a valid comparison of the performance of the MBR with that of the STR an experiment was carried out which employed a fraction of the lipase active aqueous phase generated in the enzyme loading preparative procedure i.e. the supernatant generated after centrifugation of the 10 g l^{-1} suspension of crude enzyme material.

The product concentration time profiles depicted in figure 3.20 illustrate the results of a reaction carried out in the STR at a phase ratio of 0.5 and an agitation rate of 1000 rpm. The lipase active aqueous phase being as previously described. Since interest was in the initial rate information for comparison with the results subsequently obtained from reactions carried out in a MBR, the reaction was monitored, under pH controlled conditions, for a period of 150 minutes at which time the generation of product in the reactor could be seen to decrease in relation to previous rate of generation. The results are expressed in relation to three criteria, aqueous phase concentration, organic phase concentration and concentration based on overall reactor volume.

The idea behind displaying the result for these experiments in this manner is based on the fact that in the MBR the two phases are kept separate. Effectively the

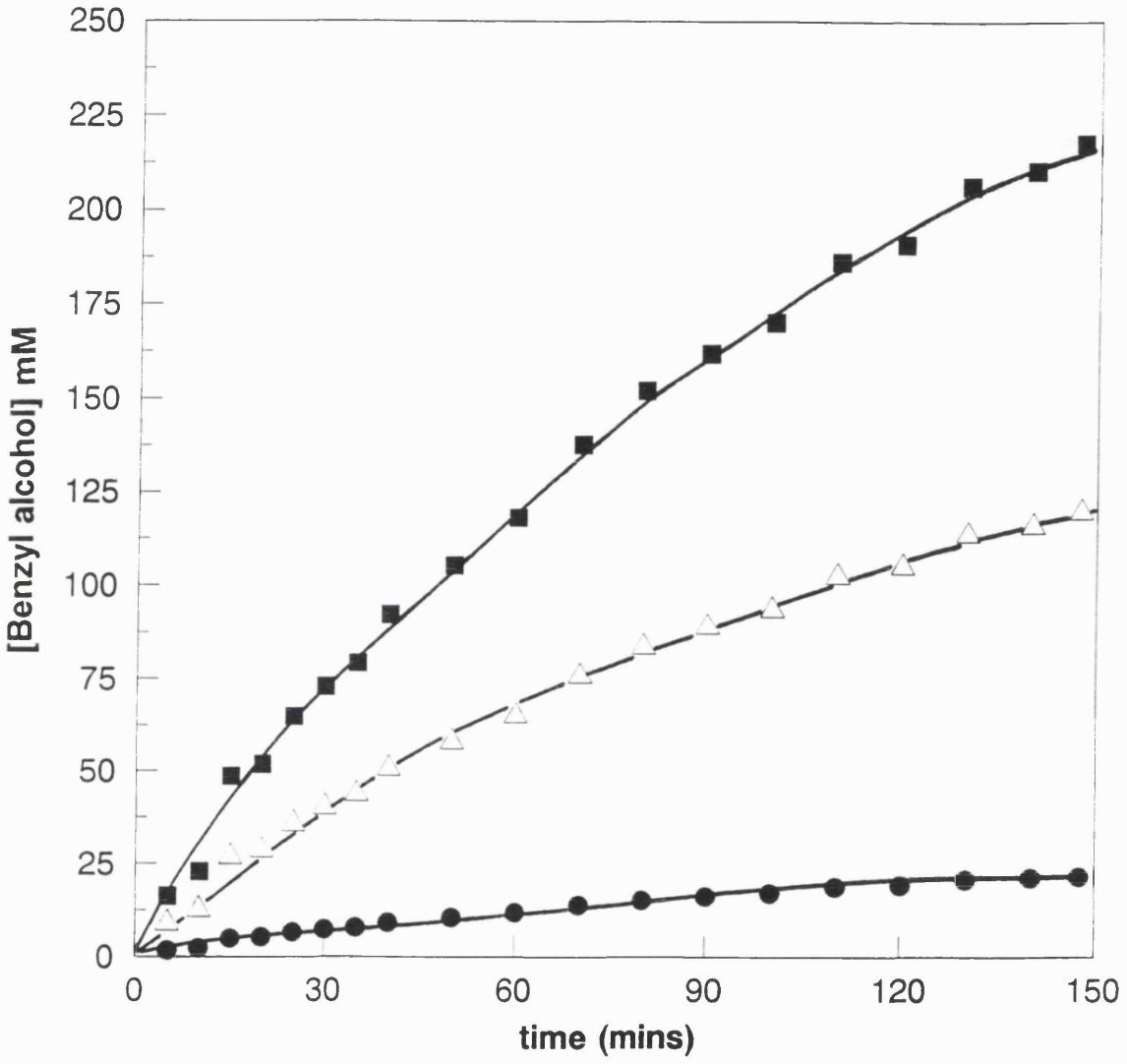


Figure 3.20 Product concentration in the STR with time for lipase hydrolysis. Reactor conditions: 0 = 0.5, n = 1000 rpm, [Ea] = 50 ml fraction from 10 g l^{-1} permeate; reactor concentration; overall (Δ), aqueous phase (●), organic phase (■).

medium volume in the reactor may, as in the STR, be said to be equal to the sum of the aqueous and organic phase volumes. Product however, assuming obedience to partitioning, will mainly be located in the organic phase (partition coefficient = 11) and will thus be concentrated up relative to a concentration if based on total medium volume. Product will also similarly partition in the STR. Such product partitioning will have particular implications in product recovery and limiting exposure of catalyst to inhibitive product. Depicting the results in this manner emphasises the different manner in which the concentrations may be expressed with respect to the reactor engineer interested in kinetic comparison and the downstream process engineer involved in product recovery.

3.3.1.2. In the MBR

The product concentration time profiles depicted in figure 3.21 show the results of a reaction carried out in the MBR using a low enzyme load (the supernatant produced after dissolution and centrifugation of the 10 g l^{-1} crude lipase preparation). The reactor was run with reservoir volumes of 400 ml for both phases at a circulation rate through the module of 500 ml min^{-1} in a cocurrent mode. The effective phase ratio can therefore be described as 0.5. The amount of enzyme loaded onto the membrane is effectively 17 times the amount used in the STR. The reaction was monitored, under pH controlled conditions, and the results illustrated in figure 3.21 are for the first 150 minute period of operation. Again results are expressed on the basis of aqueous phase concentration, organic phase concentration and concentration based on overall reactor medium volume.

3.3.1.3. Comparison of the Reaction Rates in the STR with those in the MBR.

The results of figures 3.20 and 3.21 were used to plot of figure 3.22 which shows the activity, determined in the STR and the MBR, as they appear as reaction progresses in the reactors operated under the conditions as outlined. Activities for

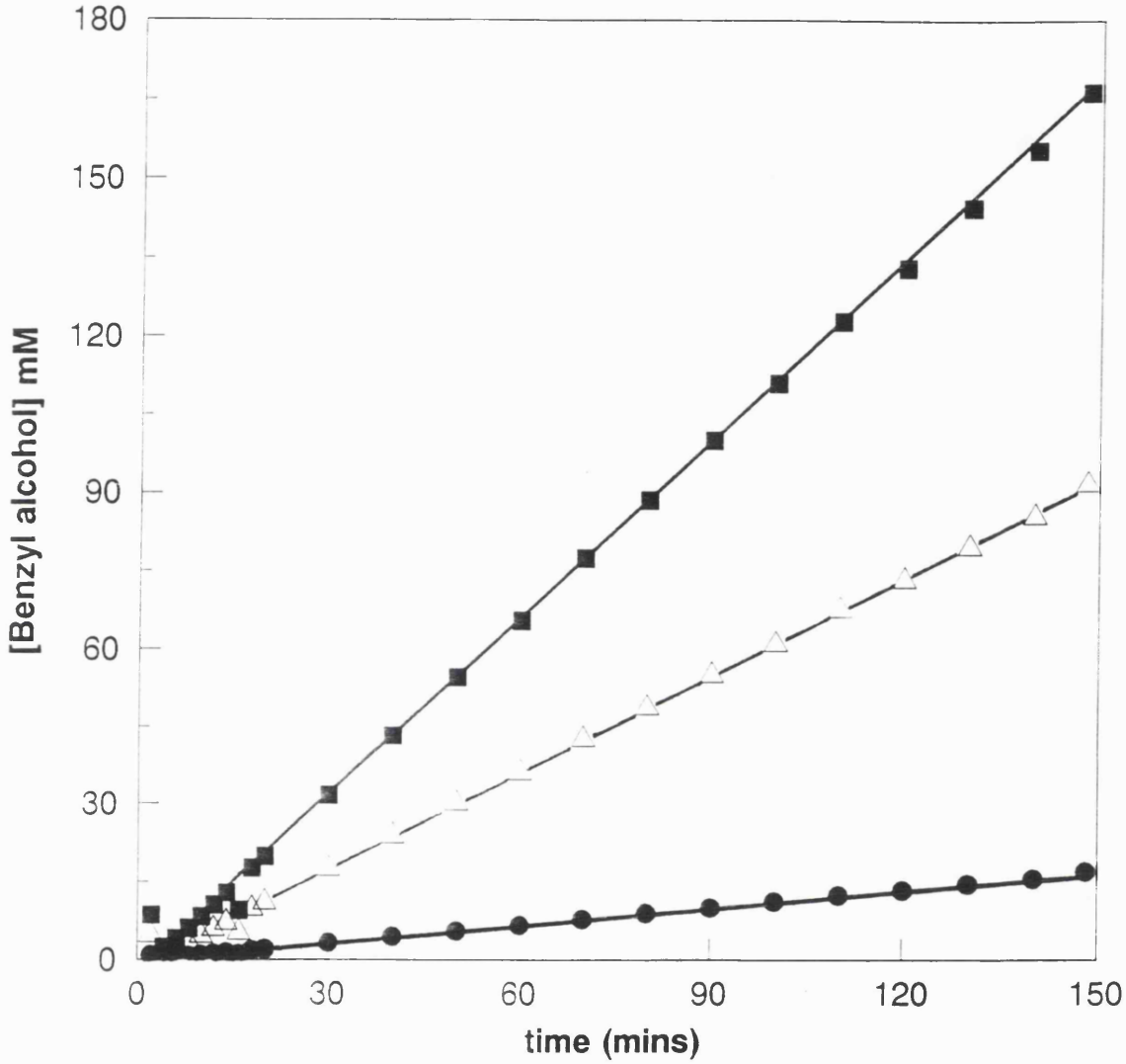


Figure 3.21 Product concentration in the MBR with time for lipase hydrolysis. Reactor conditions: Organic and aqueous phase volumes = 400 ml, Flow rates = 500 ml min^{-1}, [Ea] is that contained in the 850 ml of remaining 10 g l^{-1} permeate; reactor concentration; overall (Δ), aqueous phase (●), organic phase (■).

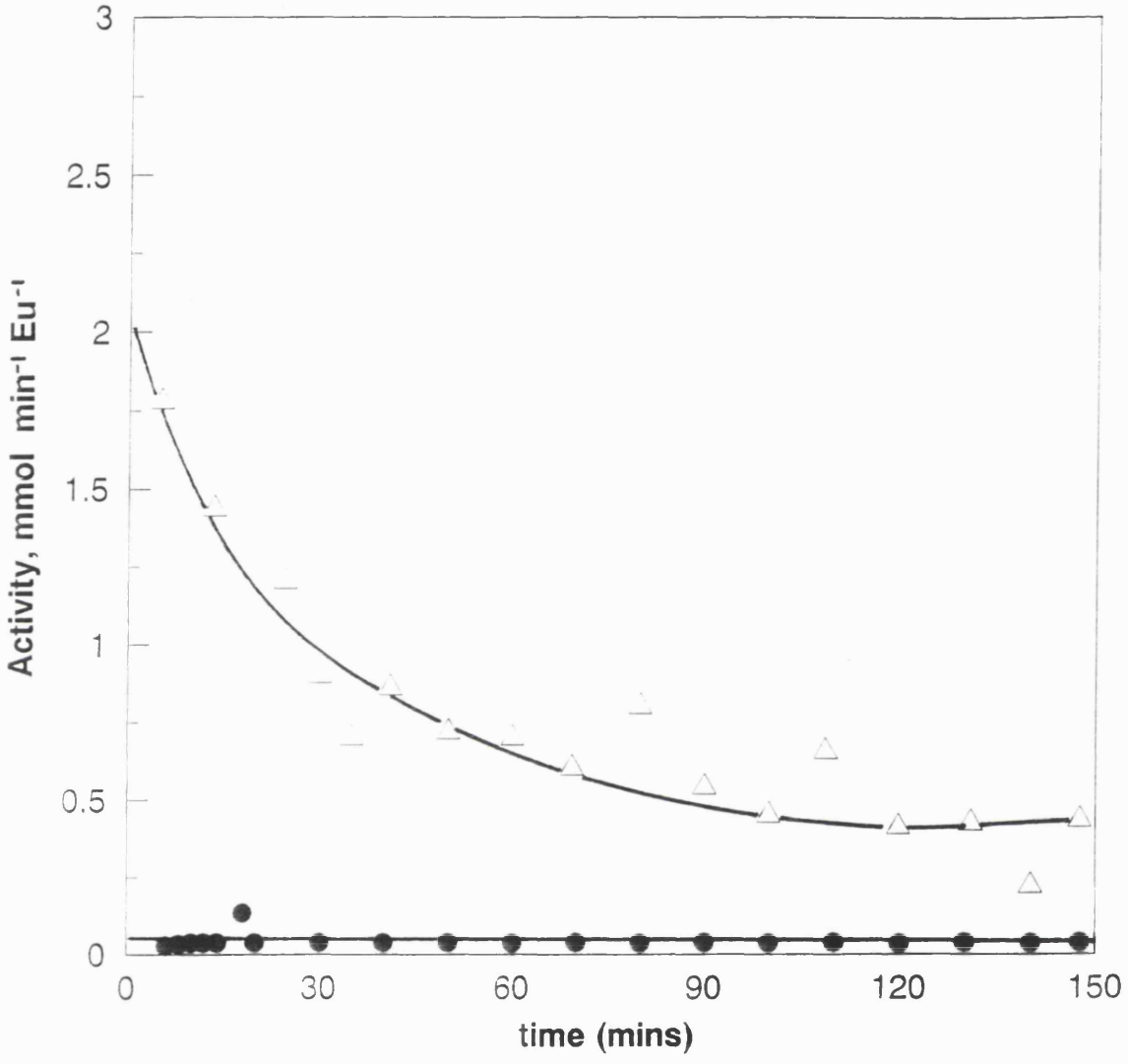


Figure 3 22 Activity as a function of time using 10 g l^{-1} permeate in the STR (Δ) and the MBR(●).

comparison were calculated on the basis of product concentration as expressed in terms of the reactor medium volume concentration. Eu was chosen as part of the rate expression, representing the quantity of enzyme contained in the 50 ml volume used in the STR, in the MBR there were 17 times more enzyme units associated with the reactor.

Observing the activity, as determined, in the STR initially the rate is comparatively high (activity at 2 minutes equals 2 mmol min^{-1} Eu^{-1}). However this activity declined rapidly in the first 30 minutes of operation (activity at 30 minutes equals 1.25 mM min^{-1} Eu^{-1}) and showed further less rapid decline in the activity over the next 120 minute period. In comparison to activity determined in the STR, activity initially measured in the MBR was initially 50 times less, at a value of 0.039 mM min^{-1} Eu^{-1}. than that initially observed in the STR. In contrast to the rate in the STR, which showed a marked decline as reaction progressed over the 150 minutes it was operated, the rate in the MBR remained constant over this period of time.

In the MBR there is a limiting factor or factors which at these low enzyme loads limit the effective activity of the enzyme as compared with its effectiveness when operated in a STR. There is however in contrast an effective increase in the stability of the enzyme when immobilised in the MBR as compared with its free form usage in the STR.

Over this initial time period of 150 minutes operation the amount of product finally generated on the basis of concentration in the whole of the liquor volume was in the STR 120 mM compared with 90 mM in the MBR. However there is effectively 17 times more enzyme in the MBR therefore the concentration of product generated per unit of enzyme available in the MBR is 5.3 mM as compared with the 120 mM generated in the STR.

3.3.1.4. Semi-continuous Operation of the MBR

The product concentration time profiles depicted in figures 3.23, 3.24 and 3.25 show the results of reactions carried out in the MBR. A number of cycles were operated

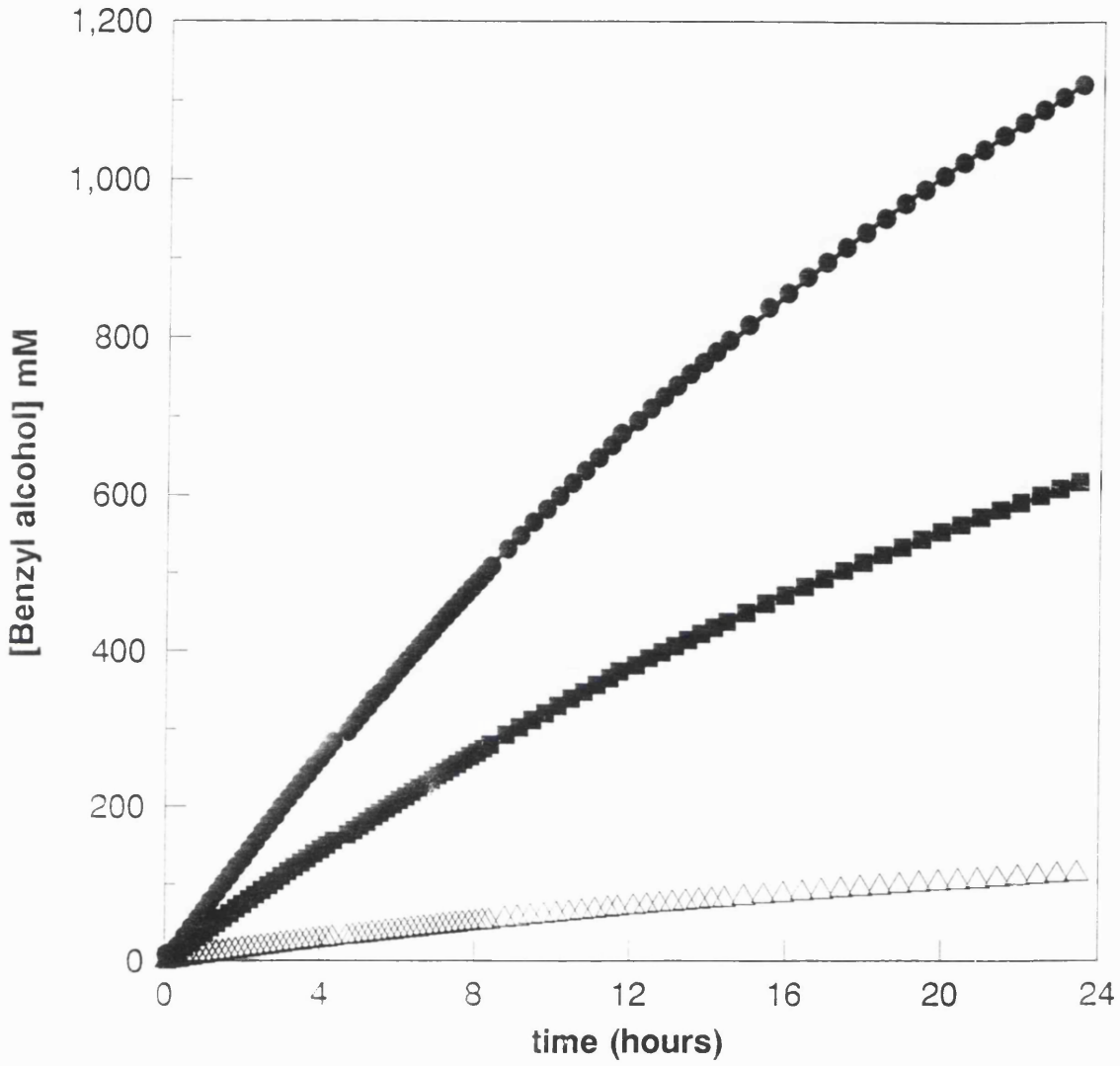


Figure 3 23 Product concentration in the MBR with time for lipase hydrolysis cycle number 1; Reactor conditions: Organic and aqueous phase volumes = 400 ml, Flow rates = 500 ml min^{-1} [Ea] is that contained in the 850 ml of remaining 10 g l^{-1} permeate; Reactor concentration; overall (■), aqueous phase (Δ), organic phase (●).

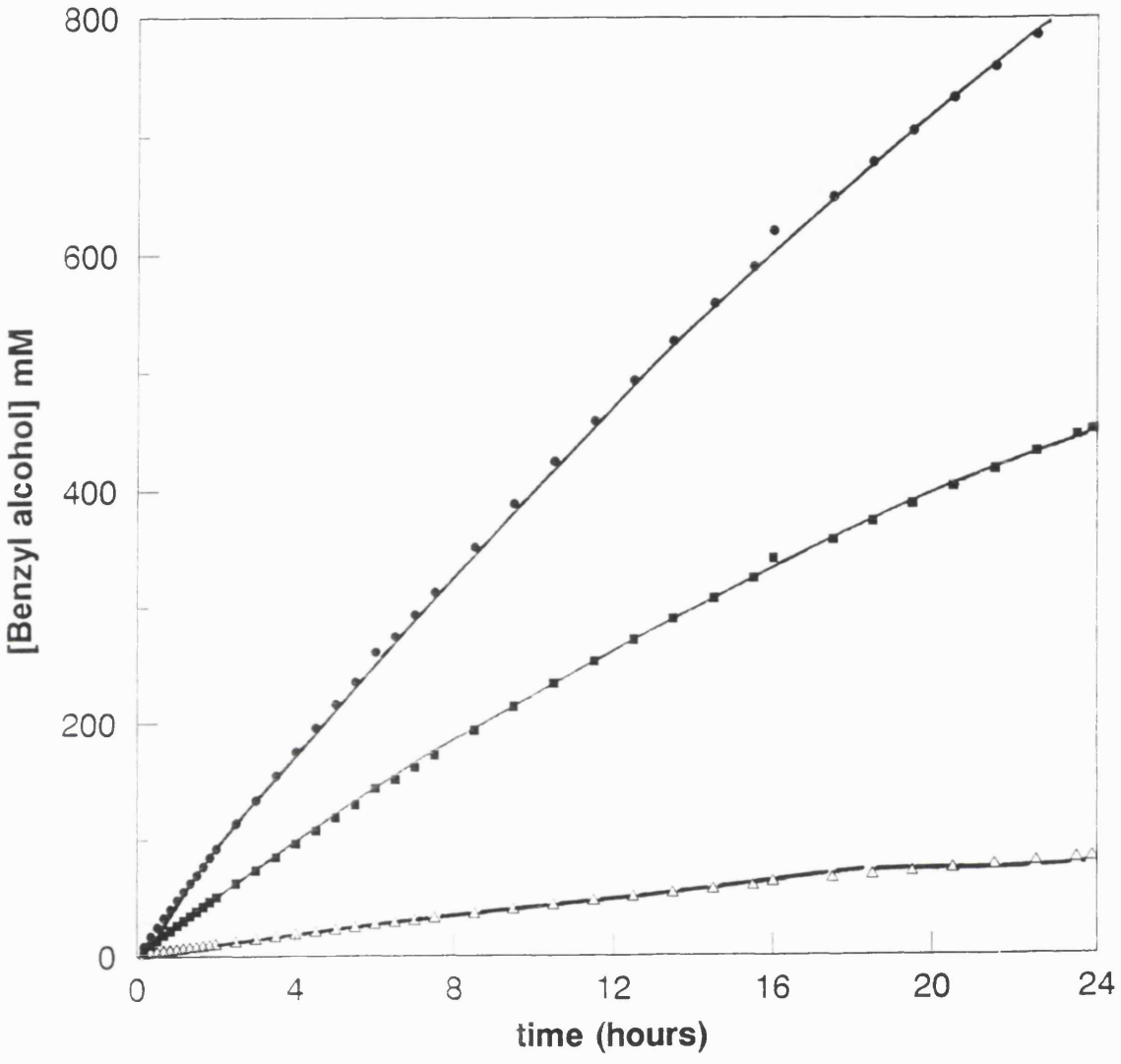


Figure 3.24 Product concentration in the MBR with time for lipase hydrolysis cycle number 2; Reactor conditions: Organic and aqueous phase volumes = 400 ml, Flow rates = 500 ml min^{-1} [Ea] is that contained in the 850 ml of remaining 10 g l^{-1} permeate; Reactor concentration; overall (■), aqueous phase (Δ), organic phase (●).

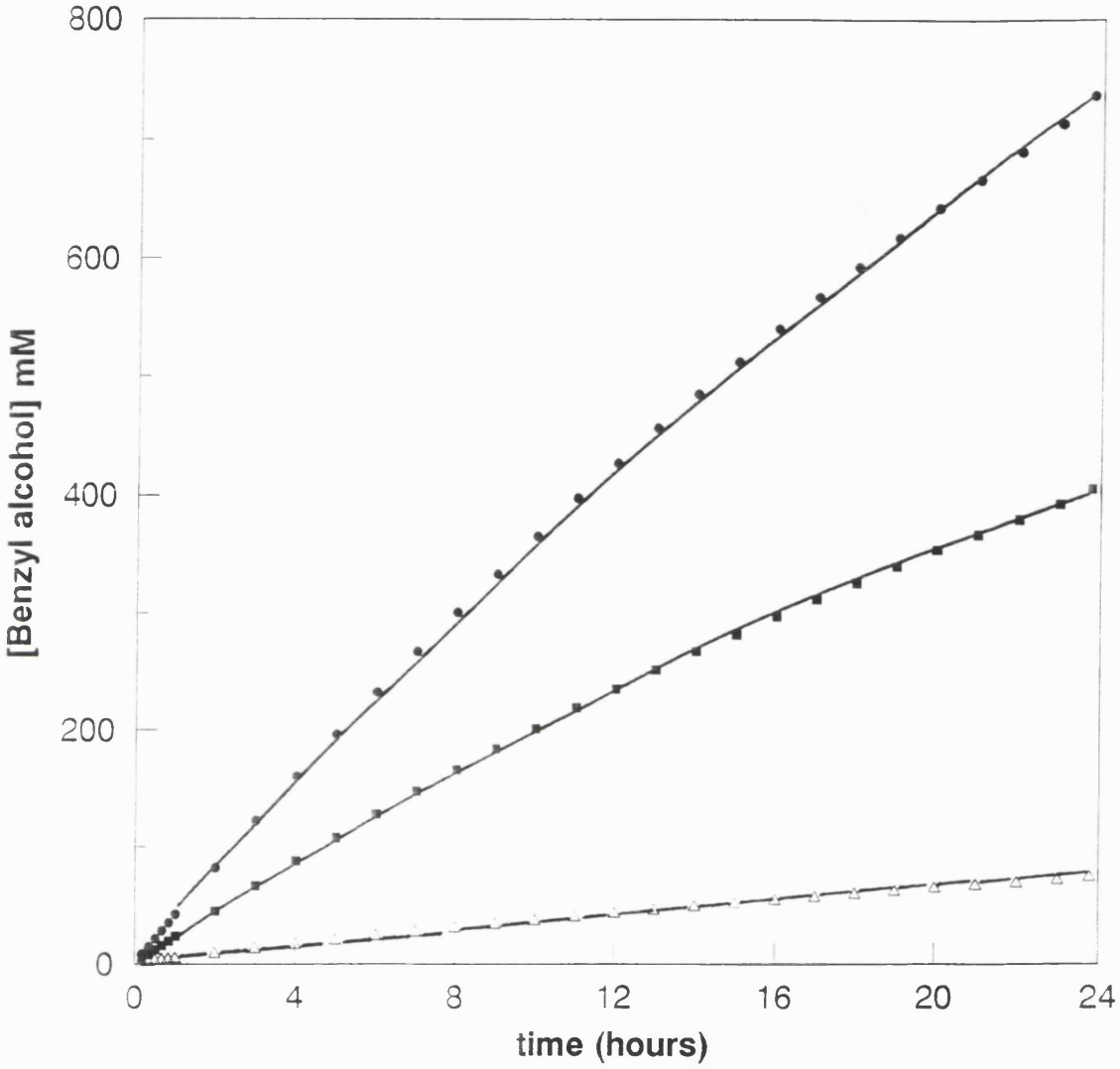


Figure 3.25 Product concentration in the MBR with time for lipase hydrolysis cycle number 3; Reactor conditions: Organic and aqueous phase volumes = 400 ml, Flow rates = 500 ml min^{-1} [Ea] is that contained in the 850 ml of remaining 10 g l^{-1} permeate; Reactor concentration; overall (■), aqueous phase (Δ), organic phase (●).

using the same enzyme load in the reactor for each repeat cycle. At the end of each cycle an amount of resevoir volume corresponding to 250 ml was removed and replenished with fresh substrate and fresh aqueous phase and another cycle begun. Since not all reservoir volume was removed after each cycle the second and third cycles were initiated with a concentration of product still in the reactor, this being 257 mM at the beginning of the second cycle and 247 mM at the beginning of the third cycle. The first two cycles were monitored, under pH controlled conditions, over a 24 hour period of time, the third cycle being allowed to continue for a fifty hour period of time. Figure 3.23 shows the result of the first cycle, figure 3.24 the second cycle and figure 3.25 the third cycle. The results are expressed on the basis of three concentrations, that potentially in the aqueous phase, that in the organic phase and that on the basis of overall reactor liquor volume.

The results of figures 3.23, 3.24 and 3.25 were used to plot figure 3.26 which shows the activities determined for cycles 1, 2 and 3 respectively in the MBR as reaction progressed. Activity was expressed as previous, based on product concentrations in the overall reactor medium volume and 17 times more enzyme than employed in the STR experiment. The activity in the first cycle began comparatively high, 0.039 mM min^{-1} Eu^{-1}. This activity was maintained for at least the first 150 minutes of operation as illustrated previously in figure 3.22 and began to decline after the reactor had been operating for approximately 3 hours. The decline in activity for this cycle continued over the 24 hour period of reactor operation, its half life under these particular conditions being approximately 18 hours, the activity at this time being 0.02 mM min^{-1} Eu^{-1}. The activity after 24 hours operation was 0.019 mM min^{-1} Eu^{-1}. The activty in the second cycle was initially determined to be 0.028 mM min^{-1} Eu^{-1}, 25 % less than that initially measured in the first cycle. The activity declined comparatively rapidly within the first three hours of operation and then decreased over the next 20 hour period to a value of 0.014 mM min^{-1} Eu^{-1}, half that of the rate determined initially. The activity in the third cycle behaved in a similar manner to the activity in the second cycle, an initial stable activity for the first 3 hours followed by a decline in the activity over the next 21 hour period. The activity in the third cycle continued to decline over

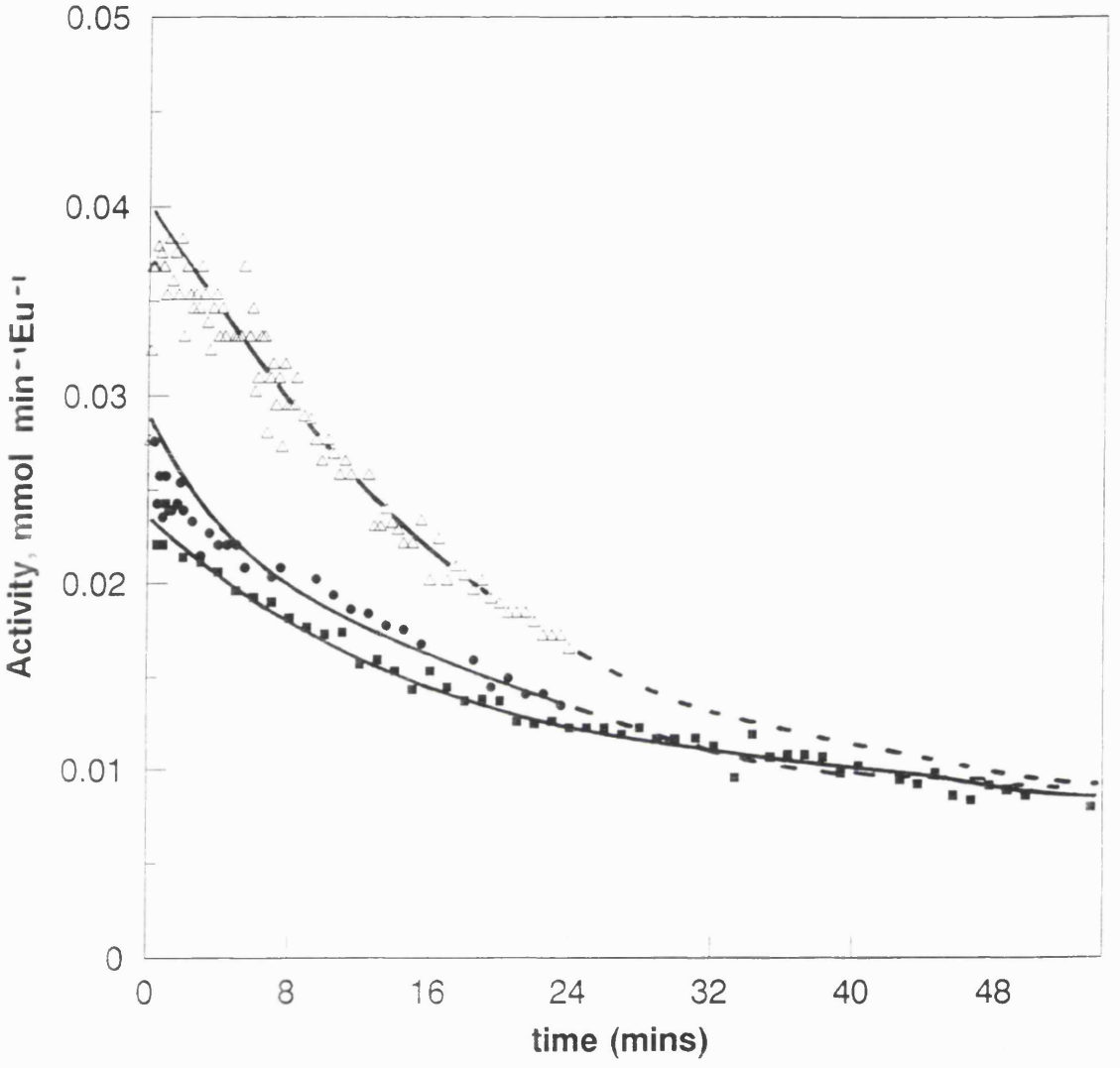


Figure 3.26 Activity as a function of time for lipase hydrolysis in the MBR at comparitively low enzyme load, cycle 1 (Δ), cycle 2 (●), cycle 3 (■).

the next 24 hours although the rate of decline was not as high as that in the previous 24 hour period, a decrease of 0.004 mM min^{-1} Eu^{-1} over the last 24 hour period compared with a decrease of 0.01 mM min^{-1} Eu^{-1} over the first 24 hour period.

The activity can be further expressed as a function of increasing concentration of product in the reactor, based on overall reactor volume, these are shown in figure 3.27 for cycles 1, 2 and 3 respectively. Since in cycles 2 and 3 not all substrate was renewed then these reactions started with a concentration of product remaining in the reactor in comparison to cycle 1 in which no concentration of product was initially present. It is interesting to note that the gradient of the decline in activity relative to the increasing product concentration is very similar in all 3 cycles, although cycles 2 and 3 show consecutively a comparative decrease in activity relative to the first and preceeding cycle. There is an effect on the activity of the enzyme linked to the concentration of product. The effect is not permanent since the restoration of product concentration levels to previous levels allowed the continued activity of enzyme almost at the previously determined rate at that concentration of product in the reactor. Comparing the rates as expressed on this basis there is a 14 % decrease in the rate in cycle 2 compared to cycle 1 and a further 8 % decrease in the rate for the third cycle.

3.3.1.5. Conversion

The concentration of the substrate available in the reactor initially for the first cycle of operation is 3.3 M, the concentration of product generated by the reaction in the first 24 hours of this cycle is 0.6 M, this represents 18 % conversion of the substrate available in the 24 hour period in which the first cycle was operating.

For the second cycle the initial reactor concentration of substrate available for conversion is 3.11 M, from which 0.45 M of product was generated, 14 % conversion of the available substrate in the 24 hour period in which this cycle was operating. The third cycle converted 10 % of the available substrate in the first 24 hours and a further 12 % in the next 24 hour period.

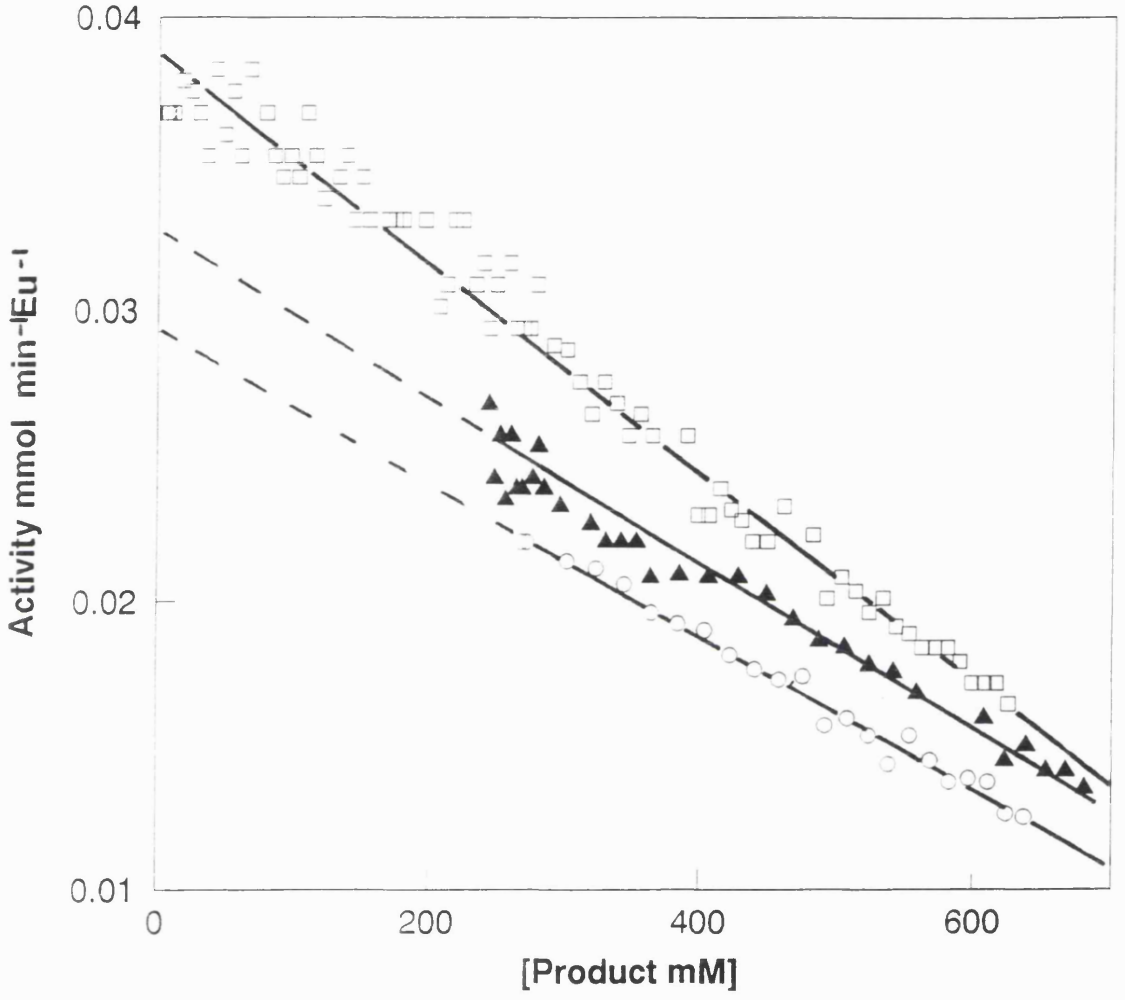


Figure 3.27 Activity as a function of product concentration in the organic phase of the reactor for lipase hydrolysis in the MBR at comparitively low enzyme load; Cycle 1 (□), cycle 2 (▲), cycle 3 (O).

3.3.2. High Enzyme Load

The quantity of crude enzyme material used for these experiments was 100 g l^{-1}, only 25 % of this material was protein, not all of which accounted for lipase activty and a further amount of activity was lost in the preparation process for enzyme loading (section 2.5.1). The amount of lipase active protein remaining in the supernatant however was, in comparison to the previous experiments, relatively high. It is the equivalent of the activity associated with 16 g l^{-1} in comparison with previous STR experiments, (section 3.2), the results of which are illustrated in figure 3.19. How much of the actual carrying capacity of the membrane being utilised is not certain.

3.3.2.1. In the STR

To make a valid comparison of the performance of the MBR with that of the STR an experiment was carried out which employed a fraction of the lipase active aqueous phase generated in the enzyme loading preparative procedure i.e. the supernatant generated after centrifugation of the 100 g l^{-1} suspension of crude enzyme material. The product concentration time profiles depicted in figure 3.28 show the results of a reaction carried out in the STR at a phase ratio of 0.5 and a stirrer speed of 1000 rpm. The lipase active aqueous phase being as previously described. Interest was in the initial activity information for comparison with the results subsequently obtained from reactions carried out in a MBR, the reaction was monitored, under pH controlled conditions, for a period of 150 minutes The results are expressed in relation to three criteria, aqueous phase concentration, organic phase concentration and concentration based on overall reactor medium volume assuming obedience to product partitioning.

3.3.2.2. In the MBR

The product concentration time profiles depicted in figure 3.29 show the results of a reaction carried out in the MBR using a comparatively high enzyme load (the

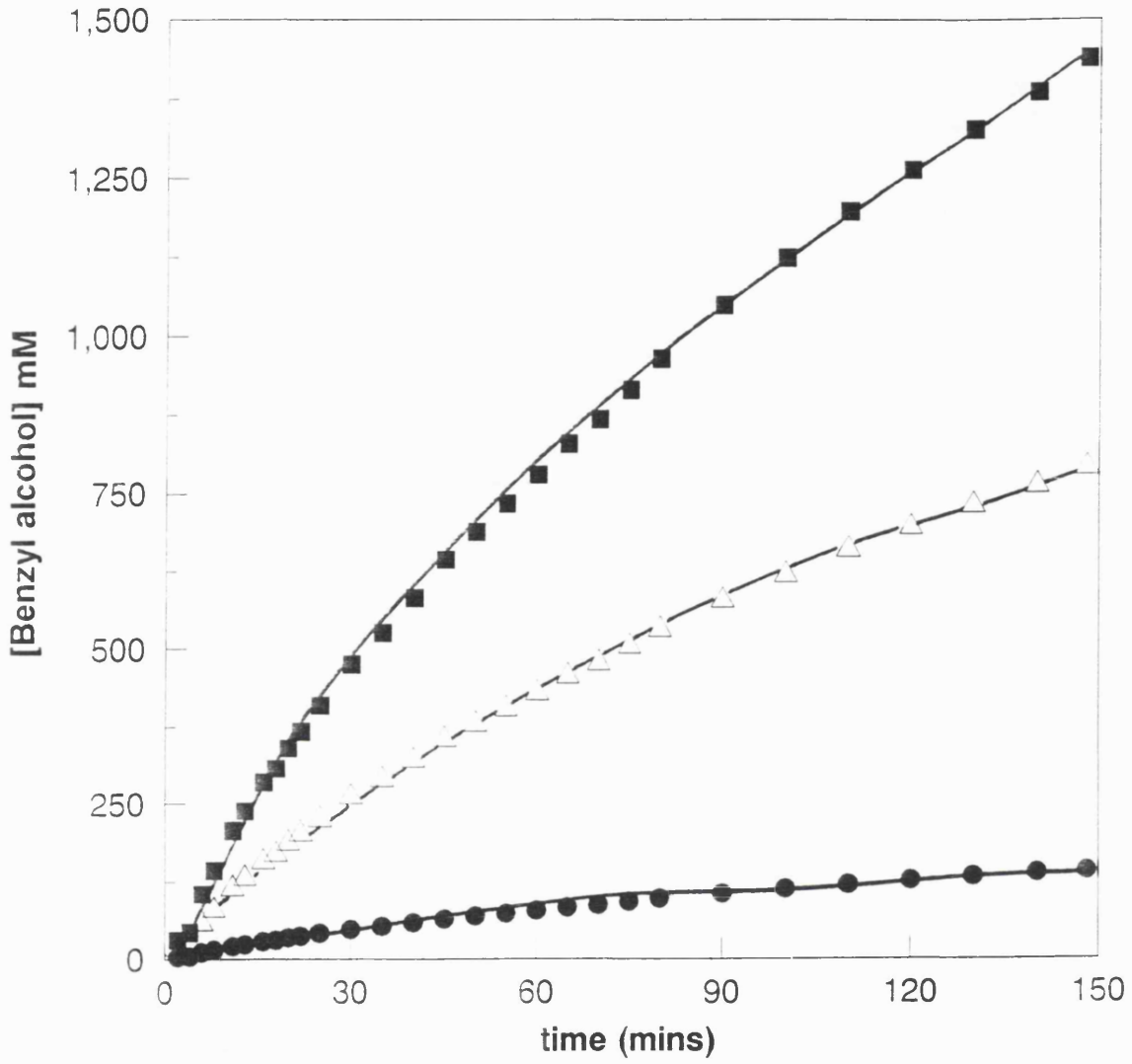


Figure 3.28 Product concentration in the STR with time for lipase hydrolysis. Reactor conditions: 0 = 0.5, n = 1000 rpm, [Ea] = 50 ml fraction from 100 g l^{-1} permeate; Reactor concentration; overall (Δ), aqueous phase (●), organic phase (■).

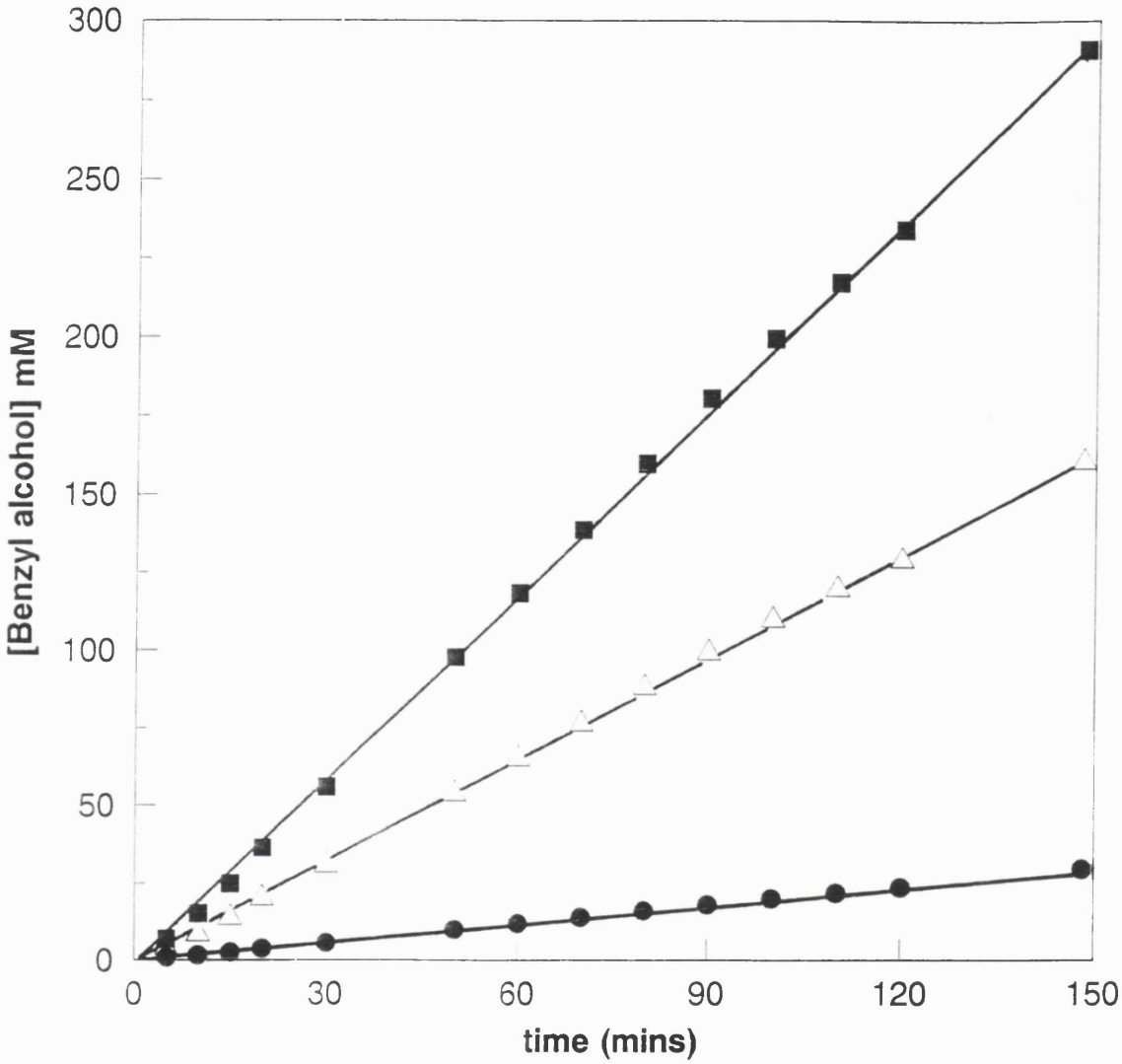


Figure 3.29 Product concentration in the MBR with time for lipase hydrolysis. Reactor conditions: Organic and aqueous phase volumes = 400 ml, Flow rates = 500 ml min^{-1} [Ea] that contained in the 850 ml of remaining of the 100 g l^{-1} permeate; reactor concentration; overall (Δ), aqueous phase (●), organic phase (■).

supernatant produced after dissolution and centrifugation of the 100 g l^{-1} crude lipase preparation). As in experiments which were carried out in the MBR at low enzyme load the reactor was run with reservoir volumes of 400 ml for both phases at a circulation rate through the module of 500 ml min^{-1} in a cocurrent mode. The effective phase ratio can therefore be described as 0.5. The amount of enzyme loaded onto the membrane is effectively 17 times the amount which was used in the STR. The reaction was monitored, under pH controlled conditions, and the results shown in figure 3.29 are for the first 150 minute period of operation. Again results are expressed on the basis of aqueous phase concentration, organic phase concentration and concentration based on overall reactor medium volume.

3.3.2.3.Comparison of the Reaction Rates in the STR with those in the MBR.

The results of figures 3.28 and 3.29 were used to plot figure 30 which shows the activities, determined in the STR and the MBR, as reaction progresses in the reactors operated under the conditions as outlined. Activity for comparison was calculated on the basis of product concentration as expressed in terms of the reactor medium volume concentration. Eu was chosen as part of the rate expression, it representing the quantity of enzyme contained in the 50 ml volume used in the STR, in the MBR there were effectively 17 times more enzyme units associated with the reactor.

Observing the activity in the STR, initially the rate was comparatively high, 17 mmol min^{-1} Eu^{-1} at 6 mins However this rate declined rapidly in the first 25 minutes of operation, 7.6 mmol min^{-1} Eu^{-1} and showed further less rapid decline in the rate over the next 30 minute period, followed by a less rapid decline in activity to reach eventually at 150 minutes an activity of 3.1 mmol min^{-1} Eu^{-1}. In comparison to the activity determined in the STR, the activity initially measured in the MBR was 283 times less at a value of 0.06 mmol min^{-1} Eu^{-1}, than that initially observed in the STR. In contrast to the rate in the STR, which showed a marked decline as reaction progressed over the 150 minutes operated, the activity in the MBR remained constant over this period of time.

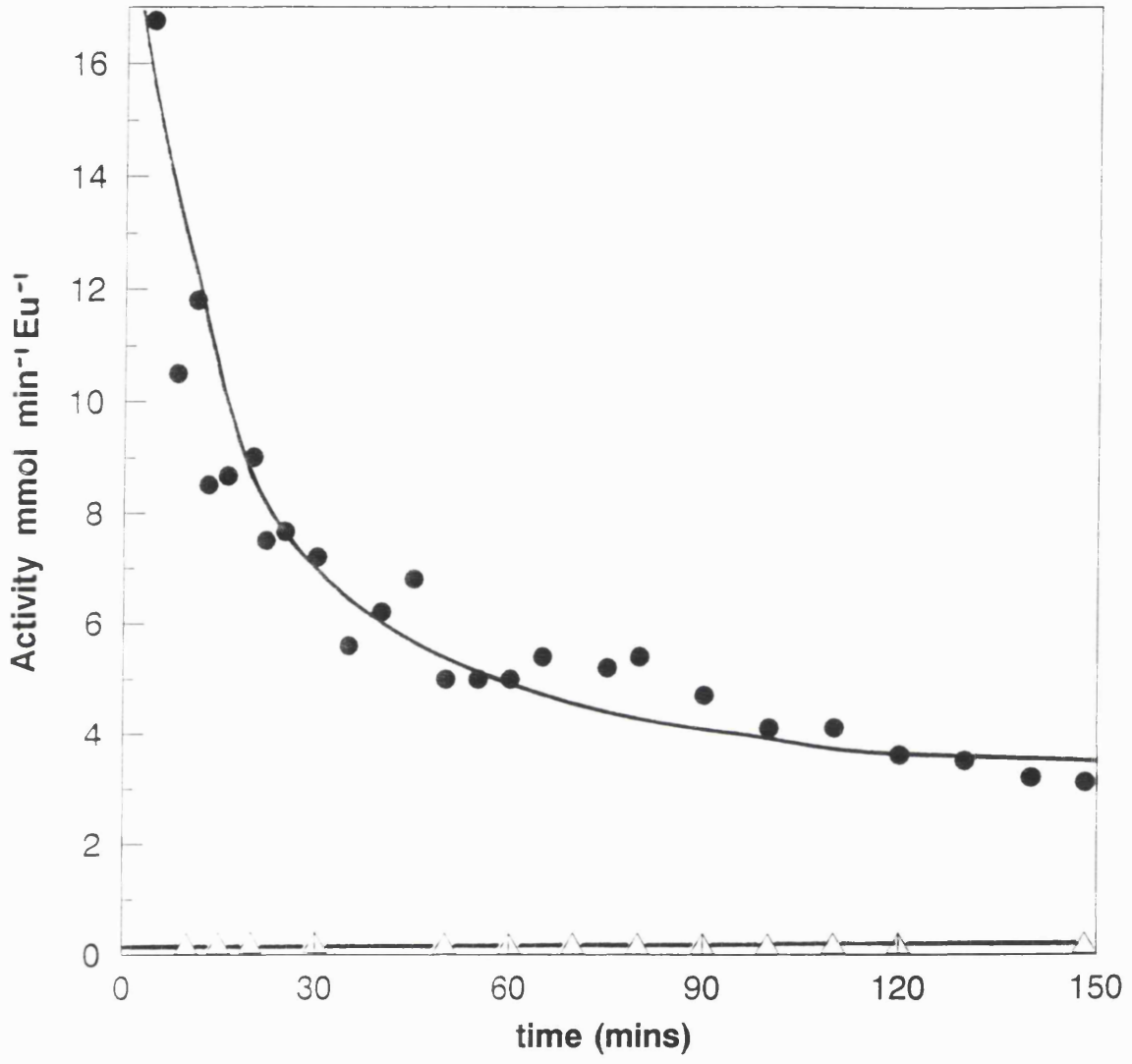


Figure 3.30 Activity as a function of time using 100 g l^{-1} permeate in the MBR (Δ) and the STR (●).

Again there is a limiting factor or factors which limit the activity of the enzyme in the MBR relative to the STR the effect being even more acute at comparatively higher enzyme load. A stabilising effect upon the enzyme of its immobilisation in the MBR is apparent.

Over this initial time period of 150 minutes operation the amount of product finally generated on the basis of concentration in the total medium volume was in the STR 800 mM, compared with 128 mM in the MBR. However in the MBR we have effectively 17 times more enzyme therefore the concentration of product generated per unit of enzyme available in the MBR is 7.53 mM as compared with the 800 mM generated in the STR.

3.3.2.4. Semi-Continuous Operation of the MBR

The product concentration time profiles depicted in figures 3.31, 3.32, 3.33, 3.34 and 3.35 show the results of reactions carried out in the MBR. A number of cycles were operated using the same enzyme load in the reactor for each repeat cycle. At the end of each cycle the complete reservoir volume of both organic and aqueous phases was replaced with fresh phase and another cycle begun. Cycles were run in each instance until a concentration of approx 2 to 2.5 M of product based on overall reactor volume had been reached, this corresponding to a concentration at which comparatively little enzyme activity remained for that cycle. All cycles were monitored for the respective length of time under pH controlled conditions. Figures 3.31, 3.32, 3.33, 3.34 and 3.35 represent cycles 1, 2, 3, 4 and 5 respectively. The results are expressed on the basis of three presumptive concentrations, that potentially in the aqueous phase, that in the organic phase and that on the basis of overall reactor medium volume.

The results of figures 3.31 and 3.32, the first two cycles, were used to the plot figure 3.36 which shows the activities determined for cycles 1 and 2. The results of figures 33, 34 and 35 were used to plot figure 3.37 which shows the activity determined for cycles 3, 4 and 5 in the MBR as reaction progressed. Activity was expressed as

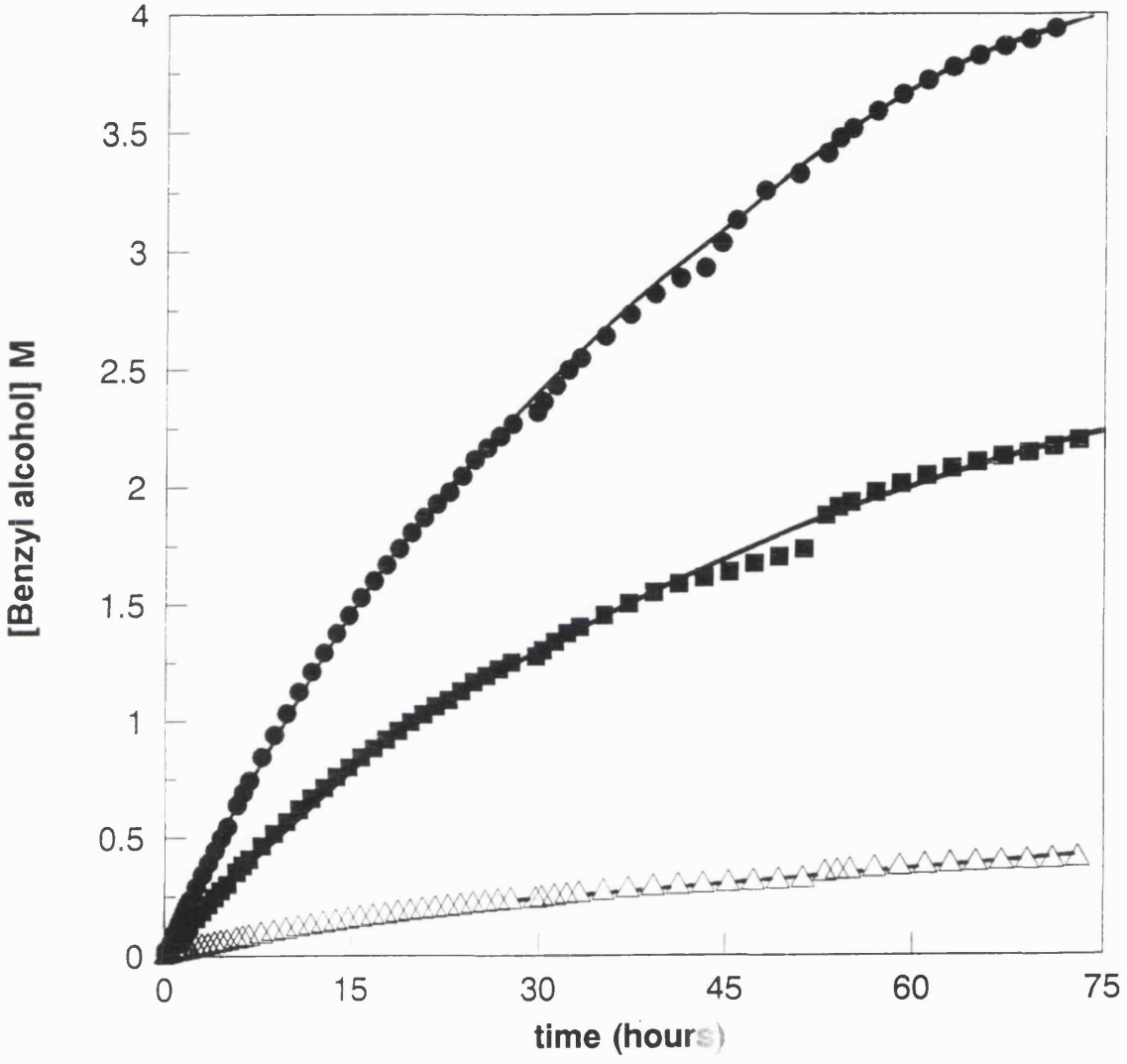


Figure 3.31 Product concentration in the MBR with time for lipase hydrolysis cycle number 1; Reactor conditions: Organic and aqueous phase volumes = 400 ml, Flow rates = 500 ml min^{-1} [Ea] is that contained in the 850 ml of remaining 100 g l^{-1} permeate; Reactor concentration; overall (■), aqueous phase (Δ), organic phase (●).

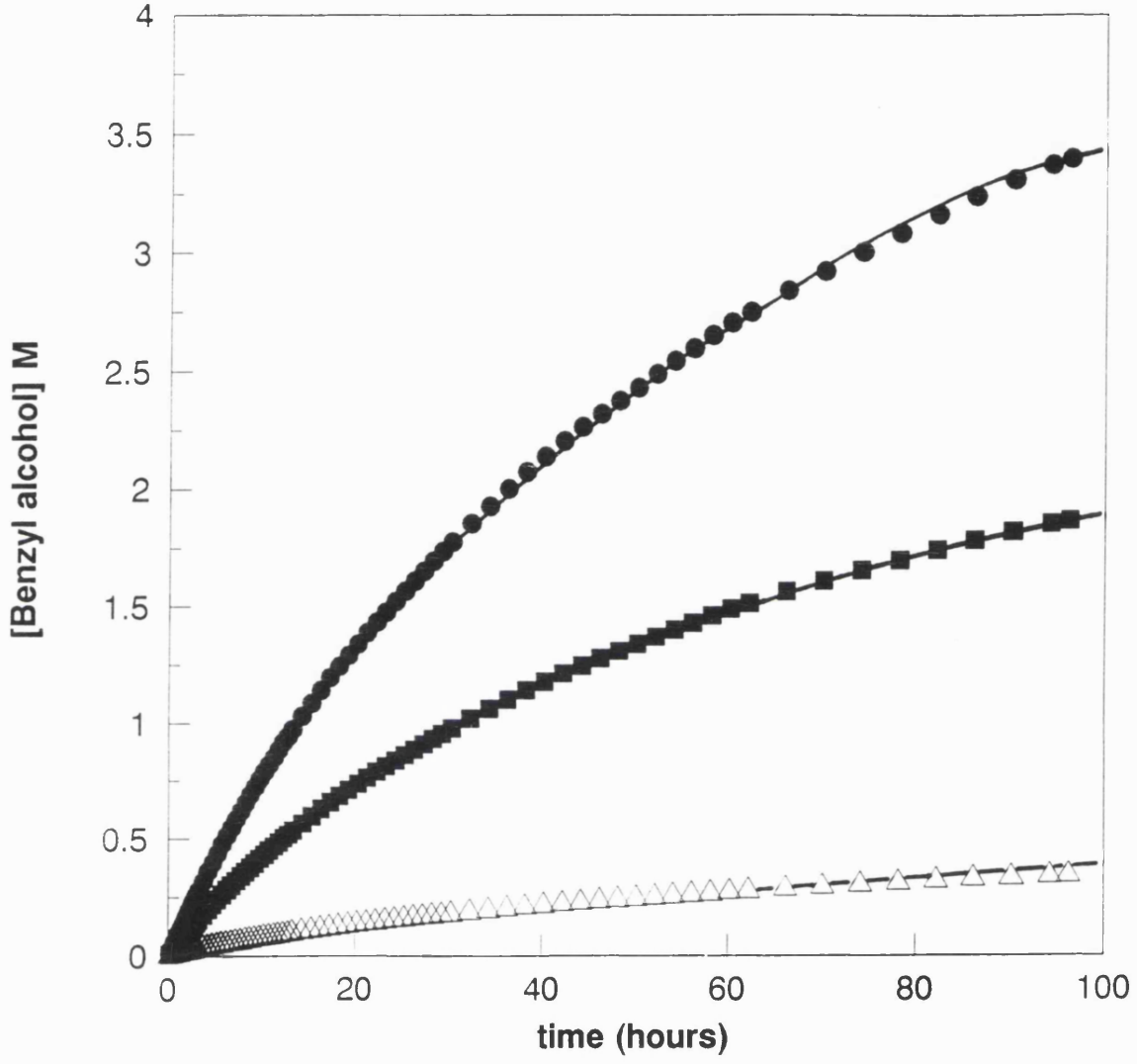


Figure 3.32 Product concentration in the MBR with time for lipase hydrolysis cycle number 2; Reactor conditions: Organic and aqueous phase volumes = 400 ml, Flow rates = 500 ml min^{-1} [Ea] that contained in the 850 ml of remaining 100 g l^{-1} permeate; Reactor concentration; overall (■), aqueous phase (Δ), organic phase (●).

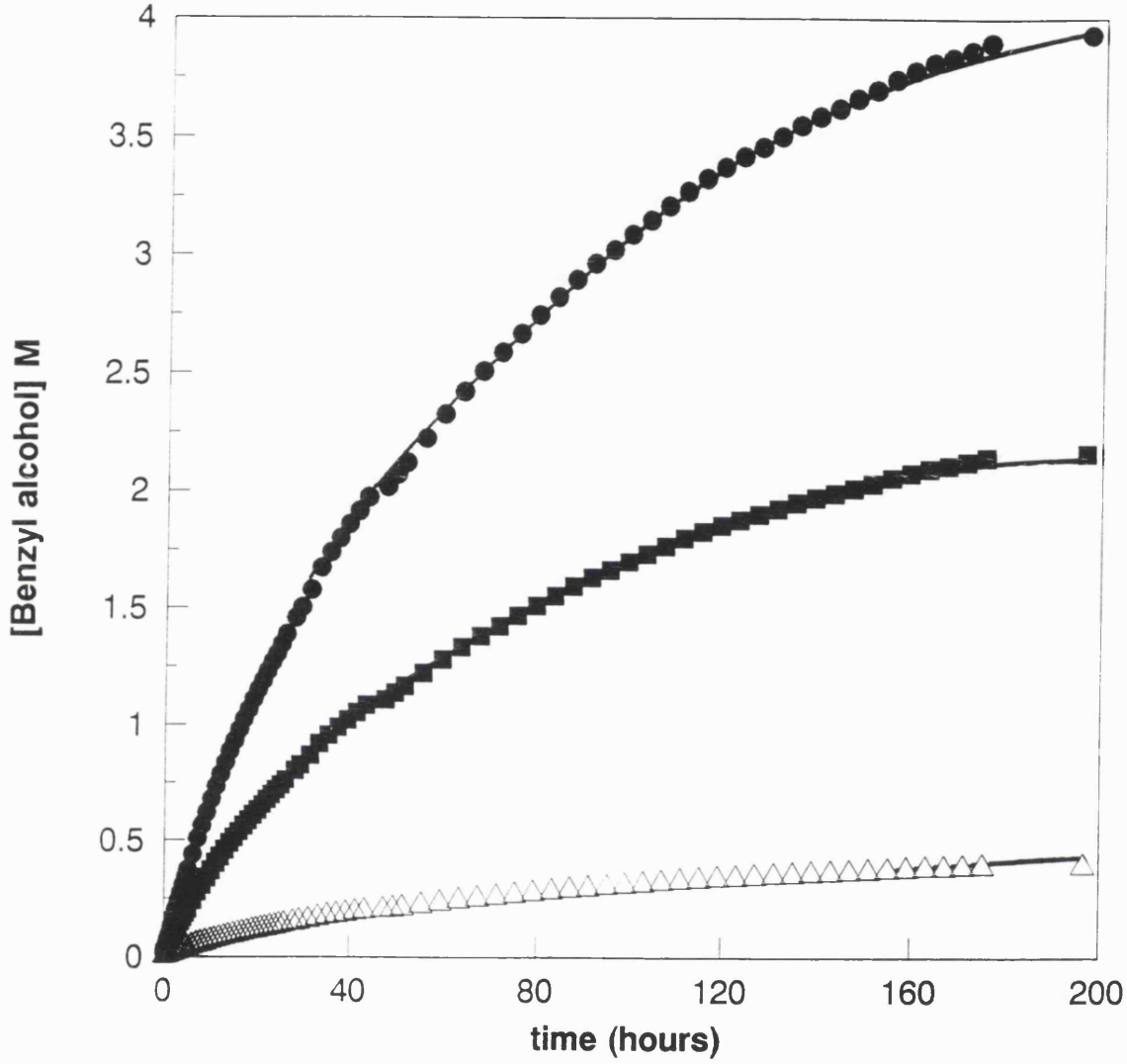


Figure 3.33 Product concentration in the MBR with time for lipase hydrolysis cycle number 3; Reactor conditions: Organic and aqueous phase volumes = 400 ml, Flow rates = 500 ml min^{-1} [Ea] is that contained in the 850 ml of remaining 100 g l^{-1} permeate; Reactor concentration; overall (■), aqueous phase (Δ), organic phase (●).

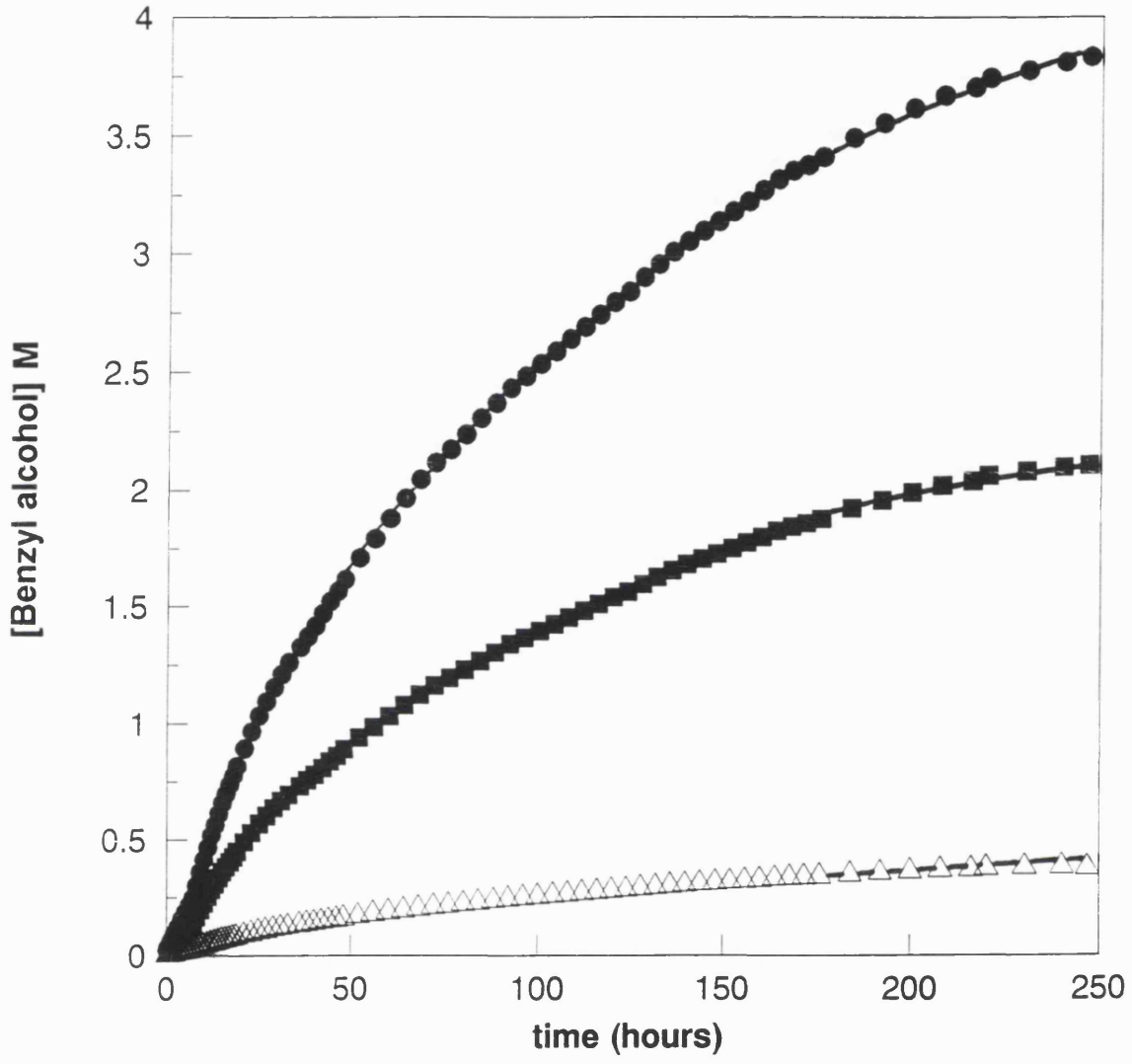


Figure 3 34 Product concentration in the MBR with time for lipase hydrolysis cycle number 4; Reactor conditions: Organic and aqueous phase volumes = 400 ml, Flow rates = 500 ml min^{-1} [Ea] that contained in the 850 ml of remaining 100 g l^{-1} permeate; Reactor concentration; overall (■), aqueous phase (Δ), organic phase (●).

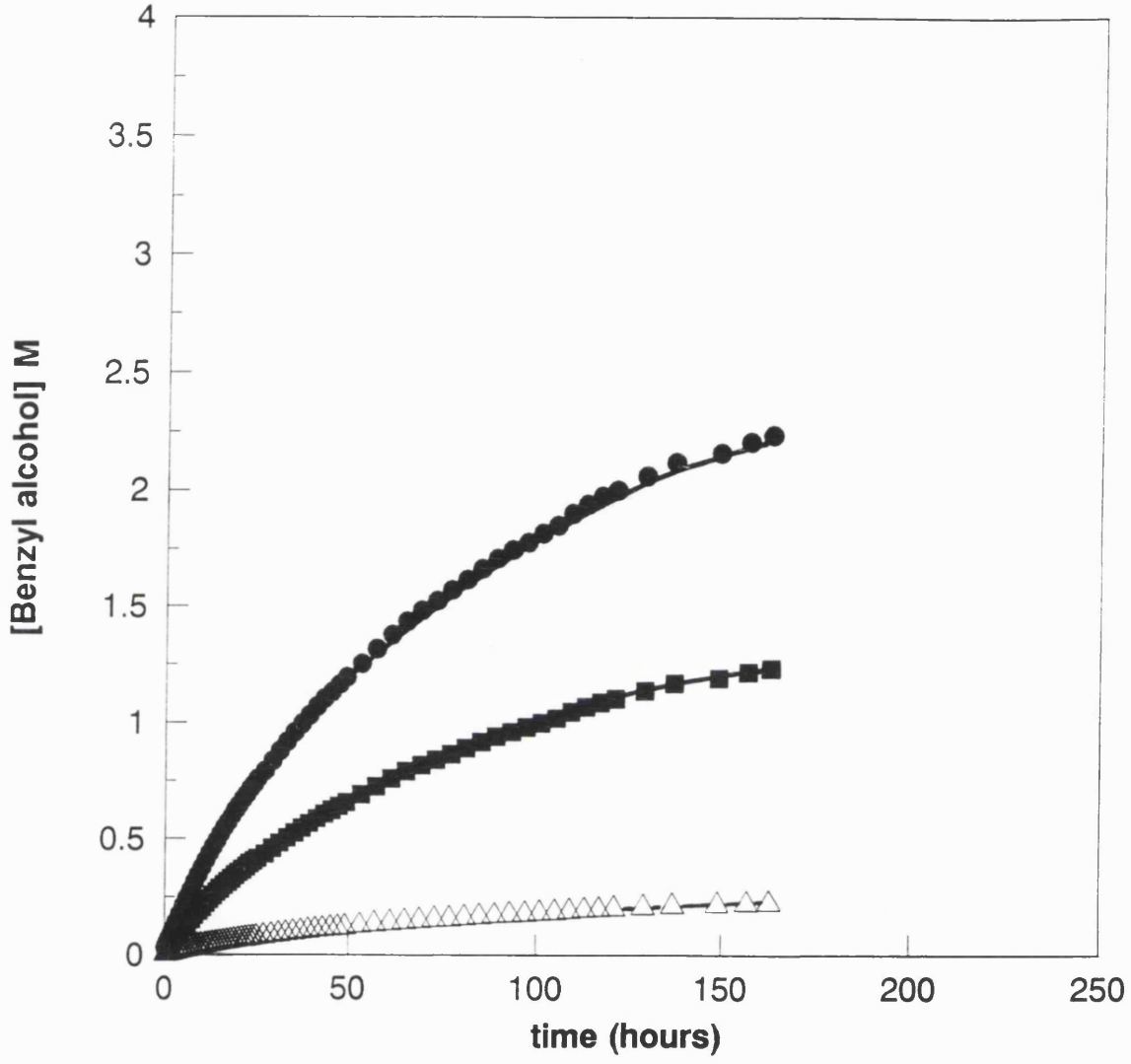


Figure 3 35 Product concentration in the MBR with time for lipase hydrolysis cycle number 5; Reactor conditions: Organic and aqueous phase volumes = 400 ml, Flow rates = 500 ml min^{-1} [Ea] that contained in the 850 ml of remaining 100 g l^{-1} permeate; Reactor concentration; overall (■), aqueous phase (Δ), organic phase (●).

based on product concentrations in the overall reactor liquor volume and 17 times more enzyme than employed in the STR experiment.

The activity in the first cycle began comparatively high, around 0.06 to 0.07 mmol min^{-1} Eu^{-1}. The rate began to decline within the first few hours of operation, initially decreasing to a value of 0.054 mmol min^{-1} Eu^{-1} within the first five hours of operation. The next 15 hour period, following this, was marked by a further decline in the activity, although not as pronounced as the decline in the first 5 hours, the rate at 20 hours equalling 0.036 mmol min^{-1} Eu^{-1}. The activity then declined further over the next 55 hours. The enzyme half life under these conditions in the first cycle is appromately 18 hours +/- 1 hour.

The rate in the second cycle was initially determined to be 0.051 mM min^{-1} Eu^{-1} 34 % less than that in the first cycle. The decrease in rate of the activity, as reaction progressed, followed a similar pattern to that observed in the first cycle with a relatively rapid decline in the first 5 hours, a less rapid decline over the next 15 hours, and a further less pronounced decline over the next 40 hours after which for the last 40 hour activity remained relatively constant. The activity differential between this and the first cycle was constant after the initial period for the first 40 hours but after this the activities began to converge. The half life was approximately 20 hours +/- 1 hour.

The activities observed for the third and fourth cycle, figure 3.37, were only marginally different in comparison to one another and were only slightly less, approximately 0.001 mmol min^{-1} Eu^{-1}, +/- 0.0005, than those observed in the second cycle. Again they followed an identical pattern to previous cycles and displayed a half life of approximately 20 hours. After 100 hours of operaration of these two cycles the low activity was maintained over the next 100 hour period of operation up until 200 hours at which time the activity markedly fell once again in the fourth cycle to an almost negligible amount.

The fifth and final cycle observed showed initially an activity of 0.031 mmol min^{-1} Eu^{-1}, this was 37 % less than had been observed in the previous two cycles. As with previous cycles the initial activity declined sharply within the first few hours of operation, declined further at a less rapid rate for the next 40 hours after which the

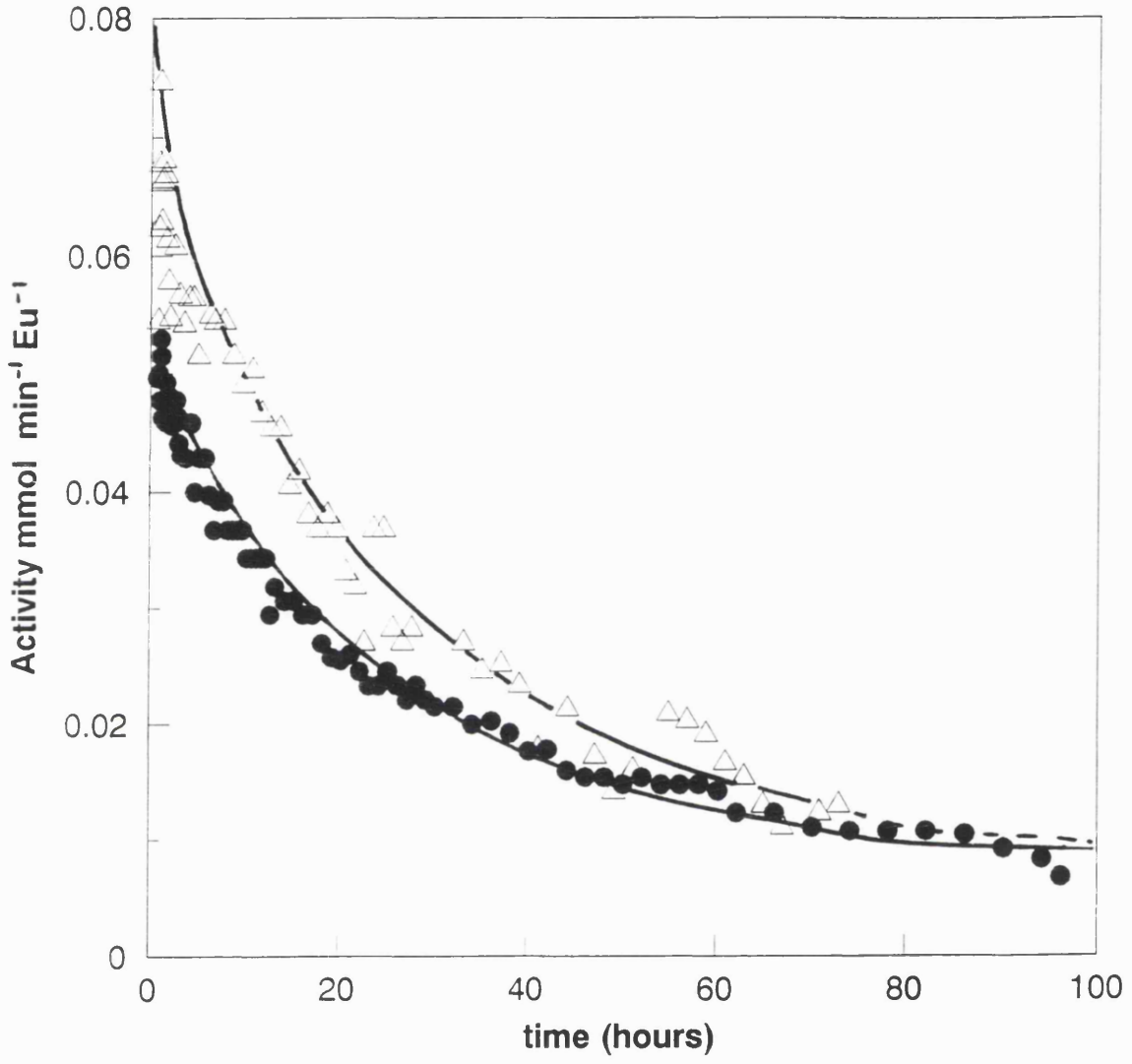


Figure 3.36 Activity as a function of time for lipase hydrolysis in the MBR at a comparitively high enzyme load for cycles 1 (Δ) and 2 (●).

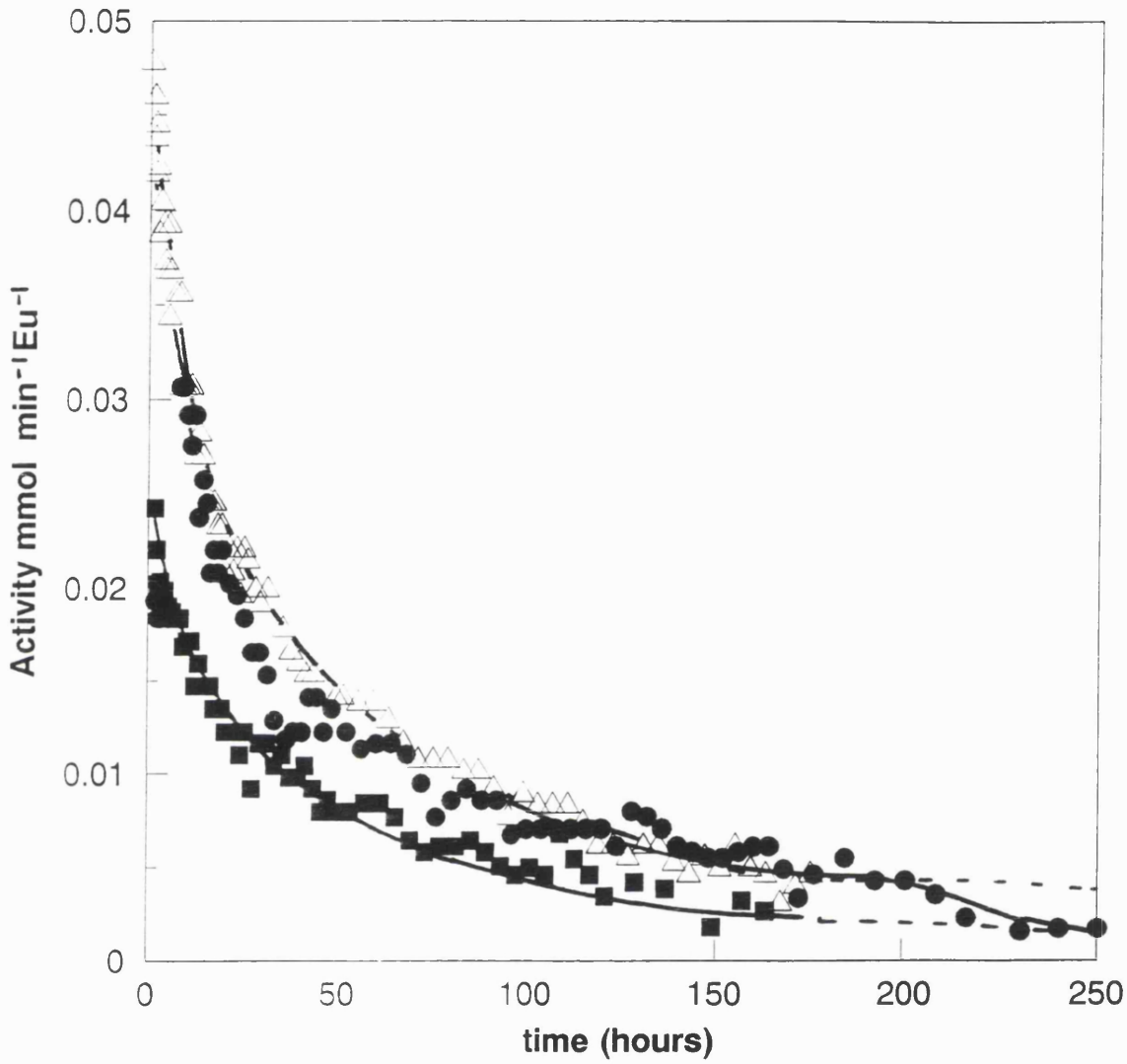


Figure 3.37 Activity as a function of time for lipase hydrolysis in the MBR at a comparitively high enzyme load for cycles 3 (Δ), 4 (●) and 5 (■).

activity remained comparatively low with little further decline for the remaining duration of the cycle. Again the half life of the enzyme in this cycle was around the 18 to 20 hour time period.

The activities were also expressed as a function of product concentration, based on overall reactor volume. These results are depicted in figure 3.38. As was observed previously at low enzyme loads, see figure 3.27, activity declined as the concentration of product increased in the reactor. The results clearly demonstrate the decrease in activity with each successive cycle, the percentage decrease with each cycle relative to the preceeding cycle being 31 %, 24 %, 22 %, and a final 50 % respectively. These percentage decreases hold well for concentrations of product up to 1.6 M above this concentration of product the low rates in each cycle begin to converge.

3.3.2.5. Conversion

Figure 3.39 depicts the conversion of substrate with operation time for each of the cycles. With each successive cycle the length of time of reactor operation required to achieve the same degree of conversion as achieved in the previous cycle increases due to the lowering of activity of the enzyme with each successive cycle. The maximum conversion observed was 66 % although if the first cycle had been continued then the level of conversion may have likely increased. However the activity of the enzyme above this level of product in the reactor is somewhat reduced. In comparison to the conversion at low enzyme load which at 24 hours was 18 % for the first cycle, the conversion at 24 hour for the comparatively high enzyme load was 38 %.

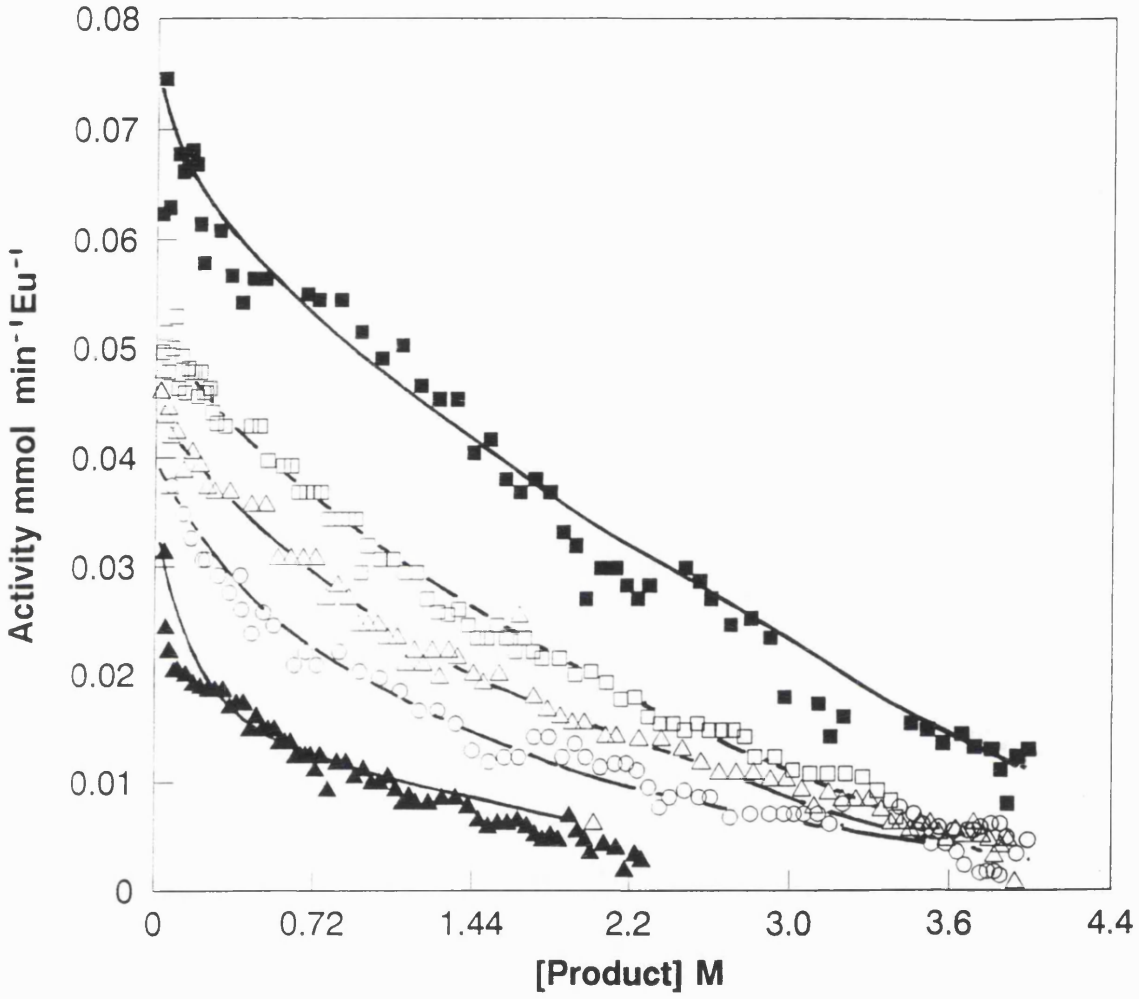


Figure 3.38 Activity as a function of product concentration in the organic phase of the reactor for lipase hydrolysis in the MBR at comparitively high enzyme load; Cycle 1 (■), cycle 2 (□), cycle 3 (Δ), cycle 4 (O), cycle 5 (Δ).

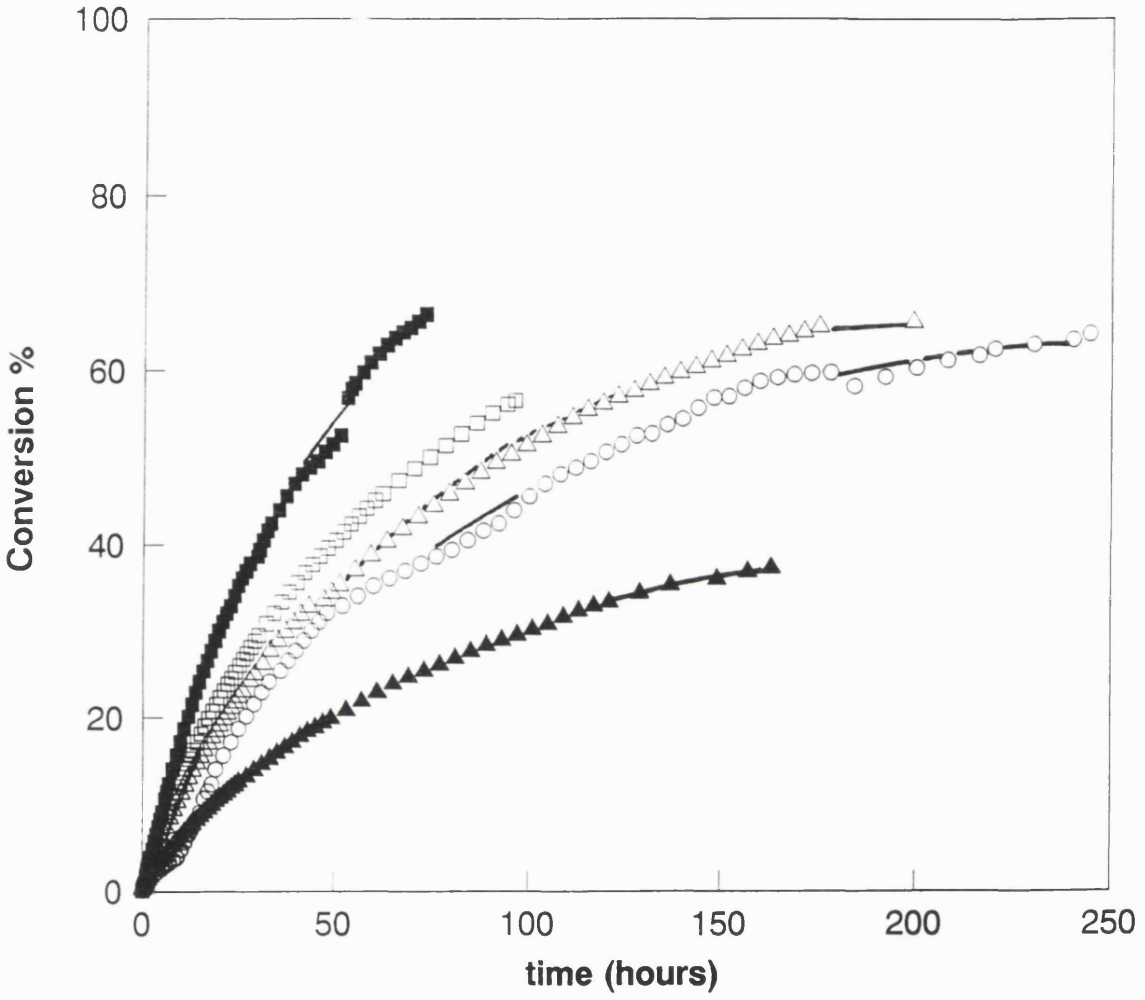


Figure 3.39 Substrate conversion as a function of time in the MBR at comparitively high enzyme loads; Cycle 1 (■), cycle 2 (□), cycle 3 (Δ), cycle 4 (O), cycle 5 (Δ).

4. Discussion

Reactions carried out both in the areas of chemical technology and biotechnology benefit from methods that lead to an understanding of the process. This givess insight into means by which the process can be efficiently operated. For a thorough understanding it is often necessary to establish influences of individual parameters and the way in which theses parameters interact. Catalysis in two-liquid phase media is of interest to the biotechnology industry as it overcomes some of the drawbacks associated with conversion of poorly soluble compounds. The effective operation of such a process will be influenced by aspects of the process and the parmeters involved therein. This study was aimed at identifying and understanding parameters for the operation of the reactor using the ester hydrolysis as a model system.

4.1. STR Operation

4.1.1. Mass Transfer in the STR

Enzymes are generally expensive catalysts, their cost can often be of primary importance in the overall cost of a process. For a process to be operated efficiently the reactor must be able to support a reasonable amount of activity from the whole amount of catalyst added to the reactor. Studies of the esterase catalysed hydrolysis of the model substrate, catalyst active in the bulk of the aqueous phase (Woodley *et al* 1991b), indicated that for a STR operated under defined conditions there comes a point at which the conditions can no longer support maximal activity of all catalyst present as indicated in figure 3.3. For the model system this represents a situation in the reactor where the steady state concentration of substrate in the aqueous phase is below the 2.5mM threshold concentration previously identified by Woodley (1990a) as that concentration required to support maximum activity. As the concentration of enzyme is increased in the reactor higher concentrations of substrate will be required to support maximum activity. Transfer of substrate across the interface must take place to supply

substrate at the necessary concentration in the aqueous phase, this is influenced by the substrate K_LA. If transfer does not supply substrate to the aqueous phase at the necessary concentration an evident decrease in the initial activity will be observed as was indicated in figure 3.3. At concentrations of enzyme which do not demonstrate maximal activity, for the model system concentrations which gave activity of less than 36.5 mmol min^{-1} g^{-1}, a transition between rate controlling regimes from one of kinetic limitation to one of mass transfer limitation is occurring. For esterase catalysed hydrolysis of the model substrate a mass transfer limited regime occured at enzyme concentrations above 1 g l^{-1} in the STR operated in the indicated manner. The efficient utilisation of enzyme in this system is therefore maximal for concentrations below 1 g l^{-1} Above this the enzyme activity is not being efficiently utilised.

Similar observations have been made with respect to the hydrolysis of menthyl acetate both by PLE and a bacterial catalyst, *Bacillus subtilis,* (Williams *et al* 1990). Both catalysts showed a reduction in activity at high catalyst concentrations believed to be due to mass transfer limitation. Similar results for the bacterial catalyst indicate the wide applicability of this theory.

4.1.2. Prediction of K_LA

Data from this study and previous studies can be used to predict the substrate mass transfer coefficient, K_LA, under specified conditions, as was indicated by Woodley and co-workers (Woodley *et al* 1991b). As a basis for the evaluation a substrate mass transfer coefficient is chosen at which the kinetics are substrate mass transfer limited at specified conditions, eg [Ea] = 2 g l^{-1}, phase ratio of 0.75 and agitation rate of 750 rpm, (figure 3.3). Using this the evaluation can be illustrated in the following manner using figure 4.1

(1) The reaction rate-aqueous phase substrate concentration profile for [Ea] = 2 g l^{-1} is plotted based on previously obtained results of Woodley and co-workers, figure 1.6 (Woodley *et al* 1990a).

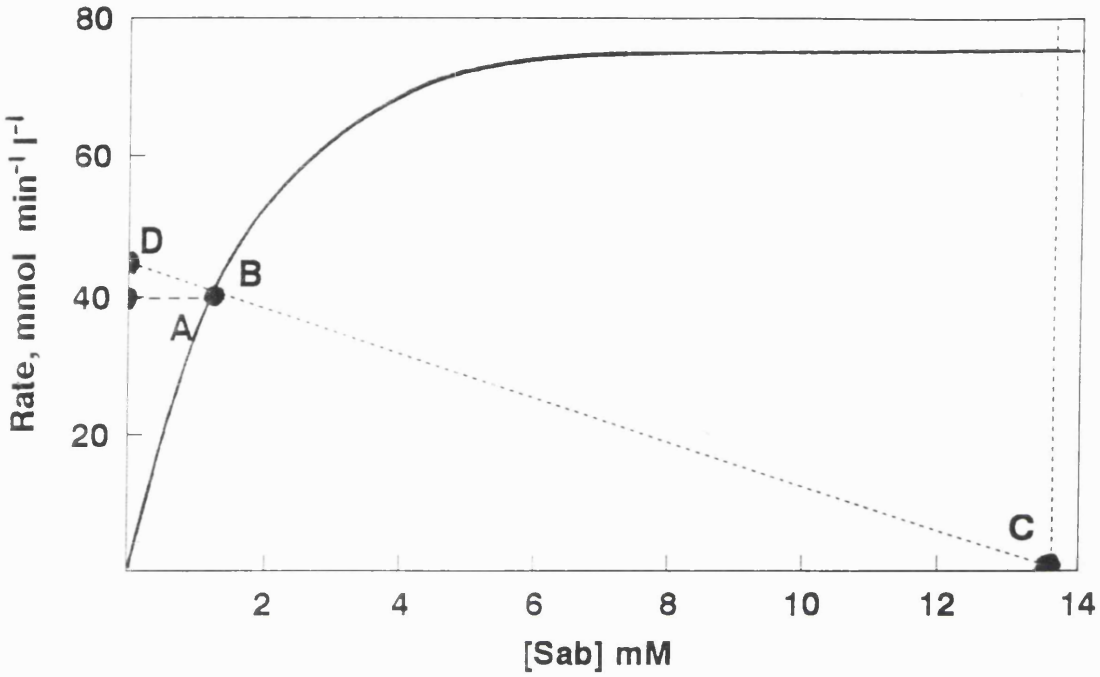


Figure 4.1 Chart for graphical evaluation of substrate mass transfer coefficient. Ra-[Sab] profile at [Ea] = 2 g l^{-1} (——); substrate transfer rate-[Sab] profile (-----), based upon a linear relationship through points B and C.

(2) At this aqueous phase enzyme concentration the activity is noted from experimental results of figure 3.3 (Point A).

(3) At steady state this measured rate must lie on both the reaction rate-aqueous phase substrate concentration profile and the substrate transfer rate-aqueous phase substrate concentration profile (point B).

(4) The assumed substrate transfer rate-aqueous phase substrate concentration profile is plotted based upon a linear relationship from point B to point C (defined by [Sab]=[Sa*] at substrate transfer rate = 0 mmol min^{-1} l^{-1}

(5) Extrapolation of this line to the vertical axis (point D) gives the maximum substrate transfer rate and the gradient of the line is the K_LA

Applying this graphical procedure to the data obtained at a phase ratio of 0.75 and an agitation rate of 750 or 1000 rpm, the K_LA was evaluated as 3.26 min^{-1}. (figure 4.1)

Alternatively if the K_LA is known for a specific set of reactor conditions, its determination being possible using the previously presented graphical procedure, then the activity can be calculated at concentrations of enzyme which are not in a kinetically limited regime. From figure 1.6 this corresponds to the region of the profile where [Sab]<2.5 mM. This region can be approximated by a first order kinetic expression, equation (1) and (2).

$$\text{Reaction rate, } Ra = K_1 [Sab] [Ea] \qquad (1)$$

$$Rs = K_1 [Sab] \qquad (2)$$

Substrate mass transfer may be described by the expression in equation (3):

$$\text{Substrate transfer rate} = K_LA ([Sa^*] - [Sab]) \qquad (3)$$

At steady state the rates of reaction and substrate transfer are equal, hence equations (1) and (3) may be equated:

$$K_1 [Sab] [Ea] = K_LA ([Sa^*] - [Sab])$$

By rearrangement:

$$[Sab] = (K_L A\ [Sa^*])/(K_L A + (K_1\ [Ea])) \qquad (4)$$

Equation (4) can be substituted into equations (1) and (2) to yield expressions for the reaction based on measurable variables in a two liquid phase biocatalytic dispersion:

$$Ra = (K_1\ [Ea]\ K_L A\ [Sa^*])/(K_L A + (K_1\ [Ea])) \qquad (5)$$

$$Rs\ [Sa^*]/((1/K_1\ ) + ([Ea/K_L A)) \qquad (5)$$

Alternatively by rearrangement the substrate mass transfer coefficient can be evaluated:

$$K_L A = (Rs\ K_1\ [Ea])/((K_1\ [Sa^*]) - Rs \qquad (6)$$

Substituting the experimentally determined parameters Rs = 20 $mmol^{-1}\ min^{-1}\ g^{-1}$ at 2 g l^{-1}, and the value of K_1 = 16.2 l $min^{-1}\ g^{-1}$ and [Sa*] 13.5 mM into equation (6), $K_L A$ was evaluated at 3.26 min^{-1}, at a phase ratio of 0.75 and agitation rate of 750 or 1000 rpm. This compares well with the result of the graphically evaluated $K_L A$.

Substituting this result for $K_L A$ into equation (5) together with appropiate parameters yields an expreesion for the specific activity, Rs, as a function of the aqueous phase enzyme concentration, [Ea]:

$$Rs = ((4.57 \times 10^{-3}) + [Ea](0.027))^{-1} \qquad (7)$$

The superimposition of this function onto the results of figure 2 showed close agreement between the model predicted rates and the measured rates in the STR. (figure 4.2). When specific activity was 36.5 $mmol^{-1}\ min^{-1}\ g^{-1}$ the reaction was in the kinetically controlled regime.

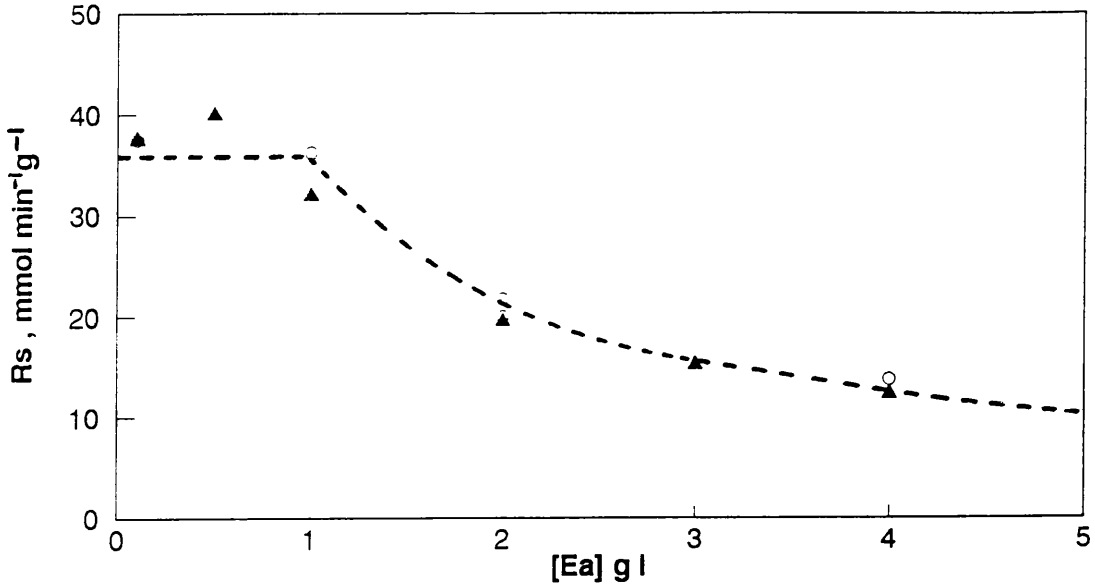


Figure 4.2 Specific reaction rate as a function of aqueous phase enzyme concentration. Reactor conditions: ∅ = 0.75; n = 750 rpm (▲), 1000 rpm (O). Model predicted function (-----).

4.1.3. Lipase Kinetics

Many workers have commentated on the difficulties of elucidating kinetic data for lipase catalysed reactions. Investigation of lipase catalysed reactions in homogeneous systems seems inappropriate as this results in interpreting esterase kinetics, (Kierkels *et al* 1990). A plot presented in their study shows absence of lipase activity for the hydrolysis of glycidyl butyrate until reactor concentration reaches an amount at which the glycidyl butyrate forms a second liquid phase. Kinetic parameters of the interfacial reaction rate for the hydrolysis of 2-naphthyl acetate are much larger than for those in a purely aqueous medium, (Miyake *et al* 1991). This study similarly showed very little activity of the lipase for reaction upon a soluble concentration of substrate in aqueous buffer, figure 2.4, thus eliminating possible activity in the crude extract as a result of esterase

Since their is a requirement for the interface, kinetic analysis will be dependent on the amount of interface as well as concentration of substrate at that interface. In the system of this study the concentration of substrate at the interface is maximal, it represents the second liquid phase. In a reaction where the substrate is dissolved in the organic phase, kinetic analysis will necessitate consideration of substrate concntration in that phase. A plot of lipase activity as a function of enzyme concentration was presented in figure 3.15. In comparison to the esterase the specific activity of the lipase for the model substrate was much less, this however represents the crude nature of the preparation used. Comparatively the plot is linear. For the esterase a linear plot was observed up to a concentration of 1 g l^{-1} reflecting a kinetic limitation of the activity. The linearity of this plot can justifiably be said to represent maximum activity for the lipase i.e. kinetic limitation rather than limitation as a result of inadequate interfacial area.

In investigations of the hydrolysis of oil by a lipase, experiments at a range of enzyme concentrations revealed increasing rates of hydrolysis with increasing enzyme concentration, (Mukataka *et al* 1987), similarly observed in this study. In contrast to this study the rates were presented as a percentage degree of hydrolysis. This makes no

indication of how effectively the available activity was being used. In a study of the interfacial kinetics of lipid hydrolysis by a lipase from Candida, maximal rates were observed up to a defined concentration of enzyme, above which rates became sub maximal, (Ekiz *et al* 1988). This effect was as a result of saturation of the interface with the lipase molecules. Above a certain concentration interface is unable to support increased amounts of enzyme and thus enzyme will remain redundant in the bulk of the aqueous phase, enzyme activity is used inefficiently. This was also reiterated in adsorption studies in which above a certain concentration lipase was detected in the bulk aqueous phase. Although in this study interfacial limitation was not recognised even at considerably high concentrations of the enzyme preparation, at the defined operating parameters, a subsequent evaluation under different operating conditions, in which the phase ratio was reduced from 0.4 to 0.2, showed decreased as a result of the reduced interfacial area and the increased relative amount of lipase in the reactor due to increased aqueous phase volume.

It was interesting to note in the work of Ekiz and co-workers, (Ekiz *et al* 1988) that at relatively high concentrations of enzyme further adsorption of lipase to the already existing monolayer took place. This had the effect of decreasing the activity of the existing monolayer possibly as a result of induced conformational changes or impediment to the diffusion of reaction products away from the interface. In this study the enzyme preparation consisted of other proteins not having lipase activity. High concentrations of these other proteins may compete for access to the interface, induce conformational changes or limit product diffusion. The results from figure 3.15 suggest that even at high concentrations this is not apparent and binding of the lipase appears to be specific. The purity of this lipase preparation is therefore not significant in terms of an effect on the activity of the enzyme. Other lipase preparations may however have different associated proteins which do have an influence.

4.1.4. STR Parameters Governing K_LA and Interfacial Area

K_LA in the reactor is dependent on the physicochemical properties of the reaction medium and operating conditions of the vessel. Similarly the interfacial area in the vessel is dependent on the physicochemical properties and operating conditions. Interfacial area has a strong influence therefore on the operation of both the lipase and esterase catalysed reactions.

4.1.4.1. Agitation

For this system interface was maintained by constant agitation of the reactor contents in the STR to create droplets which dictate the interfacial area achieved. Droplet size and thus interfacial area is a function of several variables, (Eckert *et al* 1985). The variables of interfacial tension, viscosity and density of the discontinuous phase and density of the continuous phase ,which influence droplet size, are physical properties. When considering the choice of an organic solvent in which the substrate is to be dissolved they have important implications. Often the choice of solvent may be limited for example by its biocompatibility. Alternatively it is the organic phase to be transformed and thus the use of these parameters to optimise interfacial area for mass transfer and site of activity is limited. This was the case in this study in which the model substrate benzyl acetate is the second liquid phase. These parameters were recognised for purely liquid/liquid systems. The presence of the biocatalyst, its concentration, purity and form, transformation of the substrate to product and temperature will all further influence the properties of the reaction medium These properties may undergo change as the reaction proceeds. In this study the addition of hydroxide to maintain pH resulted in an increase in the density of the aqueous phase as reaction progressed. Kierkels and co-workers (Kierkels *et al* 1990) have commentated on the amulgamating effect of crude enzyme preparations and the changes to the interfacial area once reaction has started due to this. The interface in this study was able to support high concentrations as a result of this effect, further investigation however

would be required in order to verify this. The complicated nature of assessing the mixing characteristics as a function of time is thus evident in two-liquid phase biotransformation studies.

The variables, phase ratio, power per unit volume and impeller tip speed are functions of the operating parameters in the STR and thus represent more readily investigable parameters, power per unit volume and tip speed can be simplified to represent rate of agitation. Other influences on the interfacial area as recognised in the scale up of liquid-liquid dispersions are tank and impeller geometry's. In this study the prime operating variables considered were the phase ratio and the rate of agitation. In order to be able to understand the effects of these parameters on the creation of interface Kim and Kim (1990) make claim to prediction of droplet size, this was however at very low phase ratios and not suitable for this study. Light scattering has been used as a method to determine droplet size distribution for lipase catalysed hydrolysis in a stable emulsion, (Kierkels *et al* 1990). Its application to the measurement of dispersions may prove less useful and require further investigation.

An effective means to study the influence of these parameters on mass transfer and subsequently interfacial area was to consider reactions occurring in the bulk of the aqueous phase at concentrations which are well within a mass transfer limited regime. Increased activity relative to the activity measured at different operating parameters could then be assumed to be a result of increased mass transfer and optimum conditions can then be identified. In this study it has been shown that at an enzyme concentration of 2 g l^{-1}, an operating phase ratio of 0.75 and agitation rate of 750 rpm the activity of the enzyme was mass transfer limited.

For liquid-liquid systems it is well recognised that increases in the rate of agitation generate progressively smaller droplets resulting in increased interfacial area and thus increased rates of mass transfer. Shift to a smaller droplet size and narrower size distribution at higher rates of agitation for a liquid-liquid dispersion at constant phase ratio have been shown, (Okufi *et al* 1990).

Employing mass transfer limited concentration of esterase in the reactor, increases in rate of agitation, figure 3.5, resulted in an increase of the specific activity

thus reflecting the increased mass transfer as a result of increased interfacial area. The PLE catalysed hydrolysis of menthyl acetate, in contrast to this study, has shown a decrease in the rate of product formation at higher stirrer speeds (Williams *et al* 1987). this may however be representative of detrimental effects of higher rates of agitation, their study used comparatively higher rates of agitation than in this study. For a bacterial catalyst, likely to be more resilient to higher rates of agitation, rates increase even at agitation rates comparatively greater than those in this study, (Williams *et al* 1990).

The equilibrium between droplet breakage and coalescence, and thus the characteristic droplet size distribution may not be achieved as rapidly as one might expect, (Mersmann and Grossman 1982). Initial kinetics although representative of the initial transfer conditions may not truly represent the mass transfer potential of the reactor. A study upon the hydrolysis of tributyrin with lipase observed that as reaction time increased droplet size became smaller, (Martinez *et al* 1992). The time at which equilibrium was achieved is probably a function of the physical properties of the liquids. In contrast to the liquids referred to by these workers those of this study may not be similarly characteristic, it might be expected that the mixing of liquid with greater viscosity's requires greater time to achieve the equilibrium.

Although the increased rate of agitation resulted in increased mass transfer as a result of smaller droplet size, infinitely increasing the rate of agitation will not lead to infinitely increasing mass transfer. The droplets will achieve a minimum size beyond which further breakage does not occur and thus rate of agitation will eventually be limiting. This was observed in a study of the hydrolysis of beef tallow by lipase in which at rates of agitation above 400 rpm no further increase in the interfacial area occurred (Mukataka *et al* 1985).

Since for a lipase the interface determines the amount of enzyme which can be maximally supported it can be expected that similar increases in specific activity might be observed at increased rates of agitation if the reactor is operated at known concentrations of enzyme in excess of the interfacial saturation concentration. A number of workers have identified increased activity for lipase catalysed

biotransformation of poorly soluble substrates with increasing rate of agitation (Tsai *et al* 1991, Martinez *et al* 1992, Mukataka *et al* 1985). As more interface is made available to support more of the enzyme present a decrease in aqueous phase enzyme concentration will be evident, (Tsai *et al* 1991). Increase in activity results from the increased interfacial area as a result of increased rate of agitation, (Mukataka *et al* 1985). In a study into the hydrolysis of a secondary alcohol using a lipase increasing activity with increasing rate of agitation was shown. At a defined rate of agitation no further increase in the activity was found with further increase in the rate of agitation, though the droplet size in the emulsion was progressively smaller, (Mitsuda *et al* 1989). This represented a rate of agitation which provided enough interface to support activity of all enzyme present as was found in this study.

It is interesting here to compare the hydrolytic reaction with an esterification reaction carried out for the synthesis of butyl butyrate employing a lipase as catalyst. Though essentially the phase ratio was 0.95, so as to keep the relative water content low to promote the synthetic reaction over hydrolysis, it can still essentially be recognised as a two-phase system and again increased rate of agitation resulted in increased activity of the enzyme, (Borzeix *et al* 1992).

4.1.4.2. Phase Ratio

The second operational parameter of phase ratio was similarly considered with respect to the influence upon the activity as of mass transfer and interfacial area. The result of this study are illustrated in figure 3.8. An optimum phase ratio of between 0.4 and 0.5 was found. Higher activity of the esterase was supported as a result of increased mass transfer relative to phase ratios of a lesser or greater value. In contrast to this study hydrolysis of menthyl acetate by the esterase showed a constant activity up to a phase ratio of 0.5 followed by a decline in activity as phase ratio was further increased, (Williams *et al* 1990). It must be commented however that in the study of Williams and co-workers results were expressed on the basis of overall reactor volume, no allowance was made for the changing phase ratio. Reconsidering these results, allowing in the

determination phase ratio changes, yields a similar plot to that observed in this study, figure 4.3.

As phase ratio is increased droplet size distribution is no longer completely controlled by breakdown, damping of the turbulence intensity by the dispersed droplets and increased coalescence in the regions of lower turbulence in the vessel lead to increased mean particle size in the reactor (Mersmann and Grossman 1982). The hydrolysis of tributyrin indicated that as reaction time proceeded the reduction in phase ratio as a result of the products being preferentially soluble in the aqueous phase resulted in a reduction in droplet size, (Martinez *et al* 1992). At lower phase ratios it might be expected that reduced particle size results in increased area for mass transfer, this is however not indicated in this study. At increasing phase ratio the relative volume of organic to aqueous phase is increased, although the droplet size may be increased the relative concentration of particles relative to the aqueous phase is greater and hence the specific area of interface is actually greater. A number of other workers have commented on increasing interfacial area in a two-liquid phase system as a result of increased phase ratio, where the aqueous phase is present as the continuous phase. (Lilly *et al* 1990, Eckert *et al* 1985)

As the phase ratio is increased over the optimum then inversion occurs, the point at which this occurs as well as being a function of the phase ratio will also be a function of liquid properties, agitation rate and relative impeller position in the tank (Mersmann and Grossman 1982). Once this takes place aqueous phase becomes the discontinuous phase and is present as droplets in the organic continuous phase. Further increased phase ratio results in the relative decrease of aqueous volume with a resultant decrease in the concentration of droplets and thus interfacial area. However the interfacial area relative to aqueous phase volume does not decrease and a decrease in activity might not be expected. The resultant activity decrease observed in this study, figure 3.8, for operation of the reactor at phase ratios resulting in inversion, may be a result of poor internal mixing within the aqueous phase droplets, limiting K_LA, resulting in poor supply of substrate to the enzyme and therefore reduced activity, (Hanson 1971). The higher interfacial area relative to aqueous phase volume in the

inverted system negates some of the impact of this effect and hence the decrease in activity is not as dramatic as the increase observed in the non-inverted system. Under circumstances of phase inversion increases in the rate of agitation, resulting in smaller droplets reduces the K_LA as a result of poor internal mixing within the smaller droplets, (Hanson 1971). This study, figure 3.11, revealed, in a non inverted system, increased activity reflective of increased mass transfer with an increase in the rate of agitation. At phase ratios indicative of an inverted system, increased rate of agitation resulted in decreased activity. In the inverted system as the droplets further decrease in size the mixing problems in the aqueous droplets are exacerbated and at higher rates of agitation K_LA is reduced.

Since the area of interface may also be limiting to lipases at greater than saturation concentration it would be expected that phase ratio might illicit similar effects on the activity of the lipase. Evidence for this has been provided by a number of workers. In reactions carried out on very small volumes, < 5 ml , maximum lipase activity was found at a phase ratio of 0.5, (Wang *et al* 1988). A phase ratio plot for a lipase catalysed hydrolysis of a fat similar to that observed in this study, for the esterase catalysed hydrolysis, with optimal activity being reached at a phase ratio of 0.4 and correspondingly decreasing with further increases in phase ratio was shown, (Tsai *et al* 1991). Mukataka and co-workers (Mukataka *et al* 1987*)* have presented a similar evidential plot for a reaction carried out in a baffled and non baffled vessel, it was interesting to note that in the non baffled vessel optimum interfacial area was achieved at a phase ratio of 0.3 in contrast to the baffled vessel which showed an optimum at 0.5. In the hydrolytic reaction of Kierkels and co-workers (Kierkels *et al* 1990) a limiting effect of the phase ratio was observed.

4.1.5. Modelling STR Operation for K_LA and Enzyme Activity

The consequences of these findings for the efficient operation of the two liquid phase bioreactor and its characterisation with respect to utilisation of available activity

can be illustrated using data gathered for the model system in this study and its relation with previous data.

The mass transfer coefficient (K_LA) necessary in the two phase reactor to support maximal activity of a defined concentration of esterase has been previously identified, (Woodley 1990a), and is depicted in figure 4.3a. For defined operating conditions enzyme specific activities have been elucidated by this study, figure 3.11. Substituting these values into equation (6) from the previously presented model for evaluation of K_LA, results in the plot of figure 4.3b, demonstrating the derived K_LA as a function of the phase ratio at a defined agitation rates of 1000 and 1800 rpm. The unification of figures 4.3 (a) and (b) provide values of phase ratio for a defined agitation rate and enzyme concentration to make optimum use of both reactor mass transfer and enzymatic activity. At high values of aqueous phase enzyme concentration it may not be possible to operate the reactor in a manner to support high activity of the enzyme at the defined rate of agitation. At lower values of aqueous phase enzyme concentration where the mass transfer coefficient required to support maximum enzyme activity is less than that which can be optimally achieved, two alternative phase ratios exist under which the reactor can be operated, one in a preinverted system and one in an inverted system. The process implications of choice of operating phase ratio have been discussed previously in qualitative terms, (Lilly *et al* 1990).

Alternatively the relationship of phase ratio and rate of agitation as it supports enzyme activity may defined as qualitatively illustrated in figures 4.4 (a) and (b). The qualitative depiction is based on previous assumptions with respect to the results of this study as they relate to phase ratio and agitation rate and the effective enzyme activity. Figure 4.4a is characteristic of the combined effect of agitation rate and phase ratio on the bulk aqueous phase catalyst, shape of the surface is reflective of the effects of these parameters on the activity of a bulk aqueous phase catalyst operated in a mass transfer limited regime. At non inverted phase ratios increase in agitation results in increase activity. At inverted phase ratios increased rates of agitation, above a certain agitation rate, result in decreased activity. This contrasts to the shape of the surface which might be expected for a catalyst at the interface as is depicted in fig 4.4b. The shape is similar

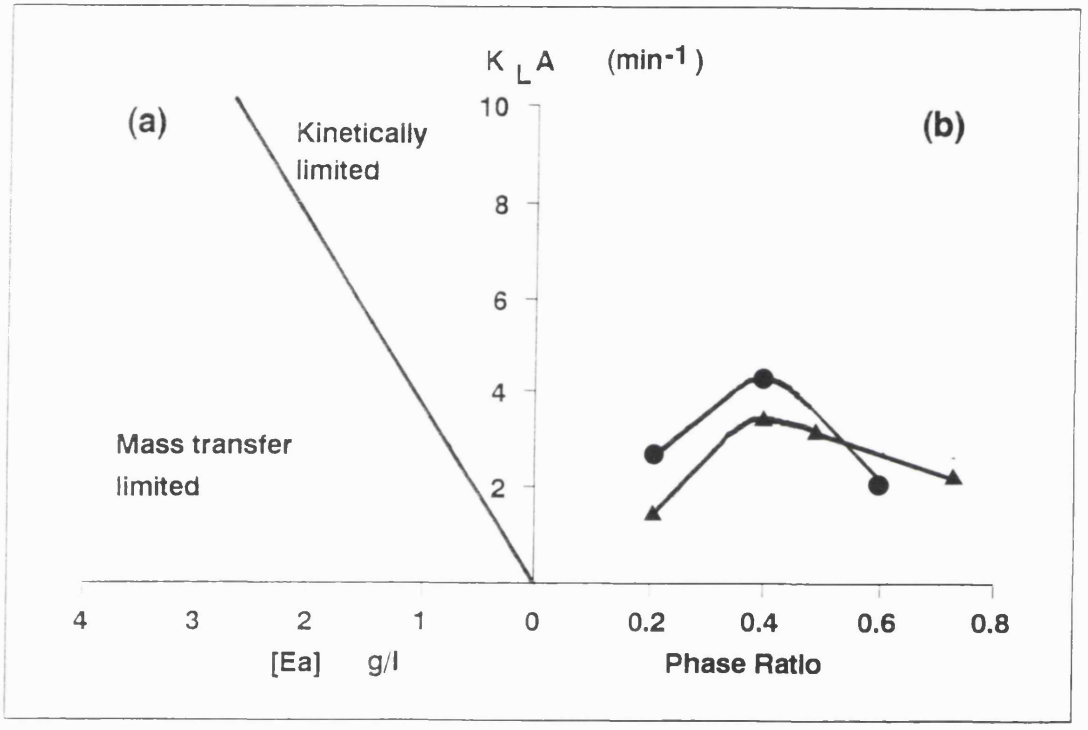
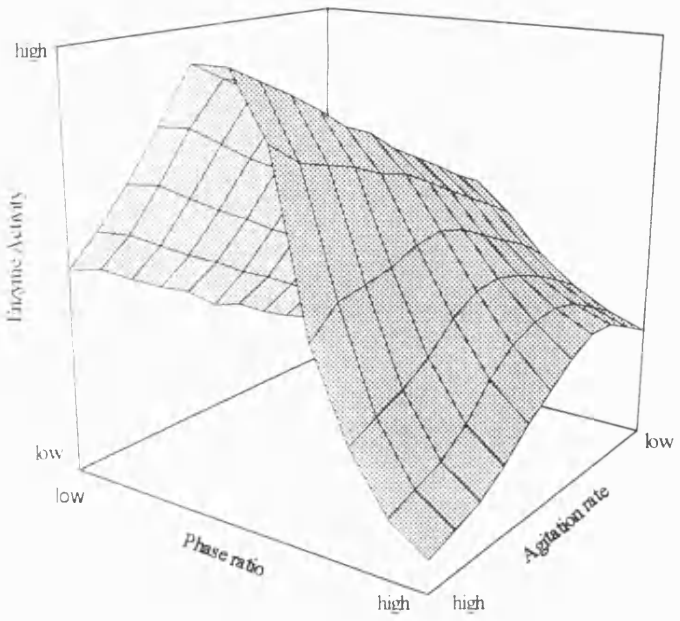


Figure 4.3 (a) Plot of aqueos phase enzyme concentration ([Ea]) against substrate mass transfer coefficient ($K_L A$) showing optimum enzyme activity (——). (b) Plot of phase ratio against experimentally determined mass transfer coefficients; Agitation Rate 1000 rpm (▲), 1800 rpm (●).

A



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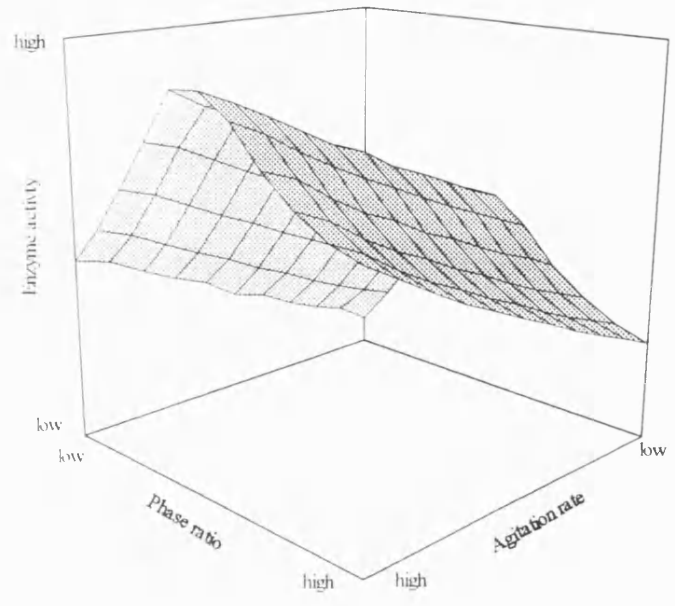

Figure 4.4 3-D surface models for the relationship of phase ratio and rate of agitation as it influences enzyme activity for esterase, (a), and lipase, (b).

at non-inverted phase ratios. However, assuming no limit to adsorption rate at the interface, mixing in the inverted system is not a limiting factor. At inverted phase ratios the increase in interfacial area as a result of increased agitation is reflected in increased activity. The peak of the surface is representative of optimal conditions of operational parameters phase ratio and rate of agitation at the defined enzyme concentration. Any contour plateauing as illustrated in figure 4.5 is indicative of exit from a mass transfer/interfacial limited regime into a kinetically limited regime.

It is of importance to note that initial optimal conditions of phase ratio may not be maintained throughout the complete time of reactor operation. Transformation of substrate will lead to depletion of the organic phase volume. Depending on the partitioning nature of the product the imbalance may be redressed to some degree. With respect to the model reaction in particular pH maintenance requires further addition of aqueous phase volume as reaction progresses. Illustrated in figure 4.6 are the potential phase ratio changes as a function of conversion for a reaction initiated at a phase ratio of 0.4, due to the addition of differing concentrations of hydroxide in order to maintain the pH optimum of the reaction. The phase ratio changes are more pronounced for a more dilute concentration of hydroxide. Using a more concentrated solution requires the addition of less volume to maintain the pH and resultingly lesser change in the phase ratio. The consequences of the reduced phase ratio as reaction progresses are a potential reduction in the interfacial area and K_LA and thus its implications to maintenance of enzyme activity as reaction progresses.

The aqueous phase enzyme concentration will similarly show a reduction as a result of its dilution by the addition of further aqueous phase volume in order to maintain optimal pH. Its dilution will be a function of the amount of aqueous phase added as dictated by concentration of the titrant. The K_LA necessary to support optimal activity of lower aqueous phase enzyme concentrations does not need to be as high as is necessary to support a comparatively higher aqueous phase enzyme concentration. The lowering of aqueous phase enzyme concentration may negate, to a degree, the effects of the lower K_LA.

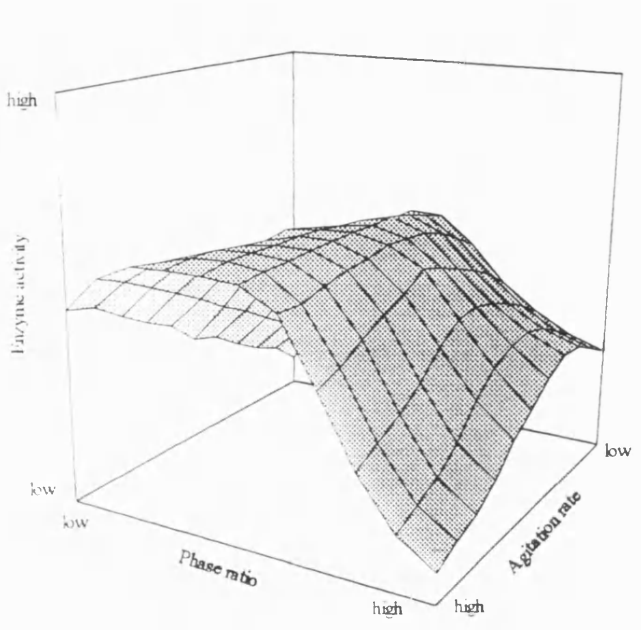

Figure 4.5 3-D surface model for the relationship of phase ratio and rate of agitation, as it influences activity, demonstrating potential contour plateauing at kinetcally limited concentrations.

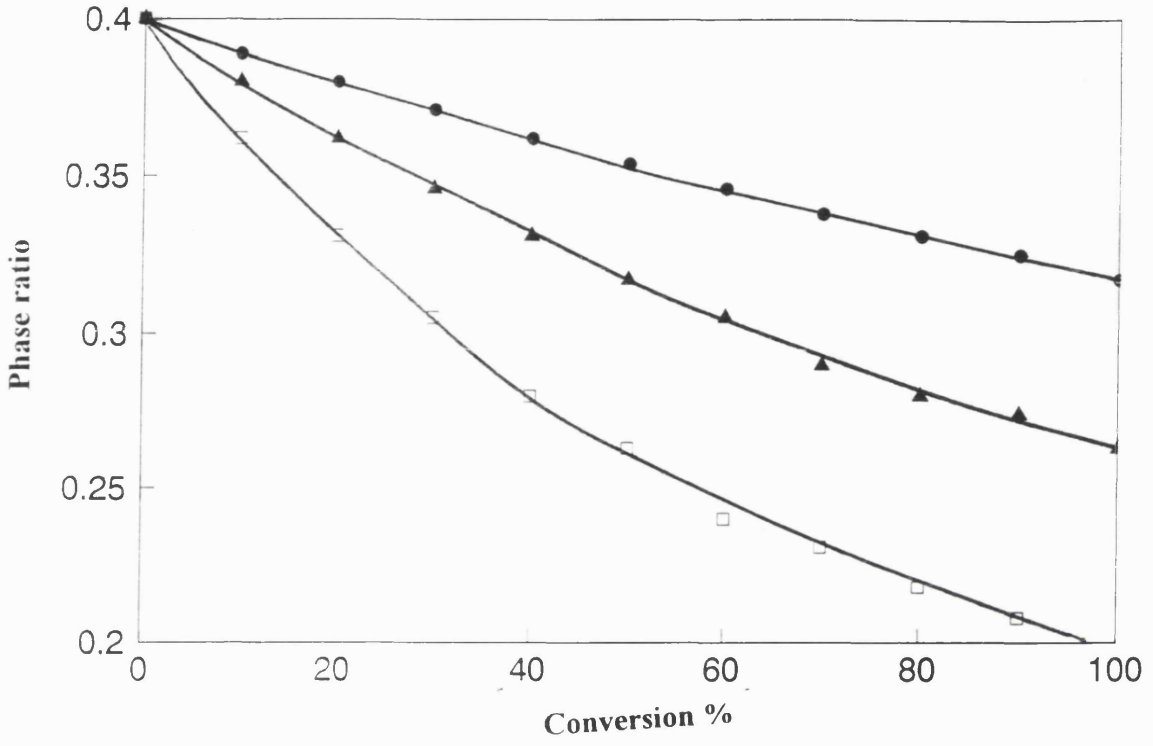

Figure 4.6 Conversion effect on phase ratio in the STR due to the addition of NaOH, of varying concentrations. to maintain pH; 10 M (●), 5 M (Δ), 2.5 (□).

4.1.6. Catalyst Stability in the STR

Implicit in the two previously represented models for the definition of optimal operating parameters is a recognition that this relates to the effects upon the initial activity and thus influence on the overall productivity. However for biocatalysed reactions productivity as it relates to overall conversion and reactor operating times will also be influenced by the stability of the catalyst.

For the esterase catalysed hydrolysis of the model substrate in this study cessation of activity was observed at an earlier operating time for reactions carried out at increased rate of agitation and phase ratio, figures 3.6 and 3.9. It is previously recognised that at the comparatively higher phase ratio and rate of agitation there is an effective increase in the interfacial area. At the higher phase ratio the increase in interfacial area is also accompanied by an effective increase in the ratio of the interface to the amount of enzyme in the aqueous phase, assuming constant aqueous phase enzyme concentration. It may be proposed that the interface is therefore affecting the stability of the enzyme, interface resulting in a premature deactivation of the enzyme.

Solvents are well known to have a deleterious effect on enzymes. Much of this work has been concentrated on bacterial catalysts and a recognition of the influence of solvent polarity as identified in a study on the microbial epoxidation of propene and 1-butene by Brink and Tramper (1985). Log P as a measure of solvent polarity has been used and in reactions carried out in solvents with a Log P of less than 4 measured activity is reduced, (Laane *et al* 1985 and 1987). Similarly the usefulness of Log P measurements for characterising solvent toxicity effects on a chymotrypsin catalysed process has been indicated, (Reslow *et al* 1987).

Much of the work in relation to solvent tolerance of enzymes has been generally in monophasic systems of a low water environment for synthesis reactions particularly with respect to lipases. In such systems the amount of solvent to which the catalyst is exposed is relatively high. Although the mechanism by which the solvent exerts its effect is not yet understood, it has been suggested that in such an environment the essential water layer present in order to maintain the enzyme in its active

conformation may be distorted or even stripped from the presence of the enzyme by polar solvents resulting in its deactivation, (Laane *et al* 1987 and Klibanov 1986). In the two phase environment, necessary to promote the hydrolytic reaction, the amount of water associated with the enzyme is comparatively large and since the esterase is contained within the bulk of the aqueous phase the poor solubility of the substrate results in the exposure of enzyme to relatively small concentrations of the substrate. Hence initial substrate toxicity might be limited in the two-phase system and should not exert influence as suggested by Klibanov (1986) and Laane and co-workers (1987). However constant exposure, over time, to small concentration may be effective in eventually inducing instability. At higher rates of agitation and phase ratio a greater K_LA, as shown in this study, results in increased steady state concentrations of substrate in the aqueous phase which may account for the relative decrease in stability with prolonged exposure.

As a result of agitation, enzyme molecules within the aqueous phase will periodically come into contact with large concentrations of solvent at the interface. Denaturation of PLE at a liquid-liquid interface has been previously supposed for the hydrolysis of menthyl acetate, (Williams *et al* 1987). For the model system the Log P of the substrate is 1.6, a Log P deemed to be potentially toxic, (Laane *et al* 1987). Contact with large concentrations of solvent at the interface may result in the distortion or stripping of the water layer associated with the enzyme molecule resulting in its conformational change and denaturation. In this study half lives were not affected by a difference in the interfacial area as influenced by agitation rate and phase ratio, figure 4.7, half life for the esterase being 12-13 minutes. However at a later stage in the reaction the stability of enzyme in the reactor, operated with a greater interfacial area, was affected in comparison to that operated in the reactor with less interfacial area, figure 3.6 and 3.9. The increased interfacial area results in increased frequency of contact of the enzyme with the liquid-liquid interface.

The effect of air/liquid interface on the stability of enzymes is documented. In a study by Lee and Choo (1989) into shear inactivation of lipase the resulting surface tension at the air liquid interface was suggested to result in a deactivation of the lipase.

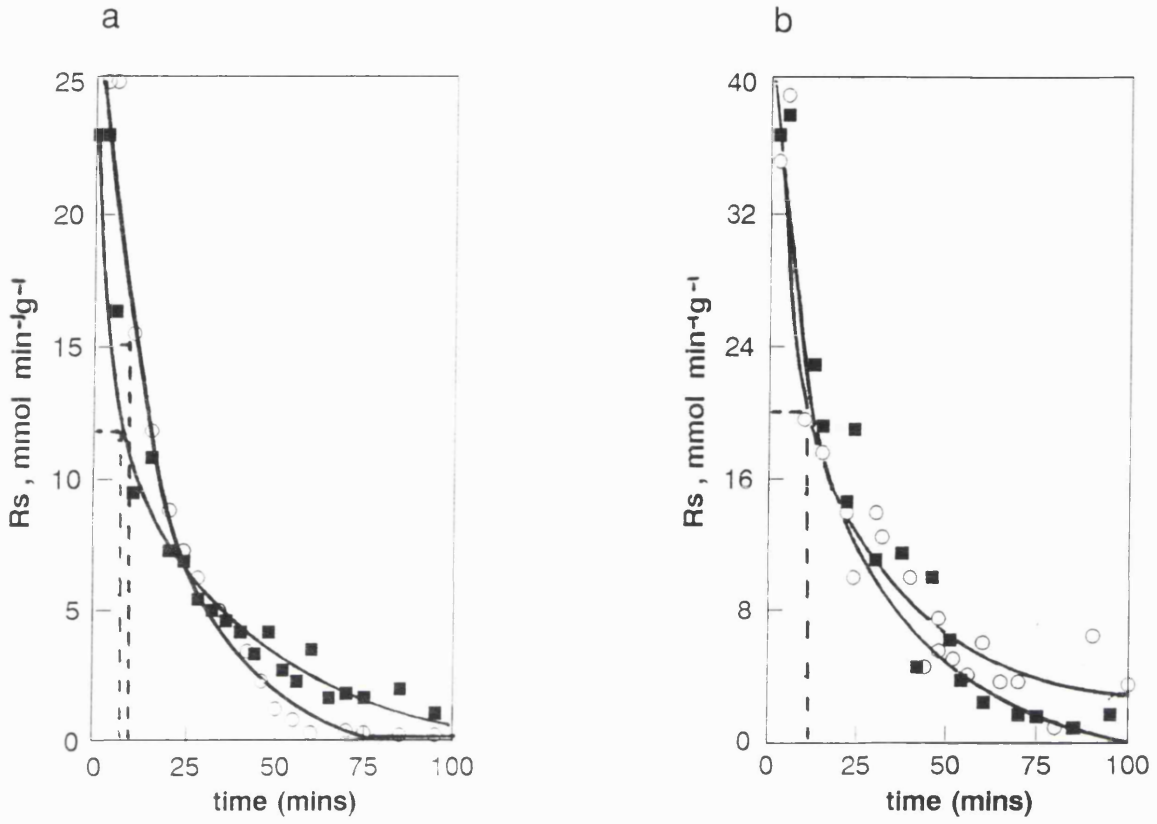

Figure 4.7 Half lives, (-----) for esterase catalysis in the STR. Reactor conditions; a: [Ea] = 2 g l^{-1}, ∅ = 0.4, n = 1800 rpm (O), n = 1000 rpm (■); b: [Ea] = 0.5 g l^{-1}, n = 1000 rpm; ∅ = 0.2 (O), ∅ = 0.4 (■)

Increased rates of agitation resulting in the intake of greater amounts of air into the system. It might be suggested that the stability differences observed at different rates of agitation in this study are due to increased air/liquid interface also. It might also be suggested that interfacial tension at the organic/aqueous interface, in the two-liquid phase medium, similarly results in deactivation of the enzyme at the interface as a result of conformational alteration.

To improve stability of the enzyme in a two liquid phase system operation, assumming instability due to solvent toxicity, will require selection of suitable solvents to limit the toxicity is desirable. If the nature is the result of interfacial tension at the interface be it air/liquid or organic/aqueous its limitation by limiting the amount of interface is relatively impractical, this results in decreased K_LA and reduced enzyme activity. Protection from the interface by immobilisation may be the only suitable means, however it is well recognised that diffusional limitations of many supports will similarly result in decreased activity. The addition of surfactants has been suggested in the work of Lee and Choo (1989). The stability of an enzyme used in the hydrolysis of cellulose in a two phase bioreactor was improved by the addition of surfactant to the reactor, (Tjerneld *et al* 1991). It was suggested the surfactant hindered migration of enzyme to the air liquid-interface, the influences on the organic/aqueous interface will also need consideration. Other proteins have been used in an attempt to improve enzyme stability by limiting exposure to interface. Bovine serum albumen, (BSA), was successfully used to reduce loss of activity for the PLE catalysed hydrolysis of menthyl acetate, (Williams *et al* 1987). Enzyme engineering may provide a means of providing more stable structures, (Arnold 1990). Enzymes can in some instances, depending on the nature of deactivation, be reactivated by certain agents (Mozhaev and Martinek 1982), the use of such agents in the reactor may result in prolonged stability. Further characterisation of the interfacial effects is obviously needed in an attempt to recognise and limit the negative effects which interfaces produce. Tools to look at enzyme structure may further provide valued insight into the effects on stability.

Similar study of potential interfacial effect on the stability of the lipase revealed comparative stability at increased rates of agitation and increase phase ratios as shown

in figures 3.16 and 3.17. It is not surprising that inactivation of a lipase is not a function of the interfacial area since there is a requirement of the lipase for the interface to support activity, the enzyme is associated with that interface. Borzeix and co-workers (Borzeix *et al* 1992) In a two-liquid phase esterification reaction using a lipase no deactivation of lipase as a result of organic-aqueous interface was found, (Borzeix *et al* 1992). It has been suggested by Klibanov (1986) that in some enzymes, (and specifically PPL), that the water necessary for activity is so tightly bound to the enzyme that its removal or distortion is extremely difficult. It might be argued that potentially as was observed in the study by Lee and Choo, (1989), air/liquid interface may be effective in limiting stability, the attachment at the organic/aqueous interface may limit exposure to an air/liquid interface. Adsorption at the interface may confer stability in a lower rate of unfolding. A decreased stability was not evident in this study for the lipase at the higher rates of agitation. The contents of the impure preparation may have influenced the intake of air into the reactor.

Although this is the case where the enzyme is not limited in its access to interface i.e. below saturation concentrations this may not necessarily reflect incidence where enzyme concentrations above saturation concentration are evident and some lipase remains in the bulk of the aqueous phase where its activity is redundant. Protective effect from the air/liquid interface may not in this instance be evident.

It may be argued that since the enzyme is redundant it is of no consequence whether it is stable or not. However it is recognised that eventually the active enzyme becomes inactive, this may be a result of its prolonged exposure to the interface. At above saturation concentration redundant enzyme in the aqueous phase, having not been exposed to the interface, is potentially still active. It may be able to replace inactive enzyme at the interface and a new for old exchange may be created in the reactor. This will be dependent on the disassociation of inactive enzyme from the interface maintaining the stability of enzyme remaining in the aqueous phase is important.

Operating the reactor at above saturation concentration allows the maintenance of rates over a longer period of time and thus achieves a higher conversion in the reactor. Illustrative of this is figures 4.8 and 4.9. For a situation at or below saturation

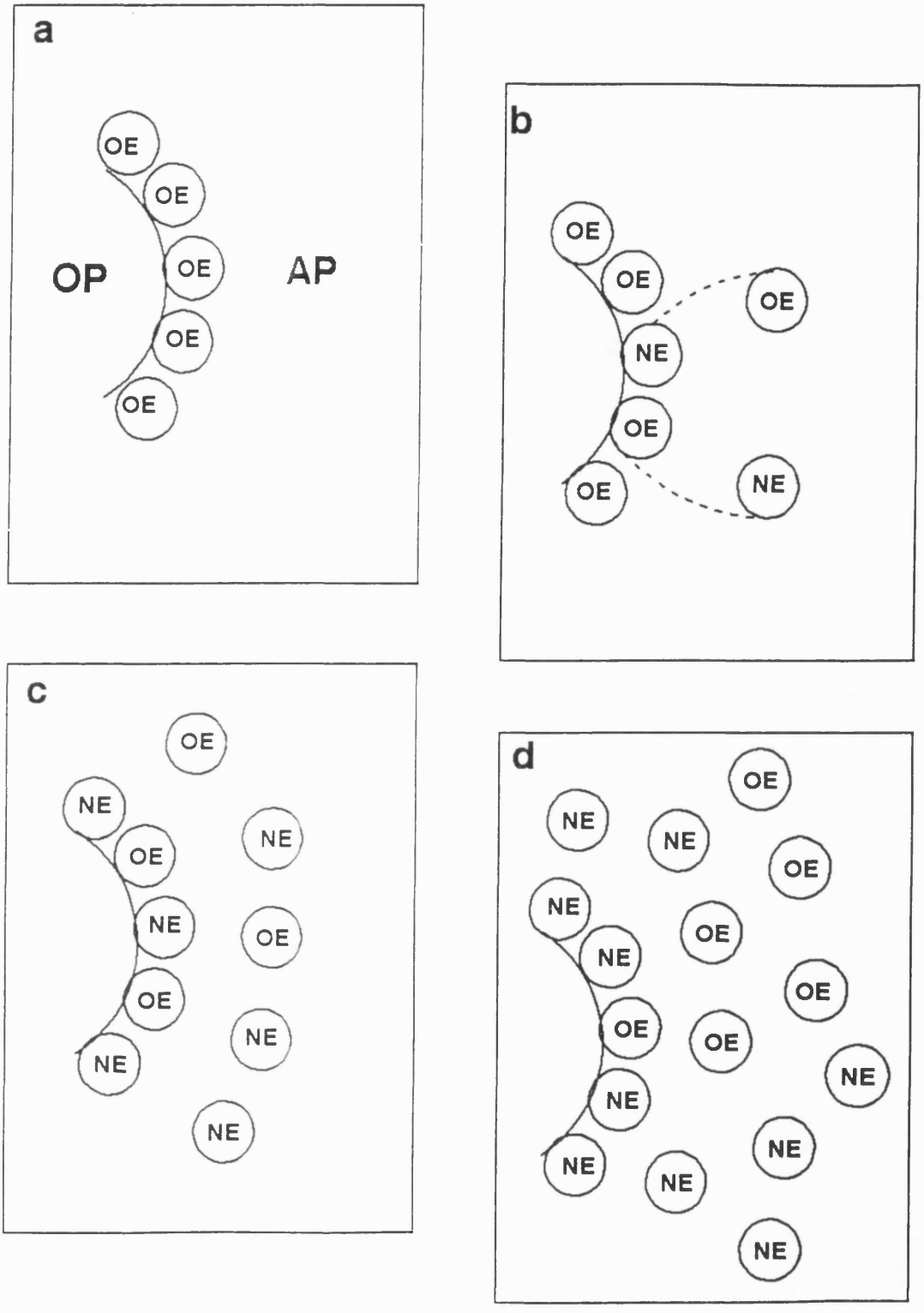

Figure 4.8 Illustrative schematic of increasing concentrations of enzyme above saturation concentration, order of increase d>c>b>a, and the interelationship of "new for old" exchange at the interface: "Old" inactivated enzyme (**OE**), "New" active enzyme (**NE**).

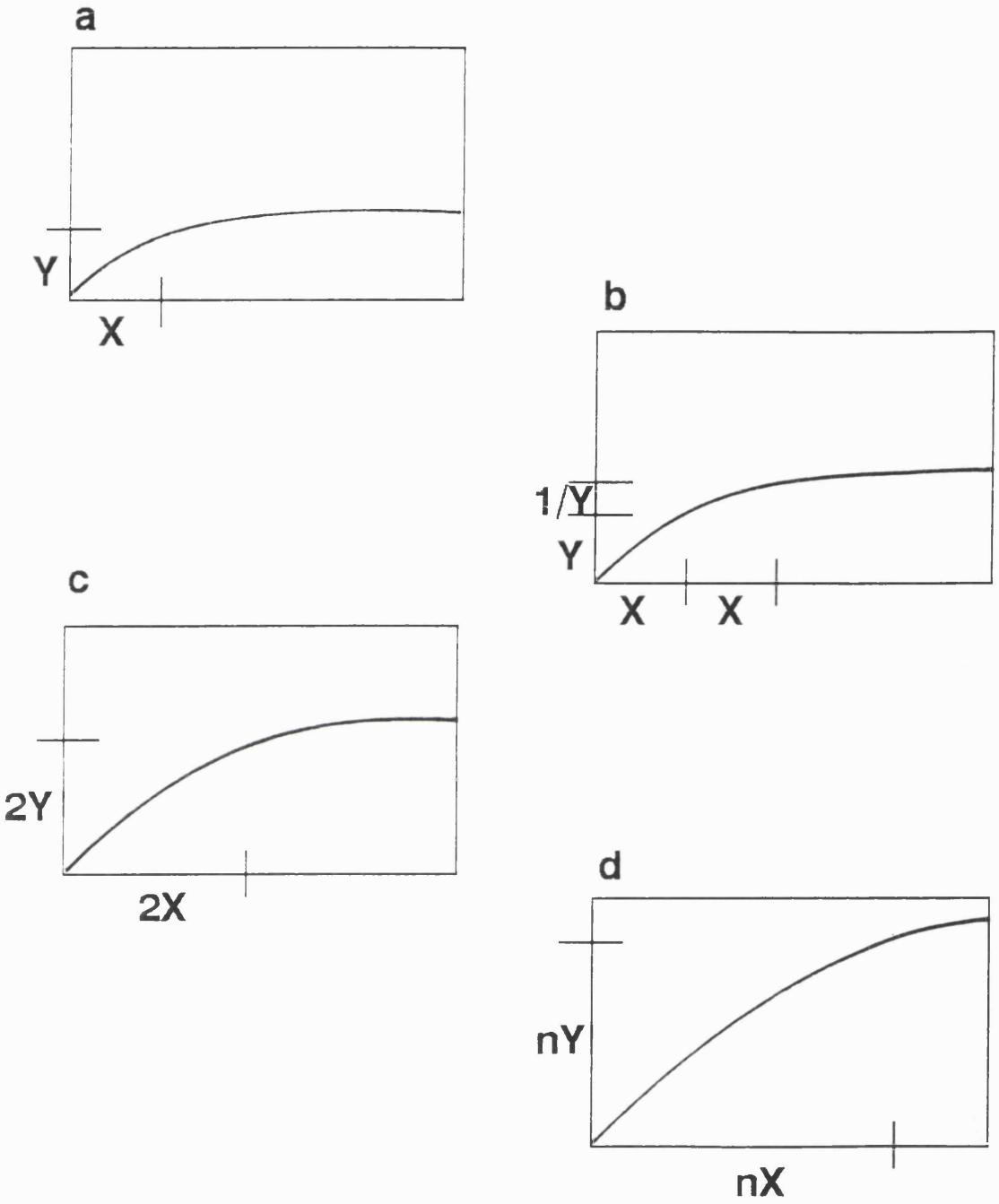

Figure 4.9 Model conversion rates as a function of time with increasing, a- b-c-d, concentrations of enzyme above interfacial saturation concentration as previously illustrated in figure 5.9: X axis = time, Y axis = % degree of hydrolysis.

concentration, (figure 4.8a), deactivation at the interface may result in minimum conversion dictated by rate Y and time X, time of deactivation (figure 4.9a). At marginally above saturation concentrations (figure 4.8b) new for old exchange will result in increased conversion but the rate maintained will be relatively decreased to a fraction of Y as a function of time X, (figure 4.9b). Where saturation concentration is equalled by redundant aqueous phase concentration (figure 4.8c) then rate Y might be maintained over time 2X. (figure 4.9c). Where redundant enzyme concentration is far in excess of saturation concentration, (figure 4.8d) rates Y may be maintained as times nX, (figure 4.9d), the value of n being a function of the relative excess of redundant enzyme as it compares with saturation concentration.

Although rates of hydrolysis will be constant expressed on the basis of % hydrolysis, specific activity of the lipase will decrease in order of a>b>c>d in contrast the degree of conversion achieved will be in the order of d>c>b>a. Although the specific activity of the lipase decreases all lipase in the reactor will eventually be optimally utilised. The potential of this is in the modelling and prediction of amounts of interfacially active enzyme necessary in the reactor to achieve a desired degree of conversion.

4.1.7. Product Effects

As well as the influence of reactor operating parameters on the stability of the catalyst biocatalysed reactions can be identified in which the products of the reaction also influence the productivity of the reactor due to inhibition. Product inhibition at low levels was not observed for the esterase catalysed hydrolysis of the model substrate, the main limiting factor in this study being the effect of pH, (Woodley and Lilly 1990). In this study overcoming the limitation of pH led to the attainment of relatively higher concentrations of product in the reactor. It was identified by relatively crude studies, (figure 3.12), that at these levels of product in the reactor, inhibition was evidenced. The concentrations of product in the aqueous phase are far in excess of those for the substrate. The concentration dependent effect relative to the amount of enzyme in the

reactor is evidenced in the results of figure 3.12. The comparative greater decrease in activity for lower aqueous phase enzyme concentration as evident in figure 3.12 demonstrates the existence of competitive inhibition. The structure of the product molecule is very similar to that of the substrate molecule.

Further purely aqueous phase studies might elucidate exact inhibitive mechanisms and these might be carried out in Lewis cell as a representation of the two-phase system, (Woodley and Lilly 1990). The difficulty exists in obtaining truly representative samples of aqueous phase product concentrations in the STR. Separation necessitates a time lapse at which product achieves partition as dictated by its coefficient. Samples may not be representative of the concentration to which the enzyme is exposed at time of sampling as dictated by the reaction rate and product mass transfer coefficient.

In order to overcome the build up of inhibitory concentrations of product more favourable partitioning is required to limit the concentration in the aqueous phase to which the enzyme is exposed. This will necessitate the use of a second solvent, into which the substrate is dissolved, which more favourably removes product from the aqueous phase and facilitates attainment of greater product concentration in the reactor. A number of issues will need to be addressed concerning a solvent suitable for this task based on the partitioning characteristics and the implications of said solvent upon reactor operation and performance.

Similar crude investigation of potential product inhibition on the lipase revealed a less pronounced effect of product concentration, figure 3.18. Since potentially the active site of the lipase is exposed at the interface a large concentration of substrate relative to the concentration of product is maintained thus competition for the active site is limited.

Initially the substrate at the interface is present in high concentrations, figure 4.10a as reaction progresses product generated in the reaction will partition between the phases and the concentration at the interface will increase, figure 4.10b. As the amount of product increases, potentially the product can form the second liquid phase in which the substrate is dissolved and the interface will predominantly be a product/aqueous

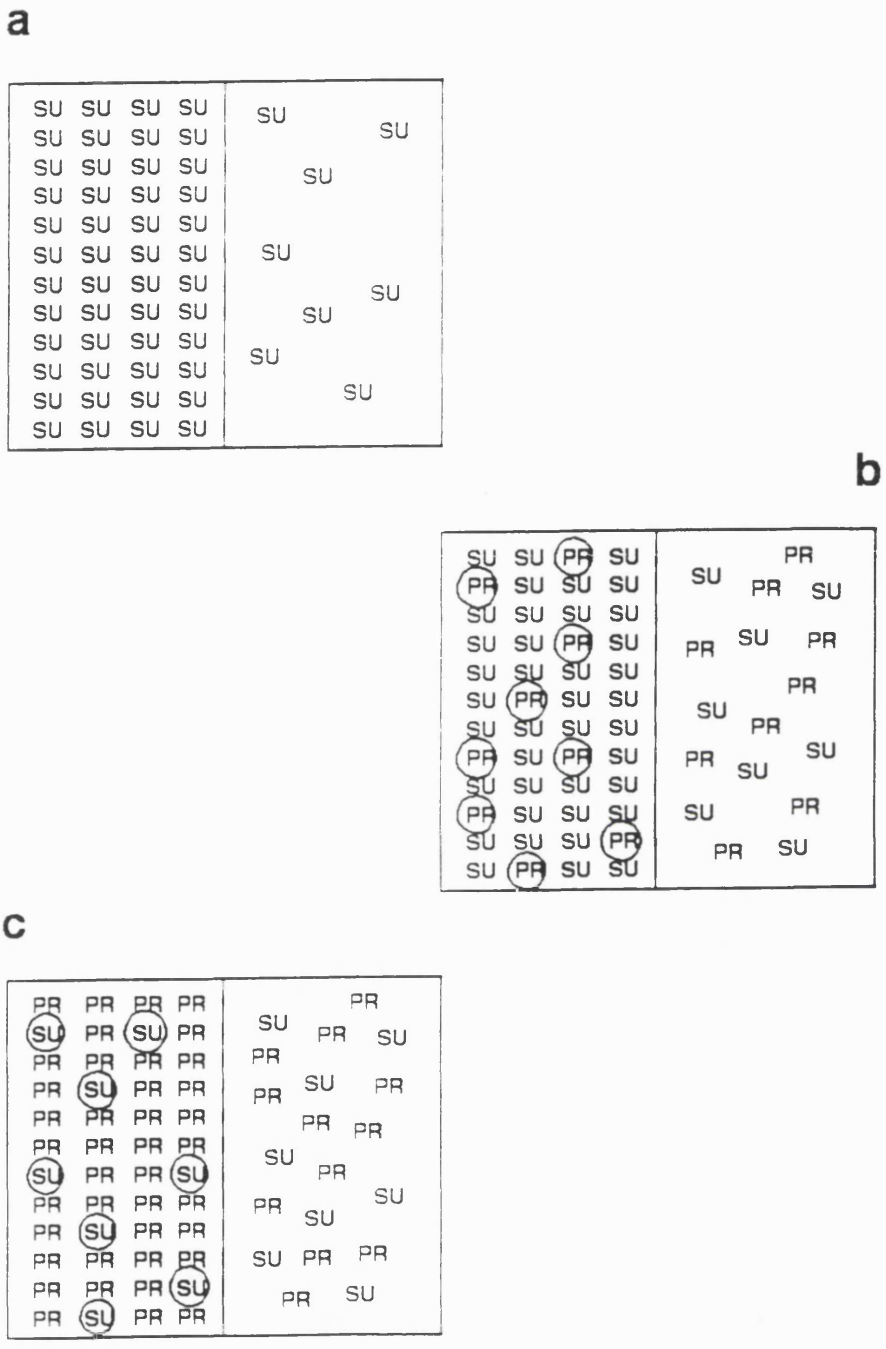

Figure 4.10 Changes in phase composition for a two liquid phase bioreaction as reaction progresses a-b-c: substrate molecules (**SU**), product molecules (**PR**).

interface, figure 4.10c. The point at which this occurs will depend on the % substrate converted, saturation concentration of product in the aqueous phase and its partitioning properties.

The results presented in section 3.2.5 indicated a reduction in the activity at high conversions. It could be argued that product inhibition is occurring to a degree since now the relative concentration of product to substrate at the interface is higher. It is interesting to note that the ratio of product concentration to enzyme at which activity ceases is the same at concentrations for the enzyme of 2, 4 and 8 g l^{-1} as indicated in figure 3.19, this may however be a result of other instability factors. Reduced activity observed at an enzyme concentration of 16 g l^{-1} at time 12 hour, relative to enzyme concentrations of 2, 4 and 8 g l^{-1} , may reflect the changing nature of the interface, and thus limitation of substrate concentration at the interface. In figure 3.18 the differences of activity between the reaction in which product was initially present at a concentration of 1M and that in which no initial concentration was present gets progressively greater as reaction progresses. This may similarly reflect the changing nature of the interface. For the process as a whole this implies increased operating times in order to achieve conversion of the final amounts of substrate remaining.

4.1.8. Optimisation

Operating parameters to achieve optimal operation of the reactor with respect to the effects of phase ratio and agitation rate as they affect interfacial area and K_LA were identified. For the esterase catalysed reaction these can be depicted in terms of an operating window which supports optimal enzyme activity, influenced by the effects of phase ratio and agitation on K_LA, figure 4.11a. The degree of instability to which the enzyme is subject will also strongly influence the choice of operating parameters for maximum productivity of the reactor. Again this can be represented in terms of an operating window showing the limits to stability of phase ratio and agitation rate, figure 4.11b. In operating the STR reactor for efficient productivity a compromise must be realised between the two. The superimposition of the two plots reveals the optimal

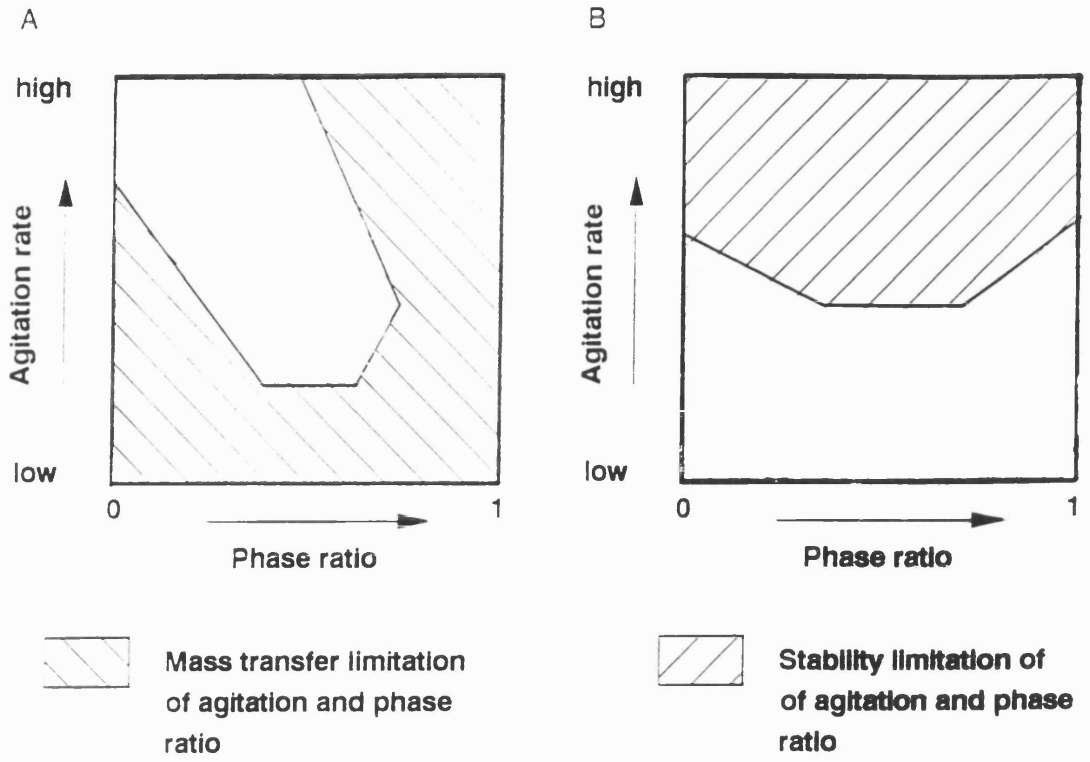

Figure 4.11 Operating windows for phase ratio and agitation rate for catalysis with the esterase. a: demonstrates the limits for mass transfer and b: the limits for catalyst stability.

window of operation to make best use of enzyme activity and stability, figure 4.12. For the lipase catalysed reaction compromise on the interfacial area is unnecessary since no interfacial effects on stability were seen.

4.1.9. Downstream Considerations

For both catalysts, problems in the separation of the phases, a result of the semi stabilisation of the dispersion after the reaction is complete, can be recognised. Upon completion of the reaction and a settling period. It was seen that although the two phases separated to a degree a distinct area of stable emulsion existed. The volume in the reactor stabilised as an emulsion might be expected to be a function of the concentration of the enzyme employed in the reactor. This volume of the reactor could also be seen to consist of coagulated enzyme as a result of extreme denaturation as the reaction progressed. Further characterisation of the problems which might be associated with product recovery, catalyst recovery and recovery of unused substrate is necessary for reactions operated in this manner. Furthermore the operation of reactions in this manner makes the recovery and subsequent reuse of enzyme impractical due to the severity of its destabilisation.

Immobilisation of the catalyst on inert supports may help to overcome some of the inadequacies associated with the downstream processing of the reactor contents and the catalyst stability and reuse. In this study a MBR was investigated as a suitable support on which to carry out the immobilisation of the lipase to facilitate the transformation of the model substrate.

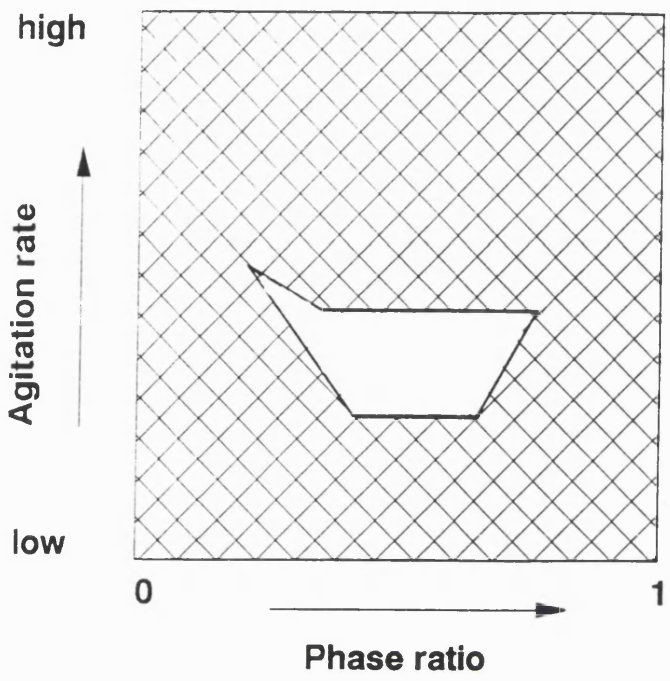

Figure 4.12 Optimal operating window for phase ratio and agitation rate as a result of the combined effects of phase ratio and agitation rate on mass transfer and catalyst stability for esterase catalysed hydrolysis of the model substrate.

4.2 Membrane Bioreactor Operation

4.2.1. Enzyme Activity in the MBR

The criteria for maximised productivity in the MBR can be similarly recognised as for a reaction in the STR, efficient support of optimal activity of amounts of enzyme resulting in high conversion in limited time and enhanced stability of the catalyst, facilitating maintenance of activity throughout the operation and catalyst reuse.

In this study the efficiency of activity for the MBR is compared with that for the enzyme in the STR. The results of figures 3.22 and 3.30 indicated that activity was lacking in the MBR as compared with the activity in the STR at comparatively low and high enzyme loads. A number of other workers have similarly observed decreased effectiveness. In studying a 2 haloalkanoate dehalogenase in a hollow fibre reactor decreased activity of the immobilised enzyme relative to its free state was observed (Diaz *et al* 1989). A decreased activity for a membrane immobilised urease in comparison to its operation in a free state was found, (Furusaki *et al* 1990), and in an earlier study Furusaki and Asai (1983) similarly found the same for an amyloglucosidase. In a chymotrypsin catalysed resolution of a racemic mixture of N-benzoyl tyrosine ethyl ester employing the identical membrane system as used in this study Matson and Lopez (1989) encountered 30 % effectiveness of the immobilised. A low ratio of activity expressed to activity loaded was detected for a lipase catalysed hydrolytic two-phase resolution of Ibuprofen trifluoroethyl ester, (McConville *et al* 1990)

In this study effectiveness factors calculated on the basis of the activity expressed in the MBR as compared with the free state enzyme in the STR were at low enzyme load 3 % and at the higher enzyme load 1.9 %. There is a decreased effectiveness of the enzyme with increasing load. Diaz and co-workers (Diaz *et al* 1989) found decreasing the protein load on the membrane gave a resultant increase in the catalytic efficiency.

In previous studies in the STR it was recognised that the limiting factor to the activity of the catalyst was the availability of substrate to the catalyst, for a bulk aqueous phase catalyst as a result of mass transfer limitation, for the interfacial catalyst availability of sufficient interface. Such phenomenon might be recognised as influential in the limitation of activity in the MBR also. The adsorption of the enzyme onto the membrane and its relationship with the organic aqueous interface of the membrane will dictate the effectiveness of the immobilised enzyme.

A number of workers have suggested diffusional limitation (Furusaki *et at* 1977, Matson and Lopez 1989, Diaz *et al* 1989 and McConville *et al* 1990). The membrane in this study is hydrophilic asymmetrically porous. The membrane will be water wet and thus the pores likely to be occupied by the aqueous phase with organic interface at the pore entrance and the surface of the membrane. The catalyst immobilised in the pores is surrounded by the wetting phase, figure 4.13. transfer of substrate at the interface must occur to make enough substrate available to support activity of the catalyst, as was recognised for the bulk aqueous phase catalyst in the STR. Increasing concentrations of enzyme will require increased support of mass transfer.

Diffusion in asymmetric pores has been described as viscous flow in the wider pore entrance and knudsen diffusion in the internal narrower parts of the pore (Van den Berg and Smolders 1990). A concentration gradient within the pore might thus be expected to exist as a result where aqueous phase concentrations are relatively greater at the pore entrance and decline with increasing depth into the pore, figure 4.13. Support of greater activity for enzyme associated at the pore entrance might thus be expected.

In a study of mass transfer in this MBR, (Cunnah and Woodley 1992), K_LA was shown to be a function of flow rate and volume of the aqueous phase of the reactor, independent of organic phase volume and flow rate. Maximum K_LA achievable, limited by highest achievable flow rate and lowest utilisable volume was shown to be 30 min^{-1}, this compares unfavourably with potential obtainable values in a STR as indicated by Doraiswamy and Sharma 1984 who report values ranging from 0.18 to 480 min^{-1}, but compares favourably with the values for the STR operated for esterase catalysed hydrolysis. However in this study the operating volumes and flow rates

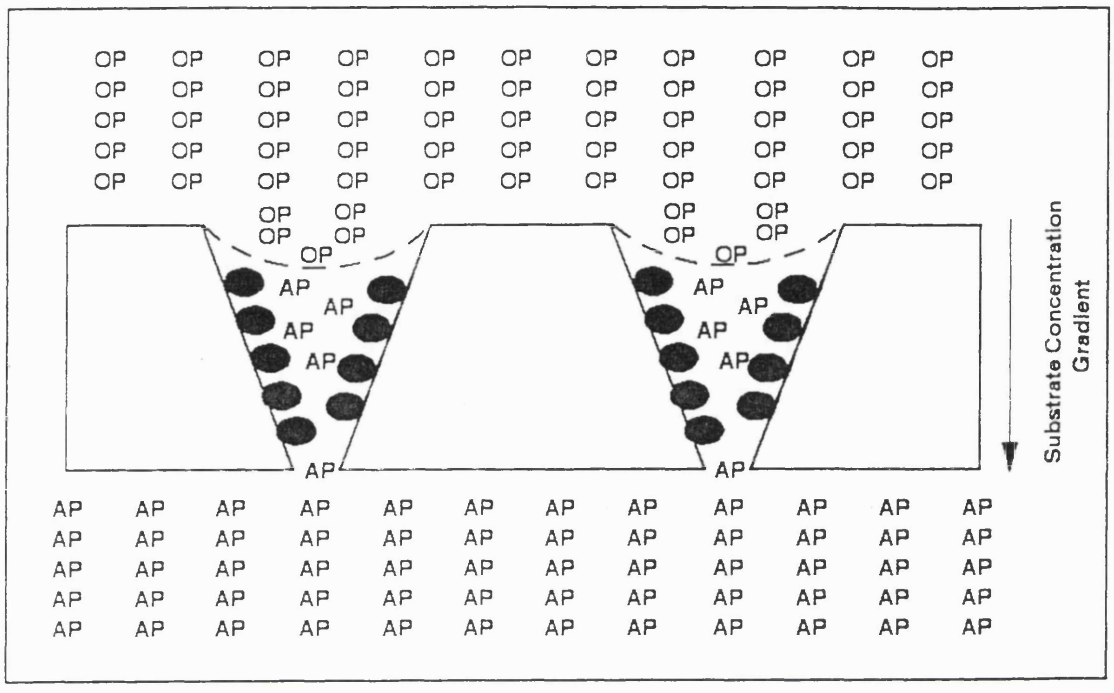

Figure 4.13 Membrane pore schematic demonstrating organic/aqueous interface at the pore entrance and hydrophilic membrane surface, enzyme immobilised on the surface within the pore surrounded by aqueous phase: Enzyme (●), organic phase (**OP**), aqueous phase (**AP**), organic/aqueous interface (------).

dictated a K_LA of less than 1.5 min^{-1}, less than was determined for the optimal operation of the STR using the bulk aqueous phase model. Further study in respect of the effect of flow rate and phase volumes is required in the presence of catalyst to provide further insight into transfer effects. The relative ratio of membrane area to aqueous phase volume will also be important, different membranes having different transfer characteristics.

The previous idea assumes that all lipase absorbed to the membrane is potentially active. In the STR the interface necessary for the lipase active conformation was provided by the organic/aqueous interface. In the MBR the interface is more likely to be solid/liquid interface. It is suggested, (Malcata *et al* 1992), that a solid liquid interface is sufficient to result in the active conformation. However it was also recognised that immobilisation to a support having hydrophobic characteristics resulted in relatively greater activity than the immobilisation to a hydrophilic support. A hydrophobic support results in an ordered packing of the enzyme molecules, the necessary orientation of all molecules resulting in the opening of the catalytic site to allow access of substrates. In an hydrophilic environment molecules will orientate themselves randomly. In this study even though enzyme is absorbed to an interface it may not necessarily all be in the active conformation. The absorption of lipase to a support is a mild treatment and it is believed that this does not result in a conformational change, (Pronk *et al* 1992). Initial contact of the protein molecule with the membrane surface of a hydrophobic support is believed to result in slight unfolding which facilitates multipoint attachment to the support, (Malcata *et al* 1992b). It is not elucidated whether this results in the necessary active conformation.

The orientation of the lipase is important to its activity. Water molecules used as substrate species are more likely to reach the active site by diffusion from the bulk of the non-aqueous phase rather than by direct contact with the organic phase, (Garcia *et al* 1992). This suggests that the active molecule is orientated with its active site at the interface. The possibility of limitations related to the transfer of the necessary water for the hydrolytic reaction can be envisioned and may require further study. The presence of lipase in the hydrophilic macrenvironment, even though the enzyme is absorbed to a

solid/liquid interface, does not facilitate the presence of the enzyme at the organic/aqueous interface, figure 4.13.

The method employed for the immobilisation of the enzyme into the membrane was ultrafiltration, in the process enzyme being adsorbed onto the support matrix. As well as adsorption within the pores enzyme may adsorb to the outer surface where there is a direct organic aqueous interface, figure 4.14, resultingly only a fraction of the enzyme will be active, evident in reduced activity, as was observed in this study. It is interesting here to note a study of Pronk and Van,t Riet (1991) who, in contrast to this study, found that activity measurements were identical in a MBR and a STR operated at enzyme loads below interfacial saturation. It appears enzyme was immobilised at the surface of the hollow fibre (Pronk *et al* 1988). In such a situation enzyme has direct access to the interface as previously illustrated, figure 4.14, and resultingly all enzyme displayed activity.

Similarly enzyme adsorbed at the mouth of the pore is in contact with the necessary interface. The degree to which the organic phase enters the pore is influential in determining the amount of lipase potentially exposed to the interface. Assuming monolayer adsorption to the support within the pore, figure 4.15, illustrates the gradation of potential activity as influence by the degree of entry of the organic, non wetting phase. Figure 4.15a depicts only slight entry of the organic phase into the pore and here the activity in relation to the potential activity will be comparatively low. In figure 4.15b, and 4.15c progressive further entry of the organic phase into the pore results in the necessary interface being made available to a greater amount of the enzyme within the pore and resultingly an increase in the amount of active enzyme, reflected in an increase in the activity. The degree to which the organic phase enters into the pore may be influenced by a number of factors, properties of the membrane material, influences of the physical properties of the phases, interfacial tensions at the pore, pore size, flow rates and applied pressure. Further study is needed to fully elucidate these potential effects.

It is however arguable whether the enzyme immobilised onto the hydrophilic support will be in the necessary orientation to take full advantage of the interface as it

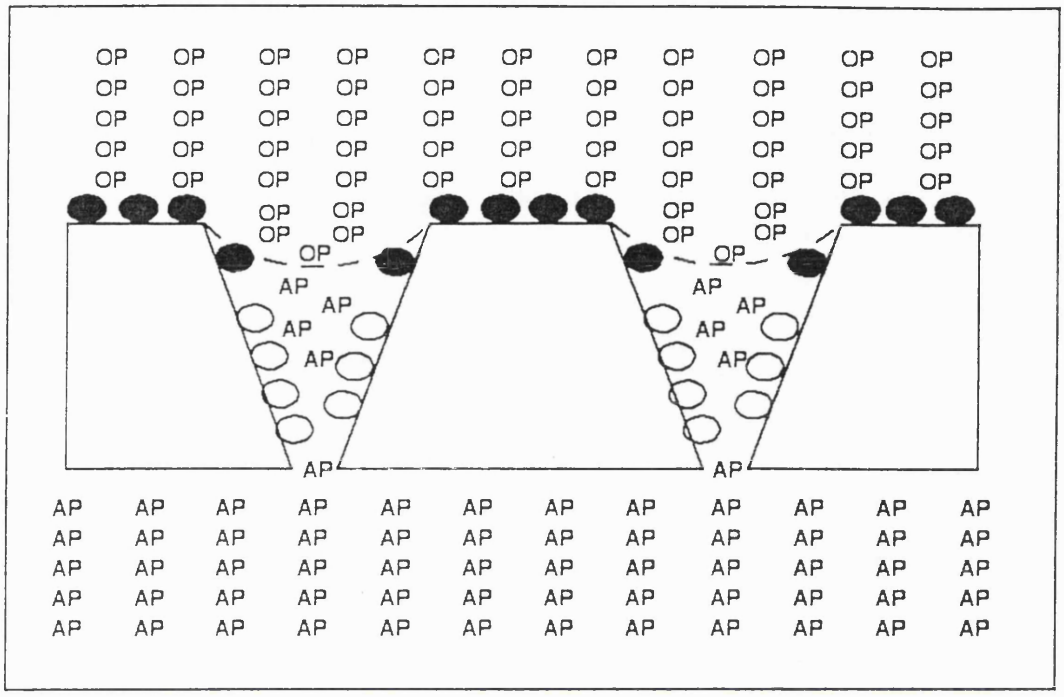

Figure 4.14 Membrane pore schematic demonstrating enzyme activity at the organic aqueous interface at the surface of the membrane and within the pore entrance: Active enzyme (●), non active enzyme (O), organic phase (**OP**), aqueous phase (**AP**), organic/aqueous interface (------).

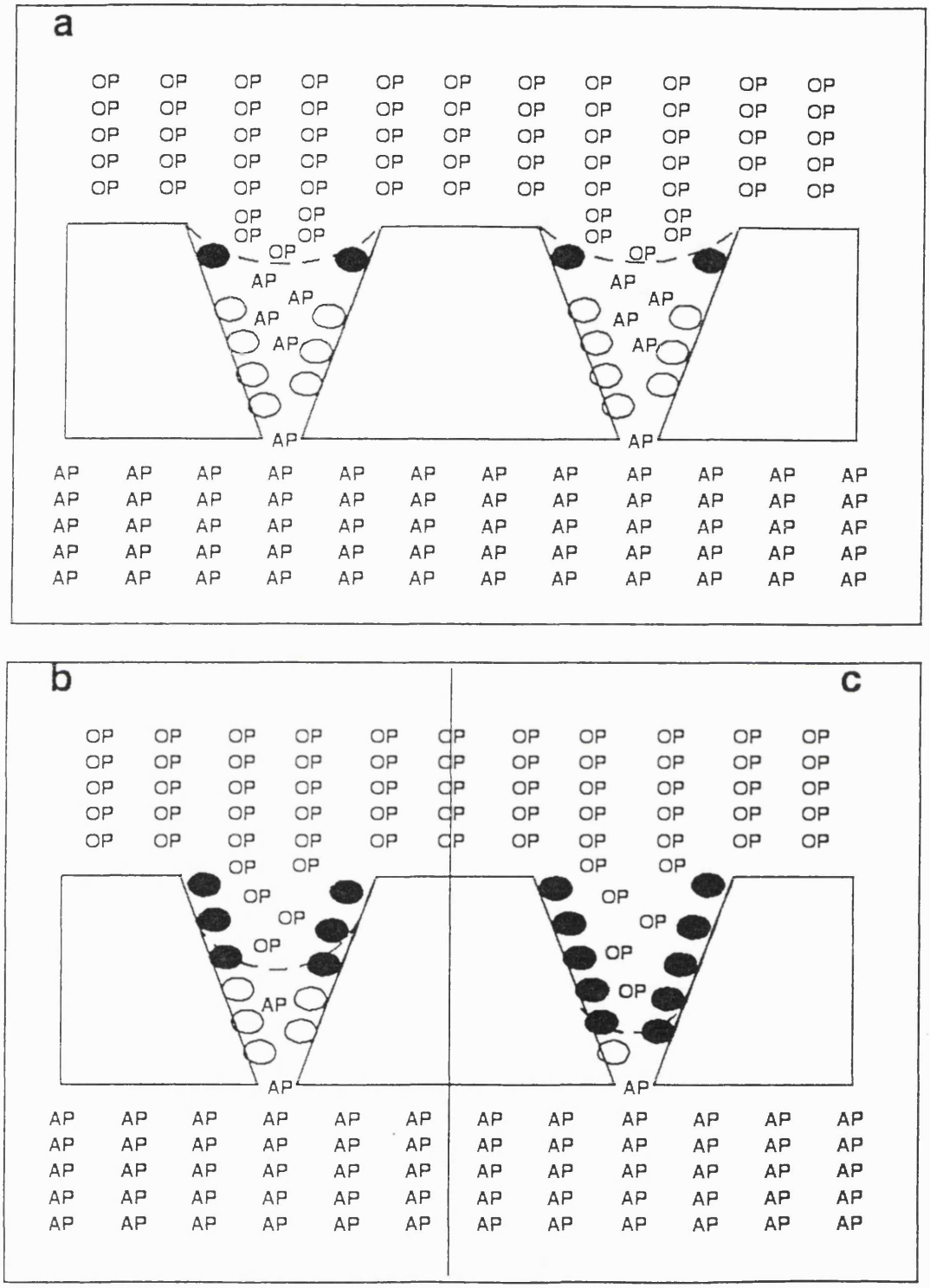

Figure 4.15 Membrane pore schematic demonstrating varying degrees of pore filling, a<b<c, influencing the interface within the pore and the proportion of active enzyme: Active enzyme (●), non active enzyme (O), organic phase (**OP**), aqueous phase (**AP**), organic/aqueous interface (------).

might exist. If the enzyme is randomly adsorbed, as previously discussed, its reorientation will be required. The multipoint attachment to the support by adsorption, although a comparatively weak interaction, may be strong enough to prevent the reorientation.

It is not clear whether the enzyme is adsorbed as a monolayer onto the surface of the support. Stable interactions between like molecules might occur. Adsorption of lipase molecules to lipase molecules, resulting in multilayer adsorbtion, has previously been identified in STR studies, (Ekiz *et al* 1988). Multilayer adsorption to the membrane surface and enzyme packing within the pores may occur. Multilayer adsorption, potentially, will lead to decreased amount of enzyme exposed at the interface, relative to monolayer adsorption assuming interfacial availability, figure 4.16.

Limiting scenarios might be recognised for enzyme packing. Figure 4.17a illustrates enzyme packing within the narrower areas of the pore, access to interface here might be completely inhibited. As the packing of the pores becomes denser then enzyme interfacial contact might be facilitated, figure 4.17b, the activity however will be limited. Pore filling can be seen to influence the degree to which enzyme packed has access to interface. Degree of enzyme adsorption and packing will be a function of the concentration of enzyme applied to the reactor.

Other proteins in crude preparations could influence the degree of adsorption and packing. It has been recognised however that adsorption is a purification process in a MBR, (Guit *et al* 1991). The degree to which other non active proteins are retained will be a function of their size relative to the membrane cut-off value, and the affinity of the protein for the immobilisation surface. Lipases have been indicated to have a greater affinity for an immobilisation surface than other proteins which are associated in crude preparations. Lipases displaced other more weakly associated proteins when impure preparations were applied to hollow fibres of a MBR, (Malcata *et al* 1992b). Enzyme adsorption at the impermeable skin layer will effectively result in the retention of other proteins, which might otherwise pass through, and thus they might be implicated in the degree of packing. A further understanding of these phenomena might

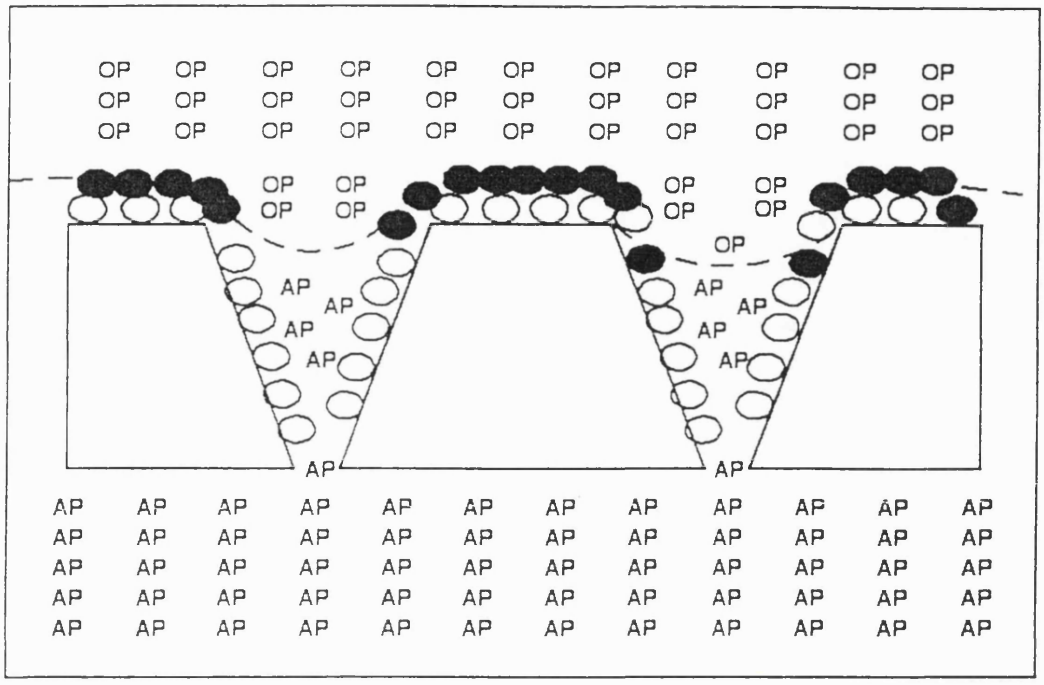

Figure 4.16 Membrane pore schematic demonstrating multilayer adsorption of enzyme at the interface influencing the plane of interface and proportion of active enzyme: Active enzyme (●), non active enzyme (O), organic phase (**OP**), aqueous phase (**AP**), organic/aqueous interface (------).

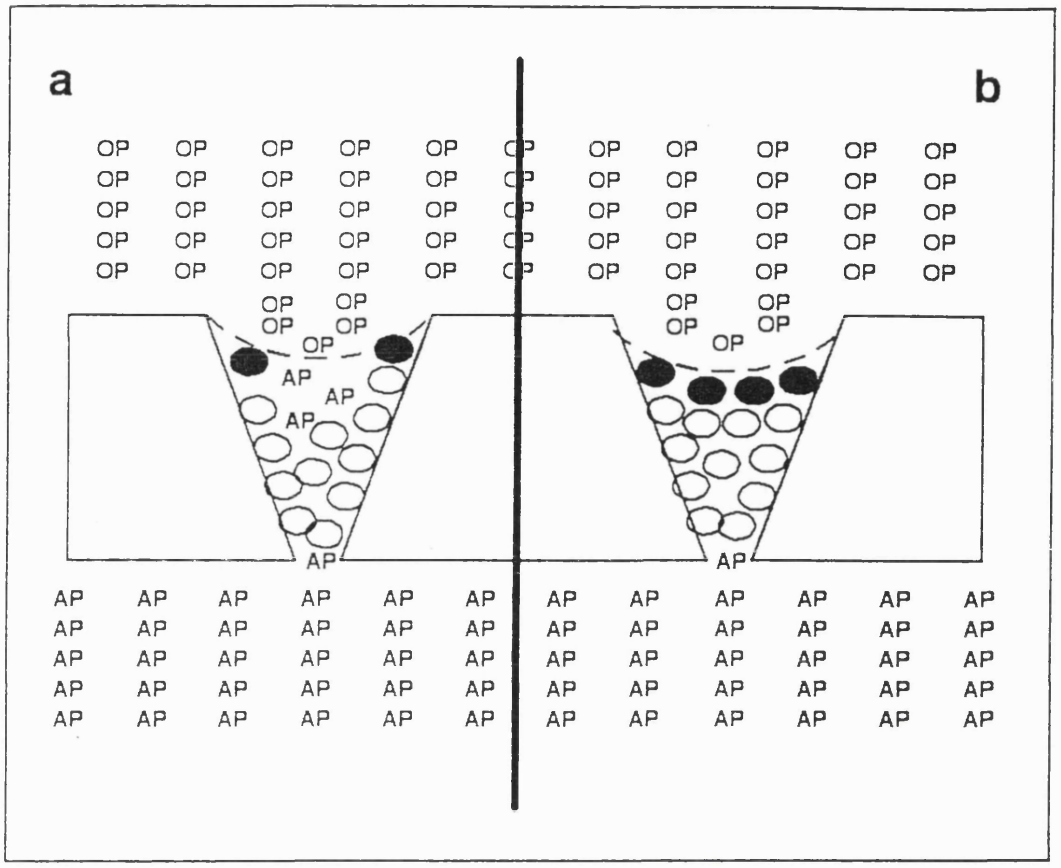

Figure 4.17 Membrane pore schematic demonstrating the degee of enzyme "packing" within the pore, a<b, and the influence of this to access at the interface and the proportion of active: Active enzyme (●), non active enzyme (O), organic phase (**OP**), aqueous phase (**AP**), organic/aqueous interface (------).

be obtained by microscopic examination of membrane bound enzyme and structural studies.

It is interesting to note as well as the limitations to enzyme activity the diffusional limitations associated with membrane bioreactors can result in decreased stereospecificity for enzymatic resolution, (Matson and Lopez 1989).

In this study the enzyme is immobilised to a hydrophilic support. This might be a suitable support for catalysts which exhibit activity in the bulk of the aqueous phase in which case mass transfer as previously discussed will be a limiting factor. For the interfacial catalyst, such as the lipase, it might be more appropriate to use a hydrophobic support. Hydrophobic supports have been applied to lipase catalysed reactions, (Hoq *et al* 1985, Koizumi *et al* 1987 and Malcata *et al* 1992). Activity limitations have been similarly evidenced, the limitations may not be so severe as was evidenced in this study. Hydrophobic membranes will be organic wet, the enzyme may now potentially be in an organic macroenvironment again interfacial availability of the organic/aqueous interface may be limited as was proposed for this study. A small amount of water will also be necessary to maintain the conformational stability of the enzyme, (Klibanov 1986). Pore structures of hydrophobic membranes have been suggested to be more open than hydrophilic membranes, (Prasad *et al* 1990). This may facilitate more direct entry of aqueous phase into the pore thus providing necessary interface. In one study an interfacially active enzyme, HLAD, was immobilised to hydrophobic microfiltration membranes of various pore sizes to transform octanol to octanal. Increasing amounts of enzyme adsorbed and increased activity for the enzyme adsorbed were observed with increasing pore size, (Hubble *et al* 1991). The potential breakthrough pressure of more open pores will however be smaller, this may lead to difficulties in maintaining separation.

A potential novel approach might be to illicit the functioning of hydrophilic membranes as hydrophobic. Pore wetting by the organic phase facilitating the interface completely within the pore and so retaining some of the advantages of the hydrophilic membrane, maintenance of a hydrophilic environment for the enzyme and potentially greater stability. It may not however overcome potential orientation problems. Prasad

and co-workers, (Prasad *et al* 1990), have illicited such functioning of hydrophilic membranes in the abscence of biocatalyst.

4.2.2. Enzyme Stability in the MBR

Although a substantial reduction in the effectiveness of the immobilised enzyme was observed it is evidenced in this study that the stability of the enzyme is much improved when immobilised in the MBR in comparison to the use of enzyme in native state in the STR. Half lives for activity of lipase in the STR were determined to be 30 minutes in comparison to a half life of 18 hours in the MBR, figure 4.18.

Increased stability is a general characteristic of immobilised catalysts. The degree to which the catalyst is stabilised varies from system to system. A number of workers have identified varying half lives for lipase catalysed reactions in MBRs, Table 4.1. The degree of stabilisation might be a function of support material and interactions, inhibition by product, toxicity of substrate or second solvent. It is interesting to note that the half lives indicated in table 4.1 are in excess of those in this study. Many of the substrates are oils, the natural substrate of lipase in it's native environment, or dissolve the oil in toluene, a solvent with a higher Log P than that employed in this study. The substrate in this study is potentially more toxic and hence lower comparative half life. In a study of a dehalogenase a half life of 4 days in a MBR compared to 2 days for the enzyme in it's native state was found, a 2 fold improvement of stability, (Diaz *et al* 1989). For this study a substantially greater improvement of stability of 36 fold was observed.

The improved stability results from the enhanced rigidity of the protein as a result of its multipoint attachment to the support and resultingly less susceptibility to conformational change, (Martinek *et al* 1977). As well as the improved stability in general, thermal protection may be afforded for operation of the reactor at increased activity as a result of increased temperature. However thermal stability is only inferred upon the enzyme initially, long term stability at higher temperatures is reduced,

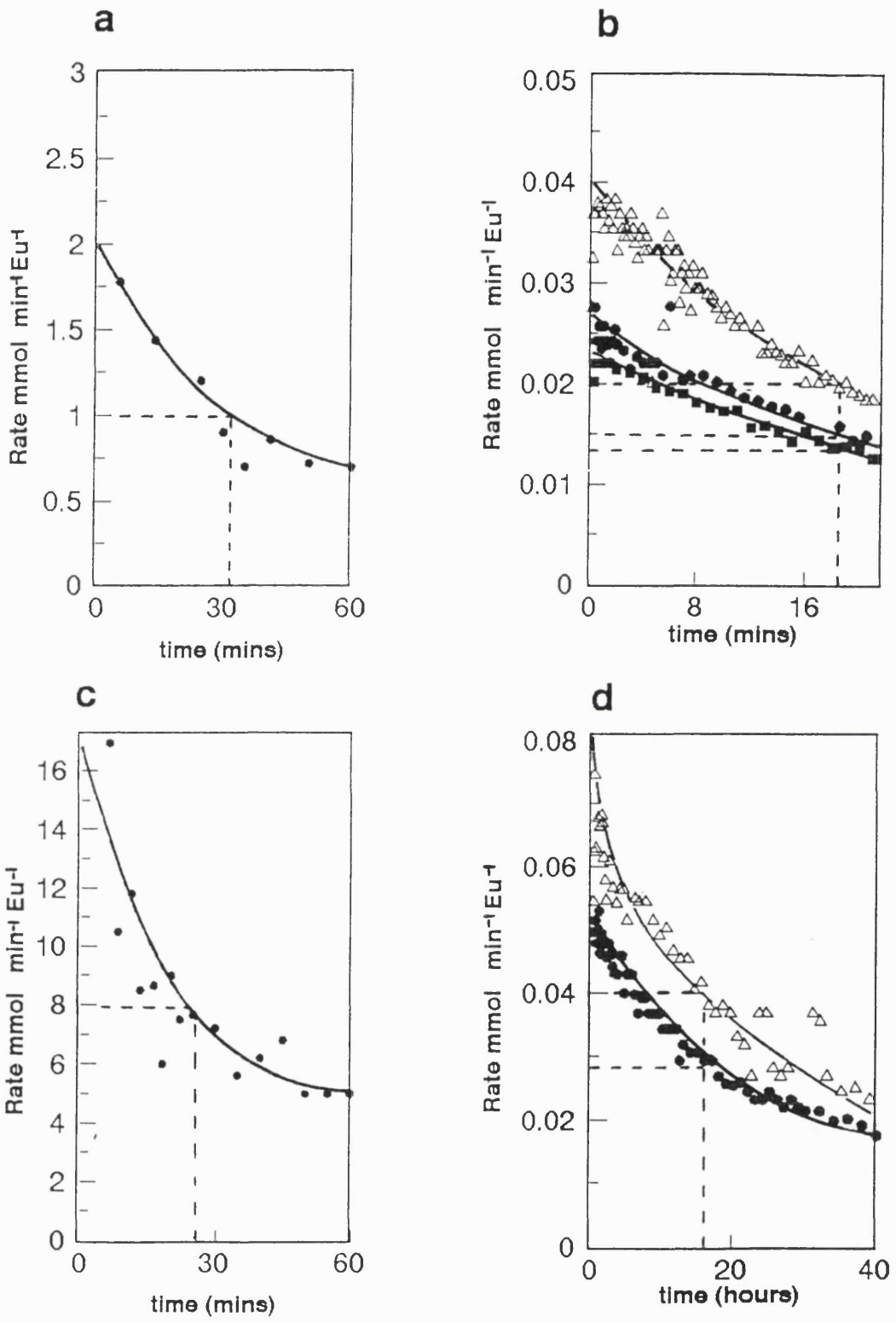

Figure 4.18 Half lives. (------), comparisons for the catalyst, lipase, at low enzyme load in the STR (a), ∅ = 0.4, n =1000 rpm; and in the MBR (b), aqueous and organic phase volumes = 400 ml, flow rates = 500 ml min^{-1}; at high enzyme load in the STR (c), ∅ = 0.4, n =1000 rpm; and in the MBR (d), aqueous and organic phase volumes = 400 ml, flow rates = 500 ml min^{-1}

	Membrane	Substrate	Half-life (days)
Hoq et al (1985)	polypropylene (hydrophobic)	Fats	2-15
Taylor et al (1986)	acrylic (hydrophilic)	Fats	30-60
Pronk et al (1988)	cellulose (hydrophilic)	Fats	43
Guit et al (1991)	cellulose (hydrophilic)	Fat in Toluene	6
Guit et al (1991)	poly-acrylono nitrile (hydrophilic)	Fat in Toluene	6
This study	poly-acrylo nitrile (hydrophilic)	Neat Benzyl Acetate	0.75

Table 4.1 Comparison of the half lives for lipase catalysed hydrolysis, in varying membrane bioreactors by a number of workers, (from Guit *et al* 1991), with the half life for the lipase in this study.

(Malcata *et al* 1992c and Pronk *et al* 1988). The thermal effects upon this system and its implications upon productivity of the reactor is an area for further characterisation.

4.2.3. Enzyme Reuse in the MBR

In semi-continuous operation of the MBR in which enzyme was reused for subsequent batch cycles, product removal, by removal of organic and aqueous phase and replacement with fresh, resulted in restoration of a degree of activity. The reusable stability of the enzyme in this study was not as great as observed for the reusable stability in a study in which a membrane reactor was operated semicontinuously for 48 hour periods up to 18 days, 9 consecutive cycles, (Tanigaki *et al* 1993). The membrane was not used as an immobilisation support but to retain the enzyme. Very little loss of activity was observed over consecutive cycles. In contrast in this study a degree of potential activity was lost with consecutively operated cycles. At low enzyme load a 14 % loss of activity between the first and second cycle with a further 8 % loss by the third cycle. At high enzyme load 31 % activity was lost between the first and second cycle followed by a further 24 % for the third cycle, consecutive further losses were observed for subsequent cycles. The percentage loss in activity was mirrored throughout the course of time over which the consecutive cycles were operating, half lives at both high and low enzyme loads and in consecutive cycles were approximately 18 hours +/- 1 hour, (see section 3.3.1.4 and 3.3.2.5). It might be suggested that the stability difference reflects the different substrates and a more deleterious effect of the substrate of this study. The substrate in the study of Tanigaki and co-workers was once again an oil, the more natural substrate of the enzyme, it also being a less polar substrate than that of this study.

Alternatively it might be supposed, even though organic and aqueous phases are removed from the reactor, an amount still remains in the vicinity of the enzyme. Enzyme will be exposed initially to product concentrations representative of those to which it was exposed at the end of the previous cycle. The results of MBR study at low enzyme load indicate that the concentration of product is influential in determining rate.

Consecutive reactions carried out for low enzyme load in the MBR were initiated with a concentration of product still present in the reactor, the rate was comparable to the rate achieved at that product concentration in the previous cycle, (figure 3.27). The amount of activity is small compared with activity evidenced by native state enzyme. It has been previously supposed that this might be due to limited exposure to interface and thus a limited amount of catalyst having the necessary active conformation. The relative ratio of concentration to active enzyme is greater in the MBR. In previous study with the STR this ratio was a factor in determining level of inhibition. The initial concentration in the vicinity of the enzyme however will be expected to be diluted out.

Although phase volumes, flow rates and pressures were maintained in consecutive runs, the character of the interface within the pore might alter changing the transfer/interfacial characteristics. Changes of these will be expected to affect activity.

Further supposition is enzyme "leakage" form the membrane by desorption. The interactions are said to be fairly stable and desorption is negligible as evidenced by long term hydrolysis experiments of a number of workers, (Malcata *et al* 1992 and Pronk *et al* 1988). Indeed the difficulty of enzyme desorption is apparent in the treatment necessary to remove spent enzyme for subsequent renewal.

For the commercial application it will be necessary to know the number of productive runs which can be achieved before enzyme renewal is required. Eventually enzyme activity will be reduced to an inefficient level. It is evident, figure 3.39, that decreased activity of consecutive cycles results in increased operating time to facilitate the same degree of conversion. It is apparent from this study that further data is required in order to identify the key causes of reduced activity in consecutive cycles and ascertain the predictability of catalyst reuse.

4.2.4. Comparative Conversion

It was suggested that the concentration of enzyme at high load was reflective of the concentration of 16 g l^{-1} applied using the native state enzyme. Figure 4.19 depicts the comparison of substrate conversion as a function of time for the STR, at this

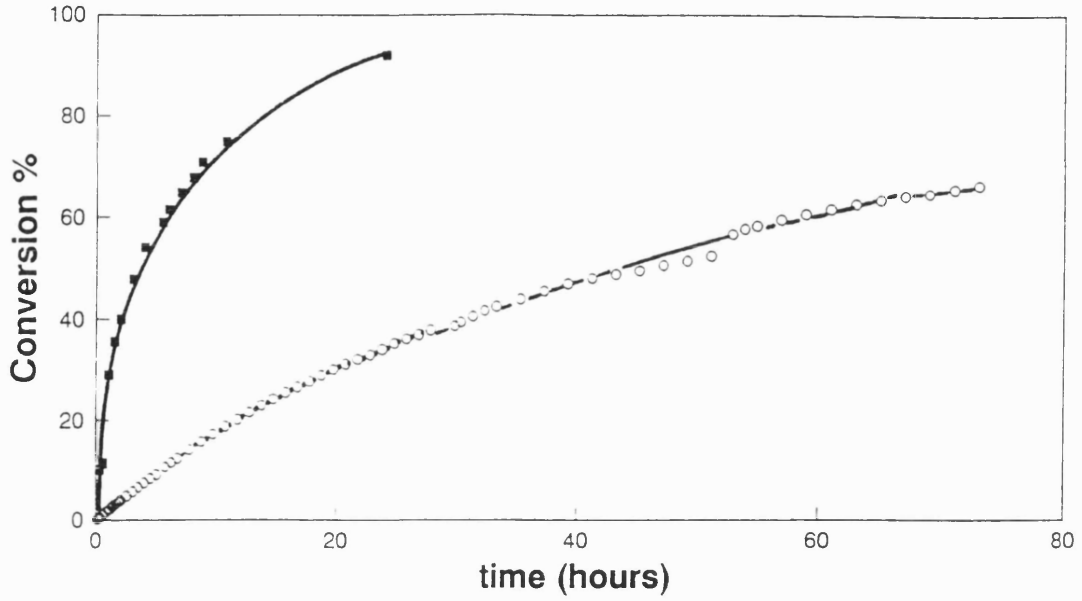

Figure 4.19 Conversion as a function of time for the STR (■) and the MBR (O) operated at an aqueous phase enzyme concentration of 16 g l^{-1}

concentration of enzyme, figure 3.19, and the MBR, cycle 1 of figure 3.39. The limitations of the MBR result in increased operating time over the STR in order to achieve the same degree of conversion and a reduction the effective degree of conversion. It is interesting to note here a comment made by van Eikeren and co-workers (van Eikeren *et al* 1992) suggesting that a MBR for the lipase catalysed hydrolytic resolution of racemic ester was more suitable than a STR since the risk of reaction going beyond 80 % conversion, over which the optical purity of the product alcohol was compromised, was avoided. Similarly in the PPL catalysed resolution of racemic glycidyl butyrate optically pure ester was collected at 67 % conversion, (Lopez *et al* 1991).

4.2.5. Downstream Considerations

Associated downstream separation of the phases in the MBR provides obvious process advantages. The separation of the two process streams eliminates the disadvantage of troublesome emulsions associated with the operation of the STR, (Stanley and Quinn 1987) Partitioning of the product into the organic phase results in a concentration of product within that phase However a relatively large fraction will still remain in the aqueous phase (see product concentration/time profiles). The MBR can be operated in a continual process in contrast to the STR in which batch reaction is carried out. Since both phases are already separated the on-line extraction of product from both phases can be facilitated thus providing a means of limiting potentially inhibitive concentration and facilitating the recycle of recovered raw materials. Bratzler, (1987), and Quinn and co-workers, (Quinn *et al* 1989), similarly suggested the usefulness of a MBR facilitating *in situ* extraction, separation and enrichment.

4.3. Implications

The potential use of both esterases and lipases for the conversion of poorly-soluble compounds is clear. For their industrial application the criteria for maximised productivity must be met in the reactor. In STR reactor operation, the creation of adequate aqueous-organic interface is required, for the support of maximal activity from maximal amounts of enzyme, for the esterase as it influences K_LA and for the lipase as a reaction site. The optimal operating conditions in a STR for the esterase were modeled. A similar approach will enable the modelling and prediction of reactor productivity for other catalysts which are active in the bulk of the aqueous phase. The model identified conditions of optimal K_LA and in so doing identifies conditions of maximum interfacial area and this, therefore, has implications in the recognition of optimal STR operating conditions for catalysts active at an interface.

For the system in this study lipase was a more productive catalyst with which to .carry out this reaction in a STR. The reactor operating conditions could support high catalyst concentrations at maximal activity and an almost 100 % conversion of substrate was achieved. In contrast the optimal operating conditions of the reactor could not support high concentrations of the esterase at maximal activity. Although the time require to achieve the equivalent degree of conversion with the lipase, as achieved with the esterase, was greater, this likely reflects specificity and purity difference rather than influences of reactor operating parameters. Esterase was further limited by its stability, as influenced by competitive product inhibition and interfacial effects, thus conversion was low. This may also be a problem for many enzymic reactions carried out in a two-liquid phase system where the catalysis occurs in the bulk of the aqueous phase. Catalysts which carry out reaction at the interface seem more appropiate in terms of stability in a two-liquid phase STR in their free state. Stability problems need to be overcome to enable the operation of the esterase and bulk aqueous phase catalysts at more productive levels.

In the STR high product yields were obtained by using the lipase in its native state, high product concentrations facilitate product recovery, however other associated

downstream problems were identified. The MBR provided a means of overcoming some of these problems and facilitates phase separation, enzyme reuse and continual operation. A drawback to the operation in the MBR was a reduction in the enzyme activity in comparison to its use in free state in the STR. The stability of the catalyst was improved and relatively high conversions are achievable although requiring a longer period of operation.. It might be more appropiate to make comparisons of the MBR with other immobilised enzyme systems in order to identify its true potential for carrying out two-liquid phase biotransformations. A further understanding of the parameters influencing the enzyme activity in the MBR is also required to enable its modelling and optimisation. For industrial application further comparison will be required in terms of the overall influence on process economics, (reactor cost, lifetime and ease of operation) and the scale of operation.

5. Conclusions

For the hydrolysis of benzyl acetate by pig liver esterase in a STR it was found that:

1. Enzyme activity could be prolonged by pH maintenance of the reaction medium

2. Aqueous phase enzyme concentrations which gave below maximal enzyme activity were limited by mass transfer conditions and were used to model the K_LA of the STR.

3. K_LA in the STR was a function of the phase ratio and rate of agitation. Optimum phase ratio supporting maximum K_LA was 0.4. At non-inverted phase ratios increased rates of agitation resulted in increased reactor K_LA. At inverted phase ratios high rates of agitation resulted in decreased reactor K_LA due to poor internal mixing within the droplets.

4. Enzyme stability was affected by liquid-liquid interface and was limited at higher rates of agitation and mid range phase ratios.

5. The enzyme was competitively inhibited by the product and thus resulting conversion of the substrate was below 40%.

For the hydrolysis of benzyl acetate by porcine pancreatic lipase in the STR.

1. The enzyme exhibited very little activity in a solely aqueous medium fully saturated with substrate.

2. In a two liquid phase medium the enzyme exhibited activity in exess of that in the solely aqueous medium due to its activity at the organic-aqueous interface.

3. Optimal phase ratio of 0.4 and an agitation rate of 1000 rpm provided adequate interfacial area to support high aqueous phase crude enzyme concentrations of up to 16 g l^{-1} . At a phase ratio of 0.2 the activity of the enzyme was limited due to inadequate interfacial area.

4. Enzyme stability was not limited by the organic-aqueous interface.

5. Product inhibition was not evident and high conversions of almost 100% of the substrate was achieved at a crude enzyme concentration of 16 g l^{-1} .

For the hydrolysis of benzyl acetate by porcine pancreatic lipase in the MBR.

1. Activity of membrane immobilised enzyme in the MBR, in comparison to activity in the STR, was limited. The activity was more severely limited for higher loadings of enzyme onto the membrane.

2. The enzyme immobilised in the MBR was more stable than the enzyme used in its free state in the STR.

3. The enzyme in the MBR, in contrast to the enzyme in the STR, was used in consecutive reactions with some loss of actvity between consecutive reactions.

4. The MBR took longer to achieve the same degree of conversion as achieved in the STR.

5. The MBR facilitated continual separation of the organic and aqueous phases of the two-liquid phase system.

6. Future Research Areas

6.1. STR Studies

(a) A more thorough understanding of the parameters of STR operation as they relate to the stability of the enzymes is required in order to further model and predict STR performance. Means to improve the stability of free enzymes in biphasic reactions requires study. This might involve structural studies to establish mechanisms of deactivation and molecular techniques in order to engineer more suitable catalysts.

(b) In relation to product inhibition of the esterase it will be appropriate to study suitable solvents in which the substrate can be dissolved and more preferentially partition product away from the aqueous phase reaction environment.

(c) For more stable catalysts issues of phase mixing, as it affects mass transfer and interfacial area and the consequent influence upon enzyme activity, as reaction progresses will need to be addressed.

6.2. MBR Studies

(a). The membrane reactor requires further characterisation with respect to operating parameters, (flow rates, phase volumes, enzyme concentration, separation pressure), for the hydrolysis with lipase in order to fully model the system. A more thorough examination of the fluid dynamics and molecular aspects of the immobilisation will help to further characterise the membrane bioreactor operation Other materials are used to manufacture hollow fiber membranes, these will need further evaluation and may prove more suitable than the material of construction in this study. Hydrophobic materials may offer advantages over hydrophilic materials for catalysts active at an interface such as lipase.

(b). The use of the esterase in the membrane bioreactor requires comparison with the operation in the STR to assess the suitability of this reactor for such a catalyst.

6.3. Immobilised Systems

Since the membrane bioreactor offers advantages in terms of downstream processing it would be appropriate to make comparisons of the reactor performance with other, more conventional, immobilised systems which facilitate catalyst reuse and limit the emulsion stabilising properties of the free enzyme.

6.4. Scale-up

Engineering aspects of reactor scale-up need to be addressed for the commercial application of these reactors.

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Abbreviations and Nomenclature

Abbreviations

STR	Stirred tank reactor
STR1	Stirred tank reactor 1
STR2	Stirred tank reactor 2
MBR	Membrane bioreactor
HPLC	High performance liquid chromatography
B.Pt.	Boiling point
F.Pt	Freezing point
MW	Molecular weight
PLE	Pig liver esterase
PPL	Porcine pancreatic lipase
BAc	Benzyl acetate
BAl	Benzyl alcohol

Nomenclature

Symbol	Definition	Units
[Ea]	Aqueous phase enzyme concentration	g l^{-1}
K_1	First order rate constant	l min^{-1} g^{-1}
K_LA	Substrate mass transfer coefficient	min^{-1}
Log P	Logarithm of partition coefficient of a given solvent in a standard two-liquid phase octanol-water system	
n	Agitation rate	
[Pri]	Initial product concentration	mM
Rs	Specific enzyme activity	mmol min^{-1} g^{-1}
[Sa*]	Aqueous phase substrate saturation concentration	mM

[Sab]	Bulk aqueous phase substrate concentration	mM
∅	Phase ratio: Ratio of the organic and aqueous phase volumes	