BIOTRANSFORMATIONS : STEREOSPECIFIC ESTER HYDROLYSIS USING *Bacillus Subtilis*

A DISSERTATION
SUBMITTED TO THE
UNIVERSITY OF POONA
IN PARTIAL FULFILMENT
FOR THE DEGREE OF
MASTER OF PHILOSOPHY
(IN BIOTECHNOLOGY)

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BY

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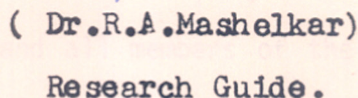
JULY 1990

CERTIFICATE

This is to certify that the work incorporated in the dissertation entitled 'Biotransformations - Stereospecific Ester Hydrolysis using Bacillus subtilis', submitted by Miss Nuzhat Pathan, was carried out by her, under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the dissertation.



(Dr.R.A.Mashelkar)
Research Guide.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my deep sense of gratitude to Dr.R.A. Mashelkar, Director, National Chemical Laboratory, for his understanding, encouraging and inspiring presence throughout the course of this investigation and his permission to submit this work in the form of a dissertation.

I am profoundly grateful to Dr. S. Rajappa, Head of Organic Chemistry Division-I, for his keen interest, constant encouragement and his invaluable help which made this piece of work possible.

I am deeply indebted to Dr. Z. Muljiani of Organic Chemistry Division (I) for her valuable suggestions, generous help and interesting discussions and also to Mr. Nene, Head of Bioengineering section (Chemical Engineering Division) for helpful discussions, and a most understanding and co-operative attitude.

Thanks are due to Ms. S.R. Gadre of Organic Chemistry Division for always lending me a helping hand and to Dr.B.V. Bapat and his group for utmost co-operation with the GC analyses.

I would also like to thank Ms. M. Natekar of Organic Chemistry Division (I) for her soothing presence and all members of the Organic Chemistry Division (I) and the Chemical Engineering Division for their help and co-operation.

Monumental thanks are due to all my friends for their unwavering support, encouragement and help throughout.

Finally, I would like to thank the Council of Scientific and Industrial Research for their financial assistance.

NPathan

(Nuzhat Pathan)

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CHAPTER 1

BIOTRANSFORMATIONS: BIOCATALYST MEDIATED ORGANIC SYNTHESIS- A BRIEF REVIEW

1. INTRODUCTION

Recent years have witnessed the emergence of biocatalysis as a new and important tool in synthetic organic chemistry[1-4]. The possible impact of biotechnology on the future of the pharmaceutical, fine chemical and commodity chemical industry is now becoming more apparent and more widely acknowledged. This rapidly developing field of synthetic technology is broadly classified under the name "microbial conversion", "bioconversion" or "biotransformation".

Biotransformation is the conversion of one substance (substrate) to another (product) by a biocatalyst. The biocatalyst may be a microorganism, a plant or animal cell or an enzyme obtained from any of these sources. Enzymes, nature's catalysts, are essential for vital life processes of cellular metabolism and reproduction. Another enzyme-based synthetic technology is fermentation, which involves complex microbial metabolism of inexpensive carbon and nitrogen sources for the production of such compounds as organic acids, solvents, antibiotics and amino acids. In contrast, biotransformations utilize enzymes only as catalysts for chemical reactions; cellular metabolism being required only for the production of enzymes.

The area of biocatalyst-aided organic synthesis is, as are most areas of practical catalysis, fundamentally a process chemistry. That is, the principal objective is the efficient and economical (both in terms of money and time) synthesis of specific compounds. Thus, in this sense, it differs from much work done in organic synthesis where the new synthetic strategy is many times more important than the specific compound being synthesized.

The vast plethora of biocatalyst-aided synthetic processes for production of biologically and chemically useful compounds along with the increasing number of commercially exploited processes[5], are no doubt an indication that biotransformations are here to stay.

2. Characteristics of Enzymes as Catalysts

Enzymes are proteins of mol.wt. ~15,000-1,00,000 which play the role of biocatalysts in plants (amylases, papain, bromelain, etc.), animals (trypsin, chymotrypsin, pig liver esterase, pig pancreatic lipase etc.) and microorganisms (amylases, lipases, catalases, etc.). The International Union of Biochemistry lists more than 2000 enzymes[6] of which more than 800 are commercially available. Of these, microbial enzymes are gaining popularity due to the ease with which they can be obtained on a large scale using techniques of fermentation technology and genetic engineering.

Chemical reactions performed by micro-organisms or catalysed by enzymes are essentially the same as those carried out in conventional inorganic or organic chemistry. Based on the type of reaction catalysed, enzymes are divided into six classes[7]:

1) Oxido reductases (dehydrogenases, peroxidases, hydrogenases and oxygenases) catalyse oxidation reactions, i.e. the removal or addition of oxygen or hydrogen atom equivalents.
2) Transferases catalyse the transfer of one-carbon groups (CH_3, CHO, CH_2OH, COOH etc.), aldehydic/ketonic residues, acyl, glycosyl, alkyl or nitrogenous groups or P or S containing groups.
3) Hydrolases (esterases, amidases, proteases, phosphatases, glycosidases, hydantoinases etc.) catalyse hydrolysis of esters, amides, peptides, glycosides, anhydrides etc.

4) Lyases catalyse additions to double bonds, such as C=C, C=N, C=O and the reverse process of eliminations.

5) Isomerases catalyse various isomerizations including C=C bond migration, cis-trans isomerizations, racemizations etc.

6) Ligases or synthetases catalyse the formation of C-O, C-S, C-N, C-C and phosphate ester bonds.

Although enzymes catalyse the same reactions catalysed by organic/inorganic catalysts and increase the reaction rate by lowering the activation energy as normal catalysts do, they possess certain properties which make them unique as compared to any other catalyst.

The most striking differences between enzymes and chemical catalysts lies in their substrate specificities. Their catalytic activity is usually restricted to a single reaction type. This high reaction specificity enables an enzyme to selectively convert a single functional group in the presence of other reactive groups. Enzymes exhibit a high degree of regiospecificity i.e. the ability to selectively transform one group among several groups of similar reactivity. Another unique property is their almost absolute stereospecificity i.e. their ability to discriminate between spatial (or) stereoisomers either carrying out the enantioselective formation of a single isomer or acting only on a single stereoisomer leaving the other stereoisomer untouched. As a result, a single product can be expected as long as only one enzyme is involved in a conversion.

High catalytic efficiency is another characteristic property of enzymes. For example, the rate of hydrolysis of an amide by chymotrypsin is about 4000 times higher than that of the corresponding base catalysis[8]; rate of formation of carbonic acid from CO_2 and H_2O by carbonic anhydrase is

10 million times faster than the nonenzymatic reaction. Enzymes increase the catalytic turnover number (moles of product per mole of enzyme per unit time) without a large energy requirement; that is only a small amount of enzyme is needed to catalyse the conversion of a large amount of substrate.

Enzymes exhibit their catalytic activity under mild reaction conditions under which normal chemical catalysts hardly function at all, such as atmospheric pressure, temperatures around 20 to 40°C and pH values near neutrality.

3. Exploitation of biocatalysts (enzymes) for organic synthesis

A major requirement in synthetic chemistry is selectivity. A number of synthetic steps may be involved in the selective transformation of a particular functional group. Biocatalysts (enzymes) come to aid where the selective conversion of one functional group among several groups of similar reactivities is required. The chemoselectivity of enzymes has been exploited for selective hydroxylations of steroids (and other complex molecules) which are of commercial importance.

Another highly desirable objective in synthesis is the development of systems that would permit selective deprotection. Here, hydrolytic enzymes give scope for considerable development because of their ability selectively to remove a protecting group in a multiply-protected substrate. Releasing a functional group (such as hydroxy or amino group) at a particular site for further chemical transformation is of great value in synthesis.

Of more general importance than chemoselectivity is the control of chirality in chemical synthesis. One of the most economic ways of synthesizing enantiomerically pure compounds is to start with chiral building

blocks. The chemical synthesis of these is almost invariably a multi-step reaction sequence, making chiral building blocks very costly starting materials for synthesis of enantiomerically pure compounds. Furthermore, the problem is compounded as the number of chiral centres in the desired molecule increases; as the stereochemical efficiency decreases with each step in the synthesis.

On the other hand, a great majority of organic biomolecules (enzymes, receptors, transport proteins etc.) are chiral. Binding of a chiral molecule with a protein is enantioselective. Consequently, biological systems usually discriminate, often absolutely in favour of one enantiomer of a chiral compound. This has profound implications for drug design, since the pharmacological activities of enantiomers of a drug are often profoundly different. For example L-DOPA used in the treatment of Parkinsonism undergoes conversion into dopamine in the brain, an enzymic process that is enantioselective and does not operate on D-DOPA. Thus a racemic mixture of a drug would have only 50% activity. In certain cases, the unwanted isomer may even have deleterious effects, e.g. the unwanted isomer in thalidomide (a tranquilizer) causes disruption of fetal development. It is thus crucially important for methods to be available for the synthesis of biologically active compounds as well as chemical synthesis in an optically pure form.

Enzymes being chiral catalysts catalyse the right asymmetric synthesis with near-perfect specificity. Enzymes, thus have a significant role to play in the development of asymmetric synthesis[9].

Enzymic transformations have been incorporated into the synthesis of intermediates for the production of pharmacologically important compounds such as prostaglandins etc. By the application of such methods, the stereochemical efficiency can be raised from an average value of 50% for each step of a chemical process to 100% with attendant savings in cost.

Biocatalyst-mediated transformations are limited by certain properties of enzymes. For example, enzymes, being proteins have delicate structures which are maintained by van der Waals forces and a network of hydrogen bonds. Drastic reaction conditions under which normal chemical catalysts exhibit good activity lead to denaturation of enzymes. Thus, mild reaction conditions becomes a condition for all enzymatic processes at least in aqueous media. These limitations can however be overcome by using more thermostable enzymes or increasing enzyme stability via immobilization, organic media or structural modifications of enzymes using techniques of protein engineering.

Broad substrate tolerance is often desired in a synthetic catalyst. Here, the high selectivity of enzyme catalysis may be of disadvantage. This however, is compensated for by the vast multitude of enzymes in nature.

A number of synthetically useful enzymatic reactions require cofactors such as ATP, $NADP^+$, etc. Cofactors are expensive thus limiting use of isolated enzymes. For economical purposes whole cells or cofactor regeneration systems may be employed.

Enzymes may be subject to regulation; that is catalytic activity may be strongly influenced by the concentrations of substrates, products or other species present in solution. Product inhibition[10] which is most often encountered may be overcome by removal of product as it is formed or using large quantities of enzymes.

Under normal circumstances, enzymes act in aqueous solutions at pH 7-8 and room temperature. This could pose limitations for conversion of water-insoluble compounds. Transformations may thus be carried out in heterogeneous reaction mixtures in which the substrate constitutes a small proportion[11], or by the addition of modest concentrations of organic cosolvents such as ethylene glycol,glycerol, dimethyl sulfoxide or dimethylformamide[12,13]. New breakthroughs in enzyme technology have made it possible to carry out transformations in almost anhydrous organic solvents[29] adding a whole new dimension to biocatalyst-mediated synthesis.

Economics of enzyme use depend upon a number of factors, the cost of the enzyme, its specific activity and operating lifetime. In cases where alternative chemical synthesis procedures are well established, a choice between the two can be made based on economic considerations and other related factors such as environmental questions, supply of raw materials etc. In some instances, however, such as in the preparation of complex biologically active compounds such as peptide hormones of large mol. wt. etc. where an alternative chemical method is not available, the enzymatic process is readily accepted. A classic example is the conversion of pork insulin into human insulin by replacement of the terminal amino acid in the B chain. The conversion is carried out only by the enzymatic procedure.

4. Designing of a Biotransformation Process

In order to design an effective biotransformation process, several aspects require careful consideration. The selection of a target compound and survey of the available substrates and routes are especially important.

4.1 Screening for a suitable biocatalyst

The biocatalyst is often the lynchpin without which new biotechnological processes and products would not exist[14]. Screening for a suitable enzyme or biocatalyst is often undertaken by adding the substrate to be converted to the growth or reaction medium followed by assaying for the desired reaction products[15]. Effective screening involves both the search for a new biocatlyst and the evaluation of existing biocatalysts for desired catalytic activity.

4.2 Choice of a suitable substrate

Any substrate can be used for a biotransformation provided the substrate molecules come into contact with the enzyme. Even gases such as methane are effective substrates when bubbled through the reaction medium[16]. Ideally, the substrate should be soluble in the reaction medium or able to pass through the plasma membrane. However, solubility of insoluble substrates may be improved by powdering of solid substrates[17], dissolving it in a water miscible organic solvent, dispersing it with the help of a solvent or a combination of these methods.

On the other hand, cells, may be permeabilized by grinding with glass beads, lyophilization, sonication etc. Cell free enzymes may be used in order to eliminate any permeability barrier.

Chemical modification of a substrate may be done for more efficient interaction with the enzyme or to avoid undesired side reactions. A change in stereoselectivity of the enzyme pig liner esterase, on substrate modification has also been reported[18].

4.3 Types of biocatalysts and reaction systems used in biotransformations

Biotransformations may be carried out with whole or treated cells, organelles, cell-free multienzyme and single cell-free enzymes.

The simplest method of carrying out a transformation is with growing cultures where the substrate is added during inoculation or growth phase of the microorganism. This method is often used for screening as well as large-scale production.

Alternately, cells may be harvested after growth by centrifugation or filtration, resuspended in appropriate medium (buffer, etc.) and substrate added with or without further treatment of cells (washing, drying etc.). This method affords independent optimization of both growth and reaction steps.

Transformations may be carried out using spores[19] which can be stored without deterioration for long periods of time.

Many transformations have been carried out with cell-free enzymes or purified enzymes[20-22]. In general, this method is expensive, but may be of use if it alleviates problems due to substrate solubility, biocatalyst permeability etc.

Whatever the biocatalyst used, immobilization techniques may be used in order to confer higher operational stabilities and prolonged reuse of the biocatalyst[23-26]. Immobilization facilitates easy biocatalyst separation, repeated usage, continuous operation, biocatalyst regeneration and easy product isolation. Special engineering design may however be required for continuous processes.

Multi-step transformations with different catalysts can also be carried out. A combination of catalysts may be used simultaneously or sequentially

to obtain desired product. Recombinant DNA technology can be used to allow the combination of such separated enzyme catalysts in a single micro-organism[27].

4.4 **Product Isolation**

Extracellular transformation products may be separated by filtration or centrifugation followed by concentration, fractionation and final purification.

Lipophilic products are usually separated by extraction with an organic phase and concentration by solvent removal in a vacuum evaporator.

Hydrophilic products may be isolated either by solid or liquid ion-exchangers or by selective adsorption to polymer resins. Insoluble products may be recovered by precipitation, volatile compounds purified by distillation.

Final purification is achieved either by crystallization, fractional distillation or column chromatography. Liquid or supercritical carbon dioxide extraction have been used to extract reaction mixtures which produce heavy emulsions with other solvents.

4.5 **Improvement of a biotransformation process**

Various approaches may be used for improvement of a biotransformation process. First of all, the environmental conditions for maximum enzyme production and maximum transformation activity can be optimized. The parameters involved are medium composition, temperature, pH and dissolved oxygen. Addition of an inducer or removal of an inhibitor may also improve any biotransformation process.

Substrate insolubility may be overcome by using multiphase systems (immiscible organic solvents etc.) which allow high concentrations of lipophilic substrates to be used, minimize product inhibition and facilitate easy recovery of product. Solid lipophilic adsorbents have been used as reservoirs in steroid conversions. This strategy helps improve the reaction rate and product yield. In addition, enzymes may be chemically modified to exhibit activity in water-organic solvent mixtures. For example, chemically modified peroxidase exhibits its activity well in benzene and n-hexane[28].

The discovery that enzymes are active in almost anhydrous organic solvents[29] opened new doors for applications in organic synthesis. Such technology can be used to shift the hydrolytic equilibrium towards water elimination facilitating synthesis of esters, amides, peptides[30] and conversion of other lipophilic compounds. In this case, substrates that are only soluble in organic solvents can also be used. In addition, it has been shown[31] that enzymes have high thermostability and sometimes, altered substrate specificity in anhydrous organic solvents[32].

Recent developments in protein crystallography and genetic engineering have significantly increased understanding of the structure, catalytic mechanisms, stability and substrate specificity of enzymes. This has resulted in the development of new, superior biocatalysts with altered specificity and increased stability. For example, a substitution of a methionine residue in subtilisin (a protease widely used in detergent formulations) made the enzyme highly resistant to oxidation at alkaline pH.

Recombinant DNA technology and mutagenesis techniques may be used for genetic strain improvement. Enzymes can be produced in much larger

quantities by high levels of expression of cloned proteins in host micro-organisms. This may be done either by increasing the gene copy number in the cell or increasing promoter strength. Use of secretion vectors generates modified strains which secrete proteins. It results in high yields of the desired product, significantly simplifies the purification procedures and consequently lowers the cost of enzyme production. To date, only in cases of a few microorganisms such as E.coli, B.subtilis and S.cerevisiae for which the genetic background and host-vector systems are well known, has recombinant DNA technology been beneficial. However, it will undoubtedly provide one of the most important keys for future development of biotransformations.

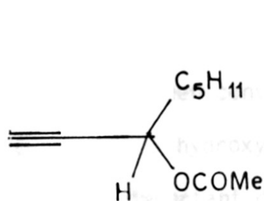
5. Biotransformations- some specific examples

Most of the research aimed at studying biotransforamtions in organic synthesis, in recent years has been conducted using simple, whole-cell systems (e.g. yeasts) or commercially available hydrolase or oxido reductase enzymes[33]. The biocatalyst mediated reaction may be a single step, often the key step in a multi-stage sequence of transformations or a multi-enzyme system may be used for the transforamtion of simple starting materials into relatively complex end-products.

5.1 Hydrolase catlysed transformations

Lipases and esterases constitute the most frequently used enzymes in this class, catalysing the hydrolysis, often stereospecific, of a wide range of substrates.

Many esters of chiral alcohols have been hydrolysed. For example, the ester (1) is hydrolysed by Mucor miehei lipase[34] or lyophilized yeast[35]



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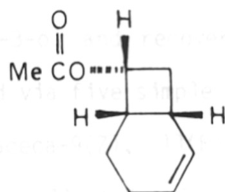

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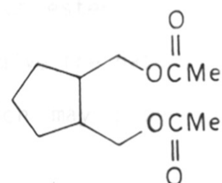

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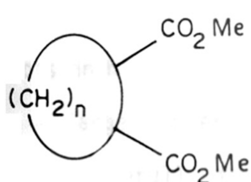

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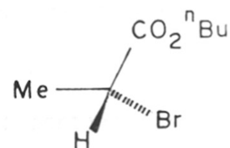

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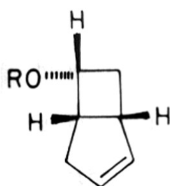
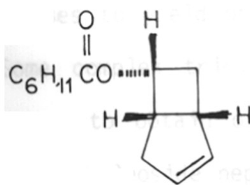


(±)-(7) R=H

(±)-(8) R=COMe



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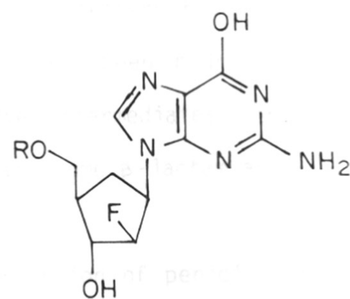

(±)-(10) $R=PO_3^{2-}$

(±)-(11) R=H

to provide 3(S)-oct-1-yn-3-ol and recovered optically active ester. The former has been converted via five simple chemical steps to give the natural product, 13-hydroxy-octadeca-9(Z), 11(E)-dienoic acid which may prove to be an important chemorepellent in vivo.

Lipase catalysed resolution of more complex esters such as (2) have also been carried out in order to obtain optically active intermediates for synthesis of potentially useful unsaturated ketones[36].

Meso-compounds, such as the cyclopentane derivative (3) have also been hydrolysed in selective fashion using hydrolase enzymes to obtain products in high optical purity and almost quantitative yield[37].

A large number of esters with chirality in the acid portion have also been usefully transformed by using enzymes. For example, racemic α-amino esters have been hydrolysed with very high stereoselectivity by using hydrolase enzymes such as α-chymotrypsin[38].

The hydrolysis of a wide range of alicyclic esters of type (4) using porcine pancreatic lipases to yield optically active monoesters have also been reported[39]. Some complex tricyclic diesters have been hydrolysed using pig liver esterase to obtain optically active intermediates. For synthesis of carbocyclic nucleoside nephalonocin A and some β-lactam antibiotics[40].

Bacterial amidases have been used for the conversion of penicillin-G into 6-amino-penicillanic acid[41]. The use of acylases for the stereoselective hydrolysis of chloroacetamides (5) have been investigated[42] as has the use of amidases from Pseudomonas putida for enantiospecific hydrolysis of racemic α-aminoamides[43].

An important breakthrough in the field of applied enzymology which contributed in a big way to enzymatic organic synthesis was the discovery that enzymes can function in organic solvents[29]. Over the past few years, lipases and amidases (proteases) have been increasingly used in low-water systems for the synthesis of esters and amides. The porcine pancreatic lipase (or yeast lipase) catalysed reaction of racemic 2-bromopropanoic acid with n-butanol in hexane containing a trace of water gave unreacted optically active acid and the ester (6)[44], Similarly, the racemic secondary alcohol (7), is esterified with high selectivity by using cyclohexane carboxylic acid and lipozyme[45], to give the ester (9) and recovered optically active alcohol. Stereoselective trans esterification is also possible and the one enantiomer of the acetate (8) reacts preferentially to furnish the ester (9).

Besides ester synthesis and trans-esterifications, proteases and lipases may also be used for the synthesis of small peptides[46]. A trypsin-catalysed transamidation process has been used to convert porcine insulin into human insulin for clinical use[47].

A viral kinase effected the synthesis of the racemic carbocyclic nucleotide (10) by regioselective phosphorylation of the 5'-hydroxyl group of the corresponding nucleoside. Enantioselective hydrolysis of the 5'-phosphate moiety was achieved by using a snake venom nucleotidase to produce optically active nucleoside (11) which proved to be an extremely potent anti-herpes agent[48].

5.2 Oxido reductase catalysed transformations

Most of this work in this area has been concentrated on the reduction of ketones into chiral secondary alcohols with dehydrogenase enzymes. The enzymes can be used as part of a whole-cell system or as partially purified protein: in the latter case, the appropriate cofactor (NADP or NADPH) must be added to the reaction mixture and cofactor recycling must be arranged. There are various pros and cons of using a whole-cell system as opposed to an isolated enzyme[49].

The achiral alkenone (11) has been converted into the optically active natural product sulcatol with a dehydrogenase enzyme from <u>Thermoanaerobium brockii</u> with NADPH as the co-factor and iso-propanol as the sacrificial alcohol in the recycling system[50]. The same reduction can be accomplished with whole cell systems also[51].

The bicyclo pentanones (13) and (14) which are complex ketones with pre-existing chiral centre(s) have also been reduced effectively. The racemic ketone (13) is reduced with high enantioselectivity by the fungus <u>Mortierella ramanniana</u> to give 1(S), 5(R), 6(S) -bicyclo [3.2.0] hept-2-en-6-endo-ol and an optically active ketone. Both products have been converted into prostaglandins by enantiocomplementary synthesis[52]. Prostaglandins and analogues of these natural products are being investigated as potential cytoprotective agents and as compounds as possible agents for the control of certain forms of heart disease. Effective enantioselective reduction of the ketone (14) has been achieved by using commercially available 3α, 20β-hydroxysteroid alcohol dehydrogenase (employing NADH as the cofactor and ethanol/horse-liver alcohol dehydrogenase as the recycl-

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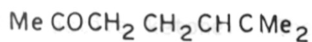

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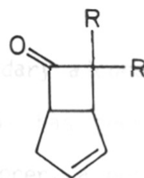
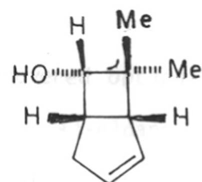


(13) R = H
(14) R = Me



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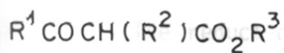

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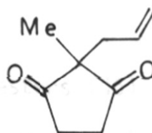

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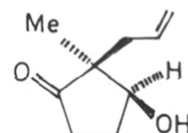

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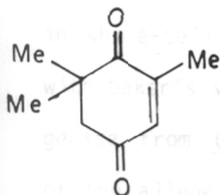

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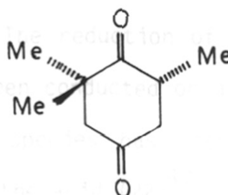

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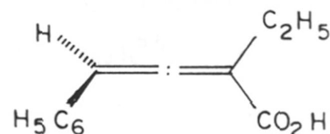

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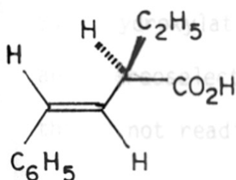

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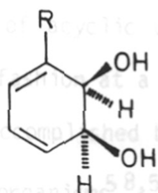

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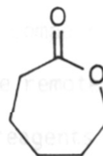

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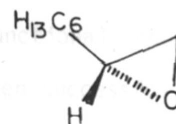


(25)

ing system) to furnish the secondary alcohol (15) and recovered optically active ketone. The alcohol (15) has been converted into (+)-eldanolide, the pheromone of the sugarcane borer, a pest infesting sugarcane and maize crops in West Africa[53]. (15) has also been converted into leukotriene-B_4, a naturally occurring substance that has been implicated as an important factor in the onset and maintenance of inflamatory disorders such as psoriasis.

The reduction of β-keto esters (16) has received a lot of attention. The reduction of ethyl 3-oxobutanoate (16) with baker's yeast provides the 3(S)-hydroxy-butanoate[54]. The achiral cyclic 1,3-diketone (17) has been reduced by yeast to give the hydroxyketone (18) which was, in turn, converted into the naturally occurring compound coriolin[55].

The reduction of carbon-carbon double bonds in αβ-unsaturated carbonyl compounds can be accomplished with reductase or dehydrogenase enzymes in whole-cell systems. The reduction of the dione (19) to compound (20) with baker's yeast has been conducted on a substantial scale[56]. A dehydrogenase from Clostridium species has been used to effect the conversion of the allene (21) into the acid (22)[57]. Reduction reactions of this type can be further exploited for the preparation of new, useful synthons.

Mono- oxygenases in whole-cell systems are capable of introducing one or more hydroxy groups at ostensibly non-activated positions in a molecule. Such hydroxylations of acyclic or alicyclic compounds in a regioselective and stereoselective fashion at a carbon centre remote from functional groups though not readily accomplished by chemical reagents have been successfully reported using microorganisms[58,59].

Hydroxylation of a cyclohexylcyclohexane to give a diol, an intermediate for synthesis of an antagonist of the leukotriene-B has also been *reported*[60].

The conversion of benzene into the diol (23) with an oxygenase system in the microorganism Pseudomonas putida has created a lot of excitement. The diol has been converted into polyphenylene[61] and into the natural product (±)-pinitol[62]. The diol [(23), R=H] has been transformed into a variety of bicyclic molecules[63]. One such bicyclic diester has been converted using pig liver esterase into a monosubstituted benzene derivative[64]. Both mono- and di-substituted benzene derivatives are also biotransformed with selected microorganisms to give cyclohexa-1, 3-diene derivatives. *Conversion of chlorobenzene into 3-chloro-cyclohexa-3,5-diene-1(S) 2(S)-*diol provided an early stage intermediate for prostaglandin synthesis.

The formation of hydroxylated derivatives of heterocyclic aromatic compounds is also being currently investigated.

Cyclohexane oxygenase from Acinetobacter sp. performs the equivalent of a chemical Baeyer-Villiger reaction on cyclic ketones. For example, cyclohexanone formed the corresponding caprolactone (24).

The stereoselective epoxidation of allylic alcohols and some homo-allylic alcohols is best done with the Sharpless method[65]. On the other hand, stereoselective epoxidation of alkene units remote from the hydroxy groups (or any other functionality) can be effected by using microorganisms: the conversion of oct-1-ene in the epoxide (25) with Pseudomonas aleovarans provides one example[66]. More fundamental research is needed in this area.

5.3 **Other Biotransformations**

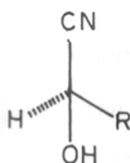
There is a vast array of other bioconversions that are used for the synthesis of new, potentially interesting substances and there are many exciting possibilities for future research[67,68] only a few have been cited here.

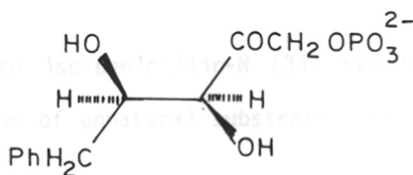
Mandelonitrile lyase, isolated from bitter almonds and immobilized on cellulose, catalyses the addition of HCN to a variety of aldehydes to furnish the corresponding (R)-cyanohydrins (26)[69].

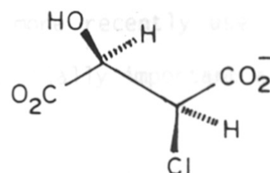
One of the most useful reactions in organic chemistry is the aldol reaction and good progress has been made in finding conditions under which aldolase enzymes catalyse the formation of the requisite carbon-carbon bond. For example, dihydroxyacetone phosphate reacts with 2-phenylethanal, under catalysis by an aldolase enzyme, to give the adduct (27); a variety of aldehydes undergo a similar transformation[69,70].

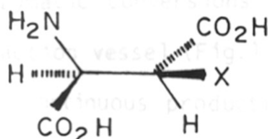
The addition of water and ammonia to $\alpha\beta$-unsaturated acids such as derivatives of fumaric acid has been studied in recent years. Thus chlorofumaric acid is hydrated to give L-threochloromalic acid (28) on catalysis with pig-heart fumarase[71]. The use of 3-methyl aspartate ammonia lyase for the conversion of halofumarates to amino acids such as (29) has been described recently[72] as has the employment of cloned E.coli aspartate transaminase for the conversion of various aromatic and aliphatic α-ketoacids into the corresponding L- -amino acids[73].

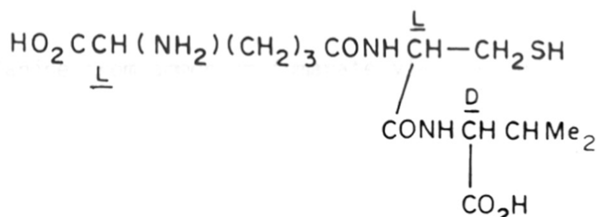
A number of multi-step enzyme catalysed reactions have also been used for synthesis. Some enzymes can perform more than one reaction on a suitable substrate. For example, the same enzyme which converts a tri-

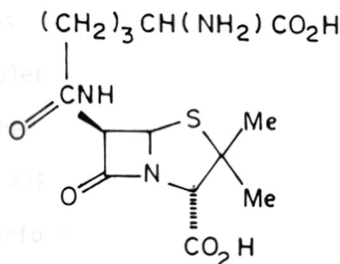












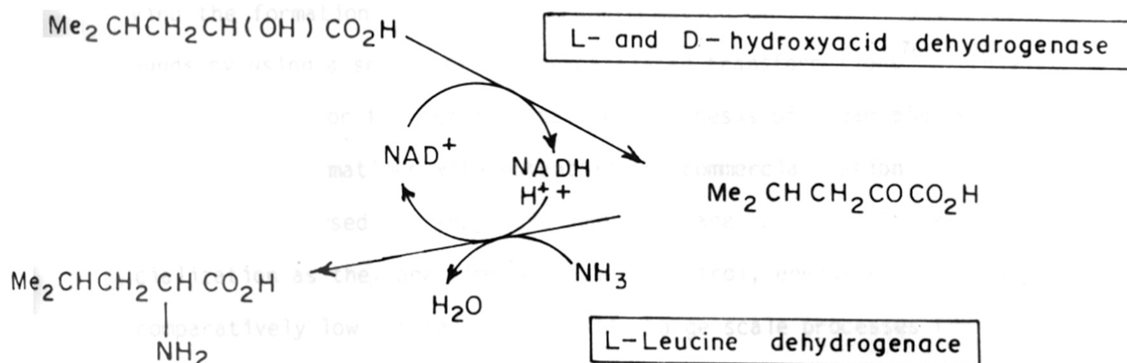


FIG. 1. A MULTIPLE-ENZYME SYSTEM FOR THE PRODUCTION OF OPTICALLY ACTIVE AMINO ACIDS

peptide (30) into iso-penicillin-N (31) has been more recently used for the transformation of unnatural substrates into potentially important antibiotics[74].

There are a number of ways of obtaining optically active amino acids by means of enzyme-catalysed reactions. One of them is using multi-step enzymatic conversions of the substrate to the desired product in the same reaction vessel (Fig.1)[75].

Continuous production of L-alanine from ammonium fumarate via L-aspartic acid as an intermediate with immobilized E.coli (aspartase) and Ps.dacunhae (aspartate decarboxylase) cells has also been carried out[77]. Other multi-enzyme processes include the production of L-tryptophan from DL-serine and indole, complete hydrolysis of racemic α-amino-ε-caprolactam[78] etc.

On the other hand, the synthesis of coenzyme A from pantothenic acid, L-cysteine and ATP (or AMP), involving five sequential steps, is being commercially performed solely with Brevibacterium amoniagens cells- made possible by extensive screening[79].

More recently, attention has been focussed on the possibilities involving the formation and further transformation of relatively unstable compounds by using a series of enzyme-catalysed transformations[76]. This strategy has been used for the enzyme catalysed synthesis of sugar mimics.

5.4 Biotransformations with potential for commercialization

Biocatalysed or enzymatic proceses are good candidates for commercialization as they are simple, easy to control, energy efficient and require comparatively low capital investments. Large scale processes for the production of optically active amino acids, peptides, antibiotics, coenzymes and other biologically useful compounds are well developed[80]

New procedures such as the use of enzymes in anhydrous organic solvents[29,81] and supercritical fluids[82,83] significantly broadened the scope of potential applications of enzymes for the production of fine and speciality chemicals.

Currently, enzymes are most widely used in the food industry (production of high fructose corn syrup, chillproofing, milk clotting, lactose hydrolysis), the pharmaceutical industry (porcine insulin modification) and the detergent industry (protein and fat hydrolysis). Other well established industrial processes include the transpeptidation of porcine insulin to produce human insulin esters[84], the resolution of N-acetyl-D,L-amino acids[85], the production of semisynthetic penicillins[86] and the synthesis of L-lysine from D,L-caprolactam[87]. Described below are a few novel processes with potential for commercialization.

5.4.1 Lipase-catalysed resolution of racemic mixtures

Lipases are abundant, stereospecific, stable and versatile enzymes. In addition to lipolysis, they also catalyse a variety of synthetic transformations such as esterifications, transesterifications, acyl exchange and oximolysis[88]. They can be efficiently immobilized and reused and they are active in both aqueous and nonaqueous environments. Under appropriate conditions, they offer remarkable efficiency and selectivity. Thus they are ideally suited for the synthesis of chiral intermediates for pharmaceutical and agricultural products.

Porcine pancreatic lipase has been used in hexane as a catalyst in esterification of racemic bromopropionic acid with butyl alcohol. (S)-2-halopropionic acids are intermediates in the production of (R)-2-phenoxy-

propionic acid, which is a potent herbicide. The enzyme was found to be specific for the L-isomer, and (S)-(-)-2-bromopropionic acid was obtained at 75% of the theoretical yield and 99.6% enantiomeric excess in 6h[89]. It also exhibits broad substrate specificity and operational stability.

Porcine pancreatic lipase has also been found to have the best combination of activity, selectivity and cost among the enzymes that hydrolyse glycidol esters[90]. Glycidol esters are used for the production of β-blockers and related compounds[91]. After 6 h of enzymatic hydrolysis of the racemic glycidyl butyrate, (R)-glycidyl butyrate is produced at 95% enantiomeric *yield and 88% of theoretical yield.* Enantiomerically enriched epichlorhydrin is often employed by the pharmaceautical industry[90,92].

The enzymatic processes have to compete with classical chemical methods, the final choice depending upon the nature of the compound to be resolved, economics of the process, the demand and the particular requirements of its purity.

5.4.2 **Refinement of fats and oils**

The lipase-catalysed refinement of palm oil for the production of cocoa-butter substitutes is a particularly attractive process. Cocoa butter *(comprising 30% of chocolate) has a very high market value and is unique* in having a melting point close to the human body temperature. The modification of palm oil is accomplished by the replacement of palmitoyl moieties in the 1 and 3 positions of palm oil triglycerides with stearoyl groups. The process for cocoa butter production is based on a transesterification reaction catalysed by 1,3-specific lipase immobilized on diatomaceous earth[93] which exhibits excellent operational stability. The alternative chemical

transesterification is random in all three positions of the triglyceride molecule, requires elevated temperatures and addition of acid catalysts and gives low yields; all these factors make it significantly inferior to the enzymatic process.

Monoglycerides, routinely used in the food and pharmaceutical industries, are produced commercially by glycerolysis of fats. Transesterification is carried out in excess glycerol at about 240°C in the presence of an alkaline catalyst. The monoglycerides are isolated by molecular distillation at high vacuum. The major drawback of the chemical process is the low yield of products resulting from thermal degradation at high temperatures during the reaction and purification. Lipase-catalysed production of monoglycerides is an exciting alternative to the traditional chemical synthesis because of the mild reaction conditions and low by-product formation. Also, highly unsaturated triglycerides can be used directly without prior hydrogenation. Fatty acid monoglycerides can be obtained by esterification of glycerol with fatty acid, partial hydrolysis of triglycerides or transesterifications of triglycerides with alcohols[90,94]. A process for the production of a mixture containing 95% monooleate and 5% dioleate glycerides based on lipase from Penicillium cyclopium has recently been developed[95].

5.4.3 **Enzymatic synthesis of peptides**

Enzymatic synthesis of peptides is an area of considerable activity. Because of its high stereo- and regioselectivity, lack of racemization and only limited need for protective groups, protease catalysis is widely used for preparative synthesis of biologically active peptides, for oligomerization, for semienzymatic synthesis, etc.[96]

One of the great commercial interests in this area is enzymatic synthesis of the dipeptide, L-aspartyl-L-phenylalanine methyl ester (aspartame), which is widely used as low-calorie sweetener. Chemical synthesis of aspartame involves the coupling of N-formyl-L-aspartic acid anhydride and L-phenylalanine methyl ester (L-Phe-OMe). The desired product results from amide bond formation between the α-carboxyl group of the aspartic acid and L-Phe-OMe. It is contaminated, however with about 20% of the peptide formed via coupling of L-Phe-OMe to the β-carboxyl group of the aspartic acid, necessitating extensive downstream processing as well as recycling of the relatively costly L-Phe-OMe.

Thermolysin coupling of N-carbobenzoxy-L-aspartic acid and D,L-phenylalanine methyl ester is highly regio- and stereoselective. Thus racemic Phe-OMe can be used and only one product- N-α-carbobenzoxy-L-aspartyl-L-phenylalanine is formed. The reaction can be carried out in both aqueous[97] and non-aqueous solvents[98]. An alternative method which does not require the use of protecting groups is based on a protease from Micrococcus caseolyticus which catalyses peptidebond formation between aspartic acid and phenylalanine methyl ester in the desired manner[99].

Despite, various advantages, the aqueous synthesis of peptides has some shortcomings; the unfavourable thermodynamic equilibrium of peptide bond formation in aqueous solutions, a narrow substrate specificity of proteases, and hydrolysis of the growing polypeptide chain. Some of these drawbacks can be alleviated by using reverse micelles[100] and non-aqueous media[101] both of which allow the equilibrium to be shifted towards product formation. Chemical modification of the existing proteases[102] and use of non-proteolytic enzymes[103,104] have helped to eliminate secondary hydrolysis

and allowed the synthesis of peptides containing unnatural or D-amino acids[103].

5.4.4 **Enzymatic Polymerizations**

Horse radish peroxidase-catalysed polymerization of phenols is of great practical importance. In an aqueous medium, peroxidase catalyses coupling of a number of phenols and aromatic amines. Its practical use as a catalyst for the production of high molecular weight polymers was not feasible until recently because of the low solubility of the phenolic dimers and trimers in water. The use of organic solvents as a medium for enzymatic transformation eliminated this drawback and yielded phenolic polymers with molecular weights between 400 and 2.6×10^4[105].

The current chemcial method of producing phenol-formaldehyde resins is based on thermal condensation of formaldehyde-based oligomers. The products formed contain a certain amount of unpolymerized and highly toxic formaldehyde. Enzymatic polymerization of phenols is an alternative way to produce phenolic resins not involving formaldehyde. Another advantage of enzymatic methods over traditional chemical process stems from the superior quality of the resin. The melting point of poly (p-phenyl-phenol) is about 100°C higher than the resin prepared by conventional means. Moreover, because of the extensively conjugated π-electron system of the polymer, it has much greater conductivity. All these characteristics significantly improve the value of the product.

6. **Concluding Remarks**

It is obvious that biotransformations have to compete with fermentation techniques and chemical synthesis. However, best results will be obtained by utilizing combinations of these methods.

Microorganisms are perhaps the richest source of different enzymes. They show great diversity and an ability to adapt to a wide variety of

environments. Therefore, they have a great potential for inducing new or novel enzyme systems capable of converting foreign substrates. It is possible to obtain and cultivate microorganisms that can survive or grow in extraordinary environments e.g. psychrophilic (stable to cold), thermophilic (stable to heat), acidophilic (stable to acid) and alkalophilic (stable to alkali) ones. Screening alone will be of great importance to obtain new biocatalysts for new conversions.[106]

Over the next few years, the employment of hydrolase and oxidoreductase enzymes for the formation of compounds, particularly optically active substances useful for the synthesis of interesting and potentially important molecules will continue to become more popular. Other transformations will also come into routine use. The use of various enzymes in low-water systems will be fully investigated.

Biocatalysts will be improved using recombinant protein and genetic engineering techniques to give better catalysts with modified activities

Biotransformations will undoubtedly become accepted as 'tools of the trade' in synthetic organic chemistry, especially for laboratory scale synthesis (less than 10 g of the compound). The number of large scale processes of industrial importance will also increase.

CHAPTER 2

STEREOSPECIFIC ESTER HYDROLYSIS USING *Bacillus subtilis*

SUMMARY

The synthesis of the potent agricultural insecticide NRDC 182 required a chiral synthon, the R(-) isomer of an allylic alcohol which is relatively unreactive and difficult to resolve using chemical methods. A strain of Bacillus subtilis obtained from NCIM, Pune was found to have the excellent ability to effect the kinetic resolution of the corresponding racemic acetate to furnish the desired alcohol.

The effect of various parameters such as cell age, temperature and inoculum size on the biocatalytic activity were investigated. It was found that B. subtilis cultivated with a 14-16 hour inoculum at 30°C for 24 hours had optimal biocatalytic activity.

Furthermore it was established that the biocatalytic activity was both associated with the cells as well as the growth medium (into which the enzyme is excreted as the culture grows). Of the two fractions, higher catalytic activity as well as enantiospecificity was assoc̄iataed with the cells. Cell disruption did not prove effective in increasing the catalytic activity.

It was observed that good conversions (≥40%) were obtained with upto 1% substrate concentration. Beyond this, there was almost a 50% fall in conversion values. It was desired to improve the biotransformation process by enhancing conversion at higher substrate concentrations (1.5-2%) maintaining high optical purities.

Varying the reaction parameters by increasing biocatalyst concentration as well as conversion time were found to be effective in achieving

this goal. Improved conversions were also obtained with cultures grown in medium with higher NaCl concentration. Surfactants, solvents and cyclodextrins were added to improve solubility of the substrate. Surfactant addition was found to be an efficient method of improving conversion. Many of the methods used showed good conversion values and enantiomeric excesses at 2% substrate concentration.

The optical selectivity of the B.subtilis was investigated by studying the rate of hydrolysis of individual R and S isomers. It was found to be highly stereoselective for the R isomer with very low rates of conversion of the S at extended time periods.

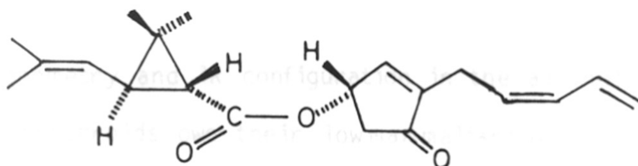
INTRODUCTION

In recent times, synthetic pyrethroids have emerged as a major class of insecticides. With their remarkable potency against economically important pests, low mammalian toxicity and rapid biodegradability, pyrethroids have made a most significant impact on the world agrochemical market.

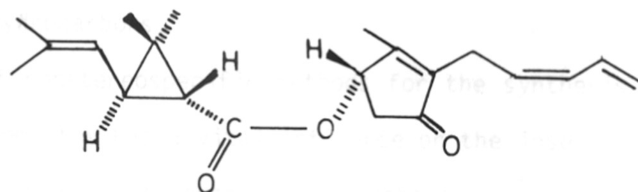
The history of pyrethroids dates back to the beginning of the century when 'pyrethrum', an extract from the flowers of the plant Chrysanthemum cinerariae was found to have potent insecticidal activity. The active components were found to be pyrethrins[107] [(1) and (2)]. The natural pyrethrins had all the above mentioned advantages of pyrethroids, but suffered the severe drawback of being sensitive to air and light.

Staudinger and Ruzicka were pioneers in establishing the main structural features of these esters as well as elucidating the structure-activity relationship. Maximum activity in these natural esters was associated with the trans geometry of the cyclopropane ring, the 1R configuration in the acid portion and the S configuration in the alcohol moiety.

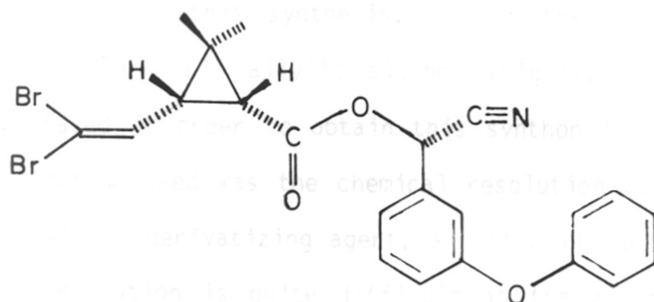
Synthetic pyrethroids have been derived from the natural pyrethrins via structural modifications[108]. Two such synthetic pyrethroids Deltamethrin (3) and NRDC 182 (4) have very high potencies[109] (being effective at concentrations as low as 1.0g ha^{-1}) in addition to remarkable chemical and photostability. Potency in these compounds is related to the cis



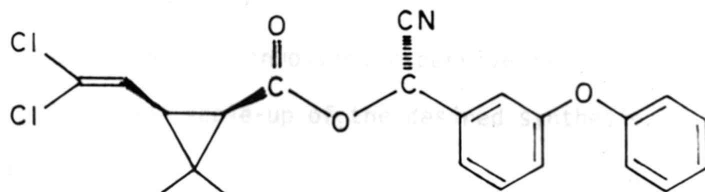
(1) PYRETHRIN-I



(2) PYRETHRIN-II



(3) DELTAMETHRIN (NRDC 161)



(4) NRDC 182

geometry and 1R configuration in the alcohol moiety. Like natural pyrethrins, pyrethroids owe their low mammalian toxicity to the presence of powerful esterases and monooxygenases in mammals which bring about their breakdown. This particular property of pyrethroids gives them a unique advantage over other classes of insecticides like carbamates, organophosphates and halogenated hydrocarbons.

There are a number of nonstereospecific methods for the synthesis of NRDC 182. Since stereochemistry has a vital influence on the insecticidal activity, efforts to develop stereospecific syntheses were made. One such synthesis of NRDC 182 developed by Hatch and Baum in 1980[110] has potential for commercial exploitation.

In this synthesis, one of the intermediates for NRDC 182 was the R(-) (1) allylic alcohol (Fig.1). Considerable difficulty was faced in order to obtain this synthon in an optically pure form. One method used was the chemical resolution of the racemic allylic alcohol with a derivatizing agent, an optically active isocyanate. The chemical resolution is quite difficult as the alcohol is very unreactuve owing to the strong electron withdrawing nature of the adjacent trichloro-methyl group. An alternative method used was the asymmetric reduction of the corresponding ketone, 1,1,1-trichloromesityl oxide. This method too is problematic as it involves stringent experimental conditions. Both methods are tedious, multistep procedures involving expensive reagents. These factors would severely limit any scale-up of the desired synthesis.

Scope of the present investigation

In the present work, efforts were directed towards finding a facile method for the resolution of (±) (1) to obtain the optically

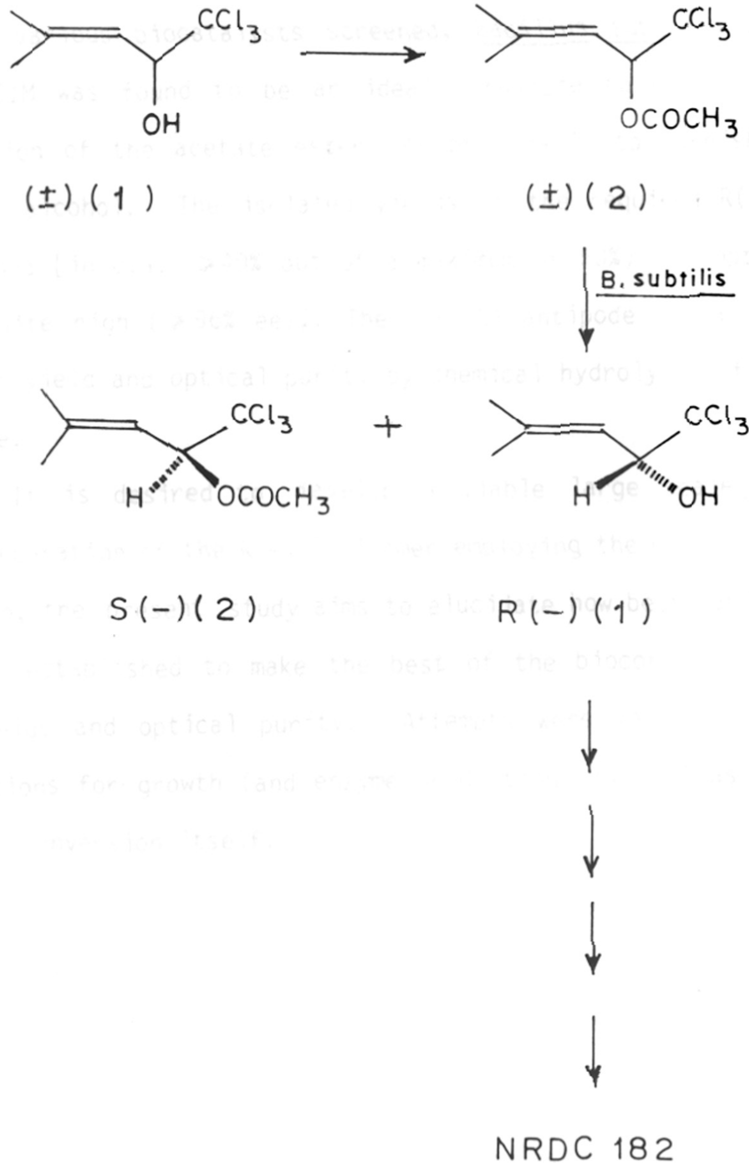


FIG. 1. PREPARATION OF CHIRAL SYNTHON FOR NRDC 182 VIA MICROBIAL RESOLUTION

pure R(-) (1) isomer in a single step.

Since the chemical resolution is difficult, alternative microbial or enzymatic resolution methods (biotransformations) were investigated. Of the various biocatalysts screened, Bacillus subtilis 2010 obtained from NCIM was found to be an ideal candidate to effect the kinetic resolution of the acetate ester (2) of (±)-(1) to furnish the desired R(-)(1) alcohol. The isolated yields of the required R(-) (1) isomer were good [in c.a. ≥40% out of a maximum of 50%) and optical purities were quite high (≥96% ee)]. The S(-)(2) antipode can also be obtained in high yield and optical purity by chemical hydrolysis of the unreacted acetate.

It is desired to develop a viable large scale procedure for the preparation of the R(-)(1) isomer employing the microbial hydrolysis. As such, the present study aims to elucidate how best various conditions can be established to make the best of the bioconversion with respect to yields and optical purity. Attempts were made both to optimize conditions for growth (and enzyme production) as well as the efficiency of the conversion itself.

MATERIALS AND METHODS

Synthesis of substrate for bioconversion

The racemic allylic alcohol (±)-(1) [1,1,1-trichloro-2-hydroxy-4-methyl-3-pentene] was prepared from isobutylene and chloral in two steps via a Prins reaction followed by acid-catalysed isomerization of the terminal olefin[111]. The acetylation of the racemic alcohol was carried out using acetic anhydride and pyridine to give (±)-(2) [1,1,1-trichloro-2-acetoxy-4-methyl-3-pentene] which is the substrate.

The R acetate was prepared via the acetylation of the R(-) alcohol of high optical purity obtained from microbial hydrolysis. The S acetate was obtained by extensive microbial hydrolyusis of (±)-(2) followed by separation and purification of the unhydrolysed substrate.

Biocatalysts used, media and growth conditions

All microorganisms used were obtained from NCIM, Pune. Pig pancreatic lipase was obtained from Sigma. Cultures were stored on nutrient agar slants at refrigeration temperatures.

Nutrient broth, the culture medium consisted of peptone (1%) beef extract (1%) and NaCl (0.5%) with pH adjusted to 7.0. Media were sterilized prior to use by autoclaving at 121°C and 15 p.s.i. for 15 minutes. Media modifications where required, were carried out prior to sterilization.

Cultures were grown in 100 ml broth in 500 ml conical flasks or 50 ml broth in 250 ml flasks. The inoculum prepared by inoculating 5 ml nutrient broth in a test tube and incubation at 30°C (with shaking)

overnight was transferred to the growth flasks which were incubated on a rotary shaker at 250 rpm at 30°C for 24 hours (Fig. 2) unless mentioned otherwise. Effect of growth temeprature was studied by cultivating B.subtilis at different temperatures in a Controlled Environment Incubator Shaker at 250 r.p.m. Wet weight of cells was determined by harvesting cells via centrifugation of culture broth at 3000 g for 15 minutes.

Reaction conditions for bioconversion

In general, the cultured broth of B.subtilis itself was used as medium for bioconversion. The substrate (±)-(2) was directly dispensed into this followed by incubation on a rotary shaker at 250r.p.m. at 30°C for 48 hours (Fig. 2) unless mentioned otherwise.

Cells which were separated by centrifugation of broth at 3000 g for 15 minutes and resuspended in 50/100 ml of 0.1M Tris-HCl buffer (pH 8.0) were also used as the medium for bioconversion. So was the supernatant obtained after centrifugation. Cells subjected to disruption via mechanical shearing in a dynomil followed by centrifugation and resuspension in the same medium or Tris-HCl buffer (pH 8.0) were also used for bioconversion.

Where required, cells harvested from one culture flask were resuspended in another cultured broth to obtain bioconversion medium with increased biocatalyst concentration. A weighed amount of β-cyclodextrins was added along with substrate into conversion medium, when required. Also, substrate dispersed in 10 ml of buffer (Tris-HCl, pH 8.0) alongwith 0.1 ml Triton X-100 and mixed thoroughly on a cyclomixer was added to conversion medium where so desired.

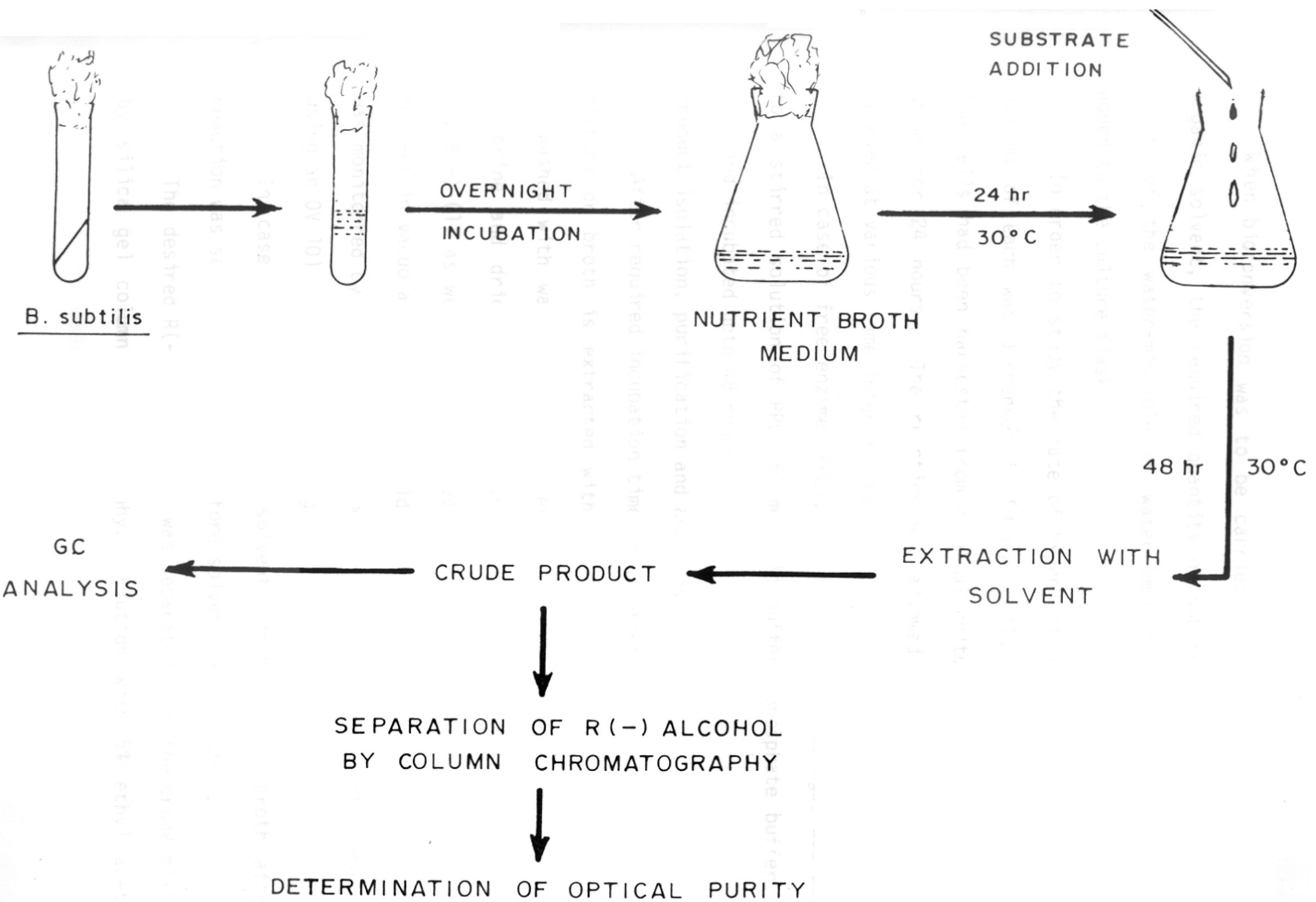


FIG. 2. GENERAL PROCEDURE FOR THE BIOTRANSFORMATION

When bioconversion was to be carried out in the presence of an organic solvent, the required quantity of substrate was dispensed into 10 ml of the water-miscible or water-immiscible organic solvent and added to the culture flask.

In order to study the rate of hydrolysis of the R and S isomers, 0.1 ml of each was dispensed in 50 ml 'cells suspended in buffer'. The cells had been harvested from a 50 ml culture broth of B.subtilis grown for 24 hours. The reaction was allowed to proceed and flasks removed at various time intervals to monitor conversion.

In case of free enzyme (PPL), the substrate (10-25 mgs) was added to a stirred solution of PPL (50 mgs) in buffer (phosphate buffer, pH 7.0) and incubated upto 48 hours.

Product isolation, purification and analysis

After required incubation time (most often 48 hours), the reaction mixture or broth is extracted with solvent ether. The ether extract is washed with water to remove residual acetic acid formed, followed by brine and dried over anhydrous Na_2SO_4. The product alcohol that is, R(-)-(1) as well as unconverted substrate was recovered by removing solvent in vacuo and the crude yield determined. The extent of hydrolysis was monitorred by subjecting the mixture to gas chromatographic analysis using an OV 101 column at 110°C (Fig.2).

In case of water-miscible solvent addition, the broth after reaction was saturated with NaCl before solvent extraction.

The desired R(-)-(1) alcohol was separated from the crude mixture by silica gel column chromatography. Elution with 5% ethyl acetate

in petroleum ether gave the unconverted acetate. Further elution gave the pure alcohol. The isolated yields thus obtained were very good (of the same order as expected from GC analysis). The alcohol had a melting point of 79-80°C.

The optical rotation of the isolated alcohol was measured with an automatic digital polarimeter DIP 181 (JASCO) at ~23°C.. Solvent used was chloroform (AR Grade). The specific rotations were estimated using the following equation:

$$[\alpha]_D = \frac{[\alpha]_{obs}}{l \times C}$$

where $[\alpha]_{obs}$ is the observed rotation; l is the light path in dms and c, concentration of alcohol in g/100 ml. The reported $[\alpha]_D$ for the R(-) alcohol is -12.1.

The enantiomeric excess (e.e.) which is the excess of the desired isomer over the undesired one is given by:

$$e.e = \frac{[\alpha]_D \text{ obtained}}{[\alpha]_D \text{ of pure isomer}} \times 100$$

and is a reflection of the optical purity of the compound.

Assay of hydrolytic activity

When the effect of various parameters on hydrolytic potential was studied, conditions for growth and enzyme production) were varied keeping conditions for conversion constant. Thus 0.1 ml of the substrate was dispensed into the reaction medium followed by incubation on a rotary shaker for 1 hour and subsequent solvent extraction. The extent of hydrolysis was then monitored by gas chromatographic analysis, the percentage GC conversion being the index of extent of hydrolysis.

RESULTS AND DISCUSSION

Screening for a suitable biocatalyst

The general strategy for obtaining a suitable biocatalyst is to screen the available biocatalysts for one which gives the highest rate of conversion and highest enantioselectivity for the desired reaction. Since it was desired to enantioselectively hydrolyse the (±)-(2) acetate which is insoluble in aqueous media, a survey of existing similar biotransformations was done. It was found that certain biocatalysts (lipases and microorganisms of Bacillus and Pseudomonas sp. effectively bring about asymmetric hydrolyses of many substrates[112-114].

Thus, some cultures were chosen and screened for hydrolysis of (±) - (2) to give (-)-(1). The reaction was carried out with 0.5 ml of the substrate each in 100 ml culture broths for 48 hours. The results obtained are presented in Table 1.

S.No.	Biocatalyst	% GC conversion
1.	Pseudomonas aeruginosa 2200	25
2.	Pseudomonas lemoneri 2060	29
3.	Bacillus subtilis 2010	45
4.	Pig pancreatic lipase	-

Table 1: Screening for a suitable biocatalyst

As seen from Table 1, the enzyme, PPL did not exhibit any hydrolytic activity whereas all the chosen microorganisms did. The culture

B.subtilis 2010 was found to exhibit the highest conversion percentage, besides showing good isolated yields and desired optical selectivity. It was therefore used as the culture of choice for all subsequent experiments. The genus Bacillus is known to secrete many hydrolytic enzymes like esterases, lipases and proteases.

Effect of age of culture on hydrolytic activity

0.5 ml of the substrate was added to B.subtilis cultures after 0 (inoculation time), 24 and 48 hours of growth. Results obtained are summarized in Table 2. All reactions were carried out for 48 hours.

Time	% conversion
0	2.3
24	45
48	10

Table 2. EFfect of cell age on extent of conversion

As seen from Table 2, best hydrolytic activity was associated with a 24 hour old culture whereas almost negligible hydrolysis was observed when substrate was added along with inoculum. The substrate seems to have an inhibitory effect on cell growth or metabolism. Therefore, it is better to separate growth of culture and bioconversion.

In order to get a better picture of the effect of cell age on hydrolytic potential of the culture, 50 ml cultures were grown for various time intervals upto 48 hours and their biomass and hydrolytic activity

monitored.

The growth pattern of B.subtilis as seen in Figure 3 shows a typical lag, log, exponential and stationary phase of growth. After an initial period of slow growth (lag) for 2 hours or so, there is a period of exponential growth upto 12 hours wherein biomass (wet weight of cells) increases continuously. The biomass is maintained constant in the stationary phase upto 24-26 hours followed by a slight decline.

Hydrolytic activity of broth as compared to that of cells suspended in buffer and supernatant is shown in Figure 4. Each curve shows an increasing activity followed by a subsequent decline. The hydrolytic activity of the broth as a whole is generally the highest with a peak at around 24 hours. Also, the cells seem to possess higher activity than the supernatant fraction.

Thus, the hydrolytic activity increases from the exponential to stationary phase after which it declines. In the initial growth phase, most of the hydrolytic activity is associated with the cells. As growth progresses, however, probably more and more enzyme is secreted into the medium, though the cells still retain a higher potential for hydrolysis. In the last stationary phase, there is almost negligible hydrolytic activity associated with the supernatant and most of the broth activity is retained by the cellular fraction. The free enzyme in solution probably denatures or is broken down by other proteolytic enzymes released by the cells in late stationary phase. Many enzymes are known to be membrane bound in young cells being released as exo-

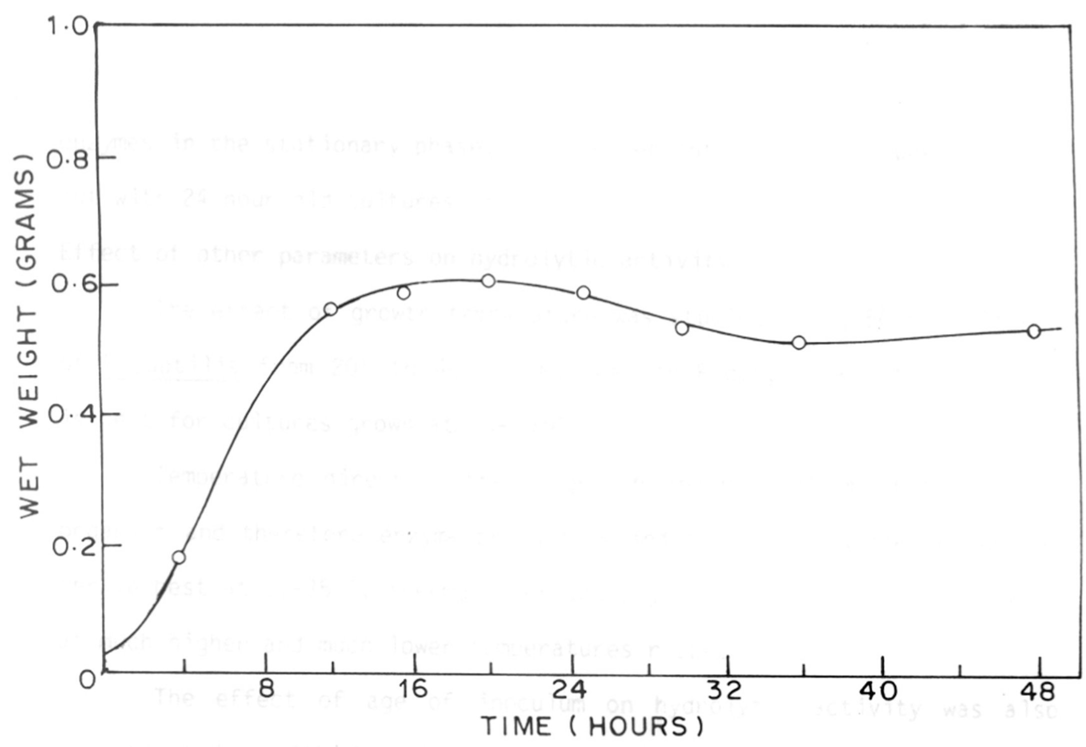


FIG. 3. GROWTH CURVE OF <u>B. subtilis</u>

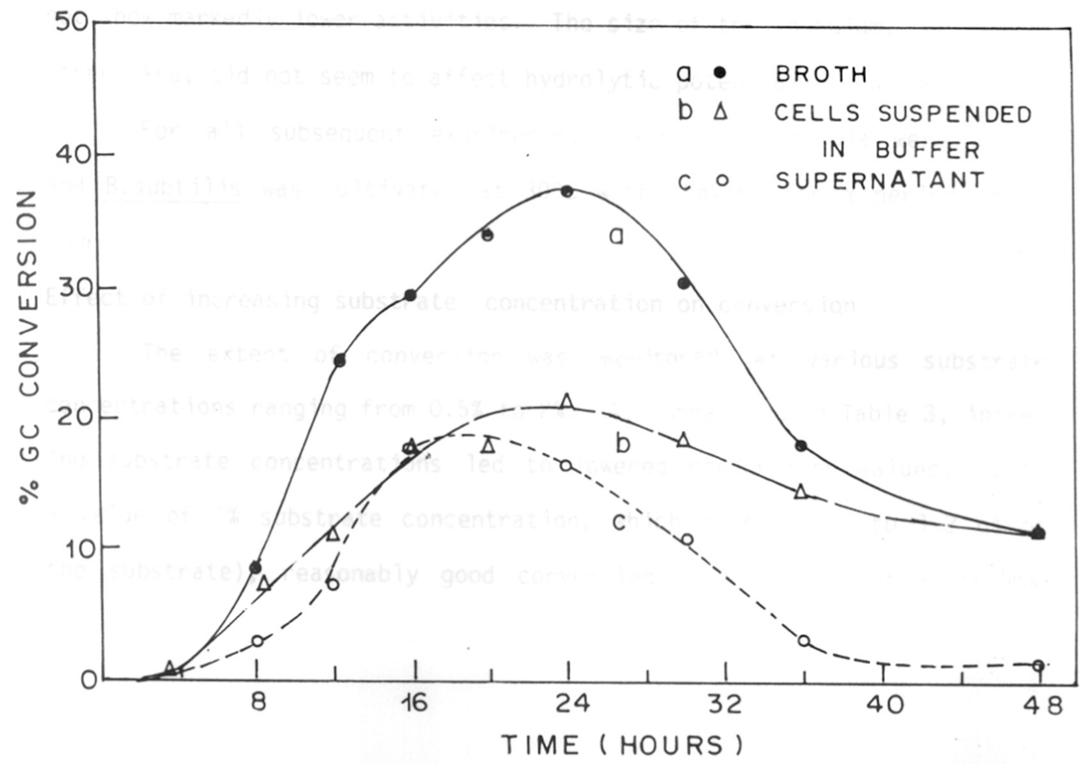


FIG. 4. DISTRIBUTION OF HYDROLYTIC ACTIVITY VS TIME

enzymes in the stationary phase. All subsequent experiments were carried out with 24 hour old cultures of B.subtilis.

Effect of other parameters on hydrolytic activity

The effect of growth temperature was studied using 50 ml cultures of B.subtilis from 20° to 40°C. As seen in Fig.5, hydrolytic activity is best for cultures grown at 25-30°C.

Temperature directly affects growth and metabolism of the micro-organism and therefore enzyme production and activity. While mesophiles thrive best at 20-35°C, thermophiles and psychrophiles are best cultivated at much higher and much lower temperatures respectively.

The effect of age of inoculum on hydrolytic activity was also investigated. With inocula of ages varying from 12 to 20 hours, best results were obtained with 14-16 hour old inocula though the rest did not show markedly lower activities. The size of the inoculum, on the other hand, did not seem to affect hydrolytic potential of culture.

For all subsequent expriments, 14-16 hour inocula were used and B.subtilis was cultivated at 30°C with shaking for a period of 24 hours.

Effect of increasing substrate concentration on conversion

The extent of conversion was monitored at various substrate concentrations ranging from 0.5% to 2%. As summarized in Table 3, increasing substrate concentrations led to lowered conversions values. Upto a value of 1% substrate concentration, which corresponds to 1.2 gs of the substrate), reasonably good conversions ($\geqslant$ 40% out of a maximum

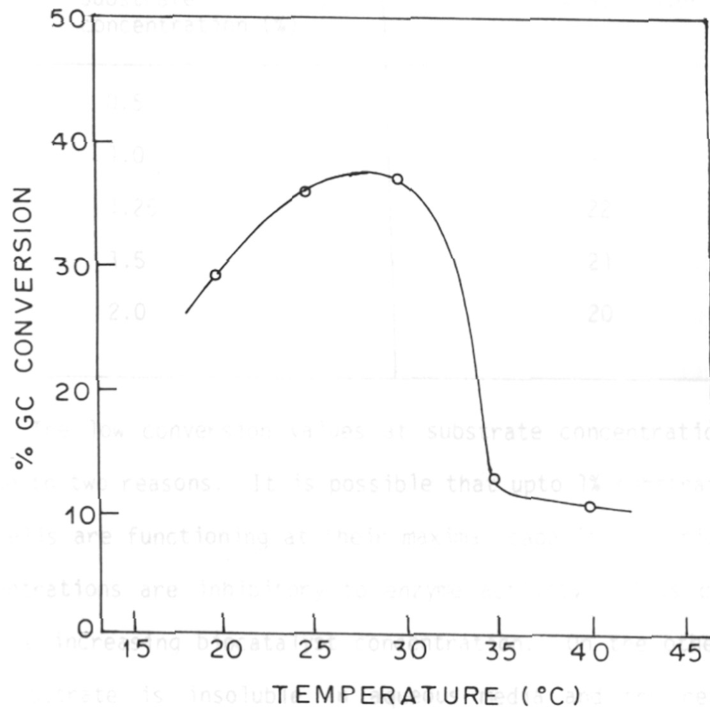


FIG. 5. EFFECT OF TEMPERATURE ON ENZYME PRODUCTION

of 50%) were obtained. The isolated yields were in close agreement with those predicted from the GC values and the optical purities remained high (⩾97% .e.e.).

Table 3. Effect of substrate concentration on conversion

Substrate concentration (%)	% GC conversion
0.5	46
1.0	40
1.25	22
1.5	21
2.0	20

The low conversion values at substrate concentrations >1 could be due to two reasons. It is possible that upto 1% substrate concentration, the cells are functioning at their maximal capacity and higher substrate concentrations are inhibitory to enzyme activity. This could be overcome by increasing biocatalyst concentration. On the other hand, since the substrate is insoluble in aqueous media and the reaction has to to occur at the aqueous organic interface, there are mass-transfer or diffusional limitations that hamper conversion. Thus, improving substrate solubility should help improve conversion values.

For any microbial resolution process to be economically attractive, product end concentration should be a few grams per litre[116]. Thus, it is of prime importance to improve the conversions at higher substrate

concentrations.

Distribution of hydrolytic activity vs substrate concentration

The distribution of hydrolytic activity in different fractions of culture broth with increasing substrate concentrations from 0.5-1.5% was investigated. As seen in Fig. 6, at low substrate concentrations (0.5%), most of the hydrolytic activity exhibited by the culture broth was associated with the cells while supernatant showed markedly lower activity. Although, this held true at and above substrate concentrations of 1%, it was observed that the conversion obtained with whole broth is approximately equal to that obtained from the sum of conversions-associated with cellular and supernatant fractions. Although alcohol obtained from all fractions showed high optical purity, the optical purity associated with the cellular fraction was in general marginally higher (Table 4).

Table 4. e.e. values of broth fractions vs substrate concentration

Substrate concentration (%)	e.e. values		
	Culture broth	Cells suspended in buffer[a]	Supernatant
0.5	98	100	98
1.0	100	100	93
1.25	97	100	-
1.5	97	-	-

a- $[\alpha]_D$ obtained were slightly higher than reported $[\alpha]_D$
This could be due to a difference in temperature

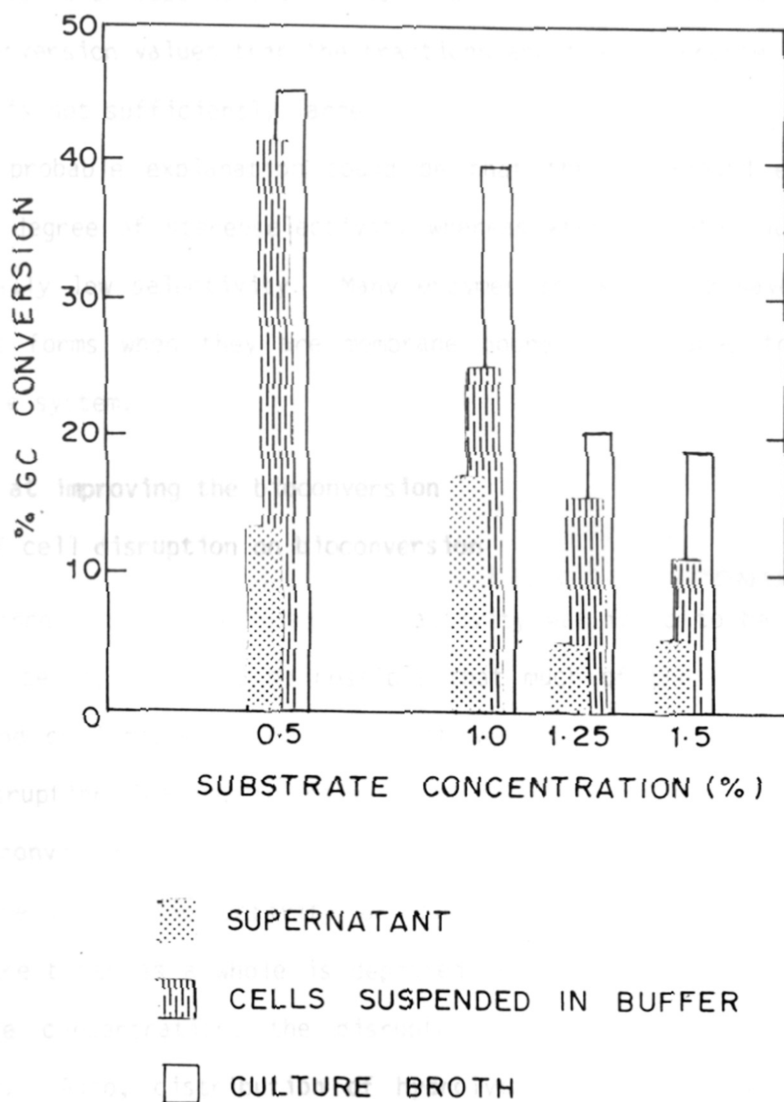


FIG. 6. DISTRIBUTION OF HYDROLYTIC ACTIVITY WITH INCREASING SUBSTRATE CONCENTRATION

From this experiment, it is clear that the broth as a whole is better as a reaction medium for carrying out the biotransformation rather than the cells or supernatant. This is because it consistently exhibits higher conversion values than the fractions and the difference in optical purities is not sufficiently large.

A probable explanation could be that the cell-bound enzyme has a higher degree of stereoselectivity whereas when secreted out, it has a marginally low selectivity. Many enzymes are known to have slightly different forms when they are membrane bound as compared to excreted out of the system.

Attempts at improving the bioconversion

Effect of cell disruption on bioconversion

Since much of the hydrolytic activity was found to be associated with the cells, it could be possible that much of the enzyme remained cell-bound or intracellular. Thus, it was of interest to know whether cell disruption leading to release of the intracellular enzyme would improve conversion of substrate.

The hydrolytic activity of disrupted cells relative to that of culture broth as a whole is depicted in Fig. 7. Both at 1 and 1.5% substrate concentration, the disrupted cells showed lower hydrolytic activity. Also, distribution of hydrolytic activity between the cell debris and supernatant obtained on cell disruption and separated by centrifugation when investigated (Fig.8) shows that there is an increase in hydrolytic activity of the supernate and a marked fall in conversion associated with the cell debris as compared with that of the cells.

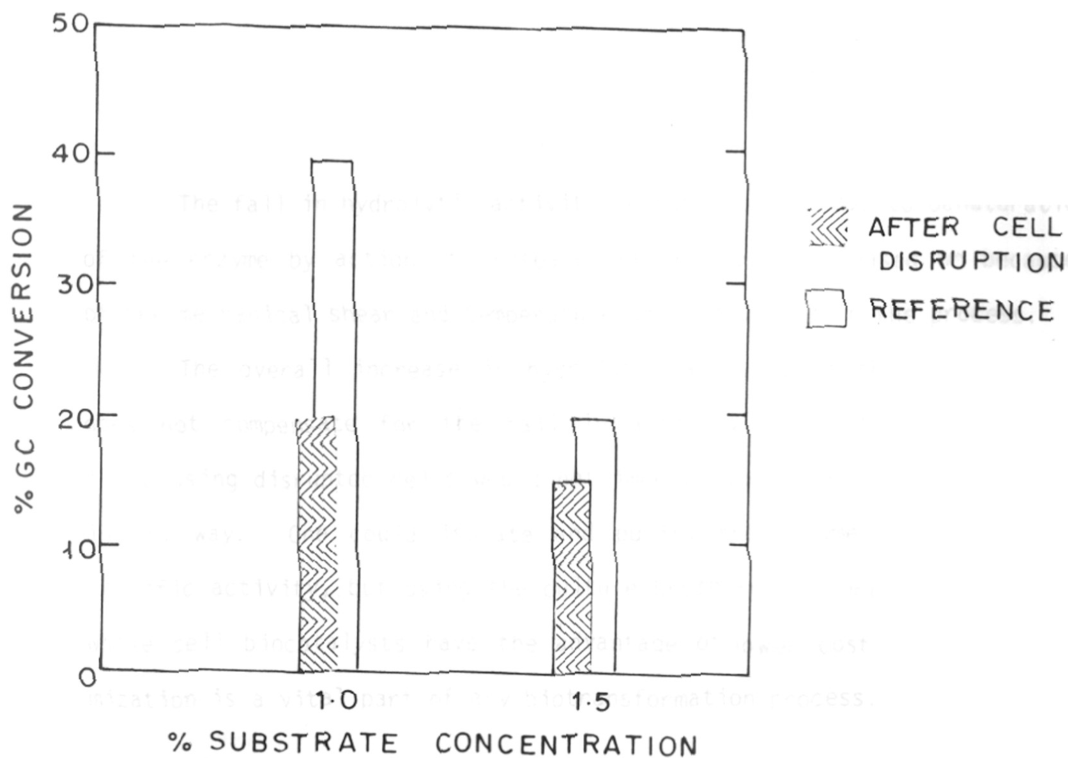


FIG. 7. EFFECT OF CELL DISRUPTION ON CONVERSION %

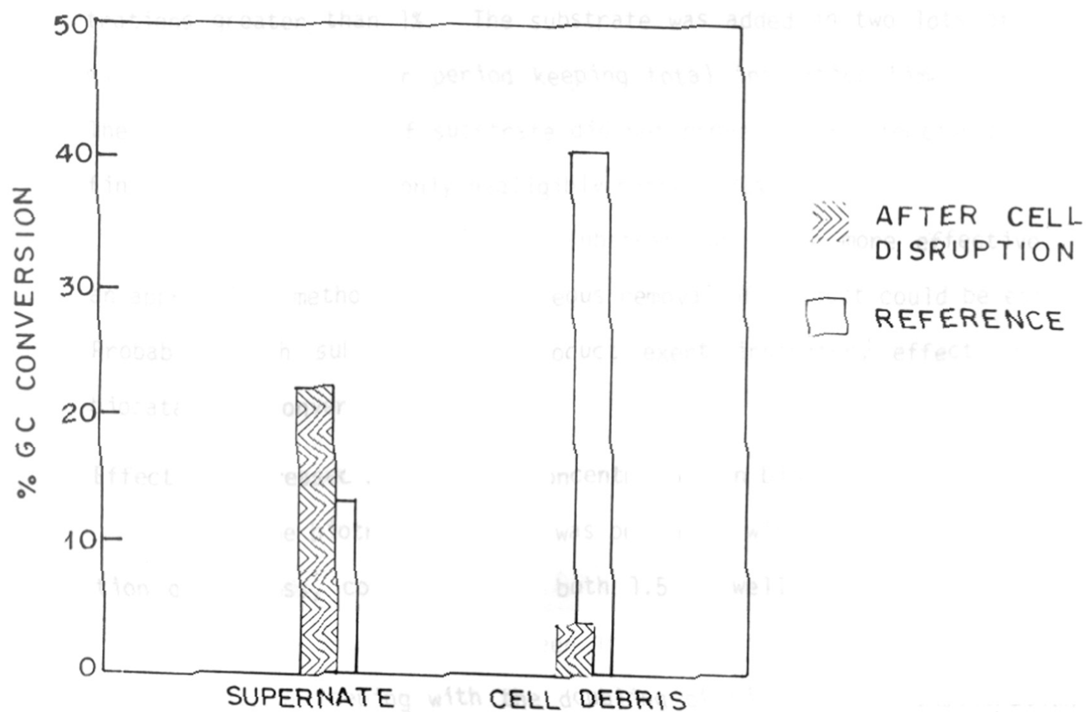


FIG. 8. DISTRIBUTION OF HYDROLYTIC ACTIVITY AFTER CELL DISRUPTION

The fall in hydrolytic activity probably occurs due to denaturation of the enzyme by action of proteases which may be released or because of the mechanical shear and temperature shock involved in the process.

The overall increase in hydrolytic activity of the supernantant does not compensate for the fall in activity associated with cells. Thus, using disrupted cells would not improve upon the biotransformation in any way. One could isolate and purify the enzyme to improve the specific activity, but using the culture broth seems adequate. Besides, whole cell biocatalysts have the advantage of lower cost and cost minimization is a vital part of any biotransformation process.

Effect of stepwise substrate addition on bioconversion

Efforts were directed at improving conversions at substrate concentrations greater than 1%. The substrate was added in two lots of 0.75 ml each over a 24 hour period keeping total incubation time constant. The stepwise addition of substrate did not prove to be effective because final conversions were only negligibly higher (22%).

The batchwise addition of substrate would be more effective if an appropriate method for simultaneous removal of product could be established. Probably, both substrate and product exert inhibitory effect on the biocatalytic conversion.

Effect of increased biocatalyst concentration on bioconversion

When the biotransformation was performed with increased concentration of biomass, conversions at both 1.5 as well as 2% substrate concentration were increased. As seen in Fig.9, the increase in conversion was twofold in keeping with the doubling of biocatalyst concentration.

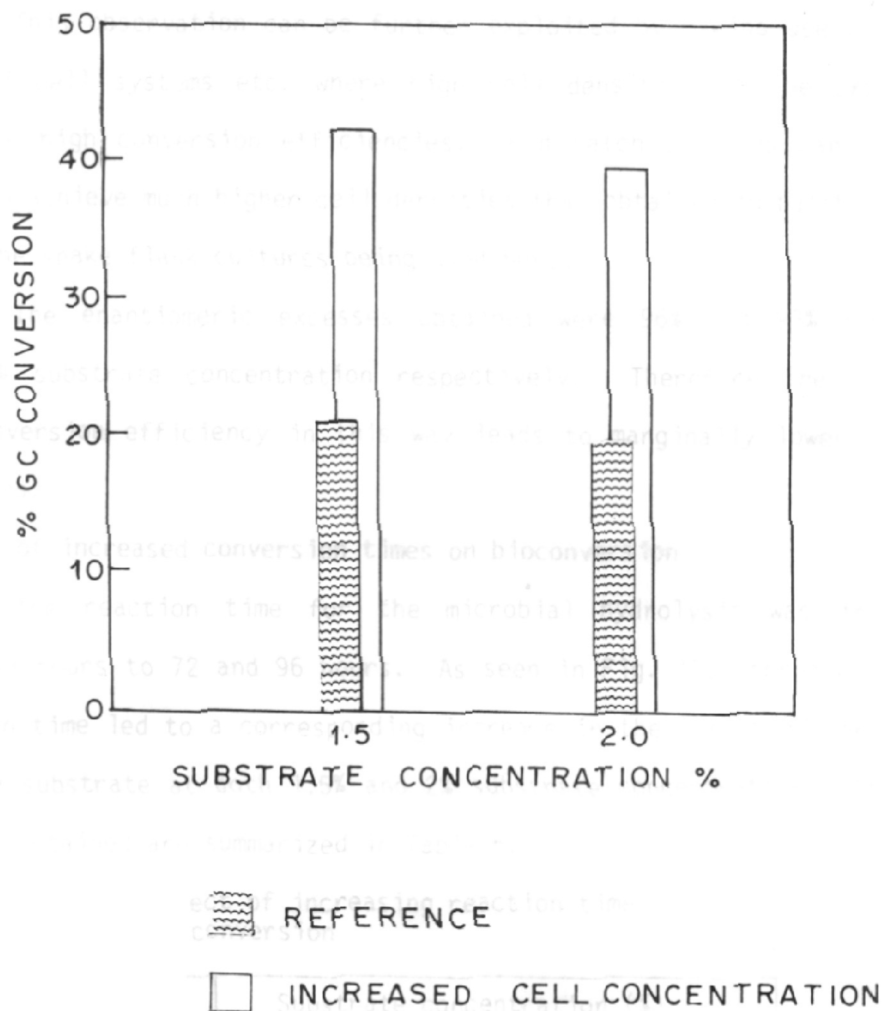


FIG. 9. EFFECT OF INCREASING CELL CONCENTRATION ON % CONVERSION

In effect, the cell:substrate ratio has been maintained constant. But the volumetric productivity(grams of product per unit volume per hour) has been improved.

This observation can be further exploited by making use of immobilized cell systems etc. where high cell densities can be exploited to give high conversion efficiencies. Fed batch cultures can also be used to achieve much higher cell densities than obtained in batch systems like the shake flask cultures being used here.

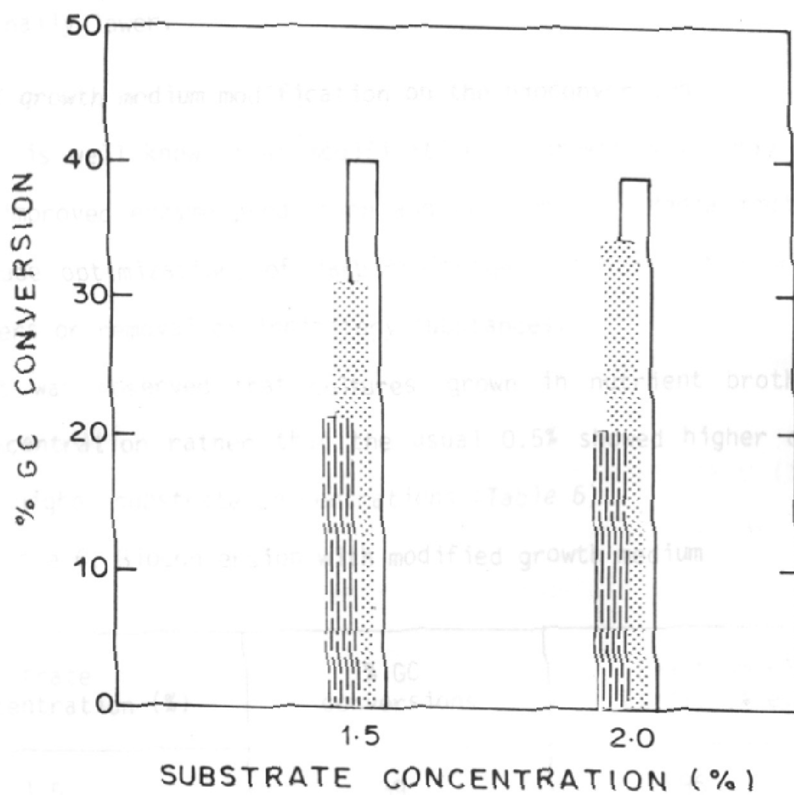
The enantiomeric excesses obtained were 96% and 93% for 1.5% and 2% substrate concentration respectively. Therefore the increase in conversion efficiency in this way leads to marginally lower optical purities.

Effect of increased conversion times on bioconversion

The reaction time for the microbial hydrolysis was increased from 48 hours to 72 and 96 hours. As seen in Fig. 10, increase in conversion time led to a corresponding increase in the extent of conversion of the substrate at both 1.5% and 2% substrate concentration. The e.e. values obtained are summarized in Table 5.

Table 5. Effect of increasing reaction time on conversion

Time	Substrate concentration (%)	
	1.5	2
48	97	95
72	96	96
96	94	95



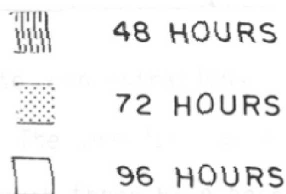


FIG. 10. EFFECT OF INCREASING CONVERSION TIME ON % CONVERSION

As seen from the Table, increasing conversion times, in general lead to lowered optical purities. Thus, although increasing conversion times improves the extent of conversion, the optical purities obtained are marginally lower.

Effect of growth medium modification on the bioconversion

It is well known that modification of growth media may sometimes lead to improved enzyme production and or biomass. Media modifications may include optimizations of carbon:nitrogen nutrient ratio or addition of inducers or removal of inhibitory substances.

It was observed that cultures grown in nutrient broth with 1% NaCl concentration rather than the usual 0.5% showed higher conversion rates at higher substrate concentrations (Table 6).

Table 6. Bioconversion with modified growth medium

Substrate concentration (%)	% GC conversions	Enantiomeric Excess (% e.e.)
1.5	39	96
2.0	38	98

Also at higher substrate concentrations (2%), the e.e. values were not adversely affected. The precise cause for this observation remains to be investigated. Though there have been reports[117] of improved enzyme production (lipases) from certain strains of Bacillus with increasing NaCl concentration, the increase may also be attributable to changes in permeability of the cell membrane.

Attempts at improving substrate solubility

Another approach used in order to improve conversions at higher substrate concentrations was to try and enhance substrate solubility. Substrate solubility can be improved by various means.

Effect of Triton X-100 addition on the bioconversion

The most commonly used stratgegy is the addition of surfactants for homogenous dispersion of the insoluble compound in the aqueous medium. Surfactants owe their unique property of solubilization to their amphipathic nature. Since they have both hydrophilic as well as hydrophobic they cause emulsion formation leading to better contact between aqueous and organic phases.

When the compound dispersed along with surfactant (Triton X -100) was added to the reaction medium, and the conversions monitored at 1.5 and 2.0% substrate concentration, a two-fold increase in conversion was obtained (Fig. 11). Similar improvements in biotransformations using surfactants have also been reported[118]. The enantiomeric excesses obtained were 97% and 98% for 1.5 and 2% substrate concentration respectively.

A number of cationic, anionic and nonionic surfactants are known. The effect of incorporating some of these on the bioconversion could be further investigated.

Effect of addition of organic solvents on the bioconversion

Very poorly soluble substrates such as steroids and fats have been enzymatically transformed in the presence of organic solvents. Moreover, both the enzymes as well as intact cells can be used as cata-

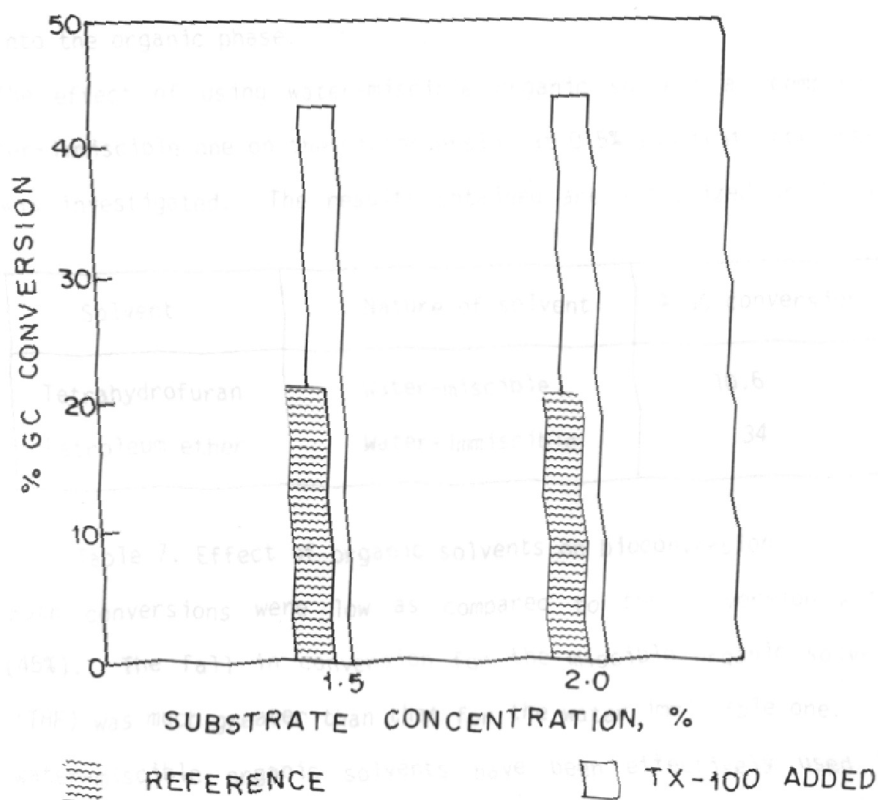


FIG. 11. EFFECT OF ADDITION OF SURFACTANT (TX-100) ON % CONVERSION

lysts[119]. In addition to solubilizing an insoluble substrate, use of organic cosolvents may help overcome substrate or product inhibition as the biocatalyst remains in the aqueous phase where the reaction occurs whereas most of the substrate as well as the product is partitioned into the organic phase.

The effect of using water-miscible organic solvent as compared to a water-immiscible one on the bioconversion at 0.5% substrate concentration was investigated. The results obtained are summarized in Table 7.

Solvent	Nature of solvent	% GC conversion
Tetrahydrofuran	Water-miscible	10.6
Petroleum ether	Water-immiscible	34

Table 7. Effect of organic solvents on bioconversion

Both conversions were low as compared to the conversion with broth (45%). The fall in conversion for the miscible organic solvent system (THF) was much greater than that for the water immiscible one.

Water-miscible organic solvents have been effectively used in synthesis of esters and peptides and also for increasing the activity of some hydrolytic enzymes[120]. They are most effective at modest concentrations (10-30%) and suppress enzyme activity at higher concentrations. Besides improving substrate solubility, they have also been used to enhance reaction rate and improve the stereoselectivity of biocatalysts[121,122].

In case of whole cell biocatalysis, miscible solvents may have a toxic effect by disruption of cell membranes and protein denaturation. Cell viability is affected by solvent polarity and properties. Immobilization of cells may prove useful for stabilization against water-miscible organic solvents.

More often, whole cell biocatalysis in biphasic sytesms i.e. water-immiscible organic solvent mixtures proves useful[123,124]. In general, the more non-polar the solvent, the less toxic it is to the biocatalyst as it has a lesser tendency to strip water molecules from the biocatalyst leading to protein denaturation.

Probably using a more hydrophobic solvent (with a higher logP value)[125] may improve the conversion efficiency. This could also be investigated. Furthermore, the advantages of immobilization and biphasic systems have been combined by using biocatalyst immobilized on photocross-linkable resins or polyurethane prepolymers of varying hydrophobicity. Stereospecific hydrolysis of d,l-methyl acetate using immobilized B.subtilis has also been reproted.

Effect of β-cyclodextrin addition on the bioconversion

More recently, a third strategy has been used to enhance substrate accessibility to the biocatalyst. This is the area of cyclodextrin-aided bioconversions.

Cyclodextrins are cyclic oligosaccharides enzymatically produced from starch, α,β and γ cyclodextrins are composed of 6,7 or 8 α1,4-linked D-glucose units respectively. Each cyclodextrin molecule is a torus (doughnut-shaped) with a hydrophilic shell and a more hydrophobic cavity.

Complete or partial inclusion of an insoluble susbtrate molecule into the internal hydrophobic cavity leads to solubilization of substrate and reduction in toxicity. Also, cyclodextrins are biocompatible and chemically inert and cause no damage either to free enzymes or to microbes. A number of cyclodextrin aided bioconversions have been reported[126].

It was observed that the addition of β-cyclodextrin alone, in the absence of the biocatalyst, did not cause hydrolysis of the substrate. Addition of increasing concentration of cyclodextrin in the bioconversion led to higher rate of hydrolysis (Table 8).

Concentration of cyclodextrin (mg %)	Substrate concentration (ml)	% GC conversion	Enantiomeric excess (e.e.)
50	1.5	32	99
100	1.5	37	99
150	2.0	30	96

Table 8. Effect of β-cyclodextrin addition on the bioconversion

As seen from Table 8 the addition of β-cyclodextrins did enhance the bioconversion though conversions obtained were not as high as those with Triton X-100. In general, the optical purities obtained were very high.

The incorporation of cyclodextrins was likewise found to enhance conversion rates with cell-free supernatant also (Table 9) at 0.5% substrate concentrations.

Concentration of β-cyclodextrin mg %	% G.C. Conversion
-	14
50	24.3
100	27

Table 9. Cyclodextrin-aided bioconversion with supernatant

Also, cyclodextrins can be recovered from the bioconversion medium by the use of suitable clathrate forming compounds to give insoluble complexes which can be heated to distill off the organic reagent leaving behind purified cyclodextrins. Use of cyclodextrins covalently bound to insoluble polymeric matrices such as cellulose would facilitate the integration of the bioconversion with product separation.

Study of the optical selectivity of the bioconversion

The initial rate of hydrolysis of the R and the S isomers was studied over a period of 2 hours. The results obtained are summarized in Table 10.

Time (mins)	% GC Conversions		
	R isomer	S isomer	Racemic acetate
20	13.3	0	13.8
40	25.2	0	26.8
80	38	0	41.8
120	97	0.2	50

Table 10. Rate of hydrolysis of R & S acetates vs racemic acetate

Even when the reaction time was increased to 3 hours and 4 hours, although the R isomer was completely hydrolysed (100%), there was negligible hydrolysis of the S acetate (0.4 and 0.6% respectively). When the same reaction with S acetate was carried out with culture broth (50 ml) for 24 hours and 48 hours, conversions obtained were 6% and 13% respectively.

The microbial resolution is highly stereoselective, in fact it can be said to be stereospecific when carried out under optimal reaction conditions. It is important to stop the reaction at 40-45% conversion. This is because, the optical selectivity is due to the difference in the rates of hydrolysis of the two isomers. The R isomer is preferentially hydrolysed over the S isomer. Even in the absence of the R isomer and at very low substrate concentrations (0.2%), the rate of hydrolysis of the S as compared to the R is very low.

This also explains the lowered optical purities with increasing reaction times.

CONCLUDING REMARKS

The efficiency of the biotransformationat increased substrate concentration was improved using increased biocatalyst concentrations, increased conversion times, modified growth medium and solubilization of the in-soluble substrate with a surfactant. Each showed reasonably good yields (very close to those estimated from the GC conversion values). The optical purities obtained were in the range of 93-99%. Best optical purities were shown by cyclodextrin-aided bioconversion but the conversions were comparatively low. In general surfactant incorporation resulted in the best combination of conversion (43%) and optical purities (97-98%).

Thus good conversions and optical purities were obtained for twice the initial substrate concentration. 2% of the substrate (2.4 gs) could thus be acted upon to obtain 0.8 gs of pure R(-) alcohol. Biotransformation processes for speciality chemicals that operate at 1% product recovery are known.

Besides, using these strategies, the substrate concentrations could be increased still further and probably good results could be obtained even with 4-5% substrate concentrations. Combinations of the various strategies could also be investigated.

Observations such as improved bioconversions with increased biocatalyst concentration could be utilized to develop immobilized systems where conversions could be carried out at much higher substrate concentrations

with higher cell densities. Polyurethane-immobilized cells have been widely used to achieve the same goal.

The microbial resolution of (±)-(1) besides being highly stereoselective (stereospecific under appropriate conditions) is also cheap, extremely simple to operate and probably amenable to easy scale-up.

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