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Ecofriendly Chirotechnologies: Approaches Utilizing Immobilized Biocatalysts

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Abstract: Study of production of L-aspartic acid using immobilized E.coli cells and synthesis of the dipeptide sweetener Aspartame by using immobilized proteases in organic media are presented.

The growing awareness of the importance of chirality in diverse fields, in conjunction with stringent environmental regulations has led to increasing demand for ecofriendly technologies for the synthesis of enantiomerically pure compounds. Amongst various possibilities, biocatalysts especially in their immobilised form have become economically and environmentally most attractive as they catalyse reactions with high regio and stereoselectivity under mild conditions and can be used repetitively.

These immobilized biocatalysts generally consist of enzymes, cells or organelles (or combination of them) which are in a state, that they can be used repeatedly. Immobilized biocatalysts are more stable than their free counter parts under the reaction conditions. Most of the techniques and principles applied to immobilized enzymes are also applicable to immobilized whole cells(ref. 1).

I. CONTINUOUS PRODUCTION OF L-ASPARTIC ACID USING IMMOBILISED E.COLI CELLS :

The utility of immobilized microbial cells for the continuous production of amino acids, the steps involved and the crucial parameters are discussed with respect to L-aspartic acid **1.**

L-Aspartic acid is used as a pharmaceutical and food additive. The world market for this amino acid is ever increasing since its direct application as a raw material for `Aspartame' a low calorie sweetener.

In principle, the synthesis of L-aspartic acid involves the conversion of ammonium fumarate to β-monoammonium L-aspartate in the presence of the enzyme aspartase (ref.2) (Scheme. 1).

The process involves the following major steps.

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*Invited lecture presented at the International Conference on Biodiversity and Bioresources: Conservation and Utilization, 23–27 November 1997, Phuket, Thailand. Other presentations are published in *Pure Appl. Chem.*, Vol. 70, No. 11, 1998.

1. Culturing of the organism under different growth conditions for maximum enzyme activity:
The cells were grown in specialised media such as Minimal, Luria-bertani (LB) Yeast extract (YT) Terrific broth (TB), corn steep liquor etc. The cell yield and aspartase activity of cells were determined at different time points to achieve maximum yield and enzyme activity after conducting various experiments in selection of the medium and also by taking into consideration the indigenous availability of the raw materials. Maximum rate of growth was supported by TB medium and a cell yield of 9 g/Lt of the medium was achieved by growing the cells to their log phase in shake flasks.

2. Harvesting of cells and study of their stability:
After harvesting the cells by centrifugation, they were suspended in a medium and stored at 4^0C before immobilisation. In some of the experiments cell lysis was observed with resuspended and/or stored cells. Since this would be an important factor, it was studied in detail with different resuspension media and storage conditions. Storage of cells was found to adversely effect immobilisition.

3. Immobilisation of E.coli cells:
Though we have made attempts to use polymers for entrapment such as agar. etc. polyacrylamide gel which is a widely used methodology was chosen in our studies.

Immobilisation of cells was carried out in presence of acrylamide-bisacrylamide, ammonium persulfate and TEMED. Conditions have been optimised for obtaining gels of consistent rigidity since gel rigidity was important for granulation.

*4. Preparation of immobilised cell column and production of L-Aspartic acid **1:***
Continuous packed bed reactor was used and fed continuously with the substrate ammonium fumarate from its top. Effect of initial substrate concentration and flow rate was investigated on the efficiency of conversion of fumaric acid to aspartic acid .The stability of the column was studied over a period of three months. In a typical experiment, the life of the column was reduced nearly one third when the space velocity was enhanced from 0.6 to 1.2.

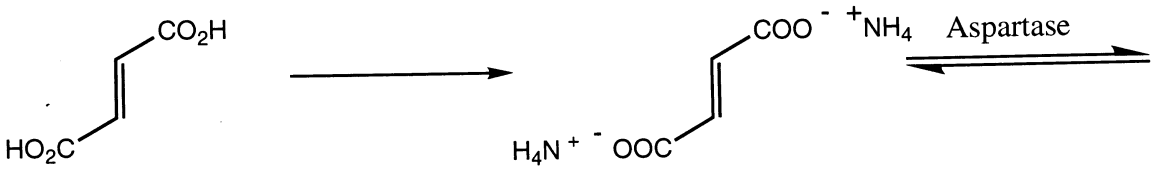
The process was standardised to prepare high purity L-aspartic acid at a rate of 30g/d/g of E.coli cells. The studies were conducted to the level of producing a total of 1.5 kg/day. Several batch preparations were made and were compared with the standard reference sample from Sigma.

II. SYNTHESIS OF PEPTIDES IN ORGANIC MEDIA:

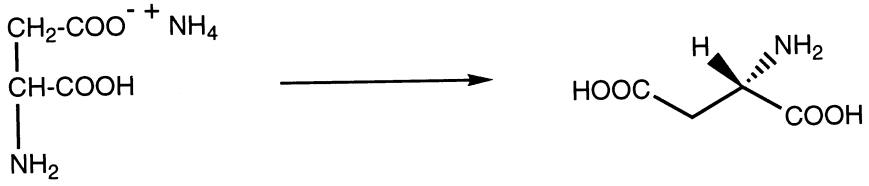
The demonstration that peptides are involved in the regulation and maintenance of many biological processes has led to advances in medicine, biochemistry and of late, biotechnology. As a result, peptides represent new opportunities as drugs, diagnostic reagents, agricultural chemicals and food additives. As a consequence of this, the area of peptides has become a growing field of interest to develop methodologies for obtaining peptides in high yield, purity and in large scale. The enzymatic methodology has become a promising area in peptide synthesis with the increasing use of proteases in organic solvents. The use of organic media offers good solubility for

hydrophobic substrates and helps for reversal of hydrolytic reactions. In addition, undesirable side reactions can be suppressed in condensation reactions. In this case, the driving force for the synthesis is the low water activity rather than the precipitation of the product.

Scheme 1



Fumaric acid



β - Mono ammonium L - Aspartate

L-Aspartic acid

1

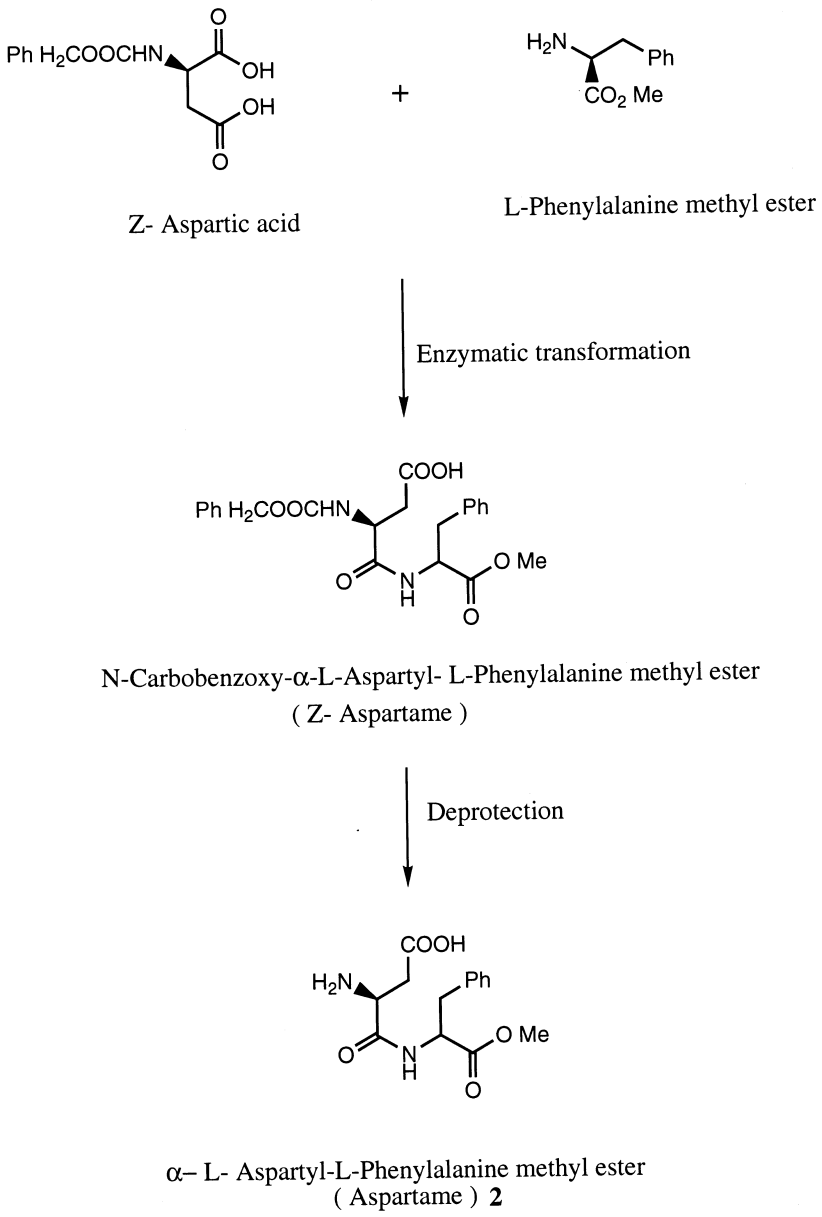
Synthesis of L - aspartic acid **1**

Having synthesised L-Aspartic acid, our attention was drawn to Aspartame **2,** a dipeptide of L-aspartic acid and L-phenylalanine methylester. It is a low calorie sweetener which is 200 times sweeter than sucrose. It is also a safe sweetener as its biotransformation follows pathway of common amino acids. The synthesis of `Aspartame' by the enzymatic method as shown on scheme 2 has received considerable attention (ref. 3) as it has several advantages over the chemical method such as less number of operations, higher yields and high selectivity.

It was shown that the enzyme 'Thermolysine' obtained from a strain of Bacillus, 'Bacillus Thermoproteolyticus Rokko' isolated from a Japanese hot spring, catalyses the formation of peptides (Ref. 3a). This enzyme shows high catalytic activity, has marked stability against heat, organic solvents and extreme pH and is devoid of any esterase activity.

It was found that Thermolysine catalyses the condensation of N-protected L-aspartic acid and L-phenylalanine methyl ester in high yield. (Ref. 3a). The reaction is specific only on the α-carboxyl group of L-aspartic acid, even though the side-chain carboxyl is unprotected. (Scheme 2)

Scheme 2



Synthesis of aspartame **2**

The utility of `Thermolysine' for the synthesis of Aspartame has been attempted by depositing it on supports with low aquaphilicity as the driving force in this case is low water activity. It was also established that the enzymes when deposited on supports with low aquaphilicity expressed higher catalytic activity than supports with higher aquaphilicity (Ref 4). Hence, in the present investigation the enzyme deposited on supports such as controlled pore glass derivatives (CPG), polyamide supports, celite etc. has been studied for the synthesis of `Aspartame'.

The synthesis of aspartame involves the following sequence of reactions.

i. Synthesis of Z-aspartic acid from L-aspartic acid
ii. Synthesis of L-phenylalanine methylester from L-phenylalanine
iii. Enzymatic transformation of Z-aspartic acid and L-phenylalanine methylester to Z-aspartame.
iv. Conversion of Z-aspartame to `Aspartame' by catalytic hydrogenation.

The reactions were carried out under the following conditions,

Phenylalanine methylester was taken in organic solvents such as ethylacetate, methanol acetonitrile, dimethyl formamide etc. containing buffers such Mcllvaine, Tris-HCl, MES etc followed by Z-aspartic acid and the immobilised enzyme. It was incubated at different temperatures for various time intervals and the ideal temperature appeared to be 40^0C. Phenyl alanine methylester was always taken in excess as these aminoacid esters are usually poor nucleophiles in low water content systems and this allows the reaction to be taken in the forward direction. Different ratios of phenylalanine methylester have also been attempted for optimisation of yields. The best results were obtained when phenylalanine methylester and Z-Aspartic acid were taken in the ration of 2:1.Various concentrations of the enzyme were immobilised to see its effect on the formation of the product. The yields of Z-aspartame obtained were in the range of 80-90%. Thus we are able to achieve the synthesis of the precursor of `Aspartame' ie., Z-Aspartame in high yields by immobilising the enzyme `Thermolysine' on supports with low aquaphilicity in organic solvents. Further work by cross linking the immobilised enzyme to increase the turnover cycles is in progress. The conversion of Z-Aspartame to Aspartame was carried out by catalytic hydrogenation in prensence of 5% Pd-C (ref-5).

We have also undertaken work with easily accessible and inexpensive proteases such as papain though it exhibits esterase activity. The reactions were carriedout at controlled water activity to overcome the esterase reactions. The enzyme was immobilised on supports with low aquaphilicity and the reactions were carriedout in various organic solvents. The formation of Z-aspartame was observed in the range of 30-35%. Further work to improve the yields and to assess the viability of the process for commerical application is under study.

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