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A NOVEL TEA-BAG METHODOLOGY FOR ENZYMATIC RESOLUTIONS OF α-AMINO ACID DERIVATIVES IN REVERSE MICELLAR MEDIA.

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Abstract: A novel tea bag methodology for resolution of methyl esters of N-acetyl-α-amino acids in reverse micellar medium of bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT) in isooctane-chloroform using immobilized enzymes or microbial cells is presented. The methodology effectively solves the problems of substrate solubility, product separation and surfactant recycling and provides products in high yields (80 to 90%) and excellent optical purities (% ee 97 to >99%).

Reverse micelles are macroscopically homogeneous and optically transperant spherical aggregates of water and surfactant dispersed in an apolar solvent . It is possible to solubilize macromolecules such as enzymes, proteins, nucleic acids and even whole cells in reverse micellar media without significant loss of their biological properties and significantly, the solubi-

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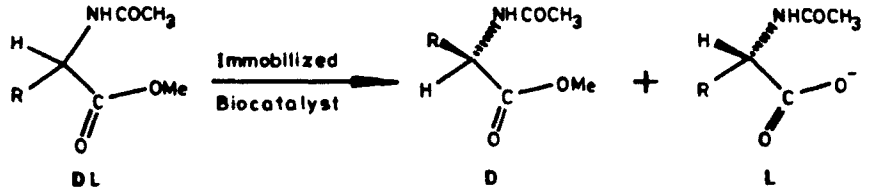
lized biocatalyst can accept highly hydrophobic substrates in these media[1-6]. This phenomenon is being exploited in our laboratory and elsewhere for biotransformations[7-9] . One of the major problems faced during enzymatic reactions in reverse micellar media, in particular the ester hydrolysis, is the pH drop in the medium during the reaction since one of the products is an acid. As the medium contains very little water (2-3%) it is not possible to maintain pH of the medium at pH-optimum (pH 7-8) even with a high concentration of buffer. Consequently, the local pH of the medium drops to a level at which the enzyme activity is almost negligible. This problem becomes a serious obstacle especially in case of preparative scale reactions. It is thus desirable to develop a methodology in which the product is continuously removed from the reaction medium while maintaining the local pH of the medium. For a possible use on industrial scale it is also necessary to separate the product from the surfactant solution easily and recycle the surfactant solution. Here we report an extremely simplified and quite a general tea bag procedure for using immobilized enzymes/microbes in reverse micellar media for resolutions with esterases and demonstrate it with a

representative example of resolution of racemic Na-acetyl-α-amino acid esters. In this novel methodology all the problems mentioned above have been solved by simple strategies. The enantiomeric purity of the products is very high (e.e. 97-99%) and the product recovery is also quite good (80-90%).

The reverse micellar system of bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT) in isooctane separates into aqueous and organic phases in presence of appropriate quantity of an electrolyte such as NaCl and KCl instead of forming a milky water-in-oil emulsion. Thus in our methodology (**Fig 1**) the L-form of the racemic ester substrate is first hydrolyzed in the reverse micellar medium with an immobilized biocatalyst highly specific for L-esters such as α-chymotrypsin immobilized in polyacrylamide[10] or baker's yeast (Saccharomyces cerevisiae NCIM 3044) immobilized in calcium alginate[8] with simultaneous extraction of the acid product into aqueous buffer containing electrolyte (Tris-HCl, pH 8.2, 1 M NaCl). This leaves optically pure D-ester in the organic layer along with the surfactant. Now, by using another biocatalyst specific for D-substrate (Pseudomonas putida IFO 12996 immobilized in calcium alginate) the D-ester present in the organic phase is hydrolyzed and the D-acid is ex-



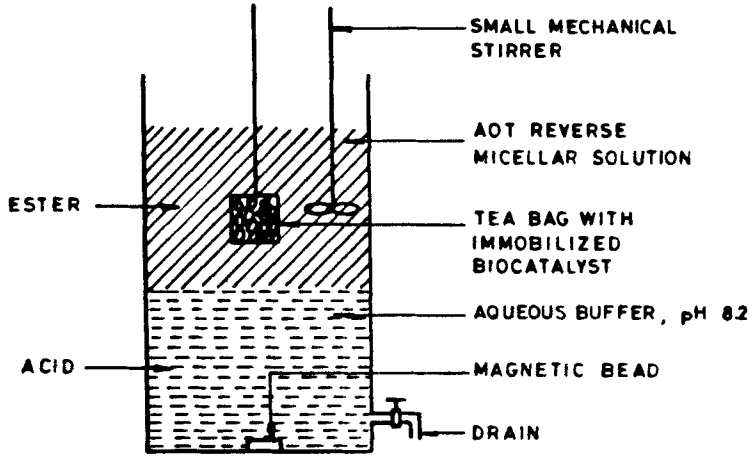


Fig.1: A Schematic Representation of Tea Bag Methodology for Resolution of α-Amino Acid Derivatives with Immobilized Biocatalyst in AOT Reverse Micellar Medium.

tracted in another batch of aqueous buffer[11]. Thus, at the end of reaction we have two resolved amino acids in separate aqueous solutions. The organic layer with the surfactant is left intact and hence can be recycled several times. The hydrolytic activity of the immobilized biocatalysts in reverse micellar media is re-

tained for a long time and hence these could also be recycled several times[8,10].

In conclusion, we may point out that the methodology has been successfully applied to the resolution of some of the common DL-amino acid derivatives to demonstrate the concept (Table). The methodology is in fact quite general and can be applied when appropriate biocatalysts are available, the substrate is hydrophobic and the product is water soluble. Further applications of this methodology to resolution of various racemic amines, amino alcohols, alcohols and carboxylic acids etc. are being investigated.

EXPERIMENTAL

α-Chymotrypsin was obtained from Sigma USA and immobilized in polyacrylamide as described earlier[10]. All other materials were of highest purity available from Spectrochem (India) and were used as received. Microbial cells were grown at room temperature (30°C) in shake flasks on an orbital shaker. For Baker's yeast (Saccharomyces cerevisiae NCIM 3044) the culture medium (1 L) consisted of peptone (10 g), yeast extract (5 g), sodium chloride (1 g), glucose (30 g) and magnesium sulfate (0.5 g). After 12 h of growth N-acetyl-L-phenylalanine ethyl ester (100 mg) was added and the cells

Table : Enantioselective Hydrolysis of Methyl Esters of Racemic N-Acetyl-α-Amino Acids in Reverse Micellar Suspension by α-Chymotrypsin Immobilized in Polyacrylamide[a].

DL-Amino acid	Isolated yield,%		ee,%	
	L-acid	D-acid	L-acid	D-acid
Phenylglycine	92 (86)	88 (82)	>99 (98)	>99 (97)
Phenylalanine	89 (82)	85 (80)	>99 (98)	>99 (98)
Homophenylalanine	85 (78)	82 (80)	>99 (97)	98 (94)
Tyrosine	86 (75)	84 (80)	>99 (98.5)	>99 (97)
Alanine	78 (72)	72 (67)	>99 (98.3)	>99 (97)
Leucine	76 (70)	70 (68)	>99 (98)	97 (96)
Valine	78 (72)	74 (69)	>99 (97)	97 (95)

a: The values in parentheses stand for reactions with baker's yeast immobilized with calcium alginate.

were grown for further 24 h. Pseudomonas putida (IFO 12996) was grown in a culture medium (1 L) consisting of peptone (10 g), yeast extract (2 g), magnesium sulfate (1 g) and glucose (30 g). After 12 h growth, urasil (100 mg) and N-acetyl-D-phenylglycine methyl

ester (200 mg) were added to induce the D-specific enzyme and growth was continued for 12 h more. The microbial cells were immobilized in calcium alginate beads (5 g wet per 15 ml of 2% sodium alginate) by a standard procedure[12].

Our typical experimental setup (**Fig.1**) consists of a volumetric cylinder (300 ml) equipped with a drain, a small mechanical and a magnetetic stirrer. Equal volumes (100 ml) of tris buffer (0.1 M, pH 8.2 in 1 M NaCl), and reverse micellar solution of AOT (0.1 M) in isooctane or isooctane-chloroform mixtures (upto 10% chloroform) containing the ester substrate (20-40 mmoles) are placed in the cylinder[13]. The lower aqueous layer is stirred slowly using a magnetic bead so that the aqueous and organic layers do not mix. After equilibrating the solutions for 10 min a tea bag containing immobilized biocatalyst (theoretical loading of 5 g wet baker's yeast in 15 ml of 2% calcium alginate or α-chymotrypsin with loading of 3 mg enzyme in 1 g dry polyacrylamide) is suspended in the reverse micellar medium with a string. The organic layer is gently stirred with the mechanical stirrer while the aqueous layer is stirred with the magnetic stirrer and the reaction is followed by reverse phase HPLC (Du Pont Zorbax C_{18} column, acetonitrile-water gradient) of the

aqueous and organic layers. When the reaction is 50% complete[14], the tea bag and the lower aqueous layer are removed from the solution. The reverse micellar solution is stirred with some more fresh buffer solution (20 ml) to extract final traces of the hydrolysis product. When all the product is removed from the reverse micellar solution (as per HPLC analysis) again aqueous buffer is added (50 ml) and a tea bag containing immobilized Pseudomonas putida (IFO 12996) is introduced (theoretical loading of 5g wet cells). After about 5% hydrolysis the aqueous layer is discarded as this contains the last traces of L-acid along with D-acid. Fresh aqueous buffer (50 ml) is introduced and the reaction is continued till all the ester is hydrolyzed to acid and is extracted in aqueous layer. Thus at the end of reaction, two aqueous solutions containing resolved Nα-acetyl amino acids are obtained. The aqueous layer also contains some dissolved D-ester which is extracted with chloroform. Its optical purity was checked with ^{1}H NMR using [Eu(tfc)$_3$] on 200 MHz NMR spectrometer and recycled if the optical purity is not satisfactory. Resolved N-acetyl acids were recovered after acidification, lyophilization and extraction of the residue with methanol. The optical purity of the

product was determined on chiral HPLC column (Machery-Nagel, Germany, Chiral-1) after removing the N-acetyl group by refluxing with 6 N HCl for 2-3 h.

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11. Substrate enantiospecificity for the second biocatalyst is quite unimportant as long as one biocatalyst shows high enantioselectivity (e.e >99%).

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13. By using mixtures of chloroform-isooctane (upto 10% chloroform) the solubility of the substrate in organic phase can be increased but the reaction times also increase with chloroform content.

14. Overall of reaction times vary between 10 to 48 h depending upon biocatalyst and its activity.

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