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OPTIMIZATION OF PROCESS FOR ENZYMATIC RESOLUTION OF RACEMIC AMINES USING CONTINUOUS FLOW BIOREACTOR

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Abstract

The optimization of enzymatic process for the resolution of chemically and pharmaceutically important racemic amines is described using a continuous flow bioreactor. Also described are the isolation and purification methods of the reaction products and the various parameters affecting the optimal reaction conditions for the enzymatic resolution to afford the products in high chemical purity and enantiomeric excess (ee) of over 90%.

Keywords: Optimization, process, continuous flow bioreactor, enzymatic resolution, racemic mixtures, chemical purity and enantiomeric excess.

Introduction

Since the early experiments reported by Klibanov[1], the use of enzymes in organic solvents rather than in their natural aqueous reaction media was widely recognized. Gutman et al described, inter alia, enzymatic lactonization of γ-hydroxyesters in organic solvents, synthesis of optically pure γ-methylbutyrolactone and γ-phenyl-butyrolactone[2], enzyme-catalyzed enantio-convergent lactonization of hydroxy diesters in organic solvents[3], lipase-catalyzed preparation of optically active γ-butyrolactones in organic solvents[4] and enzymatic formation of lactams in organic solvents[5] to name only few works that have been carried out by said group of researches in the field of enzymatic catalysis in organic solvents.

The pioneered work of practical enzymatic resolution of racemic alcohols and amines in organic solvents was also described in the literature[6]. However, the technological aspects of optimization of the process for large-scale production of chiral amines using a continuous flow bioreactor have never been published before. The technological aspects that have been optimized include, inter alia, selection of an optimal support, the support's efficiency and particle size, flow rate, mass transfer, reactants mixing, retention time, thermal control,

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viscosity of the medium and in-line purification and isolation of the reaction products and reprocessing the excess raw materials and recycling the solvents.

The continuous flow preparation of active pharmaceutical intermediates (APIs) and other important chemicals has grown significantly over the past years since the publication of said article reciting the practical enzymatic resolution of racemic alcohols and amines in organic solvents. The progress in the novel chemical methodology of enzymatic resolution using continuous flow bioreactors has resulted, inter alia, from optimization of the technology in comparison to batch-wise synthesis although immobilized enzymes in batch systems are still common.

Materials and methods

The enzymatic process which was carried out in the continuous flow bioreactor can be generally described as depicted in Equation 1:

Equation 1

Enzyme, organic solvent

R,S-amine + active ester ----------> R-amine + S-amide + alcohol

An example of the reaction is described in Equation 1A:

Equation 1A

Subtilisin, 3-methyl-3-pentanol

(R,S)-1-aminoindan + 2,2,2-trifluoroethyl butyrate ---------->

(R)-1-aminoindan + (S)-N-1-indanylbutanamide + 2,2,2-trifluoroethanol

Figure 1 below depicts a schematic scheme of the continuous flow bioreactor which consists of a column A, containing an enzyme immobilized on a fixed support. The two starting materials are separately fed into the column A by a two-piston pump H, while each piston is directed to enable a different flow rate of solutions with known concentrations of the starting materials, which are fed separately from the solvent reservoirs F and G into the column A. The two pipes connecting the pump to the column pass through a two-neck connector B, equipped with a seal through which the two solvents are allowed to uniformly drip into the column. One

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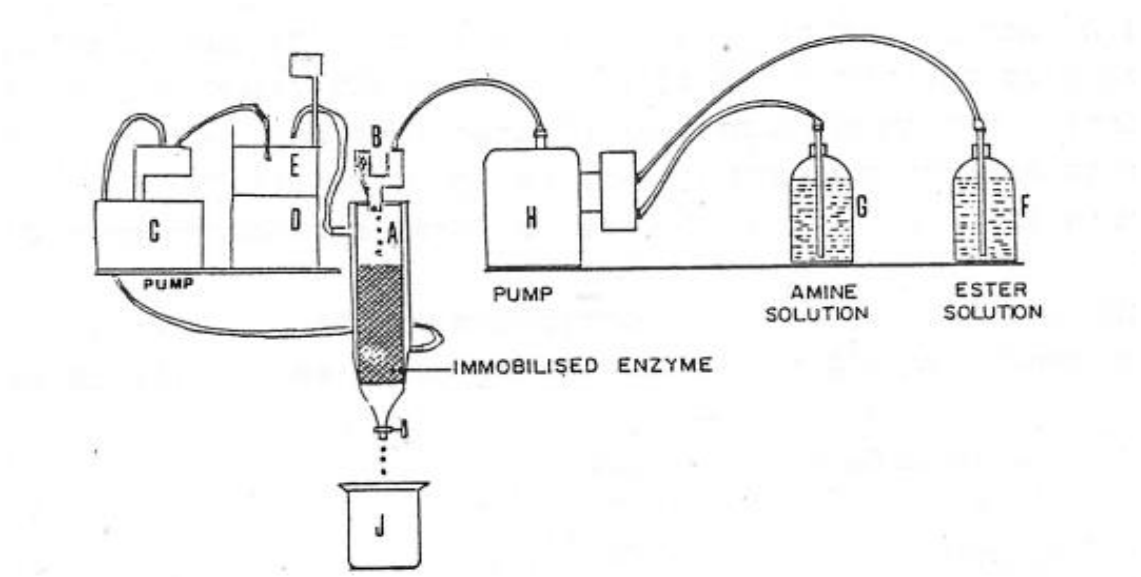
of the connector's tubes is filled with silica gel to enable drying of the system and to prevent vacuum that might not allow good dripping into the column. A peristaltic pump C transfers water at a temperature range of 25-40^{o}C form the bath E, which is heated using heating plate D. The warmed water flows through the column's sleeve, which enables warming the column at a constant temperature. The effluent drops from the column directly into a vessel J, which contains known quantity of 2M HCl solution under constant mixing.

Figure 1



Several racemic amines have been resolved using said continuous flow bioreactor including 1-aminoindan, 4-fluro-1-aminoindan, 5-fluro-1-aminoindan, 6-fluro-1-aminoindan and 1-(1-naphthyl)ethylamine**.** US patent 5,481,541 recites 1-aminoindan's monofluorinated derivatives which may serve as intermediates in the synthesis of active pharmaceutical ingredients[7]. Figure 2 below depicts the kinetics of the resolution of the four substrates 1-aminoindan, 4-fluro-1-aminoindan, 5-fluro-1-aminoindan, 6-fluro-1-aminoindan, which are marked as follows: O= 1-minoindan, Δ, ▫, and ● refer to the three other compounds respectively. It may be understood from Figure 2 that only two out of the four compounds can reach an ee closer to 100% in the experiment's specific conditions.

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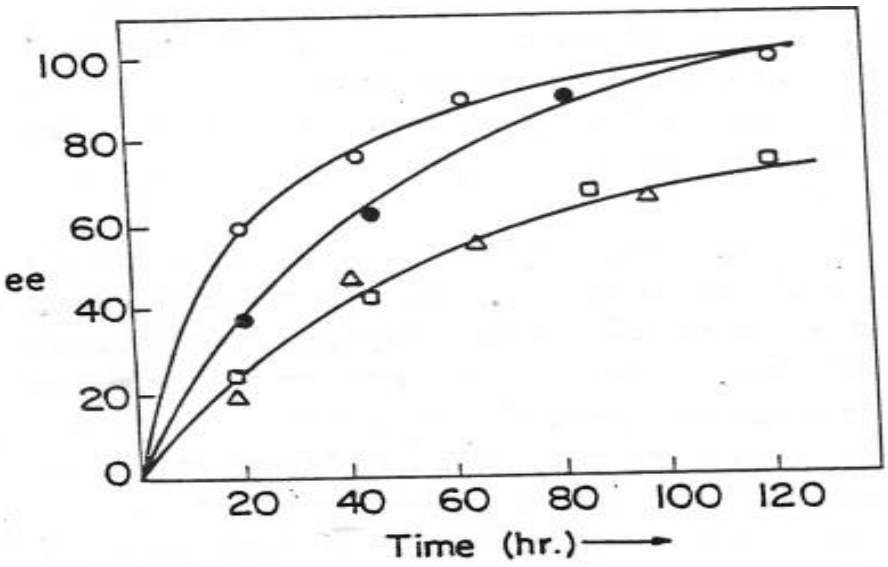
Figure 2



According to the general theory of kinetic resolution, by using enzymatic catalysis in order to reach high ee, the conversion should be about 55-60%. Since the stereospecific reaction is relatively slow, it is recommended to keep the reactants separately until they are entered into the bioreactor and thus the rate of chemical non- stereospecific reaction is reduced. In addition, by transferring the product directly into an acidic solution further chemical reaction of the resolved enantiomerically pure amine with the excess active ester is prevented. Particles having large surface area, such as glass beads, reduce the limiting factor of diffusion in the transition between the solid phase (the support containing the immobilized enzyme) and the liquid phases (containing the substrates) during the gravitational movement of the liquid phase in the continuous flow bioreactor. The flow rate is dependent on the particle size and affect both the conversion of the reaction and the enantioselectivity.

Description of the process carried out in the continuous flow bioreactor

A slurry of the enzyme immobilized on the support is produced, vigorously agitated and loaded on a glass column until the medium is settled. Then, solutions of the amine (e.g., 1-aminoindan or one of its derivatives) and active ester (e.g., 2,2,2-trifluoroethyl butyrate) at known concentrations are prepared in an organic solvent (e.g., 3-methyl-3-pentanol or tert-amyl alcohol) and stored at suitable glass bottles. The two-piston pump is operated to enable a different flow rate of said solutions into the column and the peristaltic pump is set to transfer warm water from the bath into the column sleeve. After a while, samples of effluent are withdrawn, worked out and the amine hydrochloride is isolated and tested by HPLC chromatography to determine the ee of the isolated amine salt. According to the result, the flow of the incoming stream of substrates and/or the outcoming flow may be reduced in order to increase the ee of the resolved amine. The collected effluent is subjected to extraction and phase separation, the aqueous phase is checked to verify that it is acidic and the organic phase is

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washed with water and dried. The aqueous phase is partially evaporated to afford a concentrated solution of the amine hydrochloride, which is left aside at room temperature to enable precipitation of the enantiomerically enriched amine hydrochloride. The solid is filtered out, dried in a vacuum oven until constant weight is achieved and treated with a solution of gaseous ammonia dissolved in chloroform. Titration of a withdrawn sample is carried out with HCl solution having a known concentration in the presence of the indicator methyl orange to verify the end of the reaction. The thus formed ammonium chloride solid is filtered out, the organic solution is evaporated to afford the crude product, which is distilled in vacuum to obtain the chemically pure free amine base.

The organic solution is evaporated in vacuum to obtain a "cake", which is a white solid mixture of the amine hydrochloride and the amide. Hot water is added to the solid in order to be able to transfer it to a glass vessel to cool off. Then, dichloromethane is added to complete dissolution of the solid and the phases are separated by extraction. The aqueous phase is washed again with dichloromethane and stored. The dichloromethane solvent is evaporated to afford the solid amide, which is washed 3 times with hot water to eliminate traces of amine hydrochloride, the solid is filtered out and dried in vacuum. Hydrolysis of the S-amide affords the S-amine.

Description of the process for isolation of the final product and reprocessing the excess of reactants, by-product and solvent

Figure 3 below depicts a schematic flow chart of the process for isolating the final

product and reprocessing the excess of reactants, by-product and solvent, which consists of collecting the effluent directly into an acidic solution, obtaining said "cake" and conducting a series of two consecutive distillations and collecting fractions rich in un-reacted active ester and solvent that are reprocessed back into the continuous flow bioreactor and the by-product alcohol which is used for the synthesis of the active ester. The general flow chart of isolating the final product and reprocessing the reactants is depicted in Figure 3 below.

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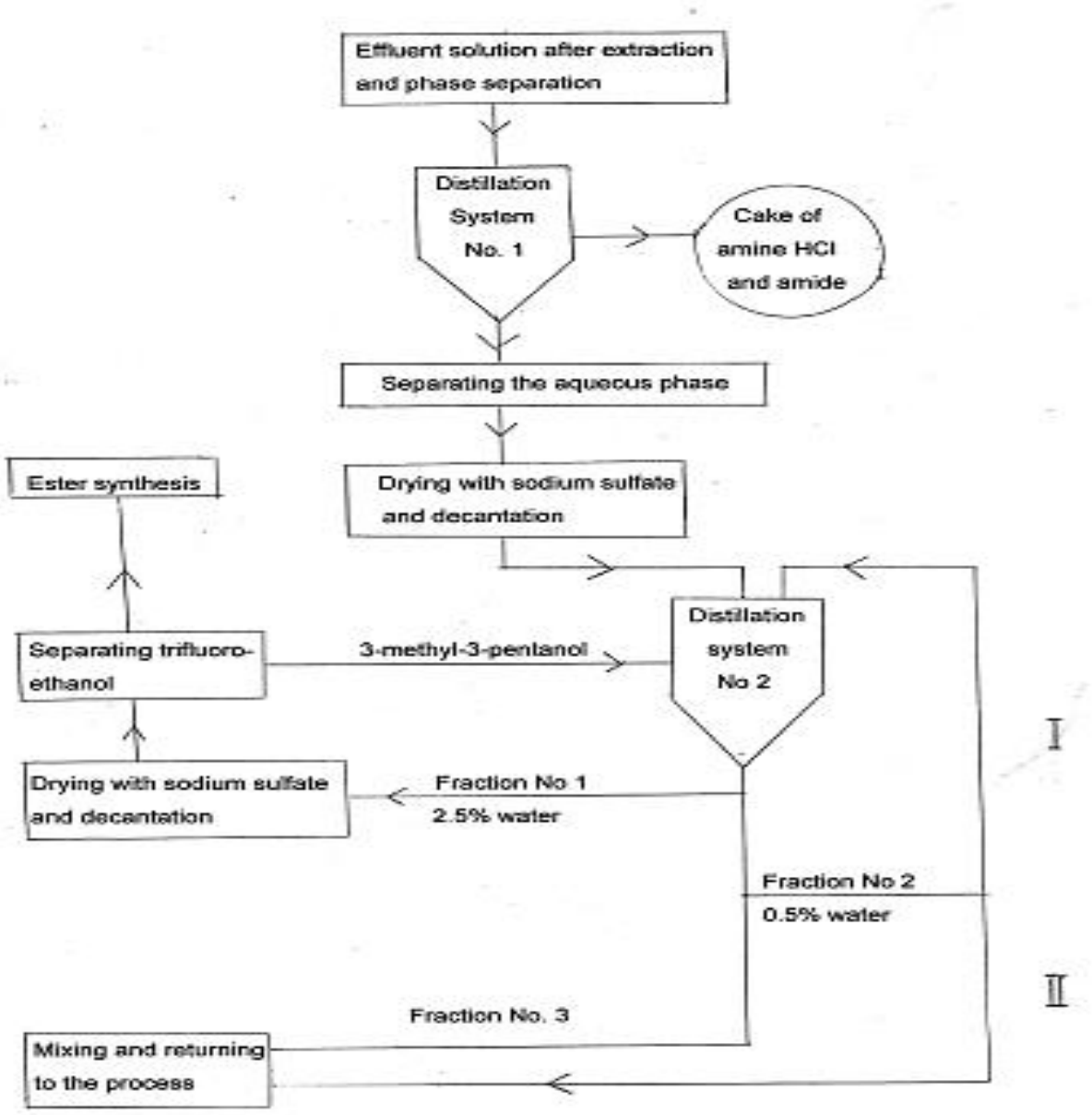
Figure 3



Preparation method of the enzyme immobilized on the support for the continuous flow bioreactor

An ammonium carbonate buffer pH 7.8 was prepared by mixing 65 mL of 0.1M ammonium carbonate solution with 35 mL of 0.1M HCl solution and 100 grams of glass beads were added to the buffer followed by addition of 1 gram of subtilisin A while mixing to afford a uniform

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slurry, which was frozen in liquid air and lyophilized for 24 hours and grounded to afford a powder.

Results and Discussion

Selecting the optimal support for the specific reaction

Sevel supports were tested batch-wise to determine which is optimal for carrying out said enzymatic reaction. 5 mg of enzyme were immobilized on 175 mg of support and placed in a glass vial. Then, 5 mL of 3-methyl-3-pentanol were added followed by addition of 100 mg (0.75 millimoles) of 1-aminoindan and 200 mg (1.75 millimoles) of 2,2,2-trifluoroethyl butyrate and reaction was carried out by placing the vial in a shaker at 200 RPM for 24 hours at 40^{o}C. After reaction completing, the mixture was filtered, the support was washed with the solvent and the reaction's conversion was determined by using HPLC chromatography. The results are described in Table 1 below.

Table 1

Support description	Particle size	Relative activity
Glass beads G-2381	< 150 microns	1.9
Aluminum oxide 90%	63-200 microns	1.4
Ottawa sand	20-30 mesh	0.7
Silica 60H	5-40 microns	0.7
Celite 545	0.02-0.1 mm	0.5
Florisil	200 mesh	0
Calcium carbonate	14 microns	0
Control	-----	1.0

The relative activity was calculated by comparing the obtained conversion to that of carrying the same reaction without support (control). It may be concluded from the results that the activity is probably not correlated to the particle size but rather to surface area of the selected support. The results demonstrate that immobilization of the enzyme on glass beads or aluminum oxide enhance the activity relative to the control.

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In order to determine which support is optimal for the continuous flow bioreactor, enzymatic reactions have been carried out in five different continuous flow bioreactors packed with the corresponding supports, as detailed in Table 2 below.

Table 2

	Glass beads	Glass beads	Sand	Al_2O_3 90%	Bentonite
Support type	Glaverbel Microperl	Sigma G-2381 **	Sigma S-9887	Merck Art. 1077	BDH 26022 *
Particle size	74-149 microns	<150 microns	70 mesh	63-200 microns	<150 microns
Number of runs needed to reach 99% ee	4	4	25	6	6
Total time (hours) needed to reach 99% ee	7	8	21	46	23
Quantity of the support, grams	27	17	23	10	10
Quantity of the enzyme, mg	140	140	140	140	140
Yield of R-amine, 99% ee	25%	27%	25%	40%	47% *
Yield of S-amide, 90% ee	73%	68%	67%	60%	39%
Total yield	98%	95%	92%	100%	86%

* The amine was isolated having only 14% ee. ** The glass beads Sigma G-2381 are acid washed while glass beads Glaverbel Microperl are not acid washed. Al_2O_3 stands for aluminum oxide.

The results demonstrate that the optimal support is glass beads.

Measurement of the substrate's retention time in the column

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1. Measuring the retention time of 1-aminoindan in a column packed with glass beads.

A glass column was packed with 17 grams of glass beads Sigma G-2381. 0.5 gram of 1-aminoindan was dissolved in 10 mL of 3-methyl-2-pentanol and loaded into the column and the dripping rate was set to 4 mL per hour. From time-to-time samples of the effluent were withdrawn, diluted in the solvent and the concentration of the amine was measured by using UV spectrophotometer. The solvent was evaporated and the weight of the residue was measured. After collecting 10 mL of effluent, 3 additional 10 mL portions each of the solvent were loaded into the column and samples of the effluent were collected. Table 3 below includes results of measures of retention time of 1-aminoindan in a column packed with glass beads Sigma G-2381.

Table 3

No	Eluent volume (ml)	Dilution coefficient	A_{266}	Molar concentration	Accumulated time	Weight of the residue, mg
1	3.0	None	2.36	0.002	45 min	1.0
2	3.2	6	2.72	0.015	1 h, 40 min	6.8
3	3.3	30	2.91	0.08	2.5 h	32.9
4	3.5	100	2.57	0.24	3 h, 20 min	112.0
5	2.5	200	2.61	0.49	3 h, 50 min	113.0
6	3.5	200	0.98	0.18	4 h, 45 min	164.0
7	3.0	100	0.98	0.09	5.5 h	86.1
8	3.4	100	0.78	0.07	6.5 h	40.6
9	2.3	100	0.34	0.032	6 h, 50 min	33.7
10	3.4	None	0.54	0.005	7 h, 45 min	None
11	3.0	None	0.28	0.0015	8.5 h	None
Total	34.1 mL					0.59 g

A266 refers to the absorbance measured with the UV spectrometer at a wavelength of 266 nm; h refers to hours and min refers to minutes.

It may be concluded from the results that it takes to the material about two hours to begin emerging from the column and highest concentration is achieved after 4 hours out of 8,5 hours

of the total run time. Therefore, the relation between the quantity of the medium and flow rate is 4.25 grams X hours/mL.

2. Measuring the retention time of 1-aminoindan in a column packed with Ottawa sand

15.8 grams of 1-aminoindan were dissolved in 300 mL of 3-methyl-2-pentanol and loaded into a glass column packed with 500 grams of Ottawa sand and the dripping rate was set to 70 mL per hour. From time-to-time samples of effluent were withdrawn, diluted in the solvent, and the concentration of the amine was measured by using UV spectrophotometer and the solvent was evaporated and the weight of the residue was measured. After collecting 300 mL of effluent, 2 additional 320 mL portions each of the solvent were loaded into the column and samples of effluent were withdrawn. Table 4 below includes results of measures of retention time of 1-aminoindan in a column packed with Ottawa sand.

Table 4

No	Eluent volume (ml)	Dilution coefficient	A_{266}	Molar concentration	Accumulated time	Weight of the residue, grams
1	97	5	0.16	0.0006	1 h	0.014
2	53	50	0.08	0.0016	2 h	0.034
3	63	100	2.05	0.008	3 h	1.32
4	67	200	1.68	0.137	4 h, 15 min	2.42
5	70	200	1.64	0.132	5 h, 40 min	2.64
6	78	200	1.54	0.126	6 h, 55 min	2.8
7	100	200	2.10	0.170	8 h, 15 min	4.48
8	75	200	1.40	0.110	9 h, 15 min	2.20
9	70	30	0.28	0.025	10 h	0.46
10	70	10	1.0	0.004	10 h, 50 min	0.07
11	75	5	1.4	0.0028	12 h	0.049
12	110	3	1.64	0.002	13.5 h	0.024

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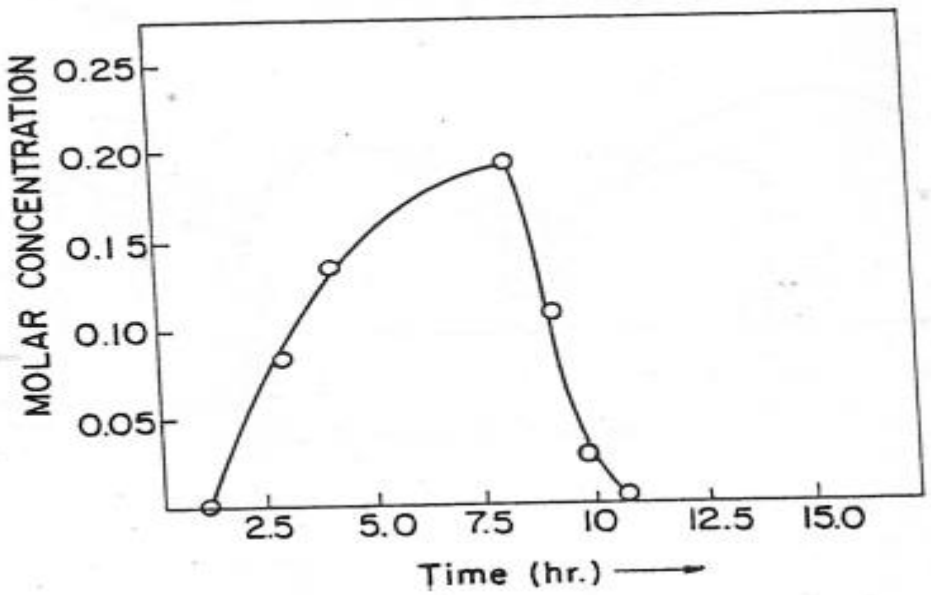
Total	928 mL					16.51 grams

A266 refers to the absorbance measured with a UV spectrometer at wavelength of 266 nm; h refers to hours and min refers to minutes.

Figure 4 below depicts the results of measurement of the retention time of 1-amindan on a column of Ottawa sand.

Figure 4



It may be concluded from the results that it takes to the substrate two hours to begin emerging the column and highest concentration is achieved after 8 hours and 15 minutes (about mid test). Therefore, the relation between the quantity of the medium and flow rate is 6.75 grams X hours/mL.

Measurements of the extent of non-enzymatic reaction

In order to optimize the enzymatic intermolecular aminolysis it was essential to determine whether or not the support itself accelerated chemical reaction, which cause reduction of the enantiomeric excess (ee) of the product. This was tested by reacting 95 µL of 1-aminoindan (0.75 millimoles) and 250 µL (1.65 millimoles) of 2,2,2-trifluoroethyl butyrate in 2 mL of 3-methyl-3-pentanol at 40^{o}C for different reaction times in the presence of two supports glass beads Microperl AF and Sigma G-1381, without an enzyme. The conversion was determined by using HPLC chromatography and the results are summarized in Table 5 below.

Table 5

Accumulated time	Conversion using glass beads Microperl AF	Conversion using glass beads Sigma G-1381
7 hours	< 1%	1.5%
24 hours	5 %	4 %
48 hours	8.5 %	7 %
5 days	22 %	21 %
7 days	34 %	35 %

The results demonstrate that there is no significant difference between the two types of glass beads. In another experiment, two types of glass beads were tested as supports, which were lyophilized from different buffers to enhance the activity of the enzyme in carrying out the aminolysis reaction. The results are summarized in table 6 below for glass beads type G-4649 (particle size < 106 microns) and table 6A for glass beads type G-2381 (particle size<150 microns) respectively. Samples were withdrawn periodically, as detailed in the tables.

Table 6

Reaction time	Conversion, carbonate buffer, pH 7.8	Conversion phosphate buffer, pH 7.8
5 hours	< 1%	< 1%
24 hours	5 %	3,5 %
48 hours	7.5 %	7 %
66 days	9.5 %	11.5 %

Table 6A

Time	Chemical conversion, carbonate buffer, pH 7.8	Chemical conversion phosphate buffer, pH 7.8
5 hours	< 1%	< 1%
24 hours	4 %	2,5 %

48 hours	7.5 %	5 %
66 days	10 %	7 %

It can be concluded from the results that while there is no significant difference between the two types of glass beads, if reaction is ceased within 24 hours at these conditions, the extent of the chemical reaction is less than 5%.

Stability of the immobilized enzyme in the continuous flow bioreactor

In order to determine the stability of the immobilized enzyme in the continuous flow bioreactor, repeated tests were carried out by passing constant quantities of reactants through the bioreactor at fixed time periods. The bioreactor contained 140 mg of enzyme immobilized on 7 grams of glass beads, thus the enzyme: substrate was 1:50, and a total quantity of 500 mg (3.75 millimoles) of 1-aminoindan, 2 mL (13 millimoles) of 2,2,2-trifluoroethyl butyrate and 8 mL of 3-methyl-3-pentanol was passed 4 times at a period of 8 hours followed by washing with the solvent. Table 7 below demonstrates the degree of conversion and ee of the isolated product from the bioreactor atter 4 consecutive runs.

Table 7

Run number	% Conversion	% ee
1	45	90
2	52	93
3	56	98.5
4	56.5	99

After each run, which included 125 mg of 1-aminoindan, 0.5 mL of 2,2,2-trifluoroethyl butyrate and 2 mL of 3-methyl-3-pentanol, a sample was withdrawn and both the conversion and the ee were determined. In another experiment, a total of 10X4 consecutives runs were carried out over a period of 9 months, during which time the enzyme was stable so that the product was isolated at high ee of 98%, while the ee at the beginning of the test was 99%. After further 4X4 additional consecutives runs the ee dropped to 90%. It may be concluded that the enzyme is stable within a period of about a year in the bioreactor.

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Stability of the stored immobilized enzyme

In order to check the stability of the immobilized enzyme after being stored for a certain period of time before use, two portions of the same batch of immobilized enzyme on glass beads were put in two separate glass vessels and the residual solvent was evaporated by blowing nitrogen gas over the immobilized enzyme layer until the color changed from grey to white. One portion was stored for 9 days and the other for 60 days in the refrigerator at a temperature of 4°C. At the end of the time period, the stored immobilized enzyme was allowed to reach room temperature, mixed with the solvent and the slurry was loaded into a glass column followed by running 4 portions each of 95 µL of 1-aminoindan (0.75 millimoles) and 250 µL (1.65 millimoles) of 2,2,2-trifluoroethyl butyrate in 2 mL of 3-methyl-3-pentanol at 40°C. After each run the ee of the product isolated from the effluent was measured. The ee of the product after 4 consecutive runs in the bioreactor was 87% after 9 days of storage in the refrigerator at a temperature of 4°C while after 60 days of storage in the refrigerator at same temperature the ee of the product after 4 consecutive runs in the bioreactor was 78%.

Measurement of the support's efficiency

As demonstrated in tables 1 and 2, glass beads and aluminum oxide demonstrated good suitability as supports for continuous flow bioreactors, which are designed for prolonged and repeated use. Thus, it is necessary to evaluate the support's efficiency during the planned working time period using these supports. For that purpose, 5 mg of subtilisin immobilized on 500 mg of glass beads and 4.5 mg of subtilisin immobilized on 315 mg of aluminum oxide were prepared and loaded into two separate columns. Each of the two columns was loaded with a mixture of 100 mg of 1-aminoindan (0.75 millimoles) and 250 µL of 2,2,2-trifluoroethyl butyrate (1.65 millimoles) dissolved in 2 mL of 3-methyl-3-pentanol and samples of the effluent were periodically withdrawn. At the end of the run time, the ee was determined, the reactants and reaction products were isolated and the support was washed with the solvent and new quantities of reactants were loaded to afford the next run. The results are summarized in tables 8 (glass beads G-2381) and 8A (aluminum oxide 90%) below.

Table 8

Run time, hours	Numbers of repeated uses	Enantiomeric excess (ee)
24	1	62
32	2	81
36	3	86
45	4	92
48	5	93

48	6	94
48	7	98
64	8	94

Table 8A

Run time, hours	Numbers of repeated uses	Enantiomeric excess (ee)
24	1	42
40	2	56
48	3	73
64	4	86
90	5	91
120	6	88
144	7	70
168	8	70

It may be concluded that glass beads show better efficiency than aluminum oxide and therefore glass beads is the optimal medium for the continuous flow bioreactor.

Factors affecting the flow rate

The ee of the amine isolated from the reaction in the continuous flow bioreactor is dependent on the outflow because the fluctuation speed of the substrates is dependent on the flow rate. If the flow rate is slower, the contact time of the reactants with the enzyme in the bioreactor is prolonged and the efficiency of the process is increased. A balance between the inflow and outflow of the liquids must be maintained at all time for optimal ee and to prevent the bioreactor from drying. The viscosity of the mobile phase has an impact on the flow. For example, higher concentrations of the active ester in the solvent lead to lower viscosity of the mobile phase and the outflow increases significantly. The factors affecting the flow rate are the following:

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1. The support's quantity loaded into the column

The flow rate can be increased if the quantity loaded into the bioreactor is diminished relatively to the dimensions of the column. In order to test which would be the optimal quantity loaded into the column relatively to the dimensions of the column and height of the solvent over the medium, different quantities of glass beads were mixed with the solvent 3-methyl-3-pentanol to form a slurry in a column having the dimensions of: height 53 cm, internal diameter of 3.8 cm and maximal volume of 600 mL. The solvent's height over the medium was 5 cm. Table 9 below includes data on the relation between the flow rate and the weight of the dry support consisting of glass beads.

Table 9

Entry	Outflow rate, mL per hour	Weight of the dry support, grams
1	78	300
2	61	400
3	56	500
4	52	600
5	48	700
6	44	800

It may be concluded from the results that as the quantity of support in the column increases, the outflow decreases.

2. The support's nature and particle size

The outflow is dependent on the permeability of the support, which is influenced by the particle size. In general, relatively small particle size will cause slower outflow because the particles are more joint together to each other, which slows the movement of the solution in the bioreactor. The flow rate was measured using the same support (17 grams of glass beads) but with different particle sizes. The outflow rate in a column packed with glass beads G-2381 (particle size<150 microns) was 19 mL per hour while the outflow rate in a column packed with glass beads G-4649 (particle size<106 microns) was 5 mL per hour.

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3. The column's structure and dimensions

The outflow rate is also dependent on the column's width, height of the solvent over the medium and the permeability of the sinter's filter. In one example, 200 grams of support (glass beads) were placed in a column having an internal diameter of 6 cm and the outflow measured was 270 mL per hour, which is about 3 times higher than the value of 92 mL per hour measured in a column carrying same quantity of support that has an internal diameter of 3.8 cm. The solvent's height over the support was 5 cm in both columns.

4. The effect of the temperature on the flow

Increase of the temperature in the bioreactor leads to increase in outflow. In one example, a column was used having an internal diameter of 6 cm, the quantity of support (glass beads) was 920 grams and the height of the solvent over the medium was 10 cm. While setting the temperature at 24°C, the flow of a 1.5 M solution of 2,2,2-trifluoroethyl butyrate in 3-methyl-3-pentanol was 240 mL per hour and while setting the temperature at 40°C, the flow of same solution was 287 mL per hour.

5. The effect of the solvent's height over the support

The flow rate increases in a column in which the solvent's height over the support is higher because of the higher pressure that the solvent applies on the bioreactor's medium. For example, if the solvent's height over the medium consisting of glass beads is increase from 5 cm to 31 cm, the flow rate will increase from 78 mL per hour to 130 mL per hour (Entry 1, Table 9).

The conclusion is that it is important to maintain a constant and low level of solvent over the support's medium in order to keep a sufficient inflow rate while diminishing to the minimum the contact time of reactants in the bioreactor and thus leading to higher ee of the product.

Description of isolation of the final products and reprocessing the ester, solvent and alcohol by- product.

As depicted in Figure 3, the acidic effluent solution was collected, extracted with dichloromethane and the phases were separated. The aqueous phase was subjected to "distillation system No. 1" to obtain a solid "cake" and a distillate. The organic solvent was evaporated and the obtained mixture was added to the distillate. Hot water and dichloromethane were added to the solid "cake" containing the amine hydrochloride and the amide to complete dissolution and the phases were separated. The aqueous phase was partially distilled and the resulting concentrated solution was left aside to enable precipitation of the enantiomerically enriched amine hydrochloride. The distillate was separated into two phases and the organic phase was dried with sodium sulfate and subjected to "distillation system No. 2", in which 3 main fractions were collected. The first fraction was collected at a temperature range of 60-87°C. It had a water content of 2.5% and contained mainly 2,2,2-trifluoroethanol (boiling point 73.6°C), which is the byproduct of the enzymatic aminolysis reaction. It was dried over sodium

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sulfate and reused for the synthesis of the active ester 2,2,2-trifluoroethyl butyrate. The second fraction had a boiling point of 90-115ºC and water content of 0.5% and contained mainly the unreacted active ester (boiling point 112ºC) and the solvent 3-methyl-3-pentanol, which were recycled back to the process. The third fraction was collected at a temperature range of 115-123ºC. It had a water content of 0.1% and contained mainly the solvent 3-methyl-3-penanot (boiling point 123ºC) and the unreacted active ester 2,2,2-trifluoroethyl butyrate, which were recycled back to

the process.

Scaling up the enzymatic process in the continuous flow bioreactor

1. The aminolysis reaction by kinetic enzymatic resolution of (R,S)-1-aminoindan, shown in Equation 1A, was scaled up to a quantity of 330 grams of the starting material. The experiment was carried out using the continuous flow bioreactor demonstrated in Figure 1 and as detailed in the description of the process carried out in the continuous flow bioreactor. Table 10 below details the different parameters of said process.

Table 10

Parameter	Description
Internal diameter of the column	3.8 cm
Medium's height	26 cm
Type of support	Glass beads
Quantity of the dry support	370 grams
Quantity of the enzyme subtilisin A	3.65 grams
Quantity of racemic amine base entered	330 grams
Enzyme : substrate ratio	1:90
Quantity of the resolved and isolated R-amine base having >99% ee	145 grams
Yield of the of the resolved and isolated R-amine base	44%
Comparative yield of the resolved and isolated R-amine in batch-wise experiments	20-25%
Volume of the medium in the column	300 mL

Total volume of the solvent	5.65 L
Total volume of the active ester	1.37 L
Concentration of the ester in the solvent	2.23 M
Concentration of the amine in the solvent	0.68 M
Density of the medium	1.23 grams/mL
Initial flow rate	120 mL per hour
Final flow rate *	70 mL per hour
Retention time	5 hours
Relation between the quantity of the medium and the flow rate	5.3 grams X hours/mL
Reaction temperature	36°C
Changes in the ee of the isolated amine as related to the time that passed from the starting time of the experiment *	Time, hours ee % 9 67 13 83 18 97 36 99

* In order to improve the enantioselectivity, the flow was gradually reduced to 70 mL per hour after 9 hours from the starting time of the experiment

It may be concluded from the data and results included in Table 10 that carrying out the process in a continuous flow bioreactor is more efficient than carrying out a batch-wise experiment because of the much shorter contact time between the reactants that leads to diminished rate of chemical reaction and hence higher yield of the isolated R-amine. Samples of isolated R-amine hydrochloride were withdrawn during the process in order to determine the ee and to adjust accordingly parameters such as the outflow rate, temperature and inflow concentration of the reactants.

2. The aminolysis reaction by kinetic enzymatic resolution of (R,S)-1-(1-naphthyl)-ethylamine, shown in Equation 1B below, was scaled up in two parts to a quantity of 5.5 Kg (32.1 moles)

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of starting material. The experiment was carried out using the continuous flow bioreactor demonstrated in Figure 1 and as detailed in the description of the process carried out in the continuous flow bioreactor.

Equation 1B

Subtilisin,

3-methyl-3-pentanol

(R,S)-1-(1-naphthyl)ethylamine + 2,2,2-trifluoroethyl butyrate ---------->

(R)-1-(1-naphthyl)ethylamine + (S)-N-1-(1-naphthalenyl)ethylbutanamide + 2,2,2-trifluoroethanol

Table 11 below details the different parameters of said process.

Table 11

Parameter	Part 1	Part 2
Column's internal diameter	3.8 cm	6 cm
Medium's height	40 cm	26 cm
Type of support	Glass beads	Glass beads
Quantity of the dry support	570 grams	920 grams
Quantity of racemic amine base entered	1.5 Kgs	4 Kgs
ee of the resolved and isolated R-amine base	95%	90%
Quantity of the enzyme subtilisin A	5.17 grams	9.2 grams
Enzyme : substrate ratio	1:290	1:435
Volume of the medium in the column	450 mL	725 mL
Total volume of the solvent	36 L *	60 L *

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Percentage of water in the solvent	0.15%	0.3%
Total volume of the active ester	4 L	6 L
Concentration of the amine in the solvent	2.07M	2.07M
Reaction temperature	38^{o}C	40^{o}C
Density of the medium	1.27 grams/mL	1.27 grams/mL
Average outflow rate	120 mL per hour	160 mL per hour
Retention time	6 hours	7 hours
Relation between the quantity of the medium and the flow rate	5.7 grams X hours/mL	7.1 grams X hours/mL

*Initially 12 liters of the solvent were used in part 1 of the experiment, which were collected, distilled and recycled back to the process and 20 liters of the solvent were initially used in part 2 of the experiment respectively.

Table 12 below details the alternations made in the inflow rates of the amine and the ester and in the ester's concentration in order to achieve optimal ee of the isolated chiral amine hydrochloride, showing that the inflow rates have to be lowered and ester's concentration in the alcohol solvent has to be increased in order to achieve higher ee.

Table 12

Change No.	Ester's concentration in the alcohol solvent, M	Inflow rate ester mL/hour	Inflow rate amine mL/hour
Starting ester concentration	1.3	160	40
1	1.5	150	30
2	1.7	112	25
3	1.7	109	22
4	1.7	110	22
5	1.9	116	28

6	1.7	125	28
7	1.7	105	22
8	1.5	100	19
9	1.9	100	23
10	1.9	108	19

The total quantity of the isolated amine hydrochloride salt was 2.55 Kgs (12.27 moles). Since the molecular weight of the salt is 207.74 and the molecular weight of the free amine base is 171.24, the calculated number of moles of the free amine base is 10.11 moles (assuming full conversion of the salt to the free base). Thus, the calculated yield of the free base is 10.17/32.12 X 100= 31.7%. The weight of the isolated S-amide was 3.9 Kgs (50%) hence the total reaction yield was 81.7%. It may be concluded from the scaling up experiments that by using an enzyme: substrate ratio of 1:90, high ee of >99% was achieved. However, increasing the enzyme: substrate ratio to 1:290 yields a final product having 95% ee and while increasing the enzyme: substrate ratio to 1:435, the ee of the final product dropped to 90%. The increased outflow rate in part 2 of the experiment is also another possible cause of the drop in ee of the final product. The pilot chart for production of chiral amines by enzymatic resolution using a continuous flow bioreactor is depicted in Figure 5 below.

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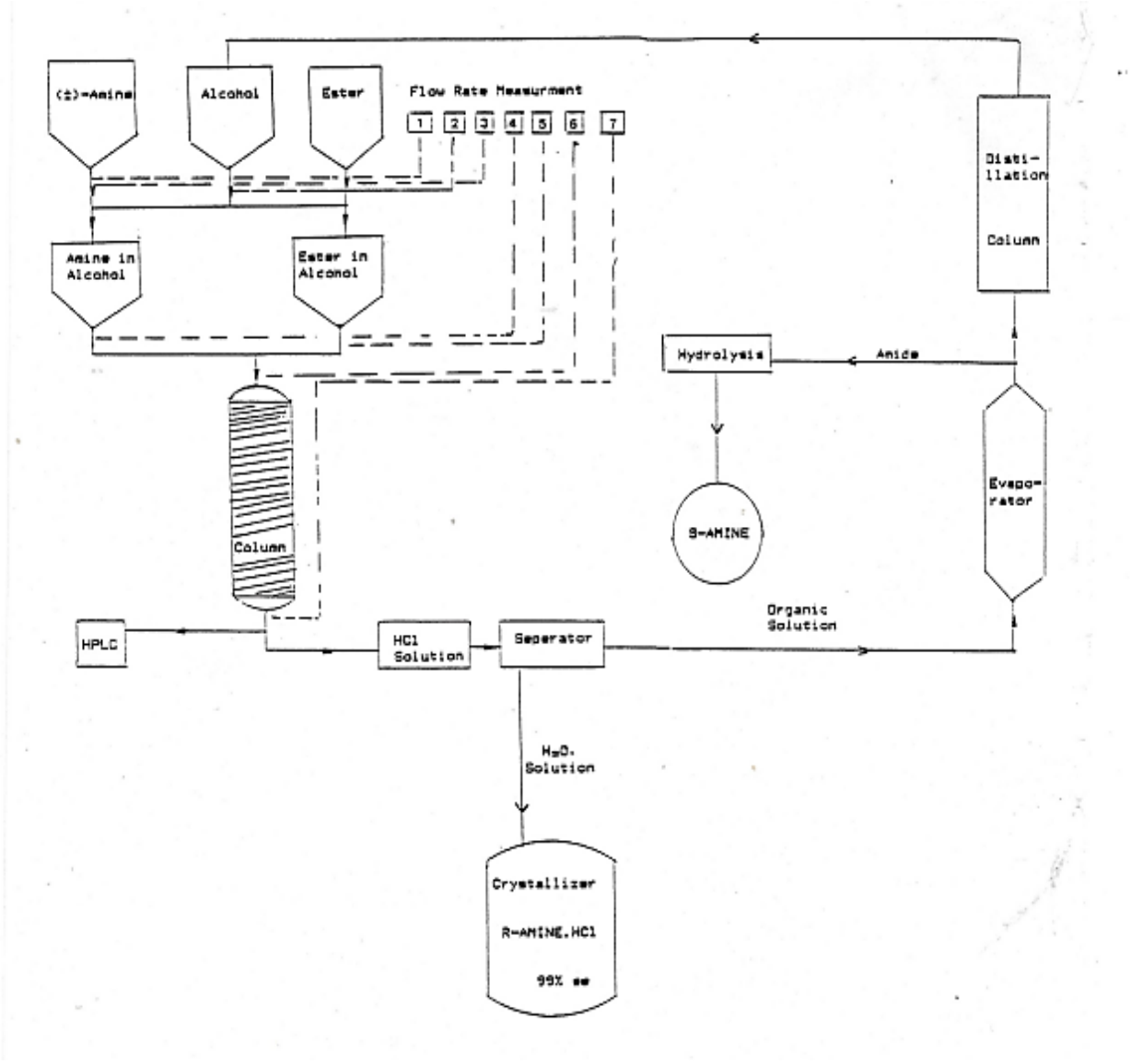
Figure 5



Conclusion

It is demonstrated in the present article that continuous flow bioreactor, packed with an enzyme immobilized on a support such as glass beads is a powerful tool for resolution of pharmaceutically important materials such as amines. Optimizing the technological aspects of the process such as selection of an optimal support, flow rate, measurements of mass transfer, efficiency of the support, particle size, reactants mixing, retention time and thermal control is crucial in order to achieve high ee and good yield. The system enables facile in-line purification and isolation of the reaction products and reprocessing of the excess raw materials and recycling the solvents.

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