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Improved Lipase-Mediated Resolution of Mandelic Acid Esters by Multivariate Investigation of Experimental Factors

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Abstract: Lipase catalyzed stereoselective acylation of butyl mandelate was studied. The determining role of solvent and acylating agent was pointed out and a considerable inhibitory effect due to mandelic acid was observed by screening different lipases. Finally, the performance of the reaction was appreciably improved thank to a multivariate approach.

The resolution of racemic mixtures of acids or alcohols can be achieved by using the enantioselective properties of hydrolytic enzymes. Optically pure compounds are obtained in aqueous solution by stereoselective hydrolysis of the racemic esters or in organic solvents by esterification or transesterification of the corresponding racemic alcohols[1-3]. Commercial lipases are inexpensive and can be used in organic solvents, but the choice of the most suitable system requires the evaluation of many factors.

In fact, many devices are commonly suggested to improve the performance of the catalytic system. For instance, the rate of certain reactions can be increased by using immobilized enzymes, and also it is well known that employing organic solvents enables the realization of transformations otherwise difficult in water. However, these systems are rather complex and usually the identification of the most profitable operating conditions is difficult and time consuming. As shown in previous papers[4,5], a multivariate study allows the experimental domain in biotechnological systems to be explored with a minimum experimental effort.

The resolution of mandelic acid and its derivatives was chosen for this study since these compounds have been utilized extensively both for synthetic purposes and in stereochemical investigations. In fact, the enantiomers of mandelic acid can be used for the resolution of alcohols[6], amines[7], etc., permitting also the determination of enantiomeric excess by HPLC[6] or NMR[8,9]. Furthermore, a significant number of syntheses employ enantiomerically pure mandelic acid to yield chiral compounds, as, for instance, the stereoselective synthesis of aldols, alcohols[10,11] and prostaglandins[12].

The stereoselective chemical- or enzyme-assisted synthesis of the mandelic acid or its derivative is widely documented[13-17] but the approaches used so far do not meet all the chemical and economical requirements appropriately, giving poor stereoselectivity or low reaction rate. The presence of a secondary hydroxy group embodies one of the main problems, due to its low reactivity, even when enzymatic catalysts are used.

This work intends to approach the resolution of mandelic acid derivatives according to the following strategy: i) choice of the most promising synthetic reaction, ii) development of the optimization study by a multivariate approach, since this enables the set of experiments required to be minimized.

RESULTS AND DISCUSSION

First of all, it was necessary to select the reaction and the catalytic system. It has been previously reported[18] that porcine pancreatic lipase and lipase from *Candida Cilindracea* are unable to catalyze the transesterification of mandelic acid using an activated ester. This was ascribed to the steric hindrance of the substrate. We tested fifteen lipases (Table 1) from various sources, without success. However when we studied the rate of the lipase-catalyzed hydrolysis of a model ester (trifluoroethyl laurate), the reaction was seven times slower in the presence of mandelic acid. This indicates that mandelic acid is responsible for enzyme inhibition and presumably steric hindrance is not the main, or at least the unique impediment to the enzymatic acylation. As a consequence, it seemed necessary to operate with esters of mandelic acid and butyl mandelate was chosen.

All the 15 lipases reported in Table 1 were screened. Vinyl acetate was chosen both as solvent and acylating agent in order to simplify the reaction system. Lipases from *Pseudomonas* are the most active and also endowed with a marked enantioselectivity towards the S-(+) isomer.

Table 1. Conversion of the n-Butyl Mandelate (0.2M) to Acetyl derivative, in Vinyl Acetate, after 48h, Catalyzed by Lipase (25mg/mL) (T=50°C).

	Lipase	Conv. (%)
1	TYPE II Crude (Steapsin, from Porcine Pancreas - SIGMA)[a]	<2
2	TYPE VII (from *Candida cylindracea* - SIGMA)	<2
3	from *Pseudomonas fluorescens* (FLUKA)[b]	12
4	from *Aspergillus niger* (FLUKA)	<2
5	from *Rhizopus arrhizus* (FLUKA)	<2
6	from *Mucor javonicus* (FLUKA)	<2
7	from *Penicillium roqueforti* (FLUKA)	<2
8	from *Porcine pancreas* (FLUKA)	<2
9	Lipoprotein lipase (LPL) (from *Pseudomonas species* - AMANO)	44
10	PS (from *Pseudomonas species* - AMANO)	56
11	M (from *Mucor javonicus* - AMANO)	<2
12	AY (from *Candida cylindracea* - AMANO)	<2
13	AP 6 (from *Aspergillus niger* - AMANO)	<2
14	FAP 15 (from *Rhizopus javonicus* - AMANO)	<2
15	N conc (from *Rhizopus niveus* - AMANO)	<2

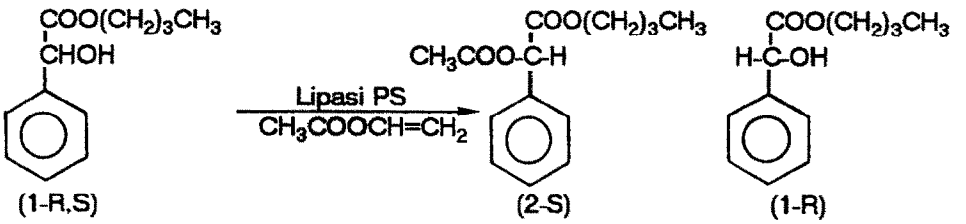
[a] This lipase was used as a crude preparation, in a concentration of 50mg/mL

[b] This lipase was used in a concentration of 5mg/mL, because of its high activity

The choice of acylating agent was effected as follows. The use of enol esters as acyl donor has been proposed as a means for overcoming the problem of the reactivity of the alcohol resulting from the reacted ester[19,20]. A comparison among three acylating agents, trifluoroethyl butyrate, vinyl acetate, acetic anhydride was carried out. When we utilized acetic anhydride, according to the

conditions proposed in ref. 21, the uncatalyzed acetylation prevailed over the lipase-catalyzed reaction thus leading to a poor enantioselectivity. On the other hand, trifluoroethyl butyrate showed a reaction rate ten times slower than using vinyl acetate. The effect of organic solvent was also examined. We found isopropyl ether to be the best solvent for our study, compared to n-hexane, tetrahydrofuran or vinyl acetate, which give either poor substrate solubility or low reactivity.

Therefore, in this preliminary setting-up of the system, the most promising system corresponds to the acylation of butyl mandelate, by vinyl acetate, in isopropyl ether, catalyzed by lipase PS, which showed the highest activity/cost ratio. Nevertheless, the reaction appears to be susceptible to improvement by means of a better choice of the operating conditions. This can be realized by a multivariate approach.



With the conventional approach ("one-variable-at-a-time", OVAT) all reaction parameters are maintained constant but the one under investigation. Unfortunately, operating in this way, the true maximum may sometimes not be reached because only a part of the experimental domain is explored. This situation can be avoided by using multivariate methods, such the factorial analysis. In this case, all parameters are changed simultaneously in a suitable, programmed manner, allowing an efficient and rational scan of the experimental range for all variables. Furthermore, by following this approach optimization is reached with fewer experiments[23,24].

The factorial analysis is an approach which does not require a complete knowledge of the kinetic and thermodynamic characteristic of a reaction system. The dependence of the y variable, which describes the system, upon the experimental variables x_i can be approximated with a polynomial equation such as:

$$y = b_0 + \Sigma_i b_i x_i + \Sigma_{ii} b_{ii} x_i^2 + \Sigma_{i<j} b_{ij} x_i x_j + \ldots \qquad \text{(eq. 1)}$$

The enantioselectivity ratio, E, offers a good description of the response system and therefore this variable has to be maximized[22].

$$E = \frac{\ln[(1-c)(1-e.e.(S))]}{\ln[(1-c)(1+e.e.(S))]} \qquad \text{(eq. 2)}$$

However, E indicates only the difference of the reactivity between the enantiomers without giving any information about the rate of the reaction. For this reason, the percentage of acylated substrate was also taken into consideration for the optimization study, since this variable measures the activity of the enzyme and has to be maximized too. However, the substrate is the enantiomerically

pure compound obtainable from the reaction. Therefore, the conversion must attain the 50%, to assure the complete acylation of the S enantiomer, but without exceeding significantly this value, since there would be to the detriment of the amount of the target compound (1-R). Finally, the enantiomeric excess can also be modelled separately. The determination of enantiomeric excess is necessary to calculate E, and no extra experimental work is required.

The variations of such responses were expressed by functions of four experimental variables, x_1: temperature (°C), x_2: substrate concentration (mM), x_3: vinyl acetate concentration (mM), x_4: time (h), (see Table 2). If we put the actual operating values in the equation, we can not compare directly the b_i coefficients, in order to know which are the meaningful variables. Consequently, it is preferable to codify the x_i values, which then assume two extreme values defining the experimental domain and corresponding to the +1 and -1 notations.

Table 2. Independent Variables, Their Levels and Responses in Factorial Design Experiments.

Independent variable	(-1)	(0)	(+1)	Dependent variable
x_1: temperature (°C)	45	50	55	y_1: Enantioselectivity ratio
x_2: [substrate] (mM)	80	120	160	$E=\frac{\ln[(1-c)(1-e.e.(S))]}{\ln[(1-c)(1+e.e.(S))]}$
x_3: [vinyl acetate] (mM)	160	240	320	y_2: % acylated substrate
x_4: time (h)	7	9.5	12	y_3: enantiomeric excess

The variation of the percentage of conversion and the enantiomeric excess with time was evaluated by measuring the responses at definite times (7, 9.5 and 12h). Experiments for determining the importance of the experimental variables were randomly performed according to a factorial design 2^3 (Table 3, experiments 1-8).

Table 3. Factorial Design Matrix 2^3.

				time = 7h		time = 9.5h		time = 12h		E
	x_1	x_2	x_3	conv.	e.e.	conv.	e.e.	conv.	e.e.	
1	-1	-1	-1	56.1	85.7	58.6	98.7	60.2	100.0	26.8
2	1	-1	-1	58.1	96.0	61.4	99.2	62.8	100.0	22.0
3	-1	1	-1	46.6	71.8	51.8	85.8	55.5	93.0	24.3
4	1	1	-1	53.2	80.5	57.0	95.4	59.7	99.0	22.5
5	-1	-1	1	51.8	81.9	55.2	96.8	57.5	100.0	33.7
6	1	-1	1	59.1	83.3	61.6	99.6	63.2	100.0	24.4
7	-1	1	1	43.2	71.7	50.7	87.4	54.6	96.5	35.1
8	1	1	1	50.4	85.0	56.0	94.2	58.0	98.5	23.3
9	0	0	0	50.6	83.1	55.2	94.3	57.3	98.5	26.4
10	0	0	0	50.8	82.4	55.2	94.5	58.1	98.9	26.9
11	0	0	0	47.3	69.6	52.3	86.8	55.4	95.0	24.0

Enantioselectivity

Estimates of the coefficients of the polynomial for the response E are given in Table 4. These estimates were compared with the experimental error in order to select meaningful coefficients. The standard deviation was calculated on the basis of the results obtained from three replicated runs of the central point of the factorial design (Table 3, exp. 9-11).

Table 4. Main and Interaction Effects upon the Enantioselectivity Ratio.

b_0	b_1	b_2	b_3	b_{12}	b_{13}	b_{23}	b_{123}
y	T	[S]	[VA]	T [S]	T [VA]	[S] [VA]	T [S] [VA]
26.5	-3.46*	-0.21	2.61*	0.06	-1.81*	0.29	-0.69

* The significance was established by comparison with standard deviation (s.d. = 1.55)

Generally speaking, large coefficient values indicate a strong influence of the variable(s) on the response. Table 4 shows that, inside the experimental domain, temperature (x_1), vinyl acetate concentration (x_3) and their interaction exert significant effects on the enantioselectivity ratio, since their values are larger than the standard deviation. It is remarkable that the application of statistical methods revealed the interaction temperature-vinyl acetate concentration and, as a consequence of this interaction, these variables can not be studied separately. The data show that the effect of the concentration of the acylating agent is great when the temperature assumes the lower coded value (-1), while it is negligible for the (+1) level. The analysis of the data suggests that the vinyl acetate concentration should be maintained at the higher level and the temperature at the lower one. It is noteworthy that, even though the dependence of E on the temperature and its independence from the substrate concentration are obvious, the dependence on the vinyl acetate concentration can not be predicted from the definition of E.

The curvature check[23] for the response surface is negative, and the surface does not present any maximum. The screening model can describe the experimental space adequately with the significant variables:

$$E = 26.5 - 3.46x_1 + 2.61x_3 - 1.81x_1x_3 \qquad \text{(eq. 3)}$$

Since there is not a maximum inside the explored region, further runs were performed along the direction of the steepest ascent in order to verify whether a higher enantioselectivity is achievable. When the variables are coded, the estimated direction of steepest ascent follows the vector of coefficient values, *i.e.* the vector resulting from moving simultaneously 2.61 ($=b_3$) units in x_3, for every -3.46 ($=b_1$) units moved in x_1, or, equivalently, -2.61/3.46 = -0.75 units in x_3 for every 1 unit in x_1. A convenient point on the path of steepest ascent is x_1 = -2.00 (T=40°C), x_3 = 1.508 ([VA]= 360mM) and the corresponding observed value for E is 39. This value is lower than the predicted one (E=43), thus indicating that a curvature should be present. It appears clear that the enantioselectivity can not be greatly improved, and the reaction time becomes longer along this direction. Furthermore, it should be emphasized that only the multivariate approach, which takes into account the interaction between x_1 and x_3, could find these conditions. This can be deduced from the following results:

T		[VA]		E
50	(0.0)	240	(0.0)	26
40	(-2.0)	240	(0.0)	34
40	(-2.0)	360	(1.5)	39
40	(-2.0)	400	(2.0)	28

The enantioselectivity ratio falls rapidly even in proximity to the direction of steepest ascent.

Conversion and Enantiomeric Excess

Another approach leading to the identification of the most favourable operating conditions consists in modelling the percentage of conversion (y_2) and enantiomeric excess (y_3) separately and then searching for a compromise solution.

The time variable was also considered, since it is an interesting operating parameter. In fact, it is advantageous to reduce the reaction time, and usually a slightly poorer yield is largely counterbalanced by a more profitable overall result.

The same set of data provides the estimates of coefficients of the quadratic models for enantiomeric excess and the percentage of conversion (Table 5). The significance of the terms must be evaluated by means of statistical tests.

Table 5. Regression Coefficients of the Second-Order Models for the Responses.

	b_0	b_1	b_2	b_3	b_4	b_4^2
Effect	y	T	[S]	[VA]	t	t^2
Conv.	56.5	2.46*	-2.66*	-0.66	3.31*	-0.91
e.e.	94.6	2.46*	-3.94*	-0.14	8.13*	-4.39*

	b_{12}	b_{13}	b_{14}	b_{23}	b_{24}	b_{34}
Effect	T [S]	T [VA]	T t	[S] [VA]	[S] t	[VA] t
Conv.	0.21	0.50	-0.45	-0.09	0.99*	0.29
e.e.	1.27	-0.17	-1.67*	0.87	1.62*	0.64

* The significance of the effects was established by means of F-test.

The model for the percentage of conversion is:

$$\text{conv.} = 56.5 + 2.46x_1 - 2.66x_2 + 3.31x_4 + 0.99x_2x_4 \qquad \text{(eq. 4)}$$
$$r = 0.962$$

and for the enantiomeric excess

$$\text{e.e.} = 94.6 + 2.46x_1 - 3.94x_2 + 8.13x_4 - 4.39x_4^2 + 1.27x_1x_2 - 1.67x_1x_4 + 1.62x_2x_4 \qquad \text{(eq. 5)}$$
$$r = 0.982$$

Both the responses vary with temperature, substrate concentration and time, but the

enantiomeric excess shows a more complex dependence, because the quadratic as well the interaction terms are significant. Since the three variables are correlated, the effect of their variation can not be evaluated independently, and thus the interpretation is neither simple nor obvious.

The effects of temperature and substrate concentration have the same sign in both the equations, and this means that in order to improve the reaction performance it is necessary to find a compromise which allows the optimal enantiomeric excess to be achieved without exceeding the percentage conversion, and therefore decreasing the amount of 1-R product.

The graphical representation of response surfaces as a function of the three factors is impracticable, nevertheless one could examine several sections at constant values. For instance, fixing the substrate concentration at -1 or +1 value, a section is obtainable in which the variation with the temperature and the time is represented (Figure 1,2). From the isoresponse diagrams it is possible to deduce which experimental conditions lead to an enantiomeric excess higher than 99% and the lowest conversion at the same time. It is noticeable that the lower the substrate concentration, the wider is the area having optical purity higher than 99%, and this results in an easier identification of conversion values which are low enough. In fact, fixing the substrate concentration at +1 level (Figure 2), the enantiomeric excess never reaches adequate values. For instance, a quite satisfying response can be obtained in the following conditions: T = 40°C (x_1=-1), [substrate] = 80mM (x_2=-1), time = 12h (x_4=+1). For this set of conditions, the predicted enantiomeric excess is equal to 100%, the conversion being 57%. Both data show an excellent agreement with the observed results corresponding to experiment number 5 of the design.

CONCLUSIONS

The reactions previously reported in literature (Table 6) propose interesting approaches to the preparation of enantiomerically pure compounds, but they present, in our opinion, some unfavourable aspects regarding the specific aim of the resolution of mandelic acid derivatives.

Table 6. Outline of the Reactions Reported in Literature

Substrate	Product	Catalyst	Time	Yield (%)	e.e. (%)	Ref.
n-butyl O-acetyl-mandelate (R,S)	n-butyl mandelate (R)	lipase from *Candida Cylindracea*	7d	45[a]	92	13
benzaldehyde	mandelonitrile (R)	mandelonitrile lyase immobilized	2.5h	47.5	99	14
benzaldehyde	mandelonitrile (R)	Compounds enzyme mimetic	8h	97	98	15
methyl benzoylformate	n-methyl mandelate (R)	Compounds NADH mimics	1h	99	98	16
n-methyl mandelate (R,S)	n-methyl mandelate (R)	lipoprotein lipase immobilized	129h	47[a]	93	17
n-butyl mandelate (R,S)	n-butyl mandelate (R)	lipase PS	12h	43[a]	>99	This work

[a] In this reaction the maximum yield obtainable was 50%.

In fact, the first method requires the previous acetylation of n-butyl mandelate and, moreover, the

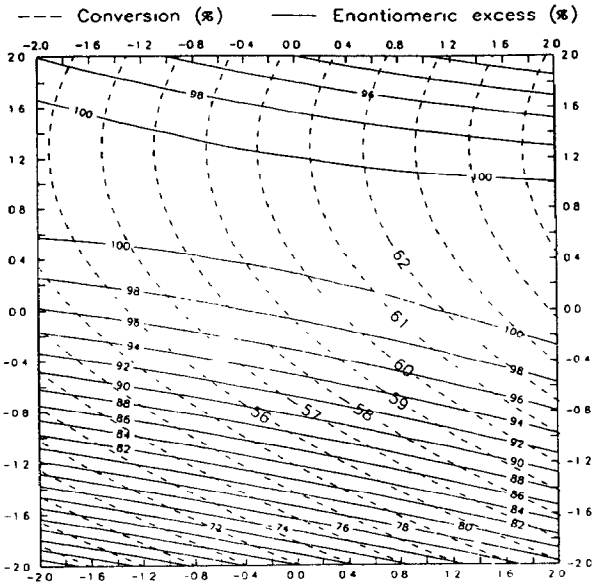


Figure 1. Isoresponse plots for the responses. The sections were obtained under the following conditions: x_2 (concentration of substrate) = -1 (80mM). y-axis = x_4 (time); x-axis = x_1 (temperature).

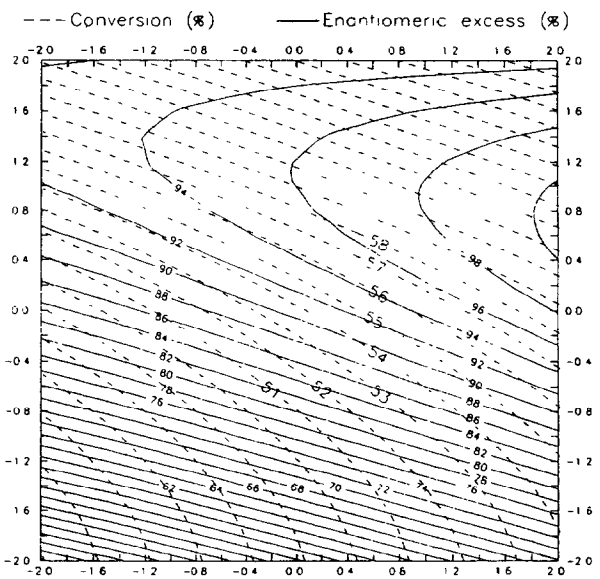


Figure 2. Isoresponse plots for the responses. The sections were obtained under the following conditions: x_2 (concentration of substrate) = +1 (160mM). y-axis = x_4 (time); x-axis = x_1 (temperature).

transformation is very slow. The mandelonitrile requires the hydrolysis (88% yield) or the alcoholysis (methanol, 50% yield) to obtain an acid or an ester. Furthermore, the employed catalysts are much more expensive than lipases and they require, respectively, to be immobilized or synthesized. The reduction of methyl benzoylformate by NADH mimics appears to be attractive, but the synthesis of NADH mimics is complex. Furthermore, the substrate is much more expensive than the (R)-mandelic acid. Finally, the method reported by Y.F. Wang et al.[17], indicates the necessity of immobilizing the lipase in order to increase the reaction rate. However, our approach shows that the optimization of the operative parameters is sufficient to yield a faster reaction, so avoiding the expense and the work for the immobilization.

EXPERIMENTAL SECTION

Materials. Butyl ester of pure or racemic mandelic acid were synthesized by using a standard procedure[25]. All the chemicals were of analytical or HPLC grade, were bottled and dried on 4Å molecular sieves (100g/L), and used without further treatments. The lipases (see Table 1) were dried under vacuum (1mmHg) for 3 days and stored on P_2O_5.

Procedure for the screening reactions. All the reactions were performed in thermostatted vessel under magnetic stirring. The conditions for the screening of the lipases were the following: temperature 50°C (±0.5), substrate concentration 0.2M, acylating agent concentration 0.8M, enzyme 25mg/mL, solvent 2mL of isopropanol. The conditions for the screening of the solvent were: temperature 40°C (±0.5), n-butyl mandelate concentration 80mM, vinyl acetate concentration 400mM, enzyme 50mg/mL.

General procedure for optimization study of the acetylation of butyl mandelate. A solution of racemic ester and vinyl acetate in isopropyl ether (5mL), according to the concentrations reported in Table 3, was supplemented with powdered lipase (50mg/mL). The suspension was thermostatted in conformity with the values of the design and magnetically stirred. Samples were withdrawn at 7, 9.5, 12h and quantitatively analyzed by HPLC and by GC.

Determination of the percentage of conversion of n-butyl mandelate. The percentage of conversion was determined by gas chromatography using a Perkin-Elmer SIGMA 3B Dual FID gascromatograph, equipped with a SE 30 column, 100-120mesh, l = 6" (N_2 carrier gas, 25mL/min, detector and injector temperature 350°C, column temperature 170°C). The retention times observed were 1.7min for biphenyl (internal standard), 3.0min for n-butyl mandelate and 4.7min for n-butyl O-acetylmandelate.

Determination of the enantiomeric excess of n-butyl mandelate. A Perkin-Elmer Series 10 Liquid Chromatograph, equipped with a Perkin-Elmer LC-85B variable wavelength UV-visible detector set a 263nm and a Regis Pirkle TYPE 1-A chiral column, was employed to measure the concentration of enantiomers. The mobile phase was n-hexane-isopropanol 95:5 and the flow rate was 1mL/min. The retention times of the enantiomers were determined by comparison with stereochemically pure compounds.

Statistical methods. Calculations for factorial analysis and response surface were performed on an IBM PS2 Model 60 microcomputer, using the REGFAC program package[26].

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