Acetylation of vitamin E by *Candida antarctica* lipase B immobilized on different carriers

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ABSTRACT

We describe for the first time the enzymatic acylation of the phenolic group of tocopherols (vitamin E) by transesterification with vinyl acetate in 2-methyl-2-butanol (2M2B). Out of 15 hydrolases screened, only the lipase B from *Candida antarctica* (Novozym 435) catalyzed the acylation. The acetylation of δ-tocopherol was faster than that of α-tocopherol, probably due to its lower methylation degree. A series of experiments using (*R*)-Trolox and *p*-cresol as competitive acceptors of tocopherols showed that reaction rate notably diminished when increasing acceptor size. To maximize the potential of this reaction, three immobilization carriers for *C. antarctica* lipase B were studied: the ion-exchange resin Lewatit (the support in Novozym 435), a biodegradable polymer (Purasorb) and polypropylene (Accurel EP100). The acetylation of α-tocopherol was faster with the enzyme immobilized in polypropylene, which was correlated with its higher porosity. A mixture hexane/2M2B 90:10 (v/v) was found to be the optimum medium composition, as it represents a compromise between substrates solubility and biocatalyst efficiency. The acylation process was no enantioselective, probably due to the fact that the chiral centers are separated from the phenolic group by a minimum of six bonds.

Key words: immobilization, lipases, transesterification, Novozym 435, tocopherols, antioxidants modification.

1. INTRODUCTION

Antioxidants protect cells against the effects of harmful free radicals and play an important role in preventing many human diseases (e.g. cancer, atherosclerosis, stroke, rheumatoid arthritis, neurodegeneration, inflammatory disorders, diabetes) and aging itself [1;2]. The study of antioxidants is of great interest for the role they play in protecting living systems against lipid peroxidation and other anomalous molecular modifications [3]. In addition, antioxidant molecules also prevent unsaturated oil products from becoming rancid during storage, thus extending its shelf life [4;5].

Among the natural antioxidants, the term vitamin E describes the beneficial biological activity on humans and animals of a group of structurally related compounds, in particular α–, β-, γ– and δ-tocopherol, plus α–, β-, γ– and δ-tocotrienol [6]. Vitamin E enhances the oxidative stability of the organisms owing to its ability to protect polyunsaturated fatty acids from peroxidation and to scavenge free radicals. Tocopherols have three chiral centers at carbons 2, 4'- and 8'-, and the naturally occurring isomer has the *RRR*-configuration (Fig. 1), whereas tocotrienols have only one anomeric center at carbon 2 [7]. It is generally accepted that the *RRR*-α-tocopherol is the most bioactive compound [8] as it is specifically recognized by membranes [9]. For example, the isomer with inverted stereochemistry at position 2 has only 30% of the biological activity of the *RRR* isomer. The synthetic vitamin E (α-tocopherol) is obtained by reaction of trimethylhydroquinone with isophytol [10], without any control of stereochemistry, and comprises a mixture of four pairs of enantiomers in equal amounts (*all-rac*). Synthetic tocopherols are not as biologically active as natural, due to non-active stereoisomers [11].

To increase its stability in the presence of light and oxygen and/or to alter its physical properties such as solubility and miscibility, vitamin E is generally administered as a prodrug in the form of *all-rac*-α-tocopheryl acetate (vitamin E acetate) or *all-rac*-α-tocopheryl succinate (vitamin E succinate). These derivatives carry an acetyl moiety at the C-6 phenolic group that blocks the antioxidant properties [12]. However, unspecific esterases rapidly

cleave *in vivo* the ester bond and release the active α-tocopherol. The vitamin E acetate is synthesized by chemical acylation of *all-rac*-α-tocopherol with acetic acid or acetic anhydride as acyl donors and a metal catalyst [13]. The enzymatic acetylacion or succinylation of vitamin E has not been described before, probably due to the large molecular size of the acceptor (tocopherol) and its steric hindrance within the active site of the enzyme.

In the present work, we have studied the enzymatic synthesis of vitamin E acetate by lipase-catalyzed transesterification and the effect of immobilization carrier and solvent composition on acylation rate. Our results suggest that *Candida antarctica* lipase B offers appropriate tools for the transformation of vitamin E under mild conditions.

2. EXPERIMENTAL PROCEDURES

Materials

Immobilized lipase from *C. antarctica* B (Novozym 435) was kindly donated by Novozymes A/S. A small sample of lipase B from *C. antarctica* adsorbed on Accurel EP100 and Purasorb was prepared by Novozymes A/S in the context of the collaborative EU project BIO4-CT98-0363 following the protocol previously reported [14]. *All-rac*-α-tocopherol was kindly provided by Biotecnologías Aplicadas (BTSA, Madrid, Spain). Tributyrin, *RRR*-α-tocopherol, methyl laurate, propyl laurate and 2-methyl-2-butanol (*tert*-amyl alcohol) were from Sigma. (*R*)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid [(*R*)-Trolox], vinyl acetate and *p*-cresol were from Aldrich. Solvents were dried over 3 Å molecular sieves. All other reagents were of the highest available purity and used as purchased.

Hydrolytic activity

The hydrolytic activity was measured titrimetrically at pH 7.0 and 30 °C using a pH-stat (Mettler, Model DL 50). The reaction mixture consisted of 0.8 ml tributyrin (64.2 mM final concentration in the vessel), 1.2 ml acetonitrile and 40 ml of 1 mM Tris-HCl buffer (pH 7.0) containing 0.1 M NaCl. The immobilized biocatalyst (5 mg) was then added and the pH automatically maintained at 7.0 using 0.1 N NaOH as titrant. Experiments were done in triplicate. One enzyme unit (*U*) was defined as that catalyzing the formation of 1 μmol of fatty acid per min.

Synthetic activity

To determine the activity in synthesis, methyl laurate (50 mM) and 1-propanol (50 mM) were dissolved in 5 ml hexane in a screw-capped test vial, and stirred for 15 min at 30 °C. Then, the immobilized biocatalyst (75 mg) was added, and the mixture incubated at 30 °C and 150 rpm using an orbital shaker (Stuart Scientific). At defined intervals, 50 μl aliquots were taken,

centrifuged and filtered using an eppendorf tube with a 0.45 μm filter (Ultrafree-MC, Millipore). The formation of propyl laurate was followed by HPLC. One enzyme unit (*U*) was defined as that catalyzing the formation of 1 μmol of propyl laurate per min.

Water activity

Water activity was determined using a humidity and temperature digital indicator Novasina Thermoconstanter TH200 (Novasina, Switzerland). The humidity sensor was calibrated with control saturated salts solutions (LiCl, a_w=0.11; potassium acetate, a_w=0.22; NaBr, a_w=0.57; NaCl, a_w=0.75) at 25 ºC.

Enzymatic acetylation of tocopherols

Tocopheryl acetate was synthesized by transesterification of vinyl acetate (100-400 mM) with α-tocopherol or δ-tocopherol (25-100 mM). Reactions were carried out in 2-methyl-2-butanol (2M2B) in sealed 30 ml dark vials at 60 ºC with orbital stirring (190 rpm). Reaction volume was 5 ml. The biocatalyst was added to a final concentration of 100 mg/ml. Aliquots (200 μl) were removed at intervals, filtered using an eppendorf tube containing a Durapore® 0.45 μm filter and analyzed by HPLC.

Competitive acetylation experiments

α-Tocopherol or δ-tocopherol (50 mM) and (*R*)-Trolox or *p*-cresol (50 mM) were dissolved in 2M2B containing vinyl acetate (400 mM). Reactions were carried out at 60 ºC with orbital shaking (190 rpm) in sealed 30 ml vials. Reaction volume was 5 ml. The biocatalyst was added to a final concentration of 100 mg/ml. Aliquots (200 μl) were removed at intervals, filtered using an eppendorf tube with a 0.45 μm filter (Ultrafree-MC, Millipore), and analyzed by HPLC.

HPLC analysis

For HPLC analysis, a ternary pump (model 9012, Varian) coupled to a thermostatized (15 ºC) autosampler (VWR Hitachi L-2200) was used. The temperature of the column was kept constant at 45 °C (oven model MEF-01, Análisis Vínicos, Spain). Detection was performed using a photodiode array detector (ProStar, Varian), and integration was carried out using the Varian Star LC workstation 6.41. For tocopheryl acetate synthesis, the column was a Lichrospher 100 RP8 (4.6 x 125 mm, 5 μm, Análisis Vínicos). Mobile phase was 95:5 (v/v) methanol:water (water contained 0.1% of acetic acid) at 1 ml/min. The reaction products were quantified by measuring the absorbance at 240 nm. For propyl laurate synthesis, the column was a Mediterranea-C18 (4.6 x 150 mm, 5 μm, Teknokroma, Spain). The mobile phase was 95:5 (v/v) methanol:water (water contained 0.1% of acetic acid) at 1.2 ml/min, and the analytes were quantified by measuring the absorbance at 216 nm.

HPLC/MS

To confirm the nature of the acetylated phenolic derivatives, samples were analyzed by HPLC coupled to mass spectrometry (using a HPLC1100 equipment, Agilent Technologies). Chromatographic conditions were as described above, except for the mobile phase contained 1% (v/v) formic acid and the flow rate was lowered to 0.6 ml/min. Ionization was performed by electrospray and the mass spectrometer had a hybrid analyzer QTOF model (QSTAR pulsar, Applied Biosystems).

Characterization of immobilized biocatalysts

Mercury intrusion porosimetry analyses of the biocatalysts were performed using a Fisons Instruments Pascal 140/240 porosimeter. To ensure that the samples were moisture free, they were dried at 100 ºC overnight, prior to measurement. Assuming a cylindrical pore model, the recommended values for the mercury contact angle (141°) and surface tension (484 mN/m) were used to evaluate the pressure/volume data by the Washburn equation. The

specific surface area (S_{BET}) of the supports was determined from analysis of nitrogen adsorption isotherms at -196 °C. The samples were previously degassed at 100 °C for 12 h to a residual vacuum of $5x10^{-3}$ torr, to remove any loosely held adsorbed species, using a Micromeritics ASAP 2010 device. Water content of the supports was assayed using a DL31 Karl-Fisher titrator (Mettler). Scanning electron microscopy (SEM) was performed using an XL3 microscope (Philips) on samples previously metallized with gold.

3. RESULTS AND DISCUSSION

3.1. Enzyme screening for acetylation of tocopherols.

Approximately 15 hydrolases (Table 1) were screened for the transesterification of vinyl acetate with *all-rac*-α-tocopherol in 2-methyl-2-butanol (2M2B). Four esterases from bovine rumen microflora [15] and one from Urania hypersaline basin [16], obtained using metagenomic techniques, were tested . An sterol esterase from the ascomycete *Ophiostoma piceae* obtained as previously described [17] was also assayed. Some of the hydrolases were previously immobilized in Celite or in epoxy-activated Dilbeads TA [18]. Screening conditions were: 50 mM α-tocopherol, 150 mM vinyl acetate, 20 mg/ml biocatalyst, 40 ºC. Among all the enzymes tested, only the immobilized lipase B from *C. antarctica* (Novozym 435) catalyzed this reaction significantly, although the acylation was slow compared with other processes involving the same biocatalyst and more simple primary or secondary alcohols. For the rest of hydrolases, α-tocopherol was probably too large to fit into the acceptor binding site and thus attack the intermediate acetyl-enzyme yielding the tocopheryl ester [19]. The lipases from *Candida rugosa* and *Pseudomonas cepacia*, despite their notable transesterification activity towards large hydroxylated substrates [20], did not catalyze the acetylation of vitamin E. By computational conformation studies, it has been demonstrated that the acceptor binding site of lipase B from *C. antarctica* is deep (compared with other lipases, e.g. from *Thermomyces lanuginosus)* [21], which partly explains the broader specificity of *C. antarctica* lipase B [22;23]. In addition, Salis *et al.* reported that the atypical lipase B from *C. antarctica* is very well adapted for catalysis in organic media [24]**.** In fact, the lipase B from *C. antarctica* is the preferred biocatalyst for many esterification and transesterification processes [25], including the modification of antioxidants [26-30].

To our knowledge, this is the first evidence on enzymatic acetylation of vitamin E. Tocopheryl acetate is normally obtained by a metal-catalyzed reaction using a great excess of acyl donor [31], but these routes do not meet the necessary requirements for food

applications. In contrast, this biocatalytic process is performed under milder conditions and thus is closer to the green chemistry [32].

3.2. Effect of acceptor molecular size.

A comparative study was carried out assaying α- and δ-tocopherol as acceptors under more drastic conditions than in the initial screening. We first analyzed the effect of the molar ratio tocopherol:vinyl acetate (data not shown). We found that a molar ratio 1:4 was optimal in terms of acylation rate without the need of increasing the concentration of acyl donor. Final conditions assayed were: 100 mM tocopherol, 400 mM vinyl acetate, 100 mg/ml biocatalyst, 60 ºC. As shown in Fig. 2, the reaction rate was higher with δ-tocopherol, which rendered 65% yield of acetylated product in approx. 2 weeks. The reproducibility of the assays was satisfactory, with standard deviations lower than 5 %. This different behaviour seems to be related with the lower methylation degree of δ-tocopherol (Fig. 1), which diminishes the steric hindrance within the acceptor binding site.

The molecular weight of the synthesized products was determined by HPLC coupled to mass spectrometry to confirm their chemical nature (Fig. 3). In the case of α-tocopherol reaction, the main peak at *m/z* 473.44 corresponded to the ($[M+H]^+$) of α-tocopherol acetate (MW 472.74). For δ-tocopherol acetate (MW 444.68), the main peak in the mass spectrum at *m/z* 462.44 corresponded to the ($[M+NH_4]^+$) with a minor ($[M+H]^+$) peak at 445.42.

It is well known that acyl-transfer reactions catalyzed by *C. antarctica* lipase B follow bi-bi ping-pong mechanism, and the alcohols used as acyl acceptors display competitive substrate inhibition [33]. A series of competitive reactions between tocopherol and other phenolic acceptors were performed to evaluate the effect of molecular size on the acylation rate. (*R*)-Trolox presents the same chromanol ring of α-tocopherol −which is responsible of the antioxidant properties− but lacks of the aliphatic chain (Fig. 1), and *p*-cresol is quite a smaller molecule. Fig. 4 illustrates the reaction progress using the following pairs of acceptors: α-tocopherol/(*R*)-Trolox, δ-tocopherol/(*R*)-Trolox, α-tocopherol/*p*-cresol, δ-

tocopherol/*p*-cresol. The formation of (*R*)-Trolox acetate and *p*-tolyl acetate was confirmed by HPLC/MS (data not shown). As depicted in Fig. 4, the reaction with Trolox was notably faster than the observed with α- or δ-tocopherol, suggesting that the main steric hindrance is probably caused by the aliphatic chain rather than the chromanol ring. When using a simple phenol such as *p*-cresol, the reaction is nearly one order of magnitude faster than with tocopherols.

3.3. Effect of immobilization support.

Immobilization is a suitable approach to expose the enzyme more efficiently to the substrate, being specially indicated for synthetic processes in non-aqueous solvents [34]. Several parameters of immobilized lipases are important to consider for industrial application: mechanical strength, chemical and physical stability, hydrophobic/hydrophilic character, enzyme loading capacity and cost, to cite some. A comparative study with the *C. antarctica* lipase B adsorbed on the ion-exchange resin Lewatit (Novozym 435), polypropylene (Accurel EP100) and Purasorb (a biodegradable polymer based on lactide and glycolide monomers) was performed in the acetylation of α-tocopherol (Fig. 5). The hydrolytic activity (measured with tributyrin) of the different immobilizates and their transesterification activity in a model reaction (methyl laurate with propanol in hexane to yield propyl laurate) are reported in Table 2. It is interesting to note that Novozym 435 presents the highest hydrolytic and synthetic activities.

For α-tocopherol acetylation, the biocatalysts were first dehydrated to reduce the total amount of water in the system and thus minimize the formation of acetic acid. Final water content was less than 1% w/w (Table 2). The hydrolysis of vinyl acetate to acetic acid is an undesirable side reaction also catalysed by the lipase, and competes with the acylation process. Most of the water in the reaction mixture comes from the biocatalyst and the solvents. A careful control of the amount of water in the system –by addition of molecular sieves, drying the biocatalyst, etc.– is crucial in terms of yield and downstream processing

(the fatty acid must be removed from the final product). It has been reported that dehydration of the biocatalyst in a vacuum dessicator usually increases the transferase to hydrolase ratio [35]. However, water activity (a_w) is preferred to water concentration for describing the hydration level of a system [14;36]. We measured the a_w of the three biocatalysts assayed, and we found that the values were in the range 0.15-0.25.

The polypropylene biocatalyst gave rise to faster α-tocopherol acetylation than Novozym 435 and Purasorb (Fig. 5). To explain these differences, the textural properties of the biocatalysts were measured by a combination of nitrogen adsorption and mercury porosimetry analyses (Table 2). Fig. 6 represents the total pore volume curves for these carriers. The polypropylene biocatalyst presented a substantially higher total pore volume (approx. 13 cm^3/g) than Purasorb (0.8 cm^3/g) and Lewatit (0.6 cm^3/g). The average pore size was also larger for Accurel EP100 and Purasorb (100-900 nm) compared with Novozym 435 (< 100 nm). The SEM pictures showed the presence in polypropylene of a channel structure (with diameters of approx. 10 μm), which facilitates the internal diffusion of substrates and products (Fig. 7). The specific surface area of the three biocatalysts was low (< 70 m^2/g) as expected for macroporous carriers. These results may help to explain the differences in acetylation of tocopherols, as large pores facilitate the contact between the enzyme and the bulky acceptor. Compared with the model transesterification reaction involving a small acceptor (propanol) in which Novozym 435 gave the highest activity (Table 2), the Accurel EP100 biocatalyst was the most efficient for acetylation of vitamin E. However, other parameters such as the local effects derived from the chemical nature of the carrier (esp. the hydrophobicity) may also exert an influence on the acylation rate.

3.4. Effect of medium composition and temperature.

The mixtures of miscible solvents have been successfully applied to the enzymatic regioselective acylation of several hydroxylic compounds [37-39]. These mixtures represent a compromise between substrates solubility and enzyme efficiency [19]. In the present work, several mixtures 2M2B/hexane were assayed in order to favour the solubilization of α-

tocopherol (Fig. 8). Using Novozym 435 as biocatalyst, the yield was higher when increasing hexane concentration up to 90% (v/v). However, a minimum of 10% (v/v) 2M2B was necessary for efficient transesterification, which seems to be related with the solubilization of vinyl acetate.

The effect of temperature (in the range 40-60 ºC) on α-tocopherol acetylation in 2M2B/hexane 90:10 (v/v) was analyzed (Fig. 9). The fastest reaction was observed at 60 ºC, at which *C. antarctica* lipase B is known to be highly stable and can be used without significant loss of activity even after several months [40].

3.5. Enantioselectivity of the process.

Attempts are being made to develop an efficient and stereocontrolled synthesis of the natural form of α-tocopherol [41]. Considering the well-known stereospecificity of lipases [20;42], and in particular of the lipase B from *C. antarctica* [40;43], one could expect different acetylation rates for the eight stereoisomers present in synthetic *all-rac*-α-tocopherol. We measured the acetylation rate of *all-rac*-α-tocopherol and *RRR*-α-tocopherol to analyze the enantioselectivity of the process. Experimental conditions were: 100 mM α-tocopherol, 400 mM vinyl acetate, hexane/2M2B 90:10 (v/v), 100 mg/ml Novozym 435, 60 °C. We found that the acylation rate was very similar with both substrates (data not shown). This implies that the degree of chiral recognition is low, probably due to the fact that the chiral centers are separated by at least six bonds from the phenolic group that is being acetylated by the lipase (Fig. 1). In this context, Zahalka *et al.* succeeded in the enantiospecific hydrolysis of α-tocopheryl acetate by a cholesterol esterase [44]. They added chiral bile salts to emulsify the substrate, which may exert influences upon the epimeric acetates induced within the mixed micelle itself, or alternatively, may arise from direct bile salt-enzyme interactions. The nature of the solvent and the length of the acyl donor may also modulate the enantiopurity of the final product [45].

ACKNOWLEDGEMENTS

We are grateful to Morten Christensen and Lotte Andersen (Novozymes A/S) for technical help. We thank Ana V. Ugidos and Soledad Peña (Biotecnologías Aplicadas, BTSA, Spain) for technical information and suggestions. We are grateful to Dr. Karl Hult (Royal Institute of Technology, Sweden) for practical suggestions. We thank Ramiro Martínez (Novozymes A/S Spain) for technical help. We thank Dr. M. L. Rojas-Cervantes (UNED, Spain) and Dr. M. Yates (ICP, CSIC, Spain) for porosity measurements. This research was supported by the Spanish CSIC (Project 200680F0132) and MEC (Project BIO2002-00337).

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Table 1. Screened hydrolases for acetylation of α-tocopherol

Enzyme	Physical state	Origin
Lipase B from *Candida antarctica*	Immobilized (Novozym 435)	Novozymes A/S
Lipase from *Thermomyces lanuginosus*	Immobilized (Lipozyme TL IM)	Novozymes A/S
Lipase from *Rhizomucor miehei*	Immobilized (Lipozyme RM IM)	Novozymes A/S
Lipase from *Pseudomonas cepacia*	Immobilized (Lipase I PS-C)	Amano
Lipase from *Alcaligenes* sp.	Immobilized (Lipase PLG)	Meito Sangyo
Lipase from *Alcaligenes* sp.	Immobilized (Lipase QLG)	Meito Sangyo
Cholesterol esterase from *Pseudomonas* sp.	Powder	Sigma
Cholesterol esterase from *Ophiostoma piceae*	Immobilized in Dilbeads TA	Fermentation
Lipase from porcine pancreas	Powder	Sigma
Lipase from *Candida rugosa*	Powder	Sigma
Protease from Bacillus licheniformes	Immobilized in Dilbeads TA	Sigma
Esterases from bovine rumen microflora	Immobilized in Dilbeads TA	Metagenome
Esterases from bovine rumen microflora	Immobilized in Celite	Metagenome
Esterase from Urania hypersaline basin	Lyophilized	Metagenome

Table 2. Properties of the *C. antarctica* lipase B (CALB) immobilizates.

Immobilizate	S_{BET} (m^2/g) [a]	Pore volume (cm^3/g) [b]	Water content (%) [c]		Hydrolytic activity (U/g biocatalyst) [d]	Transesterification activity (U/g biocatalyst) [e]
			Before	After		
Novozyme 435	68.6	0.6	2.2	0.9	2950	31.3
CALB/Accurel EP100	67.8	13	1.9	0.7	2310	29.4
CALB/Purasorb	9.2	0.8	0.9	0.3	300	21.8

[a] Measured by N_2 adsorption.

[b] By combination of N_2 isotherms and Hg porosimetry.

[c] Determined by Karl-Fisher titration before and after dehydration.

[d] Measured with tributyrin.

[e] Measured in the test reaction between methyl laurate and 1-propanol.

Figure legends

Fig. 1. Structure of α- and δ-tocopherol (the location of the three chiral centers is marked with an asterisk), (*R*)-Trolox and *p*-cresol.

Fig. 2. Kinetics of α-tocopherol (**O**) and δ-tocopherol (●) acetylation in 2M2B catalyzed by Novozym 435. Experimental conditions: 100 mM α- or δ-tocopherol, 400 mM vinyl acetate, 100 mg/ml Novozym 435, 60 °C.

Fig. 3. Molecular weight determination of α-tocopheryl acetate (A) and δ-tocopheryl acetate (B) using HPLC-MS.

Fig. 4. Competitive acetylation reactions in 2M2B between tocopherols and (*R*)-Trolox or *p*-cresol catalyzed by Novozym 435. Experimental conditions: 50 mM α- or δ-tocopherol, 50 mM (*R*)-Trolox or *p*-cresol, 400 mM vinyl acetate, 100 mg/ml Novozym 435, 60 °C.

Fig. 5. Kinetics of α-tocopheryl acetate synthesis in 2M2B catalyzed by *C. antarctica* lipase B immobilized in three different supports: (▲) Accurel EP100; (**O**) Lewatit (Novozym 435); (■) Purasorb. Experimental conditions: 100 mM α-tocoferol, 400 mM vinyl acetate, 100 mg/ml biocatalyst, 60 °C.

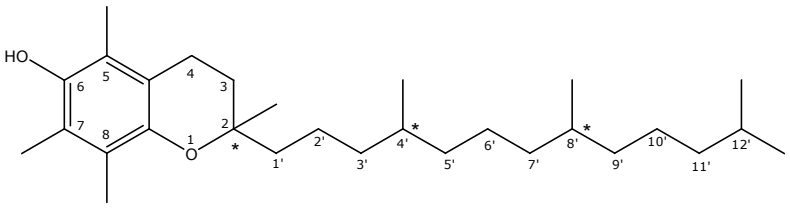
Fig. 6. Total pore volume of the biocatalysts determined by combination of N_2 adsorption and mercury intrusion porosimetry data. (A) Novozym 435; (B) CALB/Purasorb; (C) CALB/Accurel EP100.

Fig. 7. Scanning electron micrographs of the biocatalysts studied (A) 60x; (B) 500x.

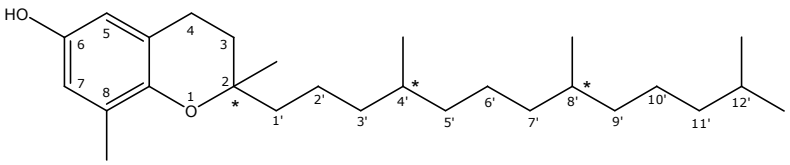
Fig. 8. Effect of hexane percentage (v/v) in mixtures 2M2B/hexane on α-tocopheryl acetate synthesis**.** Experimental conditions: 100 mM α-tocopherol, 400 mM vinyl acetate, 100 mg/ml Novozym 435, 60 °C. Yields refer to 6 days reaction.

Fig. 9. Effect of temperature on α-tocopheryl acetate synthesis in hexane/2M2B 90:10 (v/v)**.** Experimental conditions: 100 mM α-tocopherol, 400 mM vinyl acetate, 100 mg/ml Novozym 435. (●) 40 °C ; (**O**) 50 °C; (■) 60 °C

Fig. 1



α-tocopherol



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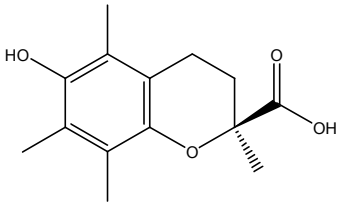
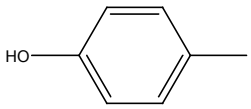


(R)-Trolox



p-cresol

Fig. 2

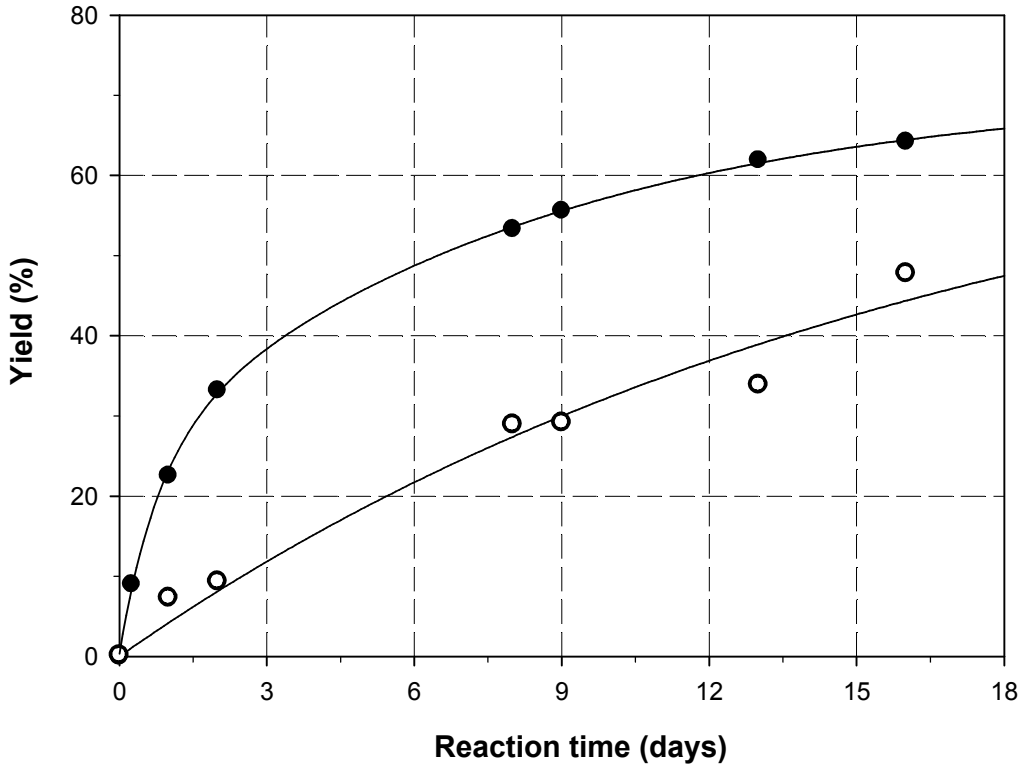


Fig. 3

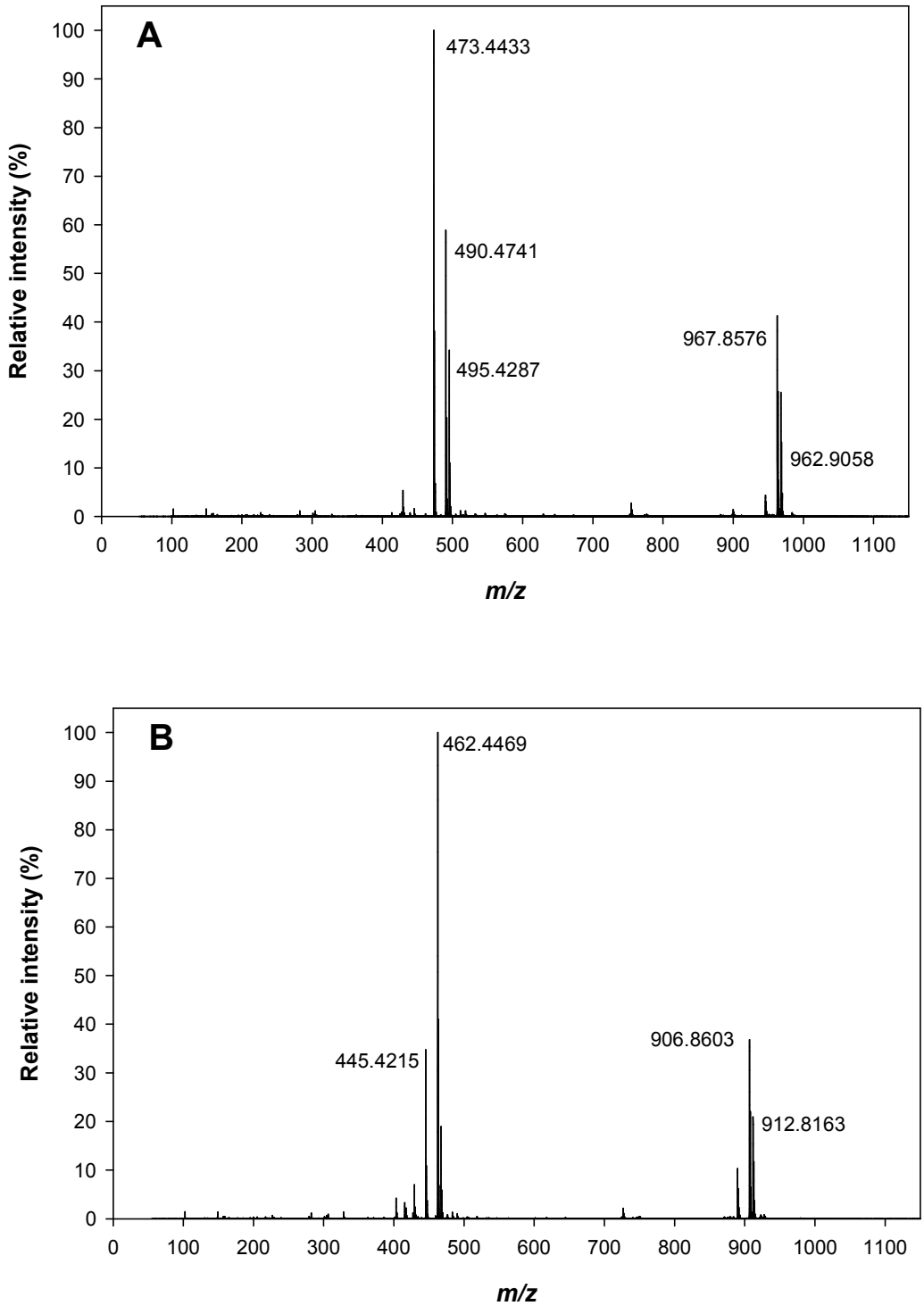

A
473.4433
490.4741
495.4287
967.8576
962.9058
Relative intensity (%)
m/z
B
462.4469
445.4215
906.8603
912.8163
Relative intensity (%)
m/z

Fig. 4

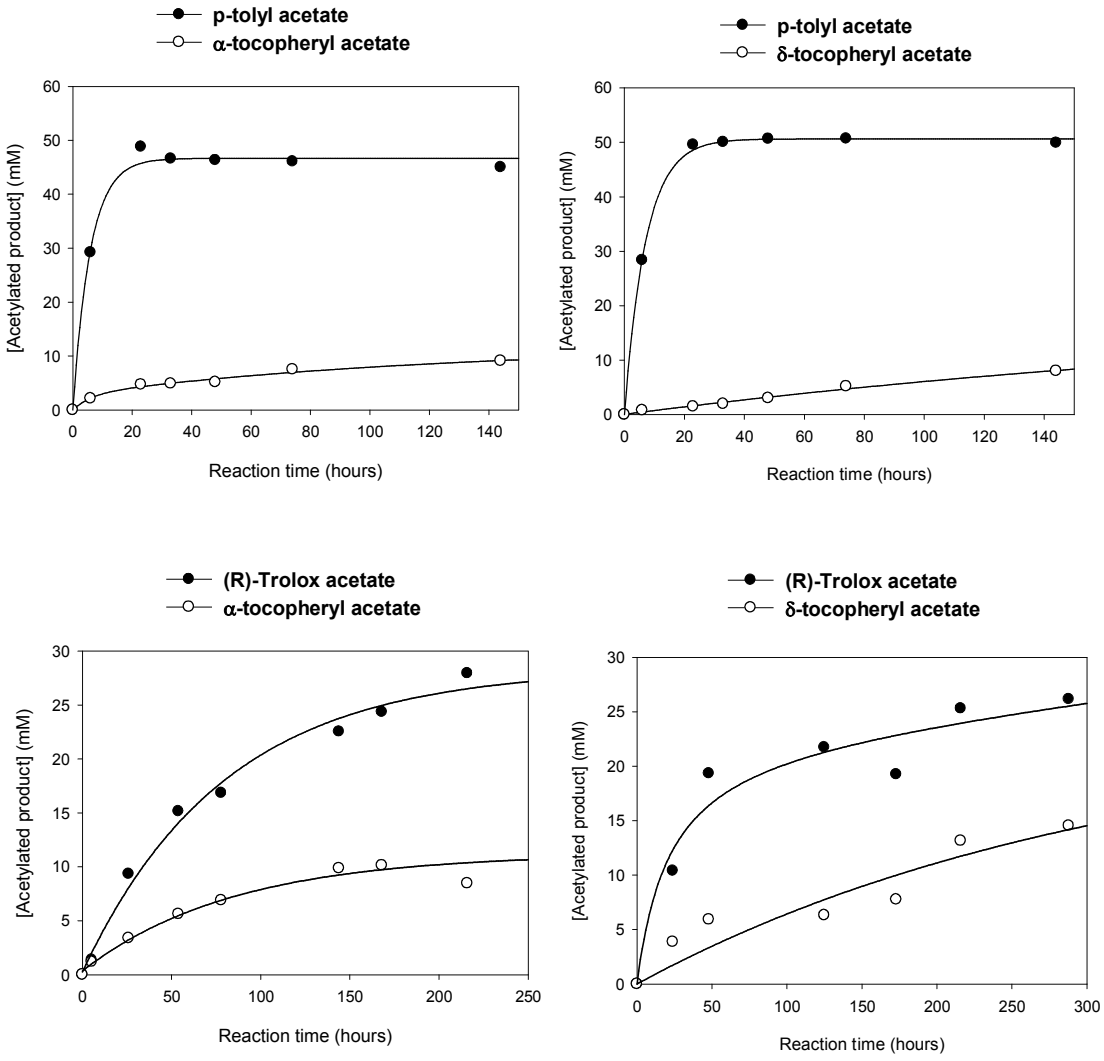

p-tolyl acetate
α-tocopheryl acetate
[Acetylated product] (mM)
Reaction time (hours)
p-tolyl acetate
δ-tocopheryl acetate
[Acetylated product] (mM)
Reaction time (hours)
(R)-Trolox acetate
α-tocopheryl acetate
[Acetylated product] (mM)
Reaction time (hours)
(R)-Trolox acetate
δ-tocopheryl acetate
[Acetylated product] (mM)
Reaction time (hours)

Fig. 5

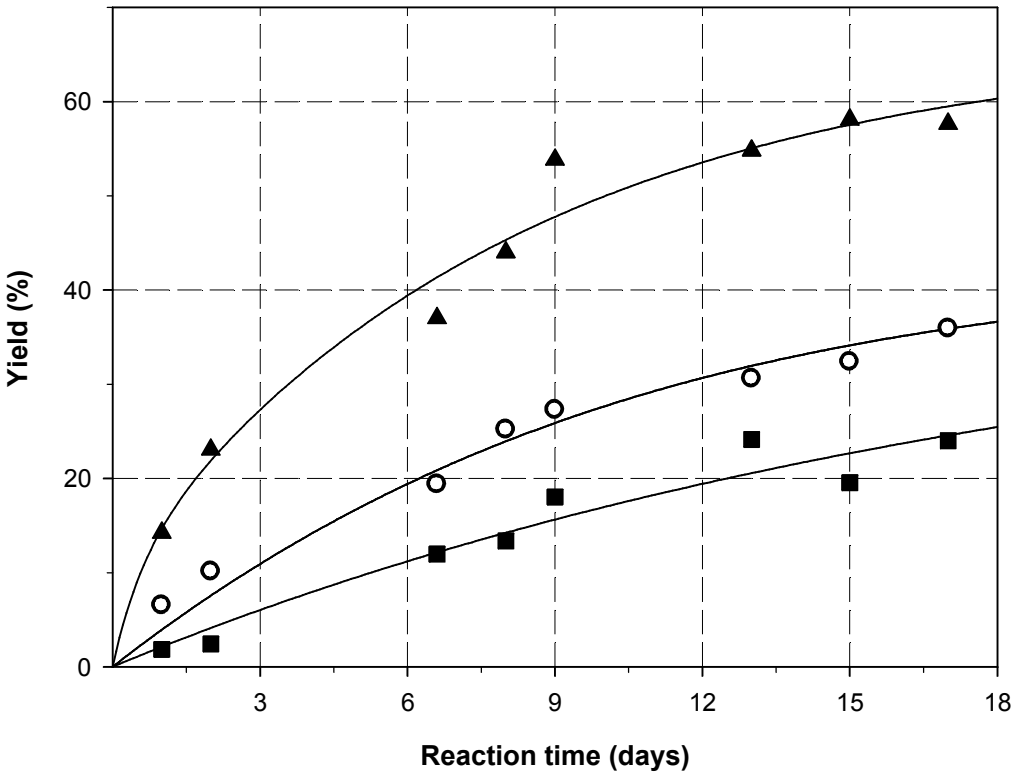
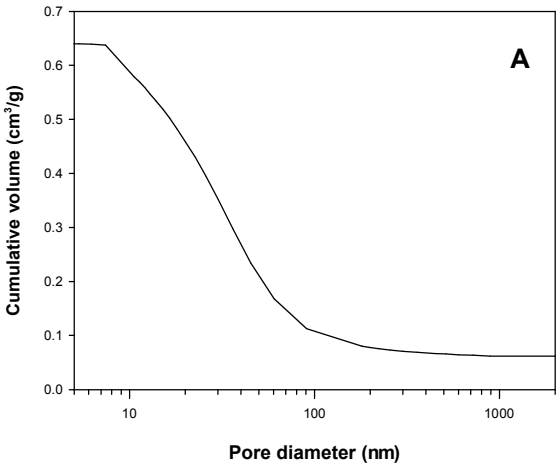
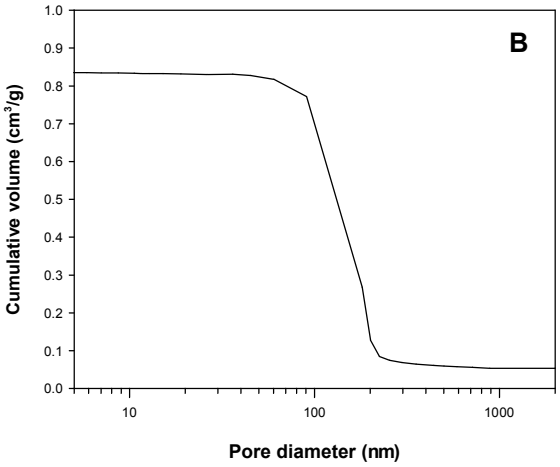


Fig. 6


A
Cumulative volume (cm³/g)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
10
100
1000
Pore diameter (nm)


B
Cumulative volume (cm³/g)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
10
100
1000
Pore diameter (nm)

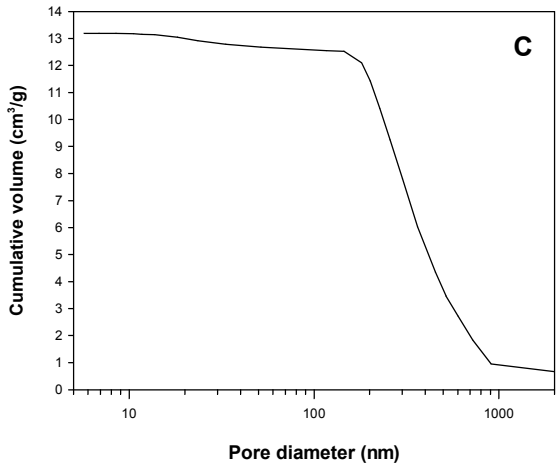

C
Cumulative volume (cm³/g)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
10
100
1000
Pore diameter (nm)

Fig. 7

Novozym 435 **CALB / Accurel** **CALB / Purasorb**

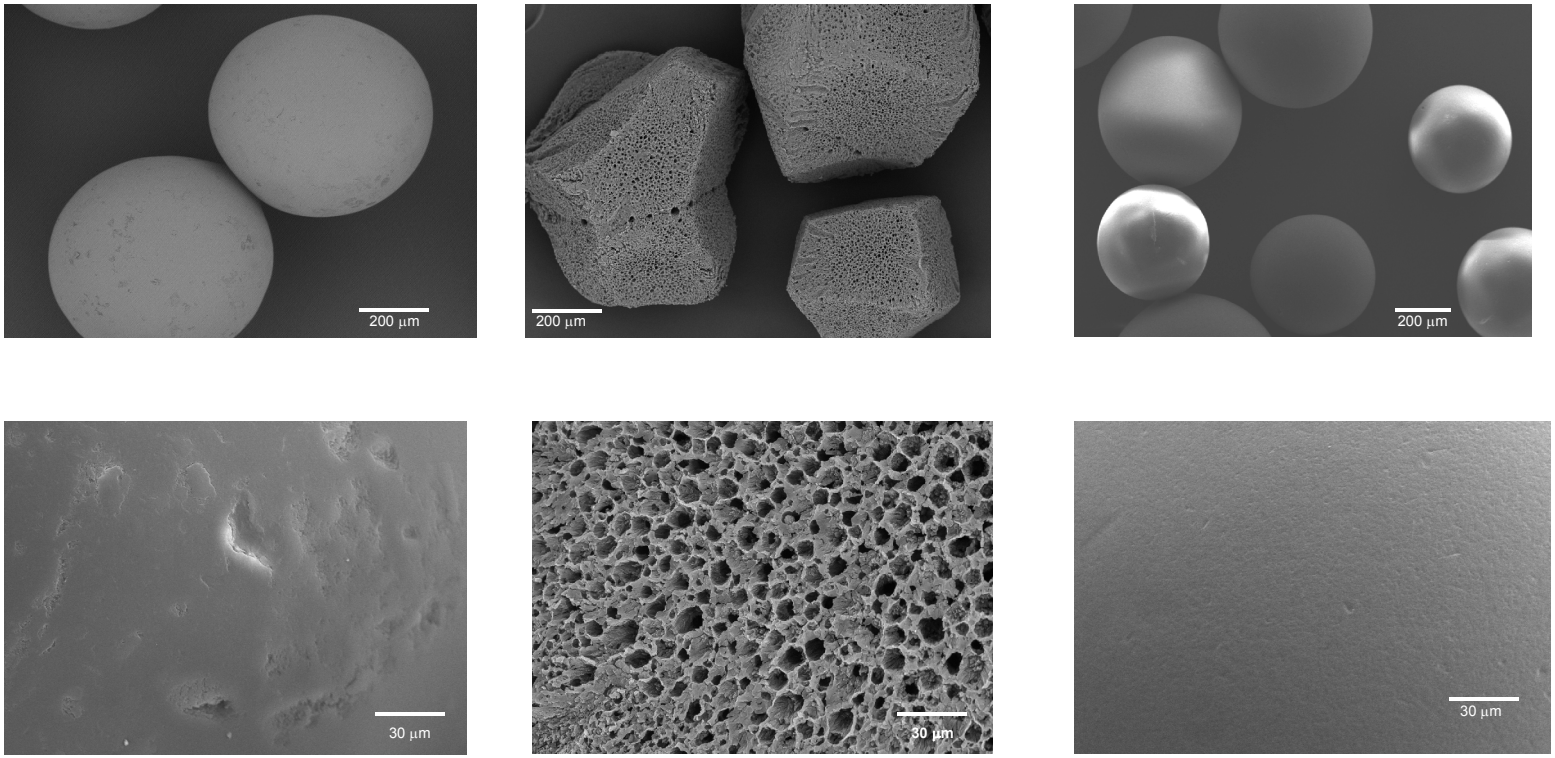


Fig. 8

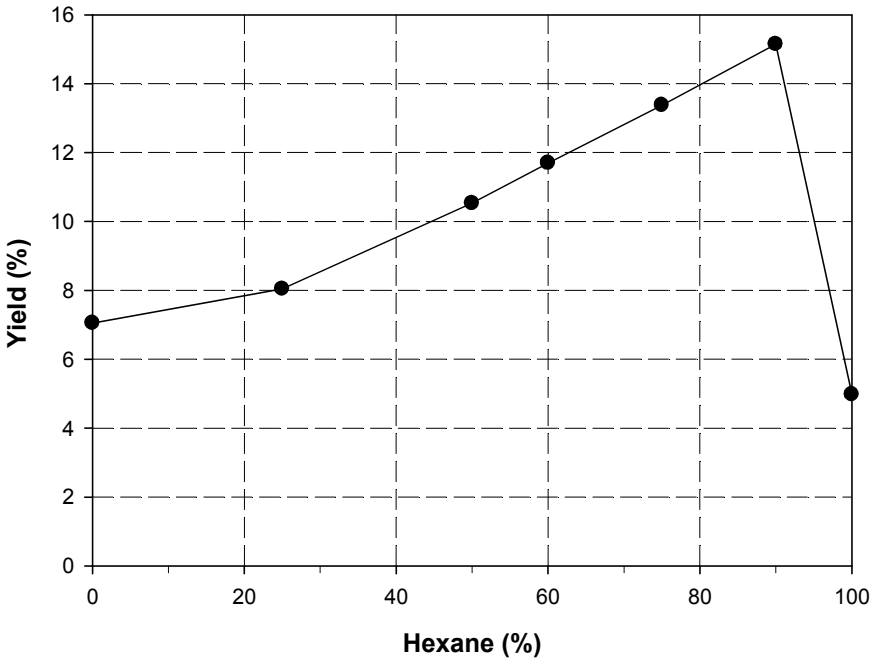


Fig. 9