รายงานฉบับสมบูรณ์

โครงการวิจัยงบประมาณแผ่นดิน ปี 2540

เรื่อง

การสังเคราะห์เอสเทอร์ที่ให้กลิ่นธรรมชาติในพืช โดยเอนไซม์ไลเพส

โดย

ผู้ช่วยศาสตราจารย์ ดร. วรวุฒิ จุฬาลักษณานุกูล

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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รายงานฉบับสมบูรณ์ฉบับนี้ขอเสนอผลงานวิจัยที่ได้ดำเนินการตลอดปี 2540 ในกรอบงานที่เสนอไว้ งานทั้งหมดได้ถูกรวบรวมและเสนอผลงานในการประชุมวิชาการ " International Conference on Biodiversity and Bioresources : Conservation and Utilization" จัดขึ้นที่ภูเก็ต ประเทศไทย 23-27 พฤษจิกายน 2540.

ผู้ช่วยศาสตราจารย์ ดร วรวุฒิ จุฬาลักษณานุกูล

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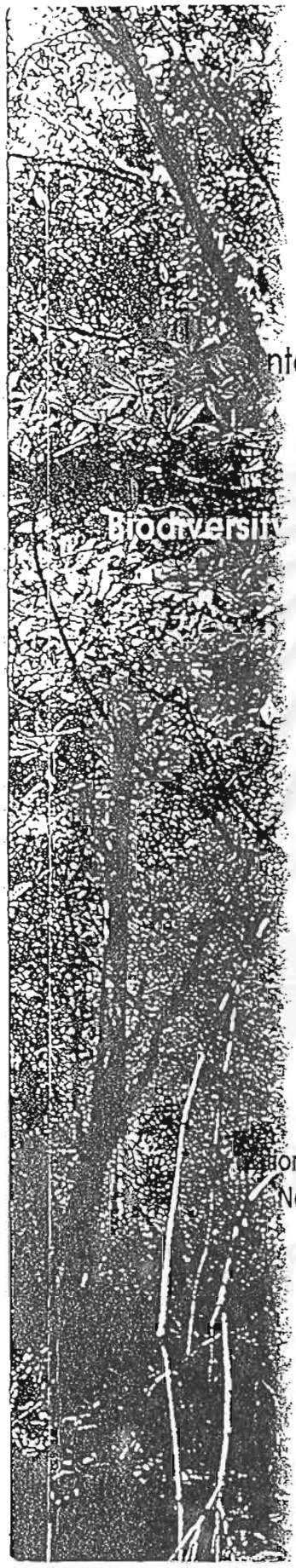

ABSTRACTS

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Synthesis of Amyl Acetate by Lipases from Various Microorganisms

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The main objective of this study is to synthesize biofragrance "amyl acetate" naturally extractable from the flowers of the Thai plant called "Nom Maew" *(Rauwenhoffia siamenesis* Scheff.) by a biotechnological method. Lipases from various microorganisms namely *Aspergillus niger, Candida cylindracea, Pseudomonas species* and *Mucor miehei* were applied to catalyze the synthetic reaction between amyl alcohol and octyl acetate through the process of "transesterification". The reaction mixture was incubated in organic solvent, hexane, at 40 °C, with continuous stirring by magnetic stirrer. The products were then sampled after 72 hours and analyzed by HPLC. From the results, the percent conversion of 41, 16.37 and 9.43 were obtained from the lipases of *Aspergillus niger, Pseudomonas spp* and *Mucor miehei* respectively. The product obtained from the reaction catalysed by lipases from *Candida cylindracea* was unmeasurably low. When the immobilized enzymes were comparatively studied between *Aspergillus niger* and *Mucor miehei,* the results obtained showed highest conversion, i.e. 64.53%, from the catalysis by lipases from *Aspergillus niger,* compared to 56.73 % obtained from *Mucor miehei.* Furthermore, the results from kinetic studies also exhibited rapid conversion to maximal yield obtained from immobilized lipases from *Aspergillus niger.* It could be seen that both free and immobilized forms of lipases from *Aspergillus niger* resulted in better conversion than lipases from other studied microorganisms. The transesterification reactions between amyl alcohol and substrates with various numbers of carbon chain length were studied. The reactions were catalysed by immobilized lipase from *Aspergillus niger* in the presence of n-hexane. Amyl acetate obtained from the transesterification was analyzed by HPLC and the kinetics of the reaction were studied. The V_{max}/K_m from the reactions containing ethyl, propyl, butyl, hexel and octyl as substrates were obtained as follows, 41.44, 32.97, 29.50, 20.22 and 6.70 μmol/mM/min/gram of enzyme respectively. From this result, it could be postulated that immobilized lipase from *Aspergillus niger* appeared to be more specific to ethyl acetate than the other studied substrates in producing the required amyl acetate in the presence of n-hexane.

จุฬาลงกรณ์มหาวิทยาลัย

ORGANIZED BY NATIONAL CENTER FOR GENETIC ENGINEERING AND BIOTECHNOLOGY (BIOTEC),
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No. BIOTEC 5202/ 3902

July 4, 1997

Dr. W. Chulalaksananukul
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Dear Dr. Chulalaksananukul,

It is our pleasure to inform you that your abstract titled "Synthesis of Amyl Acetate by Lipases from Various Microorganisms" has been accepted for poster presentation at the International Conference on Biodiversity and Bioresources – Conservation and Utilization being held in Phuket during November 23-27, 1997. The poster will be displayed (on a board, size : height x width = 1.5 x 1m) on the evening of November 25, 1997.

In order to produce a good quality "Book of Abstracts" the Committee urges you to urgently submit two original copies of your abstract (laser printer). Please consult "Guideline for Authors of Abstracts" which appears on page 7 in the Conference's Second Announcement. It is the author's responsibility to submit a well-proof and good quality copy for direct reproduction.

Since November is Phuket's high season the Organizing Committee cannot guarantee accommodation if it is not reserved well in advance. Please return the complete Registration and Accommodation Forms as soon as possible (if you have not done so).

We look forward to seeing you in Phuket.

Yours sincerely,

Professor Dr. S. Shumiratana
(Director, BIOTEC)
Secretariat

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Abstract

The main objective of this study is to synthesize biofragrance "amyl acetate" naturally extractable from the flowers of the Thai plant called "Nom Maew" (*Rauwenhoffia siamensis* Scheff.) by a biotechnological method. Lipases from various microorganisms namely *Aspergillus niger*, *Candida cylindracea*, *Pseudomonas species* and *Mucor miehei* were applied to catalyze the synthetic reaction between amyl alcohol and octyl acetate through the process of "transesterification" in organic solvent, hexane at 40° C. From the results, the percent conversion of 41, 16.37 and 9.43 were obtained from the lipases of *Aspergillus niger*, *Pseudomonas spp* and *Mucor miehei* respectively. The product obtained from the reaction catalyzed by lipases from *Candida cylindracea* was unmeasurably low. When the immobilized enzymes were comparatively studied between *Aspergillus niger* and *Mucor miehei*, the results obtained showed highest conversion ie 64.53% from the catalysis of lipases from *Aspergillus niger*, compared to 56.73 % obtained from *Mucor miehei*. Furthermore, the results from kinetics studies also exhibited rapid conversion to maximal yield obtained from immobilized lipases from *Aspergillus niger*. It could be seen that both free and immobilized forms of lipases from *Aspergillus niger* resulted in better conversion than lipases from other studied microorganisms. When the transesterification reactions between amyl alcohol and substrates with various numbers of carbon chain length were studied, the reactions were therefore catalyzed by immobilized lipase from *Aspergillus niger* in the presence of n-hexane. The Vmax/Km from the reactions containing ethyl, propyl, butyl, hexyl and octyl as substrates were obtained as follows ; 41.44, 32.97, 29.50, 20.22 and 6.70 μmol/mM/min/gram of enzyme respectively. From this result, it could be postulated that immobilized lipase from *Aspergillus niger* appeared to be more specific to ethyl acetate than the other studied substrates in the production the required amyl acetate in the presence of n-hexane.

Introduction

Most natural fragrances are esters with short chains. These are very useful in food industries and can be directly extracted from natural sources such as from various parts of plants. However, this method is rather expensive and also gives low yield in production. Chemical synthesis is also possible but the product obtained may not be sufficiently safe for consumption due to impurities (1). Therefore, the synthesis of the particular short chain esters or fragrances by using enzymes or biotechnological method which provides higher yield of safer products than the natural extraction or chemical method should be more economically applicable (2).

The enzymes applied for the synthesis are lipases which usually catalyse the hydrolytic reactions (3). However, these enzymes can also catalyse synthetic reactions via the reversible process in the presence of organic solvent with the appropriate controlled low quantities of water to prevent the hydrolysis (4). From the previous studies, the popularity of this technique has been increasing in order to produce the fragrance in industries and it is highly acceptably to be very efficient. Natural lipases

can be extracted from many living organisms and the efficiency in the synthesis has been shown to be also different according to various species (5).

The objective of this study is to compare the activities of the free form and immobilised lipases from different species of microorganisms in catalysing transesterification reactions between amyl alcohol and octyl acetate. The transesterification reactions between amyl alcohol and substrates with various numbers of carbon chain length were also studied in order to produce the fragrance, namely, amyl acetate, which can be extracted naturally from the rarely available plant at present called *Rauvenhoffia Siamensis Scheff*

Materials and Methods

Free lipases from *Candida cylindracea Pseudomonas sp* and *Aspergillus niger* were purchased from Sigma, USA. Immobilised lipases from *Mucor miehei* and *Aspergillus niger* were kindly obtained from NOVO Nordisk Denmark. Amyl alcohol, amyl acetate, octanol and other esters of analytical grade were purchased from Fluka, Switzerland. The HPLC used in the experiment is Shimadzu, LC-3A containing, Lichrocat C18 column with the size of 4.6 n.m. Idx25cm, Refracto Monior iv from LDC detector and the integrator from Shimadzu C-R1A.

The transesterification reactions were performed through the addition of 20 mg of lipases from each microorganism into the hexane solvent containing 30 mM amyl alcohol and 100 mM of ester as the substrates. The mixture was then constantly and magnetically stirred at 40 °C. The samples were withdrawn at various times to determine the concentrations of the substrate and the product by HPLC.

Results and Discussion

Synthetic activity of lipases from various microorganism

It can be seen from the result shown in Table 1 that the maximal activities of the enzymes to catalyse the transesterification to produce amyl acetate were different from the others ie; 9.43, 16.37 and 41 % from free form lipases of *Pseudomonas sp*, *Mucor miehei* and *Aspergillus niger* respectively. Moreover, the result from free lipases from *Candida cylindracea* was shown to be least active among the four enzymes that the product obtained was unmeasurably small. The result also indicated that the synthetic activities of the lipases do not directly correspond to the hydrolytic activities considering the specific hydrolysis activities from *Candida cylindracea*. The overall obtained results showed that lipases from different microorganisms can differently catalyze transesterification reactions in hexane. With respect to the types of microorganisms applied from this research, it was found that lipases obtained from fungi, namely, *Aspergillus niger* and *Mucor miehei* were more capable of catalyzing the reaction than the lipases from bacteria (*Pseudomonas sp*) and yeast (*Candida cylindracea*) respectively. The percent conversion obtained from lipases of *Aspergillus niger* was shown to be maximal ie 41% among the four enzymes. This difference obtained can be postulated from different conformation of the enzyme, lipase, in different microorganisms. The different structure in some portion of the enzyme might have shown different specificity toward the same substrates and therefore resulted in different synthetic capacity. This also can be postulated further that the 4 microorganisms might have had different genetic structures for producing the lipases.

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when the activities of the free and the immobilized forms of lipases were compared from *Mucor miehei* and *Aspergillus niger*, it was found that the immobilized form of lipases from both organisms could synthesize amyl acetate better than the free form of the enzyme. The percent conversion from the results was shown to be 56.73 and 64.53 from *Mucor miehei* and *Aspergillus niger* respectively. When the kinetics from *Aspergillus niger* immobilzed lipase which was found to give the highest yield was studied, it was shown that the rate of the synthesis was maximal among the others. The products obtained at 20, 30 and 60 minutes were 35.40, 45.87 and 56.20 % respectively. The maximal yield of the product 64.53 % was obtained within 3 hours of the incubation indicating that the steady state of the reaction was reached. This can be concluded that lipases from each microorganism were specific and were independent from the applied concentrations. From Table 1, the immobilized lipases from *Mucor mihei* and *Aspergillus niger* could synthesize amyl acetate 6 times and 1.5 times those of the free form respectively. The increased efficiency might be the result different pH, ionic strength, stability and the real concentrations of the substrates in the vicinity of the enzyme molecules or the so called " microenvironment"

Effect of carbon chain length

Transesterification reactions between amyl alcohol and substrates with various numbers of carbon chain length such as ethyl acetate were catalysed by immobilised lipases from *Aspergillus niger* in hexane and the product obtained is amyl acetate. The initial velocity of the reaction for each substrate at various concentrations was calculated and the Lineweaver Berk Plot was ploted. From the graph, the ratio between Vmax and Km was obtained and the results were shown in figure 1. The efficiency of the catalysis and the specificity of the enzyme can be analyzed from the figure 1. It was found from the results that when the 4 carbon ester (C4), amyl acetate was used as the substrates obtained the maximal ratio between Vmax and Km was substrates with ie 41.44 mol/mM/min/g. However, the ratio was shown to be reduced when the substrates were longer in order of carbon chain length such as propyl (C5), hexyl (C6) and octyl (C8) acetate. The results obtained here were consisted with Langrand et al (1990) (5) that lipases from *Mucor miehei* were formed to be mostly active in synthesizing geranyl acetate (C12) through the esterification reaction between geraniol (C10) and the acids with 4-6 carbon chain length. Moreover, Chulalaksananukul et al, 1992 (3) also found that lipases from *Mucor miehei* catalysing transesterification reaction between geraniol (C10) and propyl acetate (C5) gave maximal ratio obtained between Vmax/Km. The conclusion can be drawn that the lipases from *Mucor miehei* are mostly active when react with the substrates with the sum of carbon chain length. However, lipases from *Aspergillus niger* in the transesterification between amyl alcohol (C5) and ethyl acetate (C4) in this study produced the maximal ratio between Vmax/Km. This could be postulated that immobilised lipase from *Aspergillus niger* contain the active sites for the ethyl acetate and amyl alcohol and the appropiate sum of the number of carbon the substrates equals nine.

Acknowledgement

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Form of lipases	Sources of lipase	support	specific hydrolysis activity	Amyl acetate concentration	Conversion (%)
	C. cylindracea	-	30000 U/mg	*	-
Free	*Pseudomonas spp*	-	25 U/mg	4.91	16.37
	M. miehei	-		2.83	9.43
	A. niger		24.2 U/mg	12.3	41.0
immobilized	*M. miehei*	macroporous anion	6.9 BAUN/G**	17.02	56.73
	A. niger	resin beads	7400 BAUN/G	19.36	64.53

* The yield could not be determined because of a poor synthetic reaction
** In Danish
Table 1. The reaction was carried out with 20 mg of lipases from each microorganism in to 6 ml hexane solvent containing 30 mM amyl alcohol and 100 mM of octyl acetate as the substrates. The mixture was then constantly and magnetically stirred at 40° C

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Figure 1 Effect of Various Numbers of Carbon Chain Length on Amyl Acetate Synthesis by Lipase-Transesterification Reaction