Synthesis of Amyl Acetate by Immobilized Lipases from *Mucor miehei* and *Aspergillus niger* in *n*-hexane

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Amyl acetate, a fragrant ester compound naturally existing in the flowers of the nearly endangered Thai plant called "Nom Maew" (*Rauwenhoffia siamensis* Scheff.) was synthesized. The immobilized lipases from *Aspergillus niger* and *Mucor miehei* were applied to catalyze the transesterification reaction between amyl alcohol and hexyl acetate in *n*-hexane. The result of these two enzymes interacting was 100% conversion when the concentration of hexyl acetate, used as a substrate, was 240 mM in the reaction. When the kinetics were studied, it was found that the values of the V_{max}/K_m from the reactions catalyzed by immobilized lipases from *M. miehei* and *A. niger* were 4.17 and 3.19 ml/min gram of enzyme, respectively. This result indicated that lipase from *A. niger* is appropriate to catalyze the transesterification reaction between amyl alcohol and hexyl acetate in *n*-hexane

Key words: *Aspergillus niger*, *Mucor miehei*, amyl acetate, immobilized lipase and *n*-hexane.

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การสังเคราะห์แอมิลแอซีเตตโดยไลเปสตรึงจาก *Mucor miehei* และ *Aspergillus niger* ใน *n*-hexane

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วารสารวิจัยวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย 30(2)

งานวิจัยนี้ได้สังเคราะห์สารประกอบเอสเทอร์ที่มีกลิ่นหอมของเอมิลแอซีเตต ในธรรมชาติสารนี้สามารถสกัดได้จากดอกนมแมว (*Rauwenhoffia siamensis* Scheff) ซึ่งพืชชนิดนี้ใกล้สูญพันธุ์ การสังเคราะห์เกิดขึ้นโดยปฏิกิริยาทรานส์เอสเทอริฟิเคชันระหว่างแอมิลแอลกอฮอล์และเฮกซิลแอซีเตต ในสารละลายเอนเฮกเซนที่เร่งด้วยไลเปสตรึงจาก *Aspergillus niger* และ *Mucor miehei* จากผลการทดลองพบว่า เมื่อใช้ความเข้มข้นของซับสเตรตเฮกซิลแอซีเตตที่ 240 มิลลิโมลาร์ สามารถเปลี่ยนเป็นผลิตภัณฑ์ได้ถึง 100% เมื่อเร่งด้วยเอนไซม์จากทั้ง 2 ชนิด จากการศึกษาจลนพลศาสตร์ พบว่าค่าอัตราส่วนของ V_{max}/K_m ของไลเปสจาก *A. niger* และ *M. miehei* มีค่าเท่ากับ 4.17 และ 3.19 ml/min·g ของเอนไซม์ตามลำดับ แสดงว่าเอนไซม์ไลเปสจาก *A. niger* เหมาะสมต่อการเร่งปฏิกิริยาทรานส์เอสเทอริฟิเคชันระหว่างแอมิลแอลกอฮอล์และเฮกซิลแอซีเตตในเอนเฮกเซน

คำสำคัญ *Aspergillus niger Mucor miehei* แอมิลแอซีเตต การตรึงไลเปส *n*-hexane.

INTRODUCTION

Esters are important compounds of the natural aromas frequently used in the cosmetic and food industries. They can be directly extracted from natural sources or synthesized by fermentation. Unfortunately, the cost of processing by these methods is high and low yields of the product is obtained.[1] Chemical synthesis may be an alternative method. However, the product obtained may not be sufficiently safe for human consumption due to the possible impurities which could result in a human health hazard.[2] For these reasons, enzymatic conversion can be employed to produce the particular short chain esters or fragrances instead of these traditional methods. Products from enzyme-mediated reactions are naturally safer and cheaper.[3-4] It has been well established that hydrolytic enzymes such as lipases (triacylglycerol hydrolases, EC 3.1.1.3) are capable of catalyzing the ester-synthesis reactions in nearly anhydrous organic solvents.[5-9] The transesterification reaction is reported to be the main ester-synthesis reaction encouraging the reactor in producing the maximum yield of the transfer product. In addition, the influence of water content has also been studied in order to optimize the reaction and prevent normal hydrolysis.[10] As a consequence, hexane was found to be an appropriate solvent for the commercial extraction of flavor compounds since high substrate conversion can be obtained.[8]

At present, amyl acetate ester, commonly called banana oil, is commercially used as a paint solvent and also frequently used as an artificial fruit flavors. Naturally, it can be extracted directly from the Thai plant named, Nom Maew, *Rauwenhoffia siamensis* Scheff., but the plant is nearly endangered. Therefore, synthesizing this ester through the enzymatic transesterification reaction would help to preserve this particular Thai plant. In a 1998 study, immobilized lipases from *Aspergillus niger* and *Mucor miehei* were investigated to synthesize amyl acetate through the transesterification reaction. The results showed rapid conversion to maximal product.[11] Others reported that the immobilized lipase from *M. miehei* is not able to use alcohol with a carbon atom number less than 4 as a substrate for ester production.[8] Additional authors have demonstrated that this enzyme was more active in the presence of acids with carbon atom numbers 4 to 6 for the synthesis of flavor ester.[1] Based on these suggestions, amyl alcohol and hexyl acetate were selected as possibly suitable substrates for this transesterification reaction. Thus, the objective of this study was to synthesize the amyl acetate ester through the transesterification reaction between amyl alcohol and hexyl acetate catalyzed by immobilized lipases from two organisms, *A. niger* and *M.miehei* in *n*-hexane. In addition, the efficiency and the kinetics of these two immobilized lipases were also investigated.

MATERIALS AND METHODS

Immobilized lipase (EC 3.1.1.3) from *M. miehei* and *A. niger*, supported on macroporous anionic resin beads, were purchased from Novo-Nordisk, France. Amyl alcohol, hexyl acetate and other reagents of analytical grade were purchased from Fluka, Switzerland. The transesterification reactions were performed by the addition of 20 mg of immobilized lipases from each organism into 10 ml of *n*-hexane containing known concentrations of the two substrates, amyl alcohol and hexyl acetate. The equilibrium state was determined from the time course of amyl acetate formation by fixing the concentration of amyl alcohol and hexyl acetate as 30 mM. To study the effect of substrate concentration on ester synthesis, 30 mM amyl alcohol and various concentrations of hexyl acetate ranging from 15 mM to 240 mM were tested. The reaction mixture was magnetically stirred at 500 rpm at 40^{o}C. Five hundred micro liters of the samples were taken at various times and subjected to High Performance Liquid Chromatography (HPLC) for the determination of substrate and product concentration.[12] The initial rate of amyl acetate synthesis was determined by measuring the increase of corresponding product. Aliquot of the reaction mixture was analyzed by HPLC using Shimadzu, LC-3A system with a refractive index monitor on a Lichrocat 18 C column (250 nm in length and 4.6 nm in diameter). A mixture of acetonitrile/water/acetic acid, 782:150:3 (g/g/g) was used as the mobile phase at 40^{o}C and at a flow rate of 0.5 ml/min. Quantitative data were obtained with an integrator (Shimadzu C-R1A) after standard calibrations.

RESULTS AND DISCUSSION

The equilibrium state for the synthesis of amyl acetate

Immobilized lipases from *M. miehei* and *A. niger* were selected for catalyzing the transesterification reaction between amyl alcohol and hexyl acetate to produce amyl acetate since rapid conversion was obtained from these two organisms.[11] To determine the equilibrium state for these transesterification reactions, mixtures containing known concentrations of the two substrates, 30 mM amyl alcohol and 30 mM hexyl acetate, were prepared. After that, the reaction mixtures were incubated at 40°C to avoid the excessive evaporation of the hexane phase and to minimize the denaturation of the enzymes.[8] The time courses of the formation of the amyl acetate were observed. The corresponding product continuously increased as shown in Figure 1 for a period of 48 hours. After 48 hours no change in the percent conversion was detected from either organism indicating that the reaction had reached the equilibrium state.

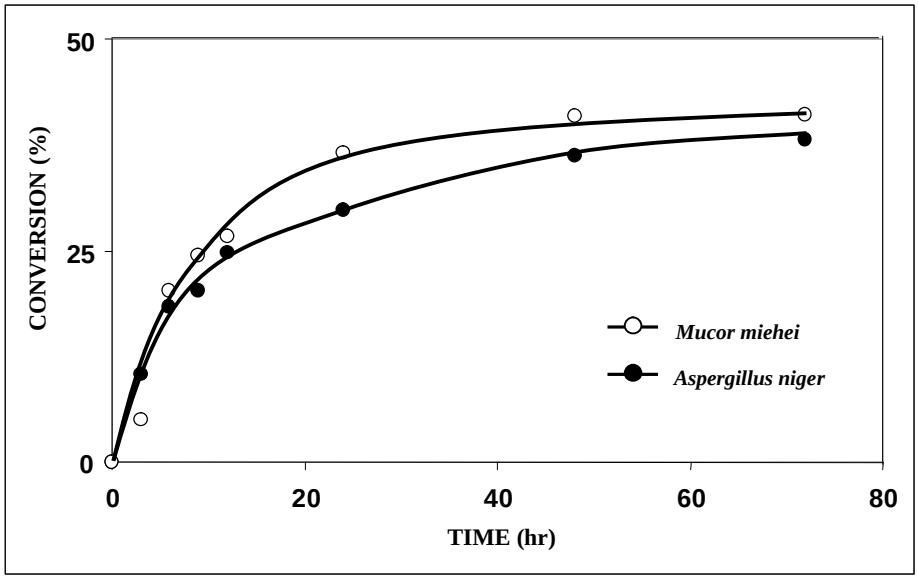
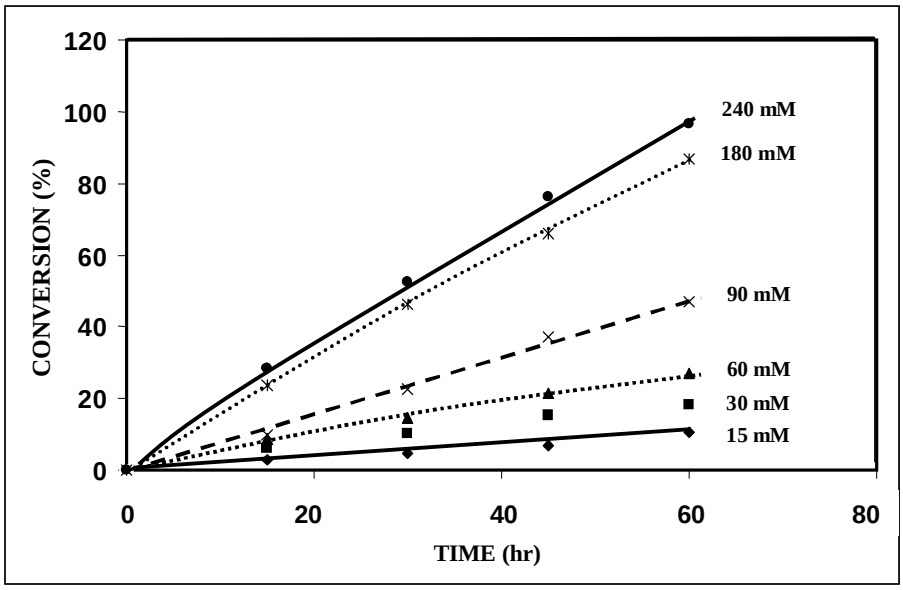


Figure 1. Percent conversion of the synthesis of the amyl acetate from transesterification catalyzed by the immobilized lipases from *M. miehei* and *A. niger*. The reaction was carried out with 20 mg of immobilized lipases in 10 ml *n*-hexane solvent containing 30 mM amyl alcohol and 30 mM of hexyl acetate as substrates and the mixture was magnetically stirred at 40°C.

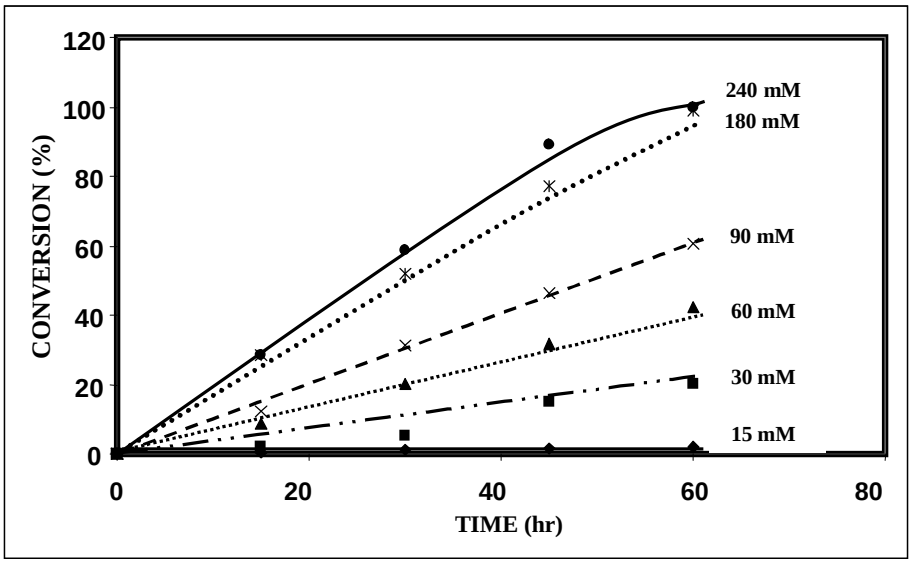
Effect of the substrate concentration

Since it had been reported that the transesterification in organic solvent was not sensitive to the carbon number of the alcohol residue,[3] the effect of the substrate concentration, hexyl acetate, on the rate of amyl acetate formation was investigated. The concentrations of hexyl acetate were varied in the range of 15 mM to 240 mM in a fixed concentration of 30 mM of amyl alcohol. Reactions were carried out under standard conditions as described in Materials and Methods. The results showed that, when the concentrations of hexyl acetate were increased, the product obtained was proportionally and similarly increased from both organisms (Figure 2). When 240 mM hexyl acetate was used as substrate, the highest percent conversion was 96.6 and 100 from *A. niger* and *M. miehei*, respectively.

For the study of the kinetics from the reactions catalyzed by immobilized lipases from both organisms, the initial velocities of the reaction for hexyl acetate at various concentrations were determined.



(a)



(b)

Figure 2. Effect of the hexyl acetate concentration on the percent conversion of amyl acetate from transesterification catalyzed by the immobilized lipases from *A. niger* (a) and *M. miehei* (b). The reaction was carried out with 20 mg of immobilized lipases in 10 ml *n*-hexane solvent containing 30 mM amyl alcohol and different concentrations of hexyl acetate as substrates and the mixture was magnetically stirred at 40°C.

The Lineweaver-Burk Plot of reciprocal velocity versus reciprocal concentrations of hexyl acetate is illustrated in Figure 3. The ratio between the values of V_{max} and K_m from the reactions catalyzed by immobilized lipases from *M. miehei* and *A. niger* were calculated as 4.17 and 3.19 ml/min gram of enzyme, respectively (Table 1).

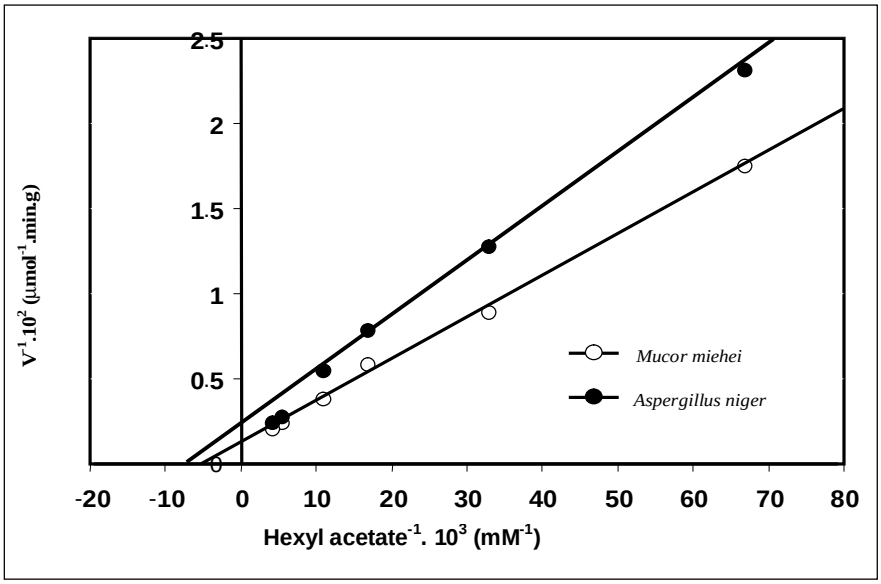


Figure 3. The Lineweaver-Burk Plots obtained from the catalytic reactions of *M. miehei* and *A. niger* immobilized lipases. The reaction was carried out in the presence of various concentrations of hexyl acetate with 30 mM of amyl alcohol in *n*-hexane.

Table 1. The efficiency of *M. miehei* and *A. niger* immobilized lipases on amyl acetate synthesis by transesterification reaction in *n*-hexane.

	Aspergillus niger	*Mucor miehei*
V_{max}(μmol.min^{-1}.g^{-1})	666.67	1000
K_m(mM)	209.21	239.81
V_{max}/K_m(ml.min^{-1}.g^{-1})	3.19	4.17

The efficiency of the catalysis and the specificity of the enzyme were then analyzed. From these results, we concluded that immobilized lipases from *M. miehei* were more efficient and more specific to hexyl acetate than immobilized lipases from *A. niger*. The results obtained here may be consistent with that of Chulalaksananukul *et al.*[11] in that immobilized lipases from *A. niger* contain the active sites for the ethyl acetate and amyl alcohol. Hence, it may also be postulated that immobilized lipases from *M. miehei* contain the more particularly active sites for the hexyl acetate and amyl alcohol than that of *A. niger*. Therefore, it can also be recommended that *M. miehei* is a more suitable organism than *A. niger* for immobilized lipase to be used in order to produce amyl acetate through the transesterification reaction.

Recently, there was a report about the use of a continuously operated packed bed reactor as the most efficient, high productivity method to obtain geranyl acetate.[13] It may also be advantageous to synthesize amyl acetate through a similar method. Also the optimal reaction conditions, for example water content, flow rate and operational stability, of *M. miehei* immobilized lipases should be thoroughly investigated and developed.

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