

Research Article

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Comparison of the performance of entrapped and covalently immobilized lipase in the synthesis of pear flavor

Tutuklama ve kovalent bağlama ile immobilize edilmiş lipaz örneklerinin armut aroması sentezindeki performanslarının karşılaştırılması

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Abstract

Introduction: Although aroma esters are produced mostly by a chemical method that requires the use of aggressive chemical catalysts, enzymatic esterification has been very favored during previous two decades. The main aim of this study was to investigate the commercially important pear flavor, hexyl acetate (HAc), by the catalysis of immobilized lipase samples and to optimize the immobilization and operational conditions in detail.

Methods: Lipases from *Candida rugosa* (CRL) and porcine pancreas (PPL) were immobilized using biodegradable polysaccharides, by entrapment in calcium alginate/chitosan composite gel (CRL_{Ca-Alg/Chi} and PPL_{Ca-Alg/Chi}) and by covalent binding onto the chitosan (CRL_{Chi} and PPL_{Chi}) carrier.

Results: Among the several organic media, the highest esterification activities were observed in heptane. HAc yield decreased when substrate concentrations were higher than 50 or 75 mM. Yield increased with increase in reaction time up to the 5th hour in the batch type reactor while it increased during the 8 h reaction time for fixed bed reactors with lower yields.

Discussion and conclusions: As well as synthesis conditions, immobilization parameters also affected HAc productivity. The amount of water in the microenvironment

of lipase is so important not only for gaining its active conformation but only for the reaction direction.

Keywords: Lipase; Hexyl acetate; Pear flavor; Alginate; Chitosan; Immobilization; Response surface methodology.

Özet

Giriş ve Amaç: Aroma esterleri çoğunlukla agresif kimyasal katalizör kullanımı gerektiren kimyasal yöntemlerle üretilmesine rağmen, son yirmi yıldır enzimatik üretimleri oldukça tercih edilmektedir. Bu çalışmanın esas amacı ticari olarak önemli armut aromasının hekzil asetat olarak immobilize lipaz katalizinde üretiminin ve immobilizasyon ile üretim şartlarının optimizasyonunun ayrıntılı olarak araştırılmasıdır.

Yöntem ve Gereçler: *Candida rugosa* (CRL) ve domuz pankreası kaynaklı (PPL) lipaz enzimleri biyobozunur polisakaritler kullanılarak kalsiyum alginate-kitosan kompozit jeline tutuklama (CRL_{Ca-Alg/Chi} and PPL_{Ca-Alg/Chi}) ve kitosan taşıyıcıya kovalent bağlama (CRL_{Chi} and PPL_{Chi}) yöntemleriyle immobilize edilmiştir.

Bulgular: Bir çok organik ortam arasından en yüksek esterleşme aktivitesi heptanda gözlenmiştir. HAc verimi 50 veya 75 mM substrat derişiminin üzerinde azalmıştır. Kesikli reaktörde 5 saat reaksiyon süresine kadar verim artmış, dolgu yataklı reaktörde ise daha düşük verimle de olsa 8 saat reaksiyon boyunca ester verimi artmıştır.

Tartışma ve Sonuç: HAc üretimini sentez koşulları kadar immobilizasyon parametreleri de etkilemiştir. Lipazın mikroçevresinde bulunan su miktarı sadece enzimin aktif formu kazanmasında değil reaksiyonun yönünde de oldukça önemlidir. Sonuç olarak, enzim molekülünü içeren taşıyıcının içinde/yüzeyinde adsorplamış olduğu su miktarı, lipaz enziminin aktif formunu kazandıracak ancak ester bağımlı hidroliz etmeyecek miktarda olması gereklidir.

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Anahtar Kelimeler: Lipaz; Hekzil asetat; Armut aroması; Alginat; Kitosan; Immobilizasyon; Yanıt yüzey metodu.

Introduction

Flavor and fragrances are complex blends designed to add either an attractive taste and aroma to processed foods and beverages or a pleasing scent to consumer products such as perfumes, toiletries and household cleaners [1]. Hexyl acetate (HAc), a short chain ester known as pear flavor, is an extremely aromatic compound and is widely used in the several industries such as food, cosmetic and pharmaceutical production. Although aroma esters are manufactured mostly by a chemical method that includes the use of aggressive chemical catalysts, development of the research area of enzymatic esterification has been very active in the last two decades [2–7]. Lipases (triacylglycerol hydrolase, EC 3.1.1.3) are a family of enzymes that in their natural environment catalyze the hydrolysis of fats. However, under appropriate working conditions, lipases have been shown to be very active catalysts in esterification, transesterification and alcoholysis reactions [8]. There are several reports about the enzymatic synthesis of flavor esters by immobilized lipases [4, 8–14]. Among the carbohydrate polymers, alginate (Alg) and chitosan (Chi), which are biodegradable polysaccharides, are widely used as a carrier or matrix to immobilize lipase enzyme [8, 9, 15–18]. Chi as a homopolymer of N-glucoseamine is obtained by deacetylation from chitin, which is a homopolymer of N-acetylglucoseamine. The amino group of Chi easily reacts with glutaraldehyde, which is a crosslinking agent due to its dialdehyde group and thus, the free aldehyde formed group on a Chi carrier make possible the covalent binding of the enzyme by free amino groups of lysine residues of protein enzymes. Alginate, commercially available as Alg sodium salt, is a linear natural copolymer of L-guluronic acid and D-mannuronic acid units. Alg can bind multivalent cations, leading to the formation of insoluble hydrogels. This anionic polysaccharide forms strong gels with Ca^{+2} , giving microspheres with good strength and flexibility. Such crosslinking stiffens and roughens the polymer, lowering swelling in water and organic media [16]. Because Alg and Chi are polyanionic and polycationic biopolymers, respectively, they can be used to prepare composite stable beads due to their electrostatically complementary ionizable groups [19]. Furthermore, these polymer matrixes have certain advantages over other materials such as low cost, ease of enzyme accessibility, crosslinking ability, stability, etc. [19].

The aim of this work is to produce commercially important HAc which is pear flavor by using immobilized lipases

from *Candida rugosa* and porcine pancreas. Immobilization was carried out by two methods, namely by entrapment into calcium Alg/Chi composite gel and by covalent bonding onto Chi. Immobilization conditions and HAc production parameters were optimized in detail. The effect of hexanol (HA) and acetic acid (AA) substrate concentrations on ester yield were investigated simultaneously using response surface methodology (RSM) by optimal design. Four immobilized lipases were applied to two reactor systems: batch-type and fixed bed reactors.

Experimental

Material

Lipase from *C. rugosa* (1170 U mg^{-1}), lipase from porcine pancrea (329 U mg^{-1}), glacial AA, HA, alginic acid sodium salt (Na-Alg), Chi, calcium chloride, glutaraldehyde (GAL), sodium hydroxide, hydrochloric acid, chloroform, heptane, hexane, xylene, toluene and all other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). GC-MS analysis was carried out using a Hewlett-Packard HP 6890 Series GC System and Innovax column at 150°C .

Method

Immobilization of CRL and PPL by entrapment in biodegradable Ca-Alg/Chi composite gel beads and also by covalent binding onto a spacer arm attached Chi carrier were well investigated in our previous study to produce isoamyl acetate (IAAc) as banana odor [9]. Optimization of immobilization conditions and characterization of immobilized enzymes in terms of IAAc production were studied in detail.

Immobilization of lipase by entrapment in Ca-Alg/Chi gel

Immobilization of CRL and PPL by entrapment in Ca-Alg/Chi composite gel was carried out according to our previous study [9]. To obtain Ca-Alg/Chi gel, the mixture of Na-Alg/Chi solution was dripped by a syringe into 2 M CaCl_2 and GAL solution at 4°C . Ca-Alg/Chi is formed by two independent cross-links: one is formed between the GAL and amino groups of Chi, the second is between Ca^{+2} ions and Alg.

To immobilize CRL or PPL in Ca-Alg/Chi gels by entrapment, CRL and PPL enzyme protein was added to the Na-Alg/Chi mixture and the gelation procedure was applied as described formerly. After gelation, CRL and PPL

were immobilized and $\text{CRL}_{\text{Ca-Alg/Chi}}$ and $\text{PPL}_{\text{Ca-Alg/Chi}}$ gel beads were obtained, respectively. $\text{CRL}_{\text{Ca-Alg/Chi}}$ and $\text{PPL}_{\text{Ca-Alg/Chi}}$ gel beads were dried at room temperature until reaching a certain weight and samples were stored in closed bottles at 4°C. The entrapped enzyme amount was calculated according to our previous study [9].

Immobilization parameters were optimized sequentially for Chi, Na-Alg and GAL concentrations while enzyme and CaCl_2 concentrations were constant as 3 mg mL^{-1} and 2.0 M, respectively, in the all experiments [9].

Immobilization of lipase by covalent binding onto Chi

Covalent immobilization of CRL and PPL onto chitosan was carried out as in our previous study [9]. Before enzyme immobilization, free aldehyde groups were formed onto Chi via GAL. Also, the Chi carrier was modified by four different spacer arms such as 1,3-diamino propane (1,3-DAP), 1,4-diamino butane (1,4-DAB), 1,5-diamino pentane (1,5-DAP) and 1,6-diamino hexane (1,6-DAH). Modified and unmodified Chi carriers were incubated with 20 mL of the CRL or PPL solution (at 3 mg mL^{-1}). After the 2 h of immobilization time, unbound lipase was removed by excessive washing. The amount of the bound lipase protein was determined as in our previous study [9]. Covalent immobilized lipases, CRL_{Chi} and PPL_{Chi} , were then dried at room temperature, and samples were stored in closed bottles at 4°C. A spacer arm was chosen according to HAC production performances. Optimal GAL concentration for activation of spacer arm modified Chi was determined by changing between 0.1% and 1.0%.

HAC production by immobilized lipases

HAC was produced by HA and AA substrates using 100 mg $\text{CRL}_{\text{Ca-Alg/Chi}}$ and $\text{PPL}_{\text{Ca-Alg/Chi}}$ or 10 mg CRL_{Chi} and PPL_{Chi} as we described in our previous study [9]. HAC producing performances were determined according to measuring the AA amount that participated in HAC synthesis by the titrimetric method. Synthetic activity was calculated as 1 μmol HAC g support $^{-1}$.

The effects of working parameters on HAC yield

Due to the water content of the microenvironment of an enzyme having a crucial role on synthetic activity, the dryness ratio of $\text{CRL}_{\text{Ca-Alg/Chi}}$ and $\text{PPL}_{\text{Ca-Alg/Chi}}$ gel beads were

optimized. To this, gels were dried to reach to 80, 40, 27 and 17% of their initial weight at room temperature as described in detail in our previous study [9]. A similar study was carried out for CRL_{Chi} and PPL_{Chi} by storing in medium with different relative humidities at 0, 10, 25, 50, 75 and 100% according to previous studies [20, 21]. Tubes containing CRL_{Chi} and PPL_{Chi} were placed in the boxes which were prepared for certain relative humidity and all of them were stored for 24 h at 25°C. Then, they were used for HAC production at the same conditions.

We synthesized HAC in different reaction media, namely xylene, toluene, hexane, chloroform, heptane, and also in a solvent-free medium in a orbital shaker at constant temperature [9]. The organic solvent that showed the highest HAC yield was selected and was used in the subsequent studies.

The mixture obtained at the end of 60 min of reaction time was analyzed using a Hewlett Packard HP 6890 Series GC System using the Innowax column to observe the synthesized HAC which was already smelled sensually.

The effect of temperature on HAC production was investigated in the range of 30–60°C for each immobilized lipase sample.

In order to evaluate the effects of HA and AA substrate concentrations on HAC yield, RSM based on a five levels-two factor optimal design was applied. Concentrations of HA and AA were chosen as 10, 25, 50, 75 and 100 mM, and 16 working runs were formed by the Design Expert 8.0.7.1 program (State Ease, Serial No: 0021-6578).

The ester producing efficiency of immobilized lipases were investigated depending on 8 h of reaction time using a batch reactor and also using a fixed bed reactor. One gram of $\text{CRL}_{\text{Ca-Alg/Chi}}$ and $\text{PPL}_{\text{Ca-Alg/Chi}}$ or 50 mg of CRL_{Chi} and PPL_{Chi} were loaded into the batch reactor for 8 h of reaction time and the mixture was stirred on an orbital shaker. In the case of fixed bed reactor, a glass column was used for the same amount of immobilized enzymes and substrate solution was fed to the reactor by a peristaltic pump.

Results and discussion

Optimization of immobilization conditions for entrapment in Ca-Alg/Chi gel

Lipase enzymes (CRL and PPL) were immobilized in Ca-Alg/Chi gel by cross-linking. Na-Alg, Chi and GAL concentrations were optimized one by one while CaCl_2 and

initial enzyme concentration were 2 M and 3 mg mL⁻¹, respectively, in all immobilization studies. HAc yields depending on Na-Alg, Chi and GAL concentrations at immobilization studies are shown in Figure 1A, B and C, respectively.

As seen in Figure 1A and B, ester yields were the highest at 1.5% Na-Alg, 1.5% Chi and 0.15% GAL concentrations for both CRL_{Ca-Alg/Chi} and PPL_{Ca-Alg/Chi}. At the optimal conditions, the amounts of entrapped lipase protein were calculated as 15.0 and 18.7 mg enzyme protein g gel⁻¹ for CRL_{Ca-Alg/Chi} and PPL_{Ca-Alg/Chi}, respectively.

It is well known that the catalytic behavior of lipases in the non-aqueous enzymatic reaction depends mainly on the amount of water present in the reaction mixture [22]. Especially, water in the microenvironment of the enzyme molecule is required for keeping the enzyme in its active conformation. Furthermore, in a non-conventional medium, the amount of water necessary for the lipase activity depends on the nature of the support, the polarity of the substrates and the solvent used [2, 12]. Due to nature of the lipase, the amount of water is also important for the catalysis direction of the lipase: ester hydrolysis or synthesis [14, 23, 24]. Because of the polar property of the Ca-Alg/Chi gel, a large amount of water was adsorbed during and after the gelation event [9]. So, we investigated the optimal dryness ratio of CRL_{Ca-Alg/Chi} and PPL_{Ca-Alg/Chi} samples (Figure 2). As seen in Figure 2, when the amount of water decreased by decreasing the weight of the gel, the activity increased slightly until they reached 40% relative weight. Then, a considerable high increment was observed of the 27% of initial weight while the ester yield lessened sharply in the drier conditions.

Optimization of immobilization conditions for covalent binding onto Chi

Firstly, the spacer arm type was chosen for the Chi support which was previously activated by GAL. After four different spacer arm attachments were incorporated to Chi support, lipase was immobilized covalently. The type of spacer arm affected both the amounts of bound lipase and esterification activity. Binding lipase amount, as well as esterification activity have the tendency to decrease by increasing the spacer arm's length [9]. According to the highest values observed, 1,3-DAP spacer arm was chosen for subsequent studies.

GAL concentration using for activation of 1,3-DAP incorporated Chi was also optimized. GAL concentration was between 0.1% and 1.0% and CRL_{Chi} and PPL_{Chi} showed the highest activity at 0.5%-GAL concentration. The bound

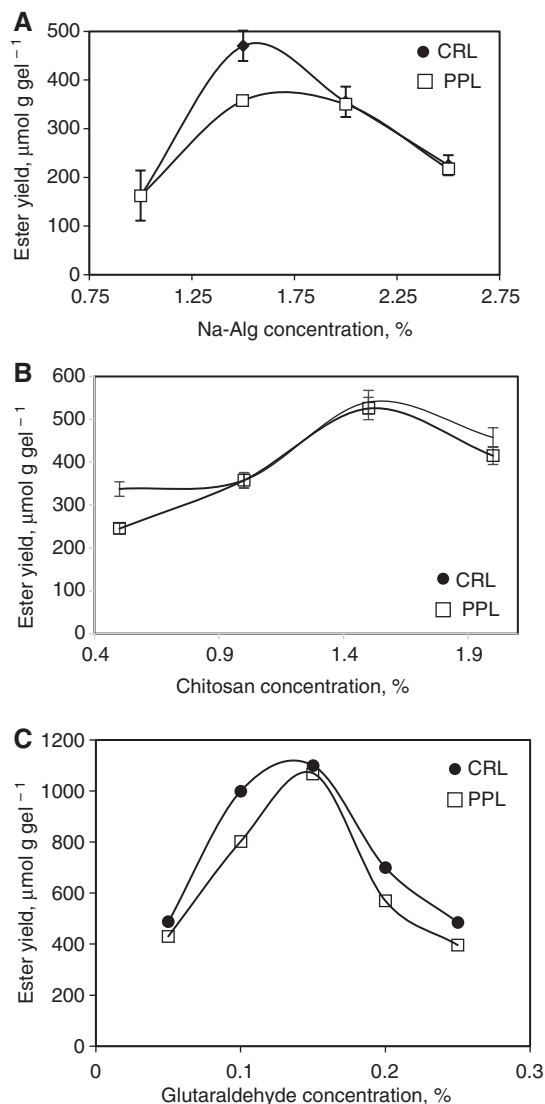


Figure 1: Optimization of immobilization parameters in terms of Na-Alg (A), Chi (B) and GAL (C) concentrations (●:CRL_{Ca-Alg/Chi}; □: PPL_{Ca-Alg/Chi}).

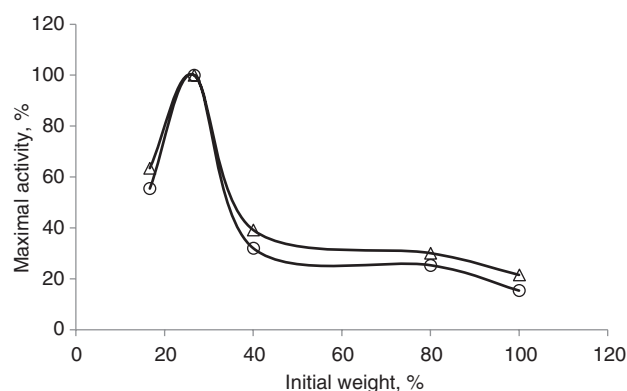


Figure 2: The effect of dryness on HAc synthesis of CRL_{Ca-Alg/Chi} (Δ) and PPL_{Ca-Alg/Chi} (O).

enzyme amount was 1.69 and 1.54 mg enzyme protein g Chi^{-1} for CRL_{Chi} and PPL_{Chi} , respectively.

Finally, the relative humidity of the medium in which CRL_{Chi} and PPL_{Chi} samples were kept before HAC synthesis was optimized. Ten milligram of CRL_{Chi} and PPL_{Chi} samples were stored in the closed system at different relative humidities 0, 10, 25, 50, 75 and 100% for 24 h and then were used for HAC synthesis. The results are given in Figure 3.

As seen in Figure 3, HAC synthesis increased with the increase in relative humidity from 0% to 25%, and then decreased by a further increase in relative humidity of the medium. The amount of water in the microenvironment of lipase is important not only for the gain of its active conformation but also for the reaction direction. It can be concluded that, the amount of water adsorbed on the Chi carrier should be sufficient to form an active form of lipase enzyme, but not to hydrolyze the ester bond. As a result, CRL_{Chi} and PPL_{Chi} samples were kept in the medium at 25% relative humidity before being used in ester synthesis for subsequent studies. Similar studies were carried out for glucose oxidase [20] and catalase [21] which were immobilized covalently onto florosil carrier and the highest activities were observed when they were stored at 60% and 20% relative humidity, respectively.

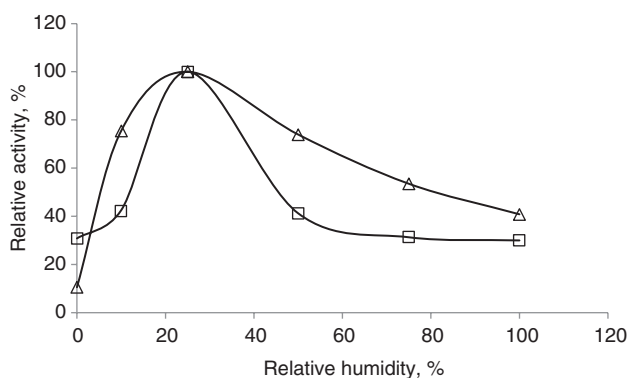


Figure 3: The effect of relative humidity of medium on HAC yield of CRL_{Chi} ($\triangle$) and PPL_{Chi} ($\square$).

Table 1: The amount of HAC produced in different organic media by immobilised lipases.

Reaction medium	HAC Yield ($\mu\text{mol g support}^{-1} \text{ min}^{-1}$)			
	$\text{CRL}_{\text{Ca-Alg/Chi}}$	$\text{PPL}_{\text{Ca-Alg/Chi}}$	CRL_{Chi}	PPL_{Chi}
Solvent free	2.7 ± 0.56	2.58 ± 113	10.2 ± 0.52	10.5 ± 0.56
Hexane	7.9 ± 0.35	7.4 ± 0.51	27.5 ± 0.19	29.2 ± 0
Heptane	10.7 ± 0.34	9.6 ± 0.48	36.3 ± 0.34	36.3 ± 0.58
Chloroform	3.6 ± 0.40	2.7 ± 0.51	31.3 ± 0.85	30.4 ± 0.69
Toluene	5.3 ± 1.19	6.1 ± 0.85	26.3 ± 0.68	27.5 ± 0.78
Xylene	4.1 ± 0.68	2.9 ± 0.52	23.8 ± 0.4	18.8 ± 0.51

Bold values indicate HAC yields were considerably higher in heptane.

HAC production by immobilized lipases

The effect of synthesis conditions

HAC synthesis was carried out in six different media, namely xylene, toluene, hexane, chloroform, heptane, and also in solvent-free medium using $\text{CRL}_{\text{Ca-Alg/Chi}}$, $\text{PPL}_{\text{Ca-Alg/Chi}}$, CRL_{Chi} and PPL_{Chi} . HAC yields are given in Table 1.

As shown in Table 1, $\text{CRL}_{\text{Ca-Alg/Chi}}$ and $\text{PPL}_{\text{Ca-Alg/Chi}}$ was observed to have the lowest activity in the solvent-free medium. The HAC yields were considerably higher in heptane (bold values in Table 1), the polarity of which is the lowest among the tested solvents, and was used as a reaction medium for all immobilized lipases. Similar studies in the literature were observed in an organic solvent system higher than solvent-free system [2, 12, 24, 25]. Bezbradica et al. [2] compared the activities of commercial lipases in the synthesis of several esters in the solvent-free medium and in isooctane. Ester yields observed in isooctane are higher than those in solvent-free systems. It is a well-known fact that a certain amount of water micro-layer around the enzyme molecule is a necessity for keeping the enzyme in its active conformation and that polar compounds readily destroy this layer [2, 26].

Immobilized lipases reacted with HA and AA substrates, in heptane solvent and, after 1 h of reaction time, the reaction mixture was analysed by GC-MS. Three different peaks, which were identified by an MS detector were observed, and the elution times of 1.76, 1.69 and 1.49 min for AA, HA and HAC were determined, respectively. Thus, the HAC synthesis was approved not only by sensorial tests due to the typical pear fragrance, but also by the MS detector of the GC-MS (Figure 4).

We investigated the optimal temperature for HAC synthesis in the range of 30–60°C. As seen in Figure 5. The highest ester production was obtained at 45°C for both $\text{CRL}_{\text{Ca-Alg/Chi}}$ and $\text{PPL}_{\text{Ca-Alg/Chi}}$ while 40°C was obtained for both CRL_{Chi} and PPL_{Chi} .

The effect of substrate concentration

The general shape of a velocity versus substrate concentration is that of a rectangular hyperbola. While reaction velocity increases by increasing substrate concentration at low concentrations, at higher concentrations, the velocity of reaction remains approximately constant and effectively

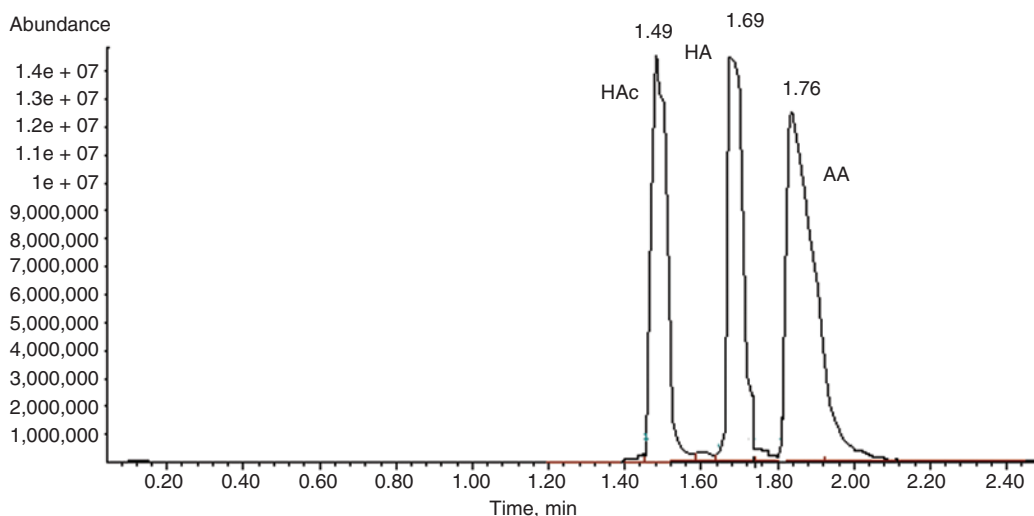


Figure 4: GC-MS chromatogram of reaction mixture after 60 min of reaction time using a Hewlet Packard HP 6890 Series GC System with Innobox column and MSD detector at 150°C isothermal conditions.

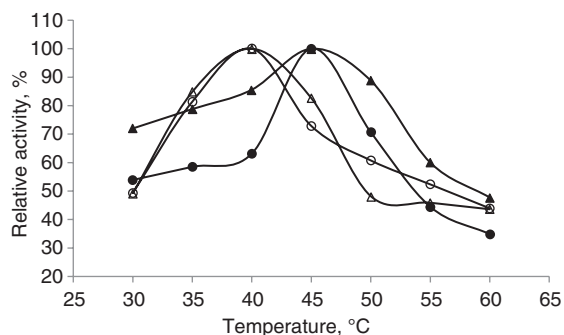


Figure 5: The effect of temperature on HAC synthesis for $CRL_{Ca-Alg/Chi}$ (●), $PPL_{Ca-Alg/Chi}$ (▲), CRL_{Chi} (○) and PPL_{Chi} (△).

insensitive to changes in substrate concentration [27]. But, in the present work, due to the nature of lipase on esterification activity, the amount of water in the microenvironment of the immobilized enzyme has a crucial role. At high substrate concentration, the high amount of water produced may cause a shift in reaction direction from esterification to hydrolysis [2, 9, 22]. Also, the hydrophobic property of hexyl alcohol and the acidic property of acetic acid may cause denaturation of the enzyme. So, to investigate the effect of HA and AA substrate concentrations on the HAC yield in detail simultaneously, five levels with a two factor optimal design of RSM was applied using Design Expert 8.7.0.1. program (State Ease, serial no:0021-6578). The RSM method used to investigate ester synthesis by immobilized lipase have been reported earlier [13, 28, 29]. The HAC yields obtained for 16 sets of worked using the program are given in Figure 6.

As seen in Figure 6, HAC yield increased with the increase of AA concentration up to 50 mM for all

immobilized samples. In the case of HA concentration, HAC yield increased to 50 mM for $CRL_{Ca-Alg/Chi}$ and $PPL_{Ca-Alg/Chi}$, up to 75 mM for CRL_{Chi} , HAC yield decreased at higher substrate concentrations for all immobilized lipase samples. This decrease can be attributed to deactivation of lipase enzyme by substrate and also to the reverse direction of ester synthesis due to the high amount of water produced at high substrate concentration. The decrease in the ester amount by immobilized lipase using alcohol and acid substrate at high concentration has been reported in several studies [9, 11, 30].

HAC production in batch and fixed bed reactors

Immobilized lipases were used in the batch-type reactor and in fixed bed reactors and HAC yields were given as depending on time in the Figure 7A and B, respectively.

As seen in Figure 7A, in the case of using a batch reactor, HAC yield increased for the first 5 h and then, a sharp decrease was observed by increase in the reaction time. However, the amount of HAC produced in a fixed bed reactor slightly increased during the whole reaction time. HAC yield was noticeably higher in the batch-type reactor than in the fixed bed reactor for each immobilized lipase.

The higher HAC production in the batch-type reactor may be related to the higher interaction probability between immobilized lipase and AA and HA molecules. The decrease, which was observed in the batch-type after 5 h of the reaction time, can be explained by hydrolysis of

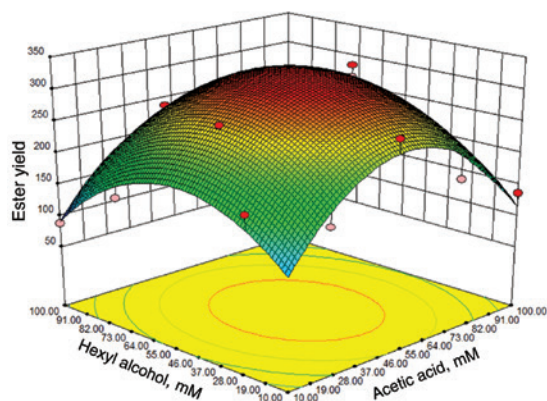
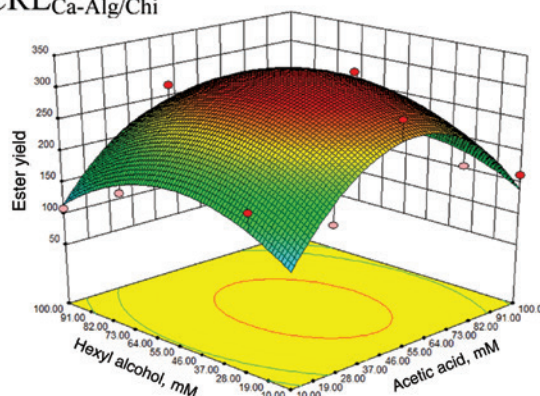
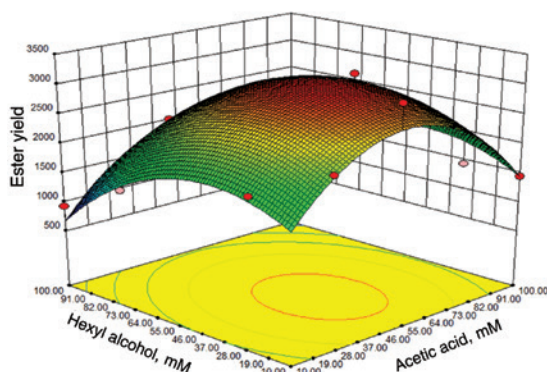
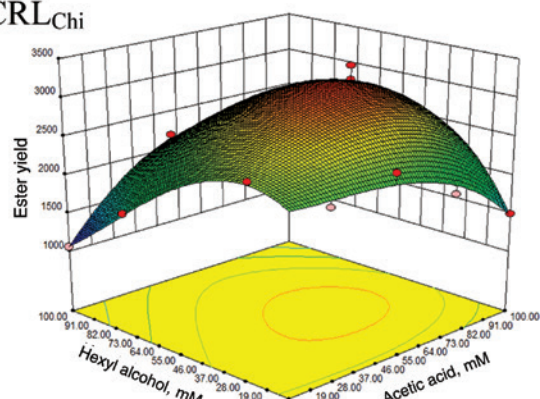
PPL_{Ca-Alg/Chi}CRL_{Ca-Alg/Chi}PPL_{Chi}CRL_{Chi}

Figure 6: Response surfaces fitted to the production of Hexyl acetate as a function of initial acetic acid and hexyl alcohol concentrations (Ester yields are represented as $\mu\text{mol g carrier}^{-1}$) ($p < 0.0001$).

the ester produced due to water formation and accumulation around of the microenvironment of immobilized lipase. So, it can be concluded that, reaction time should be restricted to 5 h for these conditions to prevent the decomposing formerly occurring ester bond. On the other hand, although the HAC amount produced in the batch-type reactor was considerably higher, a constant increase was observed when the fixed bed reactor was used to produce HAC. The HAC amount increased up to 5 h of reaction time in batch-type reactor for CRL_{Ca-Alg/Chi} and PPL_{Ca-Alg/Chi}, respectively, and decrease was observed at higher reaction times. This is mainly owing to the possibility of the enzyme and substrate interacting for a long period. Also, owing to the barrier property of Ca-Alg/Chi gel which limited the reaching of the substrate in Ca-Alg/Chi gel, the HAC production efficiency was lower for CRL_{Ca-Alg/Chi} and PPL_{Ca-Alg/Chi} especially in the fixed bed reactor. However, when PPL_{Chi} and CRL_{Chi} were used, HA and AA substrates more readily interacted

with the enzyme because the lipase was on the surface of the support.

Conclusions

It was clearly observed that, as well as synthesis conditions, immobilization parameters affected the HAC productivity. Although preparation of CRL_{Ca-Alg/Chi} and PPL_{Ca-Alg/Chi} was easier, higher HAC productivity was obtained when CRL_{Chi} and PPL_{Chi} were used. It was also revealed that the amount of water around of the microenvironment of the lipase has a crucial role in HAC production. The amount of HAC decreased at high substrate concentrations. This decrease can be attributed to deactivation of lipase enzyme by substrate and also to reverse direction of ester synthesis due to the high amount of water produced at high substrate

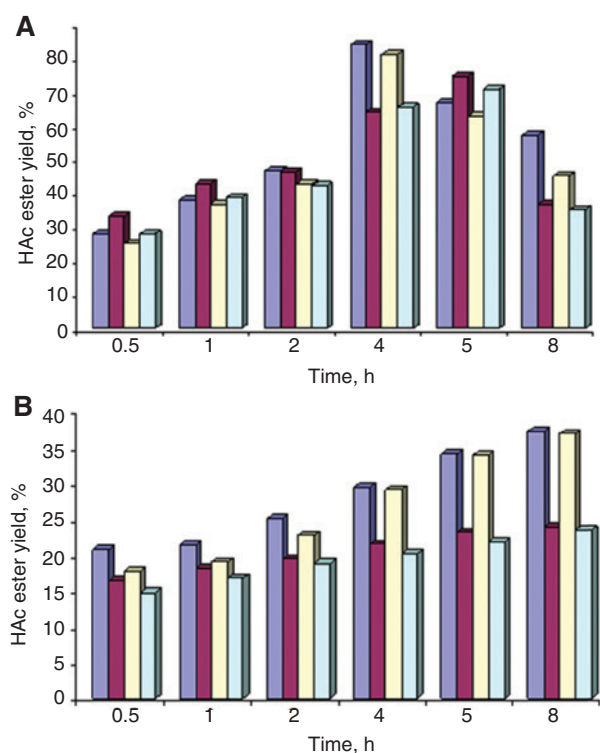


Figure 7: HAC production in batch-type reactor (A) and in fixed bed reactor (B) for CRL_{Chi} (■), CRL_{Ca-Alg/Chi} (■), PPL_{Chi} (■) and PPL_{Ca-Alg/Chi} (■).

concentration. The HAC yield was nearly 2-fold higher in the batch-type reactor than that of a fixed bed reactor. However, while HAC yield increased slightly in the fixed bed reactor during 8 h of reaction time, it tended to decrease after 5 h in the batch type reactor.

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