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Production of aroma esters by immobilized *Candida rugosa* and porcine pancreatic lipase into calcium alginate gel☆

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ABSTRACT

Candida rugosa lipase (CRL) and porcine pancreatic lipase (PPL) were immobilized into calcium alginate (Ca-Alg) gel beads by means of entrapment and were used to produce three industrially important flavour esters, namely isoamyl acetate (banana flavour), ethyl valerate (green apple flavour) and butyl acetate (pineapple flavour). Immobilization conditions were optimized in terms of sodium alginate (Na-Alg) and $CaCl_2$ concentrations by determination of the entrapped enzyme amount as well as by esterification of 4-nitrophenol and acetic acid. The best results were obtained at 2.5% Na-Alg and 2.5 M $CaCl_2$ for CRL while at 2.5% Na-Alg and 2.0 M $CaCl_2$ for PPL. On carrying out flavour syntheses in solvent-free medium and also in hexane medium, higher ester yields were obtained in hexane medium for all esters and both types of lipases. Ester esterification efficiency increased in parallel with both enzyme concentrations at immobilization medium and the immobilized lipase amount in esterification medium. Maximum ester production was observed between 40 and 50 °C for CRL and PPL. Besides, the effect of substrate concentrations on ester conversion was remarkable. The best ester yield was obtained for isoamyl acetate when immobilized PPL was used.

1. Introduction

The increasing demand for fragrance and flavour esters used in the food, cosmetics and pharmaceutical industries makes it necessary to find alternative ways instead of extraction from their natural materials which are too scarce or expensive for commercial use. The industrial ester syntheses are based on direct chemical esterification of fatty acids with alcohol in the presence of inorganic catalysts at elevated temperatures (200–250 °C). However, these chemical reactions are tedious, non-selective and consume a large amount of energy. Using enzymes and other biocatalysts for these synthesizing processes allows products with better odor and color [1].

Lipases (triacylglycerol hydrolase, EC 3.1.1.3) are a family of enzymes that are in their natural environment catalyze the hydrolysis of fats. However, under appropriate working conditions, lipases have shown to be very active catalysts in esterification, transesterification and alcoholysis reactions [2]. Lipases have been widely used for biotechnological applications in dairy industry, oil processing, production of surfactants and enantiomerically pure pharmaceuticals [3]. There are several studies about ester synthesis in non-aqueous medium using lipase by transesterification [4–8] and esterification reactions [9–18] in solvent-free medium [11,13,17,19,20] or in organic solvent medium [3,6,21,22]. Main problem appearing with organic medium when free lipase is used is denaturation and also aggregation, which prevents homogeneity in reaction medium. This problem can be solved by using immobilized form of lipase [23]. Immobilization of enzymes can offer several advantages including its reuse, ease in application of both batch and continuous systems, possibility of better control reactions, ease in removal from the reaction medium and improved stability [24].

The aim of this work is to produce three commercially important flavour esters, namely isoamyl acetate (banana flavour), ethyl valerate (green apple flavour) and butyl acetate (pineapple flavour) by using *Candida rugosa* lipase (CRL) and porcine pancreatic lipase (PPL) immobilized into calcium alginate gel (Ca-Alg). The effect of sodium alginate and $CaCl_2$ concentration on immobilization was investigated. In esterification reactions firstly flavour ester synthesis was carried out at solvent-free system and in hexane medium to compare the effect of non-polar solvent. Also investigated were the effects of various flavour synthesizing parameters such as enzyme concentration at immobilization, the amount of immobilized enzyme, temperature, concentration of the substrates and the reaction time.

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2. Experimental

2.1. Material

C. rugosa lipase (solid form 1170 U mg^{-1}), porcine pancreatic lipase (solid form 329 U mg^{-1}), isoamyl alcohol (IAA), butyl alcohol (BA), ethyl alcohol (EA), glacial acetic acid (AA), valeric acid (VA), sodium alginate (Na-Alg), $CaCl_2$ anhydrous, 4-nitrophenol (4-NP), NaOH, HCl, hexane and all other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Method

2.2.1. Lipase immobilization into calcium alginate gel

A 100 ml of Na-Alg solution containing CRL (or PPL) was dripped with syringe into 200 ml of $CaCl_2$ solution at 4 °C. As soon as the mixed solution was dripped into $CaCl_2$ solution, calcium alginate (Ca-Alg) gels were formed by cross-linking. After 2 h of hardening time at 4 °C, Ca-Alg beads were separated from $CaCl_2$ solution by vacuum filtration and washed twice with 25 ml of distilled water. Filtered $CaCl_2$ solution and washing solutions were then mixed and the total volume of the filtrate was measured. The same procedure was repeated in the absence of CRL (or PPL), upon which lipase-free Ca-Alg gel was obtained. The absorbance values of filtrate solutions were measured at 280 nm to determine the amount of non-immobilized enzyme. To convert the absorbance value to enzyme concentration value, standard curves of enzymes at 280 nm were previously constituted using CRL and PPL solutions prepared at different concentrations between 0.01 and 2.00 mg ml^{-1}. Ca-Alg gels were dried at room temperature open the atmosphere until a certain weight and samples were then stored in screwed bottles at 4 °C.

2.2.2. Optimization of immobilization parameters

Immobilization was carried out making use of Na-Alg and $CaCl_2$ solutions at different concentrations. Na-Alg concentration was changed to 1.0–1.5–2.0–2.5–3.0% while $CaCl_2$ and enzyme concentration were kept constant as 2.0 M and 2 mg ml^{-1}, respectively. Three parameters were investigated for each sample including the amount of entrapped enzyme, esterification activity by using 4-nitrophenol and acetic acid and also leakage of the entrapped enzyme to hexane medium during 180 min. After finding the best result for Na-Alg, $CaCl_2$ concentration was optimized by changing from 1.0 to 2.5 M at optimal Na-Alg concentration.

2.2.2.1. The amount of entrapped enzyme. The amount of entrapped enzyme was calculated according to equation given below:

$$C_E = \frac{C_o V_o - C_m V_m}{m_E}$$

where C_E is the amount of entrapped enzyme per gram of Ca-Alg (mg g Ca-Alg^{-1}), C_o the lipase concentration of Na-Alg solution used in immobilization (mg ml^{-1}), V_o the volume of Na-Alg solution (ml), C_m the enzyme concentration of total filtrate (mg ml^{-1}), V_m the volume of filtrate (ml) and m_E is the amount of Ca-Alg beads (g) after drying.

2.2.2.2. Esterification activity for 4-nitrophenol and acetic acid. Esterification activity was measured by spectrophotometric method described in a previous study by Ozyilmaz [25] in detail. Reaction commenced on addition of 0.2 g Ca-Alg beads to 2 ml of hexane containing 1 mM 4-NP and 50 mM AA and continued for 15 min in a shaker. On completion of reaction time, 1 ml solution was removed from the reaction mixture and added to 3.5 ml of 25 mM NaOH solution to recover the remaining part of 4-NP by vortexing for 30 s. The absorbance value of aqueous phase was measured at 410 nm. Concentration of 4-NP was determined by standard curve of 4-NP at different concentration rates prepared in hexane and extracted with 3.5 ml of 25 mM NaOH solution. Concentration difference of 4-NP between non-catalyzed and catalyzed medium was used to calculate the amount of 4-NP which participated in esterification reaction. Activity was expressed as μmol 4-NP min^{-1} mg $protein^{-1}$. All reactions were repeated three times.

2.2.2.3. Enzyme leakage. The absorbance value of hexane (5 ml) containing 0.25 g Ca-Alg beads was measured at 280 nm periodically during 180 min, where pure hexane was used as blank. The leakage percentage of enzyme from Ca-Alg beads to hexane was calculated by the equation given below:

$$\text{enzyme leakage}\,(\%) = \frac{C_L V_H}{m_L} \times 100$$

where C_L is the enzyme concentration of hexane solution, V_H the volume of hexane (5 ml), and m_L is the amount of the enzyme protein in 0.25 g of Ca-Alg beads.

2.2.3. Flavour ester synthesis

Three different industrially important esters were synthesized: isoamyl acetate (IAAc), ethyl valerate (EV) and butyl acetate (BAc) using CRL or PPL entrapped in Ca-Alg.

Flavour esters were firstly synthesized in solvent-free medium and in hexane medium to determine the proper medium. To this end, 1 ml of acid and alcohol mixture at 1:1 molar ratio was used to synthesize ester with 0.1 g of immobilized enzyme in a temperature-controlled orbital shaker. After 2.5 h of reaction time, the amount of remaining acid was determined by titrimetric method using 0.25 M NaOH. Afterwards, studies were repeated by adding 1 ml of hexane into substrate mixture before starting the reaction.

For the investigation of the ester synthesis parameters, reactions were carried out in screw-capped bottle using 0.1 g of Ca-Alg beads and 10 ml substrate mixture of 25 mM acid (AA or VA) and 25 mM alcohol (IAA, BA or EA) in hexane with shaking at 200 rpm. At the end of the 1 h of reaction time, unconsumed acid was extracted twice by 10 ml of 25 mM NaOH. Then, hexane medium was washed twice by water. Extraction and washing solutions were collected in an Erlenmayer and the concentration of remaining NaOH was determined by back titration using 25 mM HCl solution and bromocresol green as indicator. Reactions were repeated three times and the difference of HCl volumes between enzyme containing Ca-Alg and enzyme-free Ca-Alg was used to calculate the amount of acid substrate participating in the esterification reactions.

2.2.3.1. The effect of the esterification parameters. Ester synthesis was carried out by Ca-Alg beads prepared with CRL or PPL at various concentrations ranging from 1 to 5 mg ml^{-1}. After determining the best concentration of CRL and PPL at immobilization, the effects of acid and alcohol concentrations on ester production were investigated by two sets of reactions. Firstly, alcohol concentration was kept constant as 100 mM and acid concentration was selected between 10 and 100 mM. Remaining acid was extracted twice by 20 ml of 50 mM NaOH and back titration was carried out using 0.1 M HCl. Secondly, the same type of experiment was repeated with different alcohol concentrations between 10 and 100 mM at optimum acid concentration for each lipase and flavour ester.

Different effects of the amount of Ca-Alg beads (0.05–0.4 g), of the temperature (30–70 °C), and of reaction time (1–48 h) were studied to find out the best working conditions for flavour ester synthesis.

3. Results

3.1. Optimization of the immobilization conditions

Because cross-linking between alginate and Ca^{2+} ions leads to gelation, the concentrations of Na-Alg and $CaCl_2$ are major parameters for enzyme gel entrapment [26]. Therefore, the effects of alginate and $CaCl_2$ concentration on the entrapment, activity and enzyme leakage were first investigated and results were summarized in Table 1. While Na-Alg concentration increased, entrapped lipase amount decreased from 21.9 to 10.3 mg g Ca-Alg^{-1} for CRL and from 17.2 to 5.9 mg g Ca-Alg^{-1} for PPL. Activities of lipase containing Ca-Alg beads measured by spectrophotometric method [25] and maximum activities were observed at 2.5% Na-Alg concentration for both CRL (0.026 U mg^{-1}) and PPL (0.033 U mg^{-1}). In case of $CaCl_2$ concentrations, while the Na-Alg concentration was kept constant at 2.5%, a slight decrease was observed when $CaCl_2$ concentration increased. As in Table 1, there was no significant difference in the amount of entrapped enzyme. The highest activities were observed at 2 M $CaCl_2$ as 0.043 U mg^{-1} and 2.5 M $CaCl_2$ as 0.057 U mg^{-1} for PPL and CRL, respectively. Enzyme leakage lessened in parallel with an increase in Na-Alg and $CaCl_2$ concentration. At optimum conditions, after 180 min of duration time only 2.24% and 1.62% of entrapped enzyme leached out of the Ca-Alg to hexane for CRL and PPL, respectively. These results may have been observed due to the low solubility of enzymes in organic solvents causing reduction in the amount of enzyme loss from the gel material [23].

3.2. Flavour ester synthesis

To determine the effect of the reaction medium on the esterification yield, flavour esters were first synthesized at solvent-free medium and then in hexane medium. When the amounts of acid substrates used in esterification reaction were compared for CRL, it was found that 34%, 19% and 33% more ester were produced in hexane medium than those in solvent-free medium for IAAc, EV and BAc, respectively. The same tendency was observed for PPL synthesis: 14%, 42% and 6% more yield were obtained in hexane medium for IAAc, EV and BAc, respectively. Similar results related to the increment in ester yield in organic medium were reported in literature earlier. Chaabouni et al. [11] produced ethyl valerate and hexyl acetate in hexane, heptane and solvent-free medium. They found remarkably higher ester yield in both hexane and heptane medium than those obtained in the absence of organic solvents. These results showed that the presence of a solvent could shift the equilibrium towards ester synthesis possibly by a total transfer of ester into the organic phase. Similarly, Been Salah et al. [21], who synthesized butyl acetate in tert-butanol, chloroform, hexane and heptane, observed that hydrophobicity of organic solvent greatly affected and improved the esterification activity. They found higher conversion rates in heptane and in hexane media. Bezbradica et al. [19] synthesized pentyl, heptyl and geranyl esters of butyric acid in solvent-free medium and in isooctane medium. At the end of the 72 h of reaction time, ester conversions were approximately 20% in solvent-free medium and 90% in isooctane medium. However, when they used carboxylic acid with long chain as oleic acid instead of butyric acid, conversion rates in solvent-free and in isooctane medium were both nearly 90%. In this study AA and VA which are carboxylic acid with short chain were used and ester production proved to be lower in solvent-free medium as cited in the literature. Subsequent ester synthesis was carried out in hexane medium due to higher ester productivity in solvent medium.

3.2.1. The effect of enzyme concentration at immobilization

The effect of the concentration of enzyme used during the immobilization step was studied and reported in Table 2.

As seen in Table 2, not only the amounts of the entrapped enzymes but also the esterification activity of Ca-Alg gels increased

Table 1
The optimization of immobilization parameters depending on Na-Alg and $CaCl_2$ concentrations.

	CRL				PPL			
	Bound lipase[a] (mg g gel^{-1})	Activity (U g gel^{-1})	Specific activity (U mg^{-1})	Enzyme leakage[b] (%)	Bound lipase (mg g gel^{-1})	Activity (U g gel^{-1})	Specific activity (U mg^{-1})	Enzyme leakage (%)
$C_{Na\text{-}Alg}$ (%)[c]								
1.5	21.9	0.197	0.009	22.48	17.2	0.189	0.011	18.71
2.0	17.5	0.210	0.012	16.50	10.4	0.239	0.023	12.58
2.5	13.8	0.359	0.026	6.64	7.01	0.231	0.033	7.90
3.0	10.3	0.196	0.019	4.58	5.9	0.083	0.014	4.56
C_{CaCl_2} (M)[d]								
1.0	13.7	0.082	0.006	18.45	7.8	0.054	0.007	9.26
1.5	13.4	0.335	0.025	11.61	7.1	0.185	0.026	4.32
2.0	12.3	0.467	0.038	6.23	6.5	0.279	0.043	1.62
2.5	11.4	0.650	0.057	2.24	6.2	0.198	0.032	1.15

[a] The amount of entrapped lipase into Ca-Alg gel.
[b] The leakage of lipase from Ca-Alg gel (0.25 g) into hexane (5 ml) at the end of the 180 min duration time.
[c] Na-Alg concentration was changed while the concentration of $CaCl_2$ was kept constant at 2 M.
[d] $CaCl_2$ concentration was changed while the Na-Alg concentration of was kept constant at 2.5%.

Table 2
The effect of enzyme concentration used in immobilization on esterification yield.

C_o (mg ml^{-1})	C_E (mg g Ca-Alg^{-1})		Esterification yield of CRL (μmol ester h^{-1} g Ca-Alg^{-1})			Esterification yield of PPL (μmol ester h^{-1} g Ca-Alg^{-1})		
	CRL	PPL	IAAc	EV	BAc	IAAc	EV	BAc
1	6.3	4.8	100	100	200	150	83	175
2	12.3	6.2	175	165	290	250	108	250
3	20.9	8.9	250	190	375	300	168	325
4	29.9	12.9	275	196	450	325	175	375
5	31.6	13.2	308	200	475	360	210	400

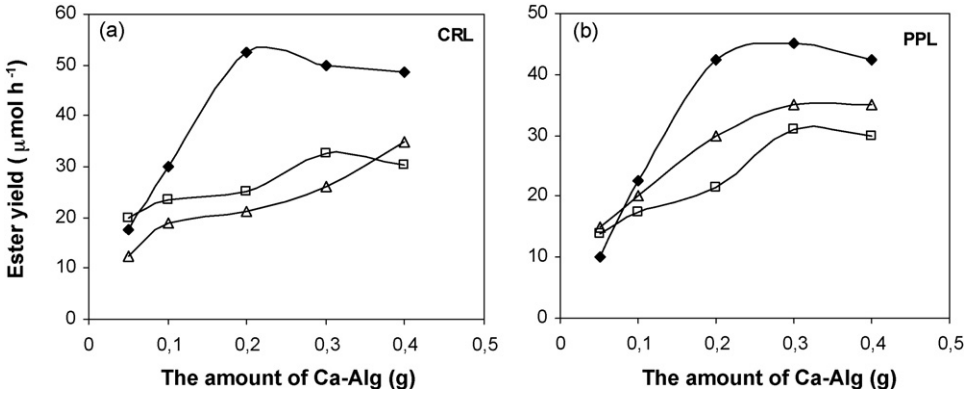

Fig. 1. The effect of the amount of Ca-Alg on the ester yield for (a) CRL and (b) PPL. ♦: IAAc, □: EV, and △: BAc.

by enzyme amount used in immobilization processes. However, this increment was not proportional to the amount of enzyme, because in the presence of high lipase amount in Ca-Alg gel, the active site may not be in close contact with the substrates and/or many molecules of the enzyme can aggregate together [11]. Generally, the increment of ester production lessened after 3 mg ml^{-1} enzyme concentration. So, immobilization of CRL and PPL was carried out using enzyme concentration at 3 mg ml^{-1} at subsequent studies.

3.2.2. The effect of Ca-Alg amount

The effect of Ca-Alg amount ranging from 0.05 to 0.4 g on the ester yield was studied and results were given in Fig. 1. The production of IAAc and EV increased in parallel with Ca-Alg amount up to 0.2 and 0.3 g, respectively, and then a slow decrease was observed at higher enzyme amount. In case of BA synthesis, ester yield was proportional to the amount of Ca-Alg used in hexane medium. Chaabouni et al. [11] who produced ethyl valerate and hexyl acetate using different amounts of immobilized lipase onto $CaCO_3$, reported that yields per milligram of lipase decreased by an increase in the immobilized amount of lipase.

3.2.3. The effect of temperature

The effect of temperature on the progress of the lipase-catalyzed esterification reactions was studied using 0.1 g of immobilized enzyme at 8 different temperatures between 30 and 70 °C, and results were given in Fig. 2.

On increasing the temperature from 30 to 40 °C, there were remarkable changes in the conversion profile of the IAAc production for both CRL and PPL, and after 40 °C the ester yield decreased dramatically. Moreover, IAAc production was observed at temperature higher than 60 and 70 °C for CRL and PPL, respectively. The minimum effect of temperature on production was observed for EV while the highest activities were observed at 50 and 45 °C for CRL and PPL, respectively. In the case of BA production, maximum activities were observed at 45 and 50 °C for CRL and PPL, respectively. As seen, each ester system has different optimal temperatures even when the same enzyme was used. Similarly, Chaabouni et al. [11] found optimal temperature for *Staphylococcus simulans* lipase immobilized onto $CaCO_3$ as 45 and 37 °C for ethyl valerate and hexyl acetate, respectively. It is safe to say that substrate thermodynamic properties determine their chemical reactivity and reaction equilibria. These properties notably depend on substrate chain-length, temperature and solvent especially for lipase [27]. Temperature also has a huge influence on the physical state of substrates such as solubility, ionization, etc.

3.2.4. The effect of substrate concentration

Ester productivity of immobilized CRL and PPL was firstly investigated depending on acid concentration ranging from 10 to 100 mM at 100 mM constant alcohol concentration and at optimal temperatures for each individual ester. As seen in Fig. 3(a) and (b), the highest amount of BAc was achieved when 25 mM acid was used for both CRL and PPL. The BAc production decreased sharply in parallel with acid concentration exceeding 25 mM. IAAc production increased with AA concentration up to 75 and 50 mM for CRL and PPL, respectively. It was observed that IAAc production was rather low at 100 mM acid. As for EV yield, it increased with VA concentration up to 100 mM. It was clearly concluded that, at AA concentration higher than 50 mM, a significant decrease in conversion yield was observed probably due to lipase denaturation by AA [11,13,21]. After investigating the effect of the acid concentration, alcohol concentration was changed from 10 to 100 mM at the most efficient acid concentration for each ester and enzyme. As can be seen in Fig. 3(c) and (d), the highest yields were obtained for IAAc at 25 and 75 mM of IAA concentration for CRL and PPL, respectively.

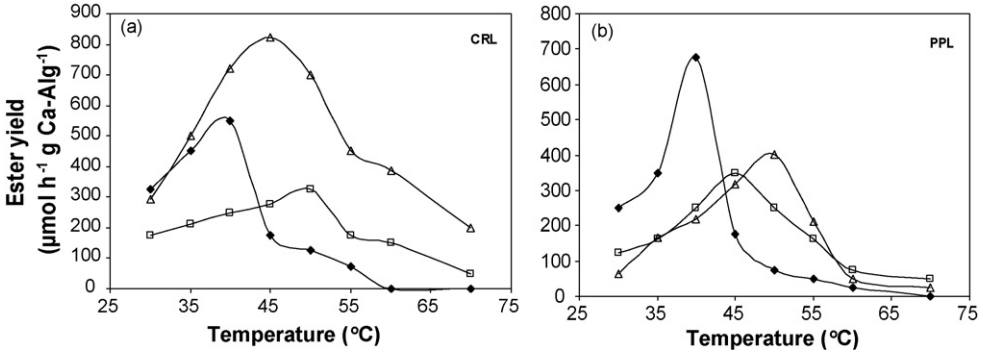

Fig. 2. The effect of temperature on the ester yield for (a) CRL and (b) PPL. ♦: IAAc, □: EV, and △: BAc.

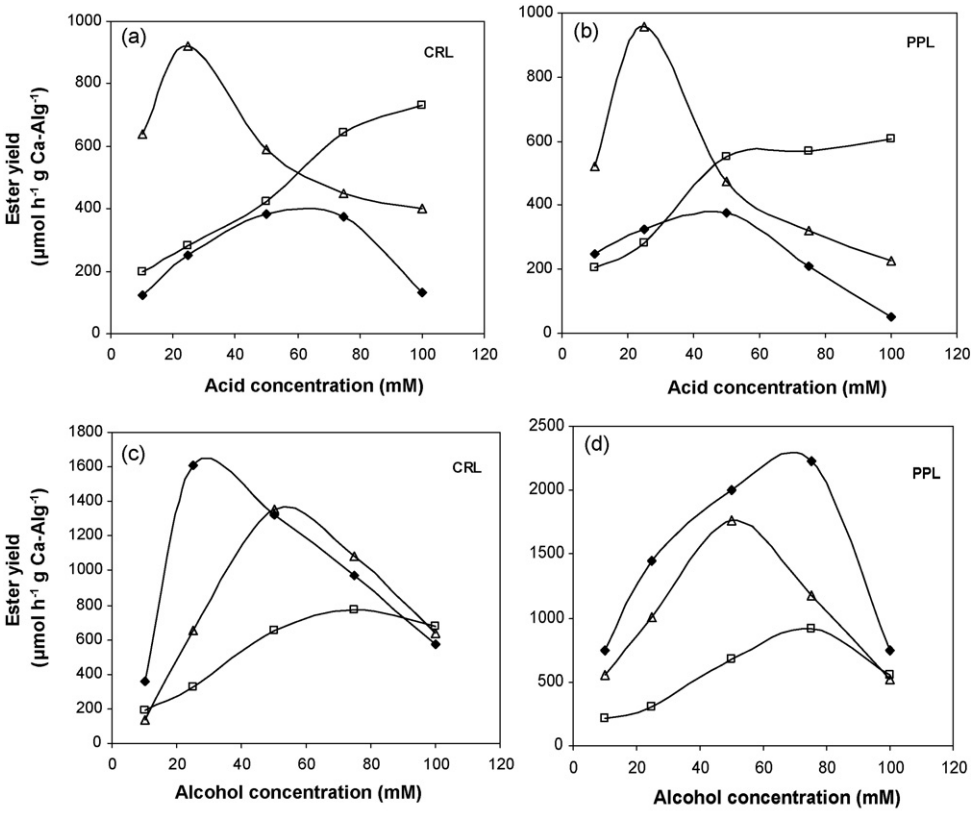

Fig. 3. The effect acid concentration on ester yield for (a) CRL and (b) PPL at 100 mM constant alcohol concentration, and the effect of alcohol concentration on ester yield for (c) CRL and (d) PPL at constant optimal acid concentration. ♦: IAAc, □: EV, and △: BAc.

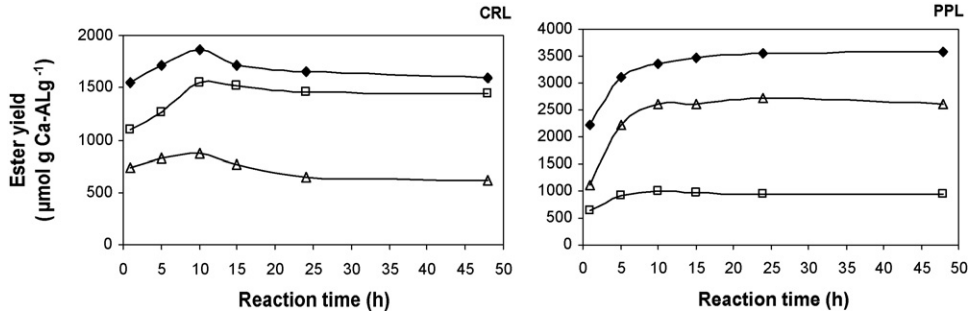

Fig. 4. Ester productivity at different reaction times for (a) CRL and (b) PPL. ♦: IAAc, □: EV, and △: BAc.

While, the highest amount of BAc was obtained at 50 mM of BA concentration for both CRL and PPL, this value was 75 mM EA for EV synthesis. The lowest yield was observed for EV synthesis. It can be seen in Fig. 3(c) and (d) that, PPL was more efficient than CRL to produce flavour esters at their optimal working conditions.

Although EV productivity increased with VA concentration up to 100 mM yield increased up to 75 mM, and then decreased in the case of increment in alcohol concentration. It may be due to more inhibitor effect of EA than those of IAA and BA [11].

3.2.5. The effect of reaction time

Ester synthesis was achieved at their optimal conditions at different reaction times between 1 and 48 h and results were given in Fig. 4. Ester production increased with time up to 10 h of reaction time and then slightly decreased while using CRL. Similar results were observed for PPL as production of EV and BAc, except IAAc. This decrease may be explained by an increment in the amount of water produced during catalysis. Large amounts of water around the enzyme in excess of the amount needed for complete hydration layer may provide some protection from denaturation by organic solvents, however, this may cause mass transfer problems for substrate. Most probably, increased water may lead to hydrolysis of ester previously produced [23].

The best result was obtained for IAAc synthesis when PPL was used. However, the minimum yield was obtained for EV flavour when CRL was used as catalyst.

4. Conclusion

The production of IAAc (banana flavour), EV (green apple flavour) and BAc (pineapple flavour) was carried out by CRL and PPL immobilized into Ca-Alg gel. Immobilization conditions were optimized in terms of Na-Alg and $CaCl_2$ concentration in detail. Ester productions were higher when synthesis was carried out in hexane medium instead of solvent-free medium. It was clearly shown that, CRL and PPL have different affinities for different substrates. The

best yield was obtained for IAAc by PPL while the yield for EV by CRL was lowest.

Nomenclature

CRL	*Candida rugosa* lipase
PPL	porcine pancreatic lipase
IAA	isoamyl alcohol
EA	ethyl alcohol
BA	butyl alcohol
AA	acetic acid
VA	valeric acid
IAAc	isoamyl acetate
EV	ethyl valerate
BAc	butyl acetate
Na-Alg	sodium alginate
Ca-Alg	calcium alginate
4-NP	4-nitrophenol

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