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Polish Journal of Chemical Technology, 25, 2, 15—20, 10.2478/pjct-2023-0012

Synthesis of isoamyl acetate using protein-coated microcrystals of different lipases

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The goal of this study was the immobilization of different lipases as protein-coated microcrystals on K_2SO_4 and their uses in the synthesis of isoamyl acetate in n-hexane medium. The optimum conditions, such as lipase variety, temperature, the initial molar ratio of vinyl acetate/isoamyl alcohol, immobilized lipase amount, and reaction time were determined. The highest conversion was obtained when protein-coated microcrystals of *Thermomyces lanuginosus* lipase (TLL-PCMCs) was used for the synthesis of isoamyl acetate. The optimum temperature, the initial molar ratio of vinyl acetate/isoamyl alcohol, immobilized lipase amount, and reaction time were determined to be 50 °C, 3.0, 30 mg, and 360 min, respectively. Under the optimized conditions, isoamyl acetate yield was obtained as 95%. TLL-PCMCs retained 90% of their initial activity after five repeat use in the isoamyl acetate synthesis. TLL-PCMCs may be used in the preparation of industrially important aroma compounds due its ease of preparation and efficiency.

Keywords: lipase; immobilization; protein-coated microcrystals; isoamyl acetate.

INTRODUCTION

Flavors and fragrances occupy an important market share in the food, cosmetic, chemical, and pharmaceutical industries[1–3]. It is expected that the sales potential of these compounds increases from 13 to 19 billion USD between 2018 and 2026[4–6]. The providing of flavors and fragrances are mainly extraction of these compounds from higher plants and/or their synthesis using chemical methods[7–10]. However, flavor compounds may exist in bound form in higher plants and the obtention of an active form of flavor compounds requires the extra purification steps that make the process economically expensive[6, 11]. On the other hand, the chemical synthesis of flavor compounds suffers from harsh reaction conditions, high production costs, low product yield, and waste problems[12]. Therefore, the production of flavor compounds using enzymes has received great attention in recent years[6, 13].

Lipases (E.C. 3.1.1.3, triacylglycerol hydrolases) catalyze the hydrolysis of triacylglycerols in water medium. Furthermore, they can catalyze various reaction types including, esterification, transesterification, and ammonolysis in low water-containing media at mild conditions[14]. Of the used biocatalysts, lipases are widely used in the synthesis of flavor compounds due to their selectivity, acceptability in the food industry, and excellent esterification activity in low water-containing media[15–19]. However, the inherent properties of free lipases, such as denaturation in organic media and single use, immobilization of lipases is one of the most used solutions to solve such inherent problems. For this purpose, different kinds of lipases have been immobilized on/in different materials to obtain stable and reusable preparations[20–24]. Protein-coated microcrystals (PCMCs) are regarded as a very simple, cheap, and effective immobilization strategy for the enzymes being used in an organic solvent medium with low water activity[25–27]. PCMCs of an enzyme are obtained by precipitating enzyme molecules over microcrystals of salts (e.g., K_2SO_4), amino acids, or sugars in an organic medium. In this way, the majority of enzyme molecules remain in an active conformation[25].

Isoamyl acetate (banana flavor) is one of the most used food additives in the food industry especially in making fruit jams, fermented alcoholic beverages, and artificial coffees[28, 29]. It is reported that the annual demand for isoamyl acetate is around 90000 kg[30]. The concentration of isoamyl acetate in beverages is reported to be 0.8–6.6 ppm[31]. Ando et al.[32] determined that low-concentration isoamyl acetate shows high antifungal, antibacterial, and antimicrobial activity and inhibits the growth of some microorganisms in Japanese sake. In this study, PCMCs of different kinds of lipases, such as *Aspergillus niger* (ANL), *Candida rugosa* (CRL), *Mucor miehei* (MML), *Rhizomucor miehei* (RML), *Thermomyces lanuginosus* (TLL) were prepared. The obtained ANL-PCMCs, CRL-PCMCs, MML-PCMCs, RML-PCMCs, and TLL-PCMCs were tested in the synthesis of isoamyl acetate in hexane medium. The optimum conditions for the synthesis of isoamyl acetate were also investigated.

MATERIALS AND METHODS

Chemicals

Lipases from *Aspergillus niger* (EC 3.1.1.3, powder (fine), ~200 U/g), *Candida antarctica* B (EC 3.1.1.3, recombinant from *Aspergillus oryzae* ~9 U/mg), *Mucor miehei* (EC 3.1.1.3, lyophilized powder, ≥4,000 units/mg solid), *Rhizomucor miehei* (EC 3.1.1.3, ≥20,000 U/g), *Thermomyces lanuginosus* (EC 3.1.1.3, ≥50,000 U/g), potassium sulfate (K_2SO_4), isoamyl alcohol, isoamyl acetate and all other chemicals used in this study were purchased from Sigma-Aldrich Chemie GmbH (Germany) and used without further purification.

METHODS

Immobilization of lipases as PCMCs

The immobilization of different varieties of lipases as PCMCs was based on the procedure described by Yildirim et al[26]. Briefly, the lipase solution (1 mL, 5 mg protein/mL in pH 7.0 phosphate buffer) was mixed with 4 mL of saturated K_2SO_4 solution at room temperature.

The obtained mixture was slowly added to 50 mL of pre-chilled acetone. Then, the mixture was stored at 5 °C for 1 h until PCMCs process is completed. After that, the immobilized samples were collected by filtration and washed with pre-chilled acetone. The PCMCs were stored at 5 °C until use.

Scanning electron microscopy analysis

The change in surface morphology of K_2SO_4 before and after lipase immobilization was analyzed by scanning electron microscopy (SEM, using an FEI Quanta 650). Samples were sputtered with a layer of gold to minimise the charging effects. SEM was performed at 15 kV, and images of the samples were taken at different magnifications (5000x–20000x).

Synthesis of isoamyl acetate

100 μL of isoamyl alcohol (0.1 M) and 100 μL vinyl acetate (0.1 M) were mixed in 800 μL of hexane in screw-capped test tubes. The reaction was started by the addition of 10 mg of TLL-PCMCs at 50 °C according to Yildirim et al[20]. After 60 min reaction time, 100 μL of aliquot was withdrawn and analyzed by gas chromatography-mass spectrometry (GC–MS). A blank reaction without TLL-PCMCs was run in parallel under the same conditions. The products were analyzed by GC–MS using the standard of isoamyl alcohol and isoamyl acetate. The column and detector used were TRMS-5 (0.25 mm, 60 x 0.25 mm, %5 phenyl-95% polysiloxane) column and electron impact (EI) detector (70 eV). GC oven was kept at 50 °C for 2 min then increased at 15 °C min^{-1} to 250 °C and kept at 250 °C for 5 min. The temperatures of the ion source, injection port, and interface were 230, 250, and 250 °C, respectively. The calibration graph for isoamyl acetate was plotted via GC-MS response versus isoamyl acetate concentration. The quantitative determination of isoamyl acetate was calculated via this graph. The isoamyl acetate yield (%) was calculated according to the following equation:

$$Isoamyl\ acetate\ yield\ (\%) = \frac{Actual\ yield}{Maximum\ theoretical\ yield} x100 \quad \text{Eq. 1)}$$

Effect of reaction parameters on the yield

To investigate the effect of lipase variety on the yield, the different PCMCs of lipase such as ANL, CAL, MML, RML, and TLL were prepared and used for the isoamyl acetate synthesis. The effect of reaction temperature on the yield was carried out at 40, 50, and 60 °C. The effect of the vinyl acetate alcohol molar ratio on the yield was investigated by performing the reaction for different vinyl acetate alcohol molar ratios, such as 1.0, 3.0, 5,0, and 7.0. To determine the effect of PCMCs amount on the yield, the reaction was performed for the different amounts of PCMCs. The effect of reaction time on the yield was investigated by performing the reaction for different reaction times.

The reuse stability of TLL-PCMCs was tested in a batch reactor. A 30 mg of TLL-PCMCs sample was loaded into a reactor and 800 μL hexane, 100 μL of isoamyl alcohol (0.1 M), and 100 μL vinyl acetate (0.3 M) were added at 50 °C. After 360 min reaction time, 100 μL of aliquots was withdrawn and analyzed by GC–MS. TLL-PCMCs were separated from the reaction mixture by filtration and washed with chilled acetone for the next cycle. These procedures were repeated 5 times.

Statistical analysis

All the experimental results are the average of triplicate measurements. Statistical analysis was achieved by using SigmaPlot software version 12.0 (Systat, USA). The significance of each factor was determined by analysis of variance (ANOVA). P values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The immobilized lipases are widely used biocatalysts in the synthesis of various aroma compounds containing ester bonds and the reaction takes place between an acyl donor and an alcohol compound. In this study, PCMCs of different varieties of lipases were prepared and used for the synthesis of isoamyl acetate from isoamyl alcohol and vinyl acetate in a hexane medium. Figure 1 shows the results of isoamyl acetate yield catalyzed by PCMCs of different lipases at the temperature of 50 °C, the molar ratio of vinyl acetate/isoamyl alcohol of 1.0, PCMCs amount of 10 mg, and reaction time of 60 min. The obtained yields were 4.5, 5.5, 8.4, 10.3, and 22.7% for CAL-PCMCs, ANL-PCMCs, RML-PCMCs, MML--PCMCs, and TLL-PCMCs, respectively. According to these results, further studies were performed by TLL--PCMCs.

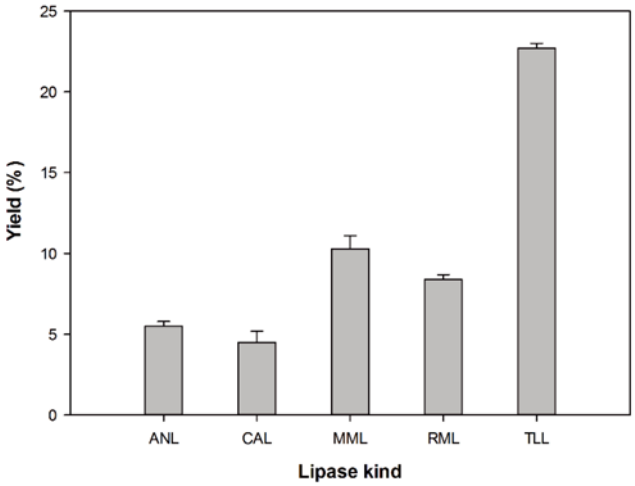

Figure 1. Effect of different lipase PCMCs on the synthesis of isoamyl acetate. The PCMCs amount, vinyl acetate/isoamyl alcohol molar ratio, reaction temperature, and reaction time were 10 mg, 1.0, 50 °C, and 60 min, respectively

The formation of microcrystals and change in surface morphology of K_2SO_4 before and after lipase immobilization were confirmed by SEM. Figure 2 shows the surface morphology of K_2SO_4 before lipase immobilization (Figure 2A) and after lipase immobilization (TLL--PCMCs, Figure 2B). As shown in Figure 2, the surface of bare K_2SO_4 crystals is rough and larger (Figure 2A), whereas the surface of TLL-PCMCs crystals (Figure 2B) was different from bare K_2SO_4 crystals and rectangular shape. As shown in Figure 2B, the lipase molecules were formed as protein aggregates on the surface of K_2SO_4 crystals. Furthermore, it was determined that the bare

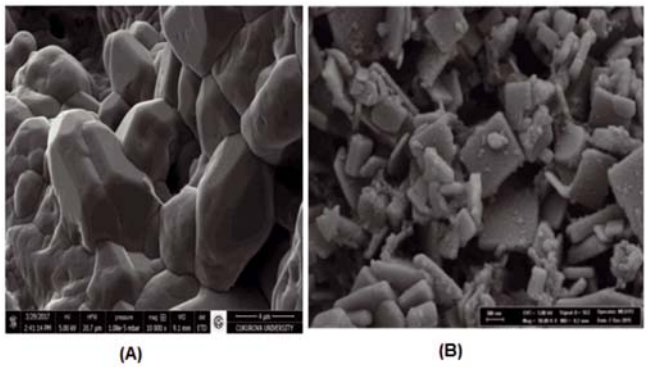

Figure 2. The change in surface morphology of K_2SO_4 before (A) and after lipase immobilization (B)

K_2SO_4 crystals (950 nm length and 505 nm width) were larger than TLL-PCMCs crystals (390 nm length and 150 nm width). Kreiner et al.[25] and Yildirim et al.[26] reported that the growth of K_2SO_4 crystals was started when its surface was coated with protein molecules and therefore smaller sized crystals are obtained.

The effect of temperature on the TLL-PCMCs catalyzed the synthesis of isoamyl acetate yield is demonstrated in Figure 3. It was observed that the product yield increased slightly when the reaction temperature was increased from 40 °C to 50 °C. Further increase in reaction temperature led to a decrease in the isoamyl acetate yield. Enzyme denaturation may occur above 50 °C and therefore activity may be decreased.

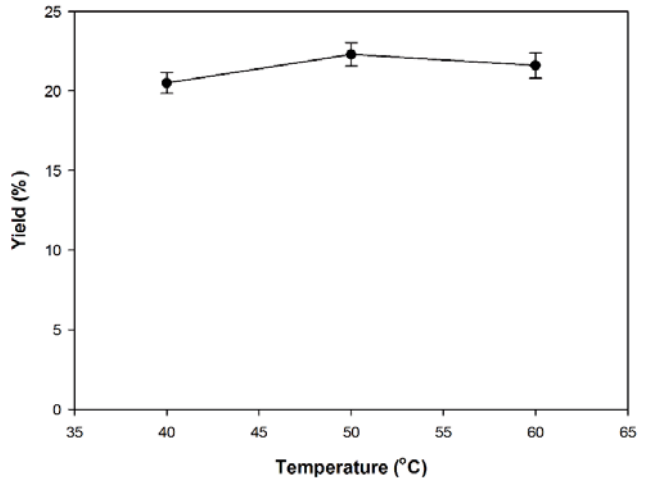

Figure 3. Effect of reaction temperature on the synthesis of isoamyl acetate. The PCMCs amount, vinyl acetate/isoamyl alcohol molar ratio, reaction temperature, and reaction time were 10 mg, 1.0, and 60 min, respectively

Vinyl acetate is a widely used acylating reagent in lipase-catalyzed acylation reactions due to its effectiveness[14]. In this study, TLL-PCMcs catalyzed the acylation of isoamyl alcohol and were investigated for the different vinyl acetate/isoamyl alcohol molar ratios at a constant isoamyl alcohol concentration of 100 mM. As shown in Figure 4, the yield increased when the molar ratio of vinyl acetate/isoamyl alcohol was increased in the range of 1.0–3.0. The yields were obtained as 22.3, 44.5, and 67.7% for vinyl acetate/isoamyl alcohol molar ratios of 1.0, 2.0, and 3.0, respectively. Further increase in vinyl acetate/isoamyl alcohol molar ratio was no effect on the yield. Kanwar et al.[33] determined that the molar ratio of vinyl acetate/isoamyl alcohol was 1.0 in the immobilized

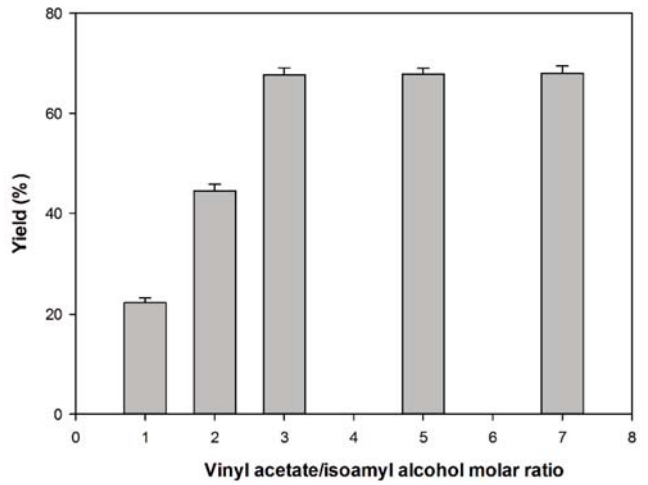

Figure 4. Effect of vinyl acetate/isoamyl alcohol molar ratio on the synthesis of isoamyl acetate. The PCMCs amount, reaction temperature, and reaction time were 10 mg, 50 °C, and 60 min, respectively

Bacillus coagulans lipase-catalyzed synthesis of isoamyl acetate in heptane. Yildirim et al.[34] showed that the initial vinyl acetate/alcohol molar ratio was 1.5 for the acylation of halostachine catalyzed by immobilized *Mucor miehei* lipase. Yadav and Borkar[35] reported that the molar ratio of vinyl acetate/citronellol was 2.0 in Novozym 435 catalyzing the synthesis of citronellyl acetate in toluene.

The enzyme amount is a crucial parameter for increasing the product yield[36]. In this study, the effect of enzyme amount on the yield was studied for the different TLL-PCMcs amounts in the range of 10–40 mg (Figure 5). The yield was 40.9% when 10 mg TLL-PCMcs was used. The increase in TLL-PCMcs amount increased the product yield for 20 mg and 30 mg of TLL-PCMcs amounts and the obtained yield was 76% for 30 mg of TLL-PCMcs amount. The further increase in TLL--PCMcs amount caused a decrease in the product yield. This may be due to the agglomeration of TLL-PCMcs at this amount. Yadav and Devendran[37] reported that the reaction rate increased with an increase in enzyme loading from 5 mg to 10 mg, while the further increase in enzyme loading was not significant effect in the transesterification of cinnamyl alcohol with vinyl acetate catalyzed by Novozym 435.

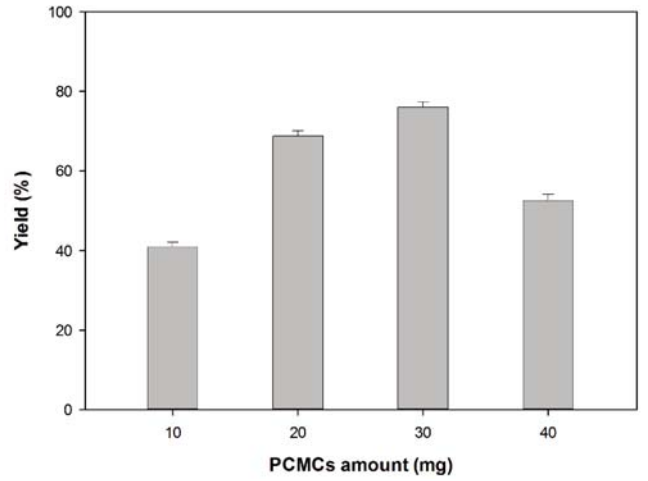

Figure 5. Effect of TLL-PCMCs amount on the synthesis of isoamyl acetate. The vinyl acetate/isoamyl alcohol molar ratio, reaction temperature, and reaction time were 3.0, 50 °C, and 60 min, respectively

TLL-PCMcs catalyzed the synthesis of isoamyl acetate and were investigated for the different reaction times ranging from 15 min to 400 min and the obtained results are given in Figure 6. The isoamyl acetate yield increased for the reaction times in the range of 15–360 min and then remained constant. The maximum isoamyl acetate yield was determined to be 95% after 360 min reaction time. The produced water during the isoamyl acetate synthesis may form a layer and this situation may arise a mass transfer problem. Furthermore, the increase in the amount of water may cause a trigger of hydrolysis reaction[38]. Figure 7 shows the time-dependent GC-MS chromatograms of TLL-PCMcs catalyzed the synthesis of isoamyl acetate. The retention times of hexane, isoamyl alcohol, and isoamyl acetate are 5.34, 6.34, and 8.12 min, respectively. As shown in Figure 7, the peak areas of isoamyl acetate increased when the reaction time was increased.

Table 1 shows the different immobilized lipase-catalyzed syntheses of isoamyl acetate in different media. As given in Table 1, the conversion of isoamyl acetate ranges from 55% to 97%. Hari Krishna et al.[39] used commercial immobilized lipase (Lipozyme IM-20) for the synthesis of isoamyl acetate in n-heptane and reported that the yield of isoamyl acetate was 95% after 72 h reaction time when the substrate concentration, enzyme amount, and reaction temperature were 0.06 M, 3 g l/L and 40 °C, respectively. López-Fernández et al.[40] obtained 55% yield for covalently immobilized *Rhizopus oryzae* lipase catalyzed the synthesis of isoamyl acetate in cyclohexane when the acetic acid/isoamyl alcohol molar ratio and reaction temperature were 1/1 and 30 °C, respectively. de Oliveira et al.[41] showed that a 96% yield was obtained in the synthesis of isoamyl acetate catalyzed by the immobilized *Porcine pancreas* lipase at the initial isoamyl alcohol/acetic acid molar ratio of 1/1, immobilized lipase amount of 0.5 g, the temperature of 40 °C, the reaction time of 4 h. Ghamgui et al.[42] achieved the synthesis of isoamyl acetate with 64% yield after 8 h reaction time when the immobilized lipase amount, acid/alcohol molar ratio, initial added water amount and reaction time were 60 IU, 2.0, 10% (w/w) and 37 °C. Padilha et al.[43] reported that the maximum yield was 92% in the synthesis of isoamyl acetate catalyzed by *Burkholderia cepacia* lipase immobilized in alginate beads. The isoamyl acetate yields were determined as 100, 82 and 80% for the commercial

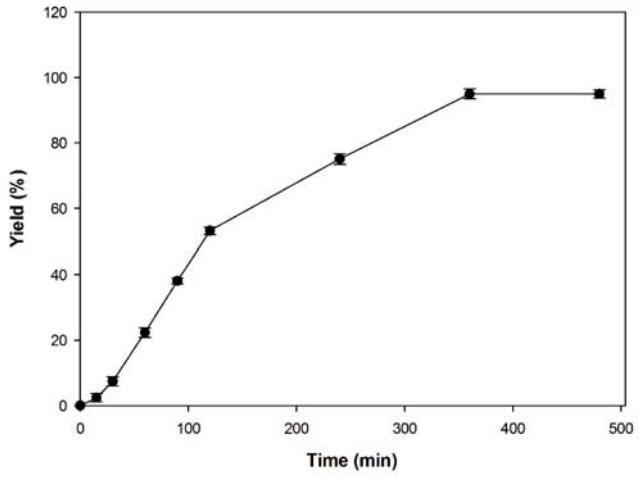

Figure 6. Effect of reaction time on the synthesis of isoamyl acetate. The PCMCs amount, vinyl acetate/isoamyl alcohol molar ratio, and reaction temperature were 30 mg, 3.0, and 50 °C, respectively

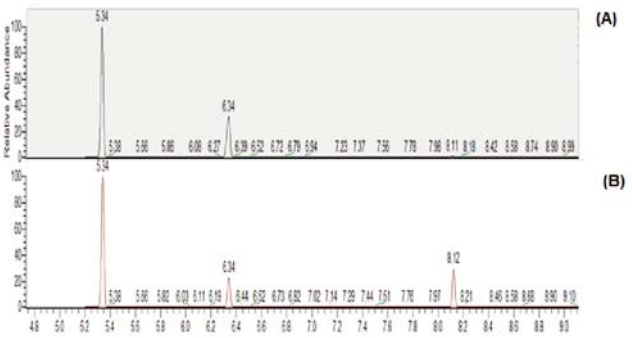

Figure 7. GC-MS chromatograms of TLL-PCMcs catalyzed synthesis of isoamyl acetate at zero time (A) and after 120 min reaction time (B)

Table 1. The different immobilized lipase-catalyzed the synthesis of isoamyl acetate in the literature

Lipase source	Immobilization type	Reaction medium	Conversion (%)	Reference
Rhizomucor miehei	Adsorption on macroporous resin	n-Heptane	95	39
Rhizopus oryzae	Covalent on Purolite	Cyclohexane	55	40
Porcine pancreas type II	Adsorption on activated carbon	n-Hexane	96	41
Staphylococcus simulans	Adsorption on calcium carbonate	Solvent-free	64	42
Burkholderia cepacia	Encapsulation in alginate beads	n-Heptane	92	43
Candida antarctica	Adsorption on macroporous acrylic resin	n-Hexane	100	44
Candida antarctica	Adsorption on macroporous acrylic resin	Solvent-free	82	45
Candida antarctica	Adsorption on macroporous acrylic resin	Glycerol triacetate	80	46
Candida antarctica	Encapsulation in polyurethane	Solvent-free	95	47
Thermomyces lanuginosus	PCMCs on potassium sulfate	n-Hexane	95	This study

immobilized *Candida antarctica* lipase (Novozym 435) catalyzed reactions in hexane[44], solvent-free[45], and glycerol triacetate media[46], respectively. Nyari et al.[47] reported that *Candida antarctica* lipase immobilized in polyurethane catalyzed the synthesis of isoamyl acetate was 95% yield in a solvent-free medium. In our study, the maximum isoamyl acetate yield was obtained as 95% by TLL-PCMcs under the optimized reaction conditions.

TLL-PCMcs was reused in the isoamyl acetate synthesis under the optimized conditions and its residual activity was 90% after five reuses (Figure 8). Krishna et al.[39] used Lipozyme IM-20 for the isoamyl acetate synthesis in n-heptane and reported that Lipozyme IM-20 retained 85% of its initial activity after 10 cycles. Kumari et al.[13] showed that the immobilized *R. oryzae* lipase protected 51% of its initial activity after 10 cycles of isoamyl acetate synthesis in a solvent-free medium.

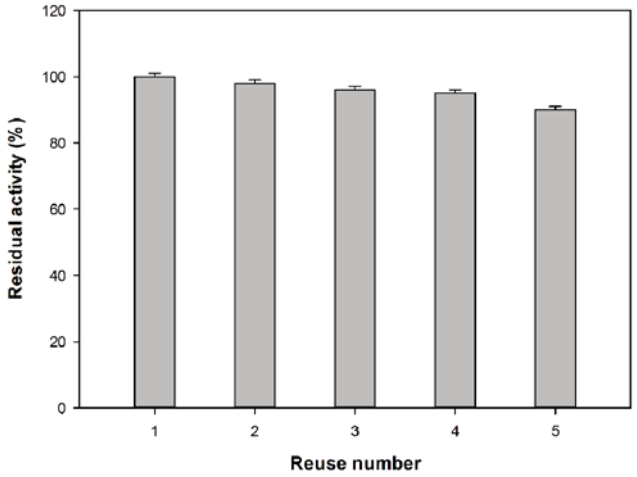

Figure 8. The reuse stability of TLL-PCMCs

CONCLUSION

In this study, PCMCs of different varieties of lipases were prepared and TLL-PCMCs showed the highest activity in the synthesis of isoamyl acetate. The optimum reaction conditions were determined as the temperature of 50 °C, vinyl acetate/isoamyl alcohol molar ratio of 3.0, TLL-PCMcs amount of 30 mg, and reaction time of 360 min. Under these conditions, the maximum isoamyl acetate yield was obtained as 95%. These results show that TLL-PCMcs may be tested for the synthesis of other food aroma compounds, such as ethyl acetate, butyl acetate, hexyl acetate, geranyl acetate, etc.

ACKNOWLEDGMENTS

This work was supported by Research Grant from Scientific Research Unit of Cukurova University with Project number FBA-2015-4932. The author states no conflict of interest. This manuscript does not contain any studies done with human or animal participants performed by the author.

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