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LIPASE - CATALYZED FORMATION OF PENTYL NONANOATE USING SCREENED IMMOBILIZED LIPASE FROM *Rhizomucor meihei*

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Abstract - Bio-catalysis has attracted the special attention of industrial flavour producers in the production of valuable ester compounds. In this study, the synthesis of pentyl nonanoate ester (a short chain ester with fruity aroma) was carried out with a commercial immobilized lipase from *Rhizomucor meihei* Lipozyme (RMIM) as biocatalyst in the esterification reaction between nonanoic acid and pentanol. Various reaction parameters such as enzyme concentration, substrate concentration, reaction temperature and reaction time in solvent-free system were screened to enhance the ester formation with the best yield. A maximum yield for pentyl nonanoate (86.08 %) in a solvent-free system was obtained within 150 min, at a reaction temperature of 45 OC, molar ratio of 1:9 M, amount of enzyme of 0.2 g, water addition of 0.2 % v/v and shaking speed of 150 rpm. This work suggests that pentyl nonanoate ester can be produced in a very high yield and in a short period by lipase-catalysed reactions of nonanoic acid and pentanol, using immobilized lipase from RMIM (lipase from *Rhizomucor miehei* immobilized on anionic exchange support).
Keywords: Pentyl nonanoate; Immobilized enzyme (RMIM); Solvent free system.

INTRODUCTION

Generally esters are obtained either by organic synthesis (i.e., esterification, transesterification or inter-esterification) or by solid liquid extraction from natural resources. For example, lubricants and plasticizers used for high-precision machinery are products of esterification processes of long-chain acids and alcohols. On the other hand, esters produced from the reaction of long chain acids with short chain alcohols are used as additives in food, detergent, cosmetic and pharmaceutical products. Moreover, esters have gained wide interest as solvents, fragrances (aromatic esters), flavours (aliphatic esters) and precursors for several processes in the food, drug and cosmetics industries (Gandhi, 1997; Abbas and Comeau, 2003; Rodrigues and Fernandez-Lafuente, 2010a). Flavour esters, are short-chain esters which belongs to the class of compounds that are widely distributed in nature known as carboxylic acid esters. These types of esters are broadly used in foods, beverages, cosmetics and pharmaceutical industries. The natural aromas in flower and fruits are produced by these flavour esters. Traditionally, these compounds have been isolated from natural sources such as flowers, fruits and vegetables. When these natural flavour esters are extracted from plant materials they are often limited and very expensive for commercial use. To make it more economical and meet consumer's demand, the use of cheaper and more widely available materials are needed to synthesize flavour esters.

Several flavour esters of industrial interest have been obtained through esterification, transesterification or inter-esterification reactions using lipases (Gandhi,

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1997; Alcantaraet al., 1998; Abbas and Comeau, 2003; Silva and Jesus, 2003; Hasan et al., 2006). These esters have been applied in juices, cheeses, baked goods, candies and beverages (Mahapatra et al., 2009). However, the demand for these flavour esters has increased continuously at the rate of 4.3% per year (Dhakeet al., 2013). In order to make it economically viable, the reaction for ester production has to be slowed down and requires the use of specific catalysts. It is well known that traditional processes use inorganic acids (e.g., sulfuric acid) and bases (e.g., sodium hydroxide) as catalysts, and this tend to lead to difficulties in operational conditions (e.g., temperature, pressure and pH) and downstream operation, due to the generation of by-products (Kasche, 1986). Rodrigues and Fernandez-Lafuente, (2010b) and Dhake et al (2013) have presented relevant reviews on the wide applications of lipases for the synthesis of flavours.

The use of enzymes as green catalyst for the esterification reactions will enhance the biomolecules to conduct the reaction under milder conditions and consequently reduce the amount of by-products due to side reactions (Rodrigues and Fernandez-Lafuente, 2010a). Since enzymatic synthesis of esters is through a biological route it has economic advantages which indirectly enhance their market value (Gabelman, 1994; Abbas and Comeau, 2003; Chang et al., 2007; Rodrigues and Fernandez-Lafuente, 2010a).

Lipases (triacylglycerol acyl hydrolases, E.C.3.1.1.3) are widely used in esterification reactions because of their ability to recognize several substrates and catalyse many different reactions (Barros et al., 2010; Rodrigues and Fernandez-Lafuente, 2010a). Among the most frequently used enzymes in organic syntheses is lipase (Davis & Boyer, 2001; Sergeeva et al., 2000). Bio-catalysis involving lipase has been well documented (Castro et al., 2000; Domiinguezs et al., 2002). Lipases are ubiquitous enzymes with different properties. For instance, one of the applications of lipases is the production of flavour esters. A wide range of flavours has been produced with this enzyme. Some of these flavours are farnesyl laurate (Rahman et al., 2010), butyl butyrate (Lorenzoni et al., 2012) and hexyl laurate (Hange et al., 2007). Furthermore, the suitability of lipase for ester synthesis has been evaluated under different conditions such as temperature and pressure (Noel and Combes, 2003), water content (Valivetyet al., 1992) and substrate molar ratio (Somashekaret al., 2007).

Free and immobilized lipases from various sources have been employed for direct esterification and transesterification reactions in organic solvents to produce flavour esters (Brault et al., 2014). Even the organic solvents are able to simplify product recovery, although there are difficulties in many applications because of solvent toxicity. Moreover, some organic solvents employed are too expensive to allow for profitable commercial scale-up. One of the major advantages of a solvent-free system is the absence of solvents which facilitates downstream processing. In addition, a solvent free system saves cost and minimizes environmental impact by eliminating solvents from the production steps. Besides that, there is the possibility of using high substrate concentrations. Few studies have reported lipase-catalysed production of flavour and fragrance esters in a solvent-free system. For instance, the synthesis of terpene esters (Yee et al, 1997) and isoamyl isovalerate are good examples.

R. meihei Lipase (RML) is commercialized in the immobilized form as Lipozyme® RM IM, where the weak anion exchange resin Duolite ES 562, based on phenol-formaldehyde copolymers, is used as support (Rodrigues and Fernandez-Lafuente, 2010a; Rodrigues and Fernandez-Lafuente, 2010b). This kind of support provides the necessary requirements for proper immobilization of the biocatalyst, such as large area for interaction with the biomolecules (Rodrigues and Fernandez-Lafuente, 2010a). To establish its industrial utilization and enhances its features, the immobilization of the enzyme, along with its stabilization, is therefore necessary. This can result in higher activity, selectivity and a decrease in inhibition, and allows for the re-utilization of the biocatalyst (Rodrigues and Fernandez-Lafuente, 2010a; Garcia-Galan et al., 2011). Non-immobilized enzymes are soluble, inhibited by substrates and products and exhibit low stability, it also has low activity for the catalysis of reactions involving non-physiological compounds (Garcia-Galan et al., 2011). Furthermore, RML is stable and remains active even at low water activity (aw). This quality allows the enzyme to be used in organic solvents, an important condition for its use in the direct esterification reaction with free acids (Rodrigues and Fernandez-Lafuente, 2010a).

The intention of the present study was to investigate the effects of various reaction parameters such as enzyme amount, acid-/alcohol molar ratio, reaction time and temperature on pentyl nonanoate esterification with nonanoic acid using immobilized lipases from *R. miehei* (Lipozyme RM IM) in a solvent-free system.

MATERIALS AND METHODS

Lipase from *R. miehei* immobilized on anionic exchange resin (RMIM) was obtained from Novo Nordisk (NOVO Nordisk A/S (Bagsvaerd, Denmark). Lipase, immobilized on Immobead 150 from *R. miehei* (based on covalent bonding), (purity $\geq$ 300 U/g) was obtained from Sigma (Aldrich, USA). Pentyl nonanoate standard (purity 90%) was obtained from BOC Sciences, (New York, USA), Pentanol, American Chemical Society (ACS) reagent (purity, $\geq$ 99%) was

obtained from Sigma (Aldrich, USA). Nonanoic acid (purity ≥ 96%); Flavour grade (FG) was obtained from Sigma (Aldrich, USA). Methanol was of analytical grade and was purchased from Sigma (Aldrich, USA). Acetone was purchased from Sigma (Aldrich, USA). Ethanol (purity, 98%) was obtained from Merck (Darmstadt, Germany). Filter paper, Grade 1 Circles, 90mm was purchased from Sigma (Aldrich, USA), sodium hydroxide was obtained from Sigma (Aldrich, USA). All other chemicals used in this experiment were also obtained from Merck (Darmstadt, Germany) and were of analytical grade. All other reagents used were of analytical grade and used as received. The experiments were carried out in a 100 ml stoppered rubber shake flask, which was incubated in an Incu-Shaker Mini (Benchmark Scientific, New Jersey) at 150 rpm. Temperature was set between 30 °C to 50 °C. Working pressure was at ambient pressure condition.

Synthesis of pentyl nonanoate

Pentyl nonanoate synthesis was performed in screw-capped vials containing 50 mM each of pentanol and nonanoic acid with different ratios (0.1 - 10 %) of additional water. The reaction was initiated by the addition of different amounts (0.1 - 1.0 g) of lipozyme RMIM. Samples were reacted at different reaction times (30 - 1440 min) in a horizontal water bath shaker at rpm (150) and temperatures (25 - 70 °C), along with the controls (samples without lipozyme RMIM). At specific time intervals, samples were withdrawn from the reaction medium and centrifuge at 1500 to remove the immobilized enzyme. The substrates were diluted with hexane (x 10) and analysed using GC.

Identification of reaction product

Detection and identification of reaction products were carried out with a QP-5050A GC-MS (Shimauzu, Kyoto, Japan) equipment with a GC-17A Version 3aru LHW with a DB- 5 column (30 m X 0.32 mm 1.0: lens thickness, 0.25 μm: Scientific, Inc., Ringoes NJ). Helium was employed as the carrier gas at 1.0 ml/min. The temperature was increased at 10 °C per min to 200 °C, and then 250 °C and the final temperature was fixed at 250 °C. The injector and detector temperatures were maintained at 250 °C and 300 °C respectively. Pentyl nonanoate eluted at approximately 17.21 min after injection.

Screening for lipase

The production of pentyl nonanoate using two different lipases (RMIM and immobead 150) was measured by the percentage conversion (%) by determining the remaining unreacted fatty acids in the reaction mixture by titration with 1.0 M NaOH solution to determine the residual concentrations of fatty acids using phenolphthalein indicator and methanol as quenching agent. Control experiments were conducted without Lipozyme under the same condition. The reaction was then terminated by dilution with 7.0 ml of ethanol/acetone (1:1 v/v). All the samples were assayed in triplicate and the experiment was repeated twice.

$$CFE = \frac{Vcontrol - Vsample}{V} \times 100 \quad (1)$$

where: CFE - conversion of flavour ester (%); Vcontrol - volume of NaOH (without enzyme) (control); Vsample - volume of NaOH (with enzyme) (sample); V - Volume of NaOH (without enzyme).

The lipase with the higher conversion yield was selected and used for the subsequent analyses.

Effect of reaction time on esterification activity

The effect of time on the ester synthesis was investigated by varying reaction time (30, 60, 90, 120, 150,360, 480, 720, 960, 1200, 1440 min) using a horizontal water bath shaker with continuous shaking speed of 150 rpm while fixing the other conditions. The percentage conversion of flavour ester was determined as described above.

Effect of reaction temperature on esterification activity

The reaction mixtures were incubated at various reaction temperatures (30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C) using a horizontal water bath shaker with continuous shaking speed of 150 rpm while fixing the other conditions. The percentage conversion of flavour ester was determined as described above.

Effect of amount of enzymes on esterification activity

The reaction mixture was catalysed by varying amounts of enzyme RMIM and Immobead 150 (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 g) using a horizontal water bath shaker with continuous shaking speed of 150 rpm while fixing the other conditions. The percentage conversion of flavour ester was determined as described above.

Effect of molar ratio on esterification activity

The reaction mixtures were reacted with different molar ratio of substrates, mmole pentanol/ mmole nonanoic acid (molar ratio = 1, 2, 3, 4 and 5) using a horizontal water bath shaker with continuous shaking speed of 150 rpm while fixing the other conditions. The percentage conversion of flavour ester was determined as described above.

RESULTS AND DISCUSSION

Screening for the immobilized enzymes

The screening of immobilized enzymes (lipozyme RMIM and immobead 150) for the synthesis of penthyl

nonanoate was carried out in screw-capped vials. A conventional method of varying one parameter-at-a-time was applied to obtain the optimum operating conditions. The effect of various parameters that influenced the reactions such as temperature, time, enzyme amount and molar ratio were studied. Preliminary screening of immobilized enzymes at different reaction conditions showed that lipozyme RMIM gave a higher percentage conversion compared to immobilized lipase, on immobead 150 (Figure 1). An enzymatic reaction with a maximum yield of 88.08 % for pentyl nonanoate was obtained using lipozyme RMIM.

Lipozyme RMIM, which uses acid-regenerated cation resin exchangers as catalysts for effecting esterification, offers distinct advantages over conventional methods. Several types of cation-exchange resins can be used as solid catalysts for esterification (Polichnowski et al, 1986). The low molecular weight acids and alcohols and, in most cases, the resin structure produce little effect on the yield of the esterification. Lipozyme RMIM is a catalyst which contains strongly acidic groups. Its catalytic activity is dependent on the water content of the resin, the rate determining step of the surface reaction of the chemisorbed acid and adsorbed alcohol, respectively. The activity of the covalent bonded enzyme depends on the size and shape of carrier material, nature of the coupling method, composition of the carrier material and specific conditions during coupling.

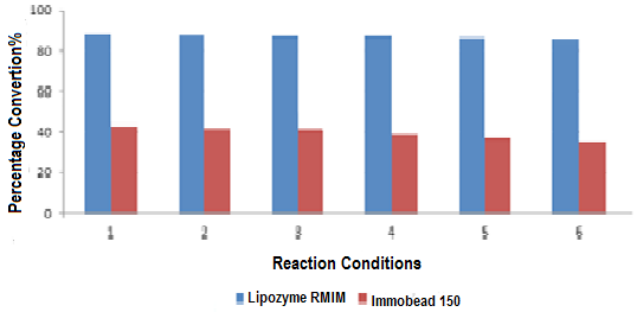

Figure1. Effect of different types of immobilized lipase (lipozyme RMIM and immobead 150) on the synthesis of pentyl nonanoate. 1, 2, 3, 4 and 6 were the different reaction conditions employed as listed below: 1. Reaction time 150 min; temperature 45 o C; amount of enzyme 0.2 g; molar ratio 1:9 M and agitation speed of 150 rpm; 2. Reaction time 120 min; temperature 35 o C; amount of enzyme 0.1 g; molar ratio 1:1 M and agitation speed of 150 rpm; 3. Reaction time 90 min; temperature 40 o C; amount of enzyme 0.3 g; molar ratio 1:5 M and agitation speed of 150 rpm; 4. Reaction time 60 min; temperature 50 o C; amount of enzyme 0.4 g; molar ratio 1:13 M and agitation speed of 150 rpm; 5. Reaction time 480 min; temperature 60 o C; amount of enzyme 0.5 g; molar ratio 1:17 M and agitation speed of 150 rpm; 6. Reaction time 1440 min; temperature 70 o C; amount of enzyme 1.0 g; molar ratio 1:21 M and agitation speed of 150 rpm.

Effect of amount of immobilized enzyme on the esterification reaction

The effect of varying the amount of enzyme on the esterification reaction is shown in Figure 2. Results have shown that the percentage conversion increased from 94.03 % to 96.34 % when the amount of immobilized enzyme was increased from 0.1 g to 0.2 g, respectively. Further increase of the immobilized lipase resulted in a decrease in the conversion. Therefore, the amount of enzyme at 0.1 g to 0.2 g was sufficient to catalyse the esterification reaction. In addition, the excess amount of immobilized lipase did not contribute to the increase in the percentage conversion. Similar results have been reported by Garlapat and Banerjee (2013), on the synthesis of methyl butyrate and octyl acetate through immobilized *Rhizopus oryzae* NRRL 3562 lipase mediated esterification. The probable reason for the observed decrease in the percentage conversion of pentyl nonanoate with increase in amount of lipase might be due to the difficulty in sustaining uniform suspension of the biocatalyst at higher immobilized lipase concentration and the agglomeration of immobilized lipase (Karra-chaabouni et al, 2006).

Since all the substrates are bound to the enzyme, any added enzyme molecule could not find any substrate to serve as a reactant when it reaches the saturation point, thus, when the enzyme concentration is increased more than the saturation point it will cause a decline in reaction due to the steric hindrance produced by excessive enzyme. This shows that substrate was the limiting factor.

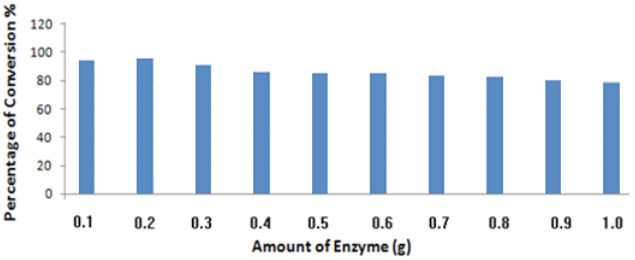

Figure 2. Effect of RMIM enzyme on the synthesis of pentylnonanoate. Reaction conditions: reaction time (150 min), temperature (45 ºC), molar ratio (1:9 M) and agitation speed (150 rpm).

Effect of molar ratio of substrates on the esterification reaction

The effect of pentanol / nonanoic acid percentage concentration on the molar ratio of pentyl nonanoate was investigated in a solvent free system (Figure 3). Results revealed a maximum molar conversion of pentyl nonanoate (85.55 %) at a molar ratio of 1:9.

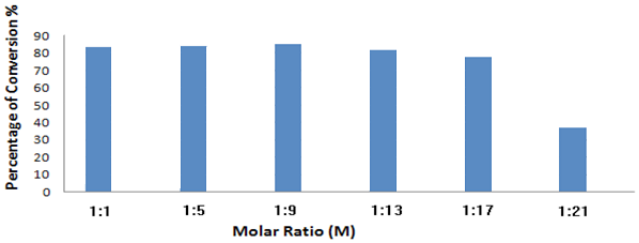

Figure 3. Effect of molar ratio on the synthesis of pentylnonanoate. Reaction conditions: reaction time (150 min), temperature (45 ºC), amount of enzyme (0.2 g) and agitation speed (150 rpm).

Increasing the molar ratio resulted in lower molar conversion of pentyl nonanoate. When the acid level was in excess, the conversion decreased from 85.55 % to 82.10 %. This finding is in agreement with previous studies on the enzymatic synthesis of isoamyl acetate with immobilized *Candida antarctica* (Romeo et al., 2005) and the production of flavour esters catalysed by Lipase B from *C. antarctica* (Souza et al., 2017).

The observed decrease in conversion rate with increasing excess of nonanoic acid implies that the substrate was inhibited by the excess acid. Souza et al. (2017) suggested that the acid and alcohol inhibit lipases through similar mechanisms of competitive inhibition. In addition, the increase in acid concentration increases the proton content in the system, and this subsequently reduces the enzymatic activity through detrimental protonation (Nordblad and Adlercreutz, 2008).

Effect of reaction temperature on the esterification reaction

The effect of temperature on the esterification reaction is shown in Figure 4. Results showed that the percentage conversion of pentyl nonanoate increased with an increase in temperature from 25 ºC to 50 ºC. The conversion remained slightly constant at maximum range of 45 ºC to 55 ºC (80.35 % to 86.08 %) and it subsequently dipped at 60 ºC to 74.34 %. It is worthy of note that Novozyme 435 is known to be stable up to 60 ºC and thus there was no denaturation of the enzyme (Yadav and Trivedi, 2003). To confirm that Novozyme 435 was not deactivated during the conversion of pentyl nonanoate, the immobilized catalyst was filtered off, washed with n-hexane and reused. It was found that esterification activity remained unaffected after the third reuse.

In addition, the observed decrease in conversion after 60 ºC might be due to the vibration and movement of the enzyme molecule, which probably affected the hydrogen bonds and other bonds in the lipase structure, thus resulting in enzyme molecule unfolding and the alteration of the tertiary and quaternary structure of the enzyme (Radzi et al., 2005).

Effect of reaction time on the esterification reaction

The reaction time or time course study is a good indicator of enzyme performance and reaction progress. This gives an insight into the performance of an enzyme as the reaction progresses, it helps to determine the shortest time necessary to obtain good yield and also enhance the cost-effectiveness of the process reaction conditions (Yee et al. 1997).

In the present study, the effect of reaction time on the esterification reaction of nonanoic acid with pentanol is shown in Figure 5. The experiments at different times (between 30 min and 1440 min) were carried out to analyse its influence on the esterification reaction. Generally, the relative percentage conversion (extent of reaction) of pentyl nonanoate increased with increasing reaction time. The highest conversion was obtained for pentyl nonanoate (86.08 %) at an incubation time of 150 min. There was no further increase in conversion with increased incubation time.

Different studies (Dave and Madamwar., 2005, Aragao et al. 2011, Grosso et al. 2012) have reported similar observation with several different immobilized lipases in the production of ethyl butyrate. For example, Aragao et al. (2011) reported a conversion of 88 % to ethyl butyrate in 3 h of reaction, using lipase from *M. meihei*, immobilized on commercial resin beads. After this the percentage conversion remained slightly constant, which might be due to the effect of the reactions attaining equilibrium.

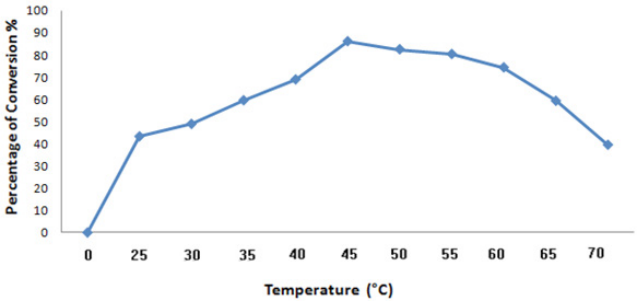

Figure 4. Effect of temperature on the synthesis of pentylnonanoate. Reaction conditions: reaction time (150 min), amount of enzyme (0.2 g), molar ratio (1:9 M) and agitation speed (150 rpm).

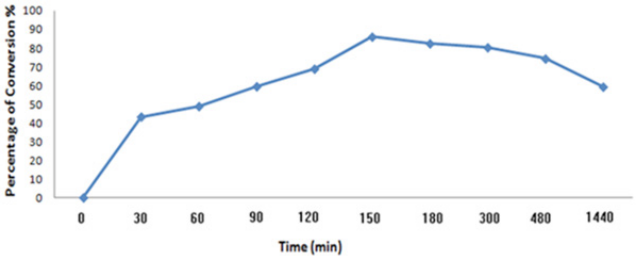

Figure 5. Effect of reaction time on the synthesis of pentylnonanoate. Reaction conditions: temperature (45 ºC), amount of enzyme (0.2 g), molar ratio (1:9 M) and agitation speed (150 rpm).

Effect of water addition on the esterification reaction

Water has been shown to affect the dimensional structural integrity of lipase in many lipase mediated reactions (Paez et al., 2003). In addition, water plays an important role in providing optimal catalytic activity for the enzyme during reaction (Paez et al., 2003). In the present study, addition of water ranging from 0.1 % to 10 % (v/v) to the reaction mixture produced varying responses. For example, addition of 0.2 % water resulted in maximum conversion to pentyl nonoate while addition of higher amounts of water >0.4 % (v/v) resulted in decreased conversion. The probable reason for this observation is that an increase in water level tends to isolate the enzyme molecules from the substrate thus reducing the catalytic activity of the enzyme (Paez et al., 2003).

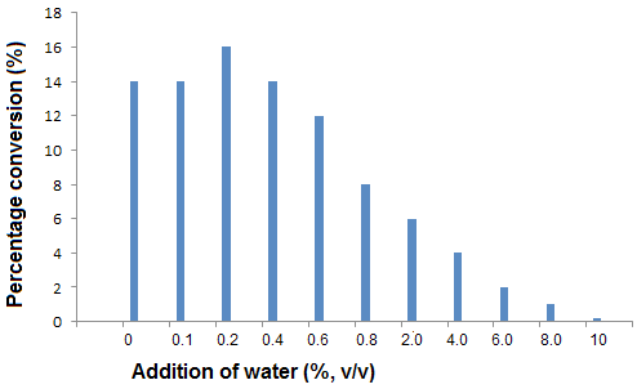

Figure 6. Effect of water addition on percentage conversion of pentylnonanoate. Reaction conditions: reaction time (150 min), temperature (45 ºC), amount of enzyme (0.2 g), molar ratio (1:9 M) and agitation speed (150 rpm).

CONCLUSION

In this study, an initial experiment on the effectiveness of two different immobilised enzymes (lipozyme RMIM and immobead 150) in catalysing pentyl nonanoate synthesis under solvent free conditions was carried out. Results revealed that lipozyme RMIM gave a better percentage conversion (88.08%) of pentyl nonanoate compared to immobead 150 (40.0%). Lipozyme RMIM was subsequently chosen for the synthesis of pentyl nonanoate under solvent-free conditions (i.e., reaction time, reaction temperature, molar ratio of substrates, water addition and amount of enzyme). The maximum yield of pentyl nonanoate (86.08 %) in a solvent-free system was obtained within 150 min, at a reaction temperature of 45 ºC, molar ratio 1:9 M, amount of enzyme of 0.2 g, water addition 0.2 % v/v and shaking speed of 150 rpm. This work suggests that pentyl nonanoate ester can be produced at in very high yield and in a short period by lipase-catalysed reactions of nonanoic acid and pentanol using immobilized lipase from RMIM (lipase from *R. miehei* immobilized on anionic exchange resin). The high percentage conversion is also essential for possible large scale synthesis.

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