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# Lipase mediated synthesis of rutin fatty ester: Study of its process parameters and solvent polarity

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ABSTRACT

Lipophilization of antioxidants is recognized as an effective strategy to enhance solubility and thus effectiveness in lipid based food. In this study, an effort was made to optimize rutin fatty ester synthesis in two different solvent systems to understand the influence of reaction system hydrophobicity on the optimum conditions using immobilised *Candida antartica* lipase. Under unoptimized conditions, 52.14% and 13.02% conversion was achieved in acetone and *tert*-butanol solvent systems, respectively. Among all the process parameters, water activity of the system was found to show highest influence on the conversion in each reaction system. In the presence of molecular sieves, the ester production increased to 62.9% in *tert*-butanol system, unlike acetone system. Under optimal conditions, conversion increased to 60.74% and 65.73% in acetone and *tert*-butanol system, respectively. This study shows, maintaining optimal water activity is crucial in reaction systems having polar solvents compared to more non-polar solvents.


## 1. Introduction

Phenolic compounds are secondary metabolites produced by plants for their growth, reproduction and defence against pathogens. These compounds constitute one of the most important classes of natural antioxidants (Brewer, 2011). These natural phenolic compounds have been gaining increasing importance in food industries as additives, for improving the stability and flavour of foods (Brewer, 2011). They are found to be beneficial additives for foods with high lipid content, as they impart stability through different mechanisms owing to their variable structure (Vaisali, Belur, & Regupathi, 2016). Present dietary trend for n-3 polyunsaturated fatty acid (PUFA) rich oils has increased the commercial demand for stable and nutritionally rich bulk oil systems. Thus, producing nutritionally rich and oxidatively stable n-3 PUFA oil through the addition of efficient natural antioxidant has taken a centre stage.

The effectiveness of an antioxidant depends on their chemical reactivity and their interaction with food components, which in turn depends on their physical location in the food system (Ramadan, 2012). The "polar paradox" theory signifies the importance of physical location of the antioxidant. According to this hypothesis, hydrophilic antioxidants are effective in bulk oil, while lipophillic antioxidants perform well in emulsions (Zhong & Shahidi, 2012). However, this theory has recently faced challenges, as some studies report inconsistent results. This was attributed to the wide variation in the fatty acid and triglyceride content in bulk oil systems (Vaisali, Charanyaa, Belur, & Regupathi, 2015). Further study of bulk oil revealed colloidal structures with many hydrophilic and hydrophobic regions. As oxidation was said to proceed at the interface of such micro- or nano-environments (Shahidi & Zhong, 2011), it is critical to design a more effective antioxidant strategy that could interact with such complex structures.

Since the biggest concern in using natural antioxidants is their solubilisation in lipid systems, the best approach will be to modify the solubility properties of the antioxidant, to interact better at the site of oxidation. This could be achieved by bringing together a fatty acid and the phenolic antioxidant into a single entity (Roby et al., 2015). The resulting phenolic esters were proven to improve solubility properties in oil (Figueroa-Espinoza, Laurre, Villeneuve, & Lecomte, 2013). Though chemical synthesis of phenolic esters is feasible, the non-regioselective nature of acylation and high reaction temperature (Xiao-na et al., 2012), has rendered this chemical method unpopular. Enzymatic production of natural products relevant to various industrial applications has been widely studied in organic media. Hence, application of this process for the synthesis of phenolic lipids becomes an interesting alternate to chemical.

Lipases are diverse group of enzymes that have the ability to reverse hydrolysis reaction to form esters in organic media under

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controlled conditions. Based on the nature of substrate used, two types of reaction can be performed for ester synthesis, viz. esterification and transesterification. Depending on the type of reaction, water, methanol or acetaldehyde are formed as by-products (Chebil, Humeau, Falcimaigne, Engrasser, & Ghoul, 2006). Though water is essential for the enzyme to exhibit its catalytic prowess, water beyond a certain concentration can switch the ester synthesis to hydrolysis reaction (Ma, Persson, & Adlercreutz, 2002). Hence, maintaining optimum concentration of water is critical. If the choice of reaction medium is a hydrophilic solvent, the importance of water becomes more crucial, in view of the intense competition for water prevailing between enzyme molecules and solvent system (Halling, 1984). Significant number of reports on the effect of reaction medium hydrophobicity and the water activity on esterification of several phenolic acids with different acyl donors are available (Humeau, Girardin, Coulon, & Miclo, 1995; Karboune, Safari, Lue, Yeboah, & Kermasha, 2005; Roby et al., 2015; Wang, Zhang, Zhang, Chen, & Zhi, 2013).

More attention has been given recently to flavonoids that are important group of polyphenolic antioxidants found in plants. Though flavonoids are known to have several biological activities, their application is restricted due to their low solubility. Hence, for their application in PUFA rich oils, several studies on enzymatic acylation with fatty acids and aromatic acids have been performed (Chebil et al., 2006). The major works on enzymatic acylation of flavonoids has been done with glycosylated flavonoids with meagre reports on aglycon form. The literature on enzymatic synthesis of flavonoidester is discontinuous. While some studies focused on the regioselectivity of acylation (Chebil et al., 2007a; Kontogianni, Skouridou, Sereti, Stamatis, & Kolisis, 2001; Kontogianni, Skouridou, Sereti, Stamatis, & Kolisis, 2003), several others aimed at understanding the nature of substrates, type of lipase origin and operating conditions including water activity of the system on esterification (Ardhaoui et al., 2004; Chebil et al., 2007a; Duan, Du, Yao, Li, & Wu, 2006). However, the technical gap on the high significance of solvent polarity was never addressed. Such a gap was also reported in a review paper by Chebil et al. (2006) and still remain unaddressed. This emphasize the need for a detailed study on the effect of process variables on the conversion of flavonoid to its corresponding ester with reference to solvent polarity used. As there is a large choice of solvent systems available to carry out enzymatic synthesis of antioxidant esters, finding the influence of solvents polarity on the overall synthesis, helps in arriving at a solvent system which is optimal.

Hence, the current work aims at maximizing the synthesis of a flavonoid fatty ester in two closely similar organic solvent systems. The influence of process parameters on the synthesis of ester was studied. Further, attempt has been made to comprehend the influence of each parameter on the solvent polarity of the reaction system and its effect on product formation. This study for the first time, attempts at establishing a relationship between solvent system and its influence on each process parameter.

## 2. Materials and methods

### 2.1. Materials

*Candida antartica* lipase immobilised on acrylic resin with ≥5000 U/g was purchased from Sigma Aldrich, India. Quercetin was purchased from Sigma Aldrich, India and rutin was obtained from CDH chemicals India. Decanoic acid, acetone and *tert*-butanol were purchased from Loba chemie, India and were of analytical grade. Molecular sieves 3 Å were purchased from Sisco Research Laboratories, India. All solvents were purchased from Merck, India and were of HPLC grade. Water employed for HPLC and LC–MS analysis was of MS grade and purchased from Sigma Aldrich, India.

### 2.2. Drying of reaction components

Both substrates were dried using silica gel in desiccators for more than one week. Acetone and *tert*-butanol was dried for 5 days using 150 g/L of molecular sieves 3 Å. Enzyme was used without any drying.

### 2.3. Synthesis of phenolic esters

The synthesis of phenolic esters was carried out similar to Ardhaoui et al. (2004) with slight modifications in 30 mL glass vials with screw cap tubes. Measured quantities of previously dried rutin (50 mM) and decanoic acid (200 mM) were dissolved in known quantities of dried solvents so that the final molar ratio was 1:4 (antioxidant:fatty acid) and the total reaction volume was 5 mL. The catalysis reaction was initiated by the addition of 75 mg of immobilised lipase enzyme to the reaction mixture. The mixture was then kept at constant agitation at 150 rpm and 55 °C for a period of 96 h in orbital shakers. These conditions were maintained throughout the study, unless otherwise specified. All experiments were done in duplicates. Care was taken to avoid solvent evaporation by sealing the vials. The samples were analysed using HPLC and HPLC–ESI-MS. While studying the effect of process parameters, single parameter was altered at a time by keeping the other conditions constant.

### 2.4. Water activity adjustment

The water activity of the system was altered by pre-equilibration of the reactants and enzyme prior to synthesis. The reactants and enzyme were placed in a large closed container consisting of small volume of saturated salt solutions based on the required water activity: KOH ($a_w = 0.07$), $CH_3COOK$ ($a_w = 0.23$), NaCl ($a_w = 0.75$). The reactants were kept in respective containers for a period of three days to reach equilibration.

### 2.5. Analytical methods

The synthesis of esters was monitored using HPLC and further confirmed by HPLC–ESI-MS. HPLC analysis was done using Shimadzhu HPLC unit equipped with online degasser, column heater and a RP-C18 column (250 mm × 3 mm and 5 μm particle size). The injection volume was kept at 20 μL. The separation of the reaction components was performed using the following gradient system: water (A), acetonitrile (B) and methanol (C) at a flow rate of 1 mL/min: 0 min (52.5% A, 30% B and 17.5% C), 5 min (4% A, 56% B and 40% C), 7.5 min (4% A, 56% B and 40% C), 10 min (52.5% A, 30% B and 17.5%C) 15 min (52.5% A, 30% B and 17.5%C). The quantification of the reactants and products were done at 254 nm. The percentage conversion was calculated as the area of ester peak divided by combined area of ester peak and antioxidant peak, multiplied by 100. Following quantification, the product was ascertained by HPLC–ESI-MS detector system (Shimadzhu) with electron spray ionisation (ESI) at both positive and negative mode for identification of fractions. The MS conditions were as follows: interface temperature 350 °C, DL temperature 250 °C, nebulising gas flow 1.5 L/min., heat block temperature 200 °C and drying gas flow 15 L/min. Sampling was averaged over a $m/z$ range of 100–1000 amu.

## 3. Results and discussion

Of the several flavonoids available in nature, rutin was chosen for this particular study as it performed well in imparting oxidative stability to sardine oil (Vaisali et al., 2016). Quercetin also had fared well in imparting oxidative stability. However, in spite of our best efforts, quercetin esters could not be synthesized using *Candida antartica* lipase. Though several literature are available on the successful enzymatic synthesis of quercetin esters (Kumar, Jahan, Mahajan, & Saxena, 2016; Xiao-na et al., 2012), no reports are available on the synthesis of quercetin esters using *Candida antartica* lipase. This strengthens the fact that *Candida antartica* lipase is effective in acylating only glycosylated flavonoids, while lipase from *Pseudomonas cepacea* and Rhizopus *oryzae* were able to acylate aglycon forms (Kumar et al., 2016). Hence, rutin was chosen for further studies on esterification reaction.

The enzymatic synthesis of rutin fatty esters (Fig. 1) was performed similar to several existing methods with slight modifications (Ardhaoui et al., 2004; Kontogianni et al., 2003). It has been reported that the conversion rate of phenolic compounds decreased with increasing chain length of the acyl donor (Katsoura, Polydera, Tsironsis, Tselepis, & Stamatis, 2006; Lue, Guo, Glasius, & Xu, 2010). Also, the free radical scavenging activity of the long chain esters were also found to be low. Hence, a medium chain fatty acid, namely decanoic acid (C10) was chosen for the current study. The reaction was monitored using HPLC, and the typical HPLC chromatograms of the reaction mixture are shown in Fig. 2. The rutin peak was identified using standard rutin. Rutin eluted as dual peaks (Fig. 2), at a retention time of 2.8 min. This is similar to the results obtained by Duan et al. (2006), who claimed that one peak corresponded to rutin while the other was an impurity of the flavonoid in the raw material of rutin. The shift in the retention time of rutin after reaction with decanoic acid was an indication of the product formation (Fig. 2). The retention time of the product was found to be 9.7 min. Rutin fatty ester was relatively hydrophobic than pure rutin, that makes the retention time longer in case of a reverse phase chromatography.

The product was further ascertained by HPLC-ESI-MS (Fig. 3). Based on the electron spray ionisation mass spectroscopy (ESI-MS), the synthesized product was found to be monoacylated rutin ester (Fig. 3).The MS data of the rutin peak indicated a principle signal at $m/z$ 609 and two significant fragments at $m/z$ 301 and $m/z$ 463 (Fig. 3) indicating the presence of a quercetin and quercetin 3-*o*-rhamnoside respectively in rutin structure. The analysis of the product peak gave a strong signal at $m/z$ 763, indicating that rutin was acylated with single decanoic acid molecule, preferably at the hydroxyl group of the second rhamnoside group (Fig. 1) (Mbatia, Kaki, Mattiasson, Mulaa, & Adlercreutz, 2010; Razak & Annuar, 2015).

### 3.1. Effect of solvent

In case of non-aqueous reactions, the nature of solvents used as reaction medium is of paramount importance. The type of solvent determines the medium hydrophobicity and they affect the enzyme catalysis directly or by influencing the solvation of reactants and products (Adlercreutz, 2008). In addition, the influence of the type of organic media depends on the type of substrates used (Chebil et al., 2007b). For biocatalytic synthesis of rutin ester, the primary concern is to find a solvent that will solubilise both polar rutin and non-polar fatty acid without denaturing lipase. Hence, two solvents viz. acetone (log*P* value −0.23) and *tert*-butanol (log*P* value 0.6) were chosen based on the solubility data of flavonoids and fatty acid (Chebil et al., 2007b).Considering the adverse effect of excess water content in non-aqueous reactions, all reaction components except lipase were dried prior to ester synthesis. Water activity of the reaction system was not controlled during the course of reaction, as molecular sieves were not used.

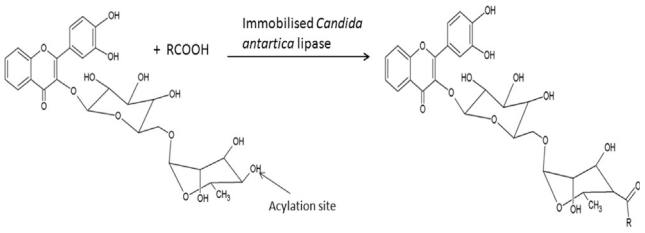


**Fig. 1.** Schematic representation of esterification of rutin with fatty acid.

The reaction rates of rutin ester synthesis varied depending on the solvent, with acetone showing higher reaction rates that increased till 96 h of reaction after which it remained constant (Fig. 4). Additionally, the percentage conversion was 52.14% and 13.02% in acetone and *tert*-butanol solvent respectively after 96 h of reaction (Fig. 4). The main determining factors on solvent influence are the log*P* and dielectric constant. It was noted that there was a negative correlation of solvent polarity with conversion yields which is consistent with the results obtained by Li et al. (2015) when dihydromyricetin was esterified with vinyl acetate using immobilised lipase from *Penicillium* sp. Furthermore, it has been indicated that high dielectric constant is favoured for higher conversion (Chebil et al., 2007a). These observations in literature are consistent with the results obtained in the current study, as the more polar *tert*-butanol with lower dielectric constant gave lower reaction rate and lesser conversion in comparison to acetone.In acetone, the conversion to rutin fatty ester increased during first 96 h of reaction after which it remained constant (Fig. 4). This is similar to the results obtained by Karboune et al. (2005), where the conversion to cinnamic ester increased during the first 3 days of reaction. Nevertheless, the synthesis of rutin ester increased in the first 24 h in *tert*-butanol media (Fig. 4) after which it remained constant with no significant change.

### 3.2. Reaction process parameters

Effect of various process parameters such as enzyme load, molar ratio of substrates, reaction temperature, initial water activity and addition of molecular sieve to the reaction system was studied in both acetone and *tert*-butanol solvent systems. The process parameters were varied one at a time, incubated for 96 h and the percentage conversion was estimated.

#### 3.2.1. Effect of enzyme load

The effect of enzyme load (2.5–20 mg/mL)on the acylation of rutin with decanoic acid was evaluated. With increasing lipase load from 2.5 to 10 mg/mL, enhancement to the extent of 42.36–58.63% conversion in acetone system was noticed(Fig. 5A). This was due to the increase in the active sites available for reactants. However, with further increase in the enzyme load to 15 mg/mL, conversion reduced to 40.9% (Fig. 5A). This could be attributed to the overcrowding of immobilised enzyme beads that result in reduction of effective collision among the molecules (He et al., 2012; Wang, Zhang, Chen, & Zhi, 2015). Thus, the mass transfer rate between substrates and enzymes reduces drastically, yielding poor esterification. Another possible reason could be the increase in by-product water formation with higher concentration of lipase, resulting in the inability of hydrophobic acyl donor to reach the enzyme active site (Giraldo et al., 2007).The optimal concentration of lipase for acetone was found to be 10 mg/mL. In case of *tert*-butanol system, ester formation remained same with no significant difference from 2.5 to 10 mg/mL unlike acetone system (Fig. 5A). Maximum con-

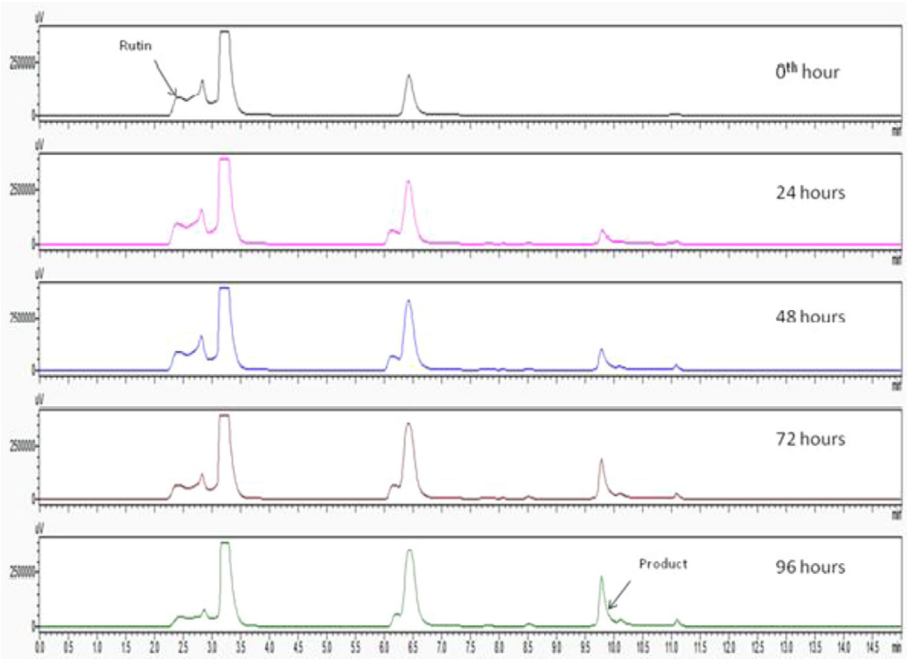


**Fig. 2.** HPLC chromatograms at 254 nm of reaction mixture of lipase catalysed esterification of rutin and decanoic acid over a period of 96 h. Peaks were identified as follows: rutin (2.8 min) and rutin decanoic ester (9.7 min).

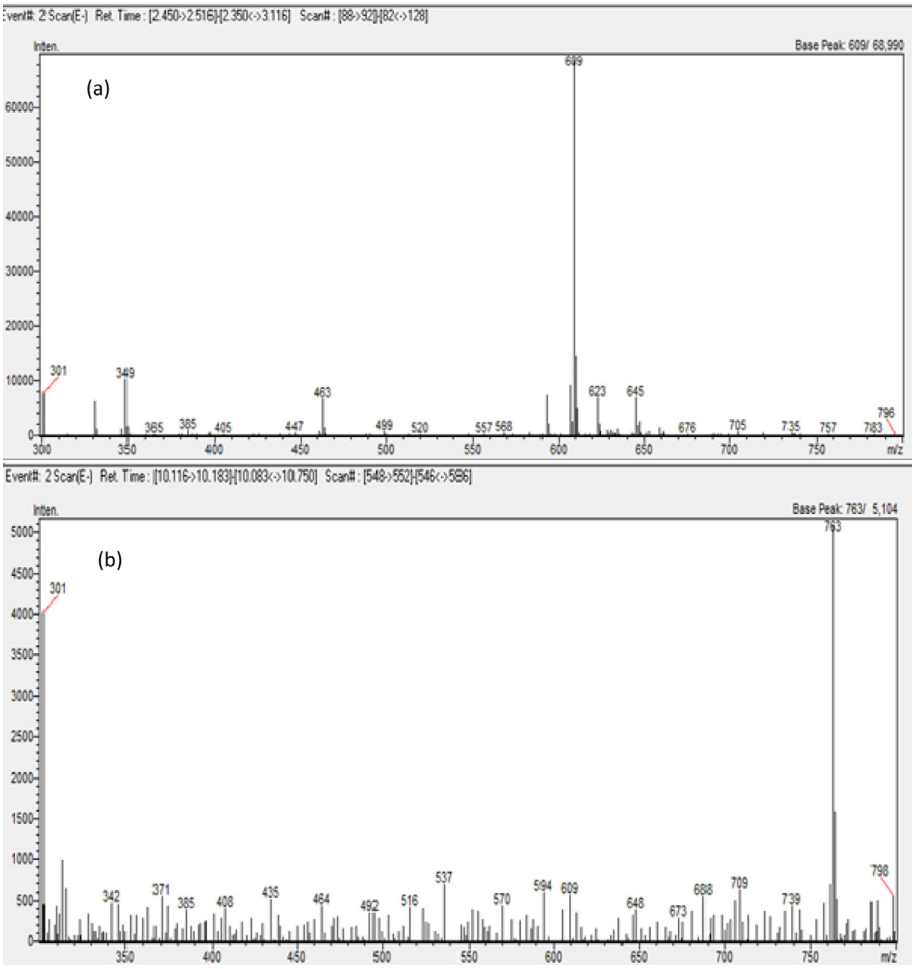


**Fig. 3.** Mass spectra of (a) rutin (b) rutin decanoic ester scanned at the negative mode using HPLC–ESI-MS.

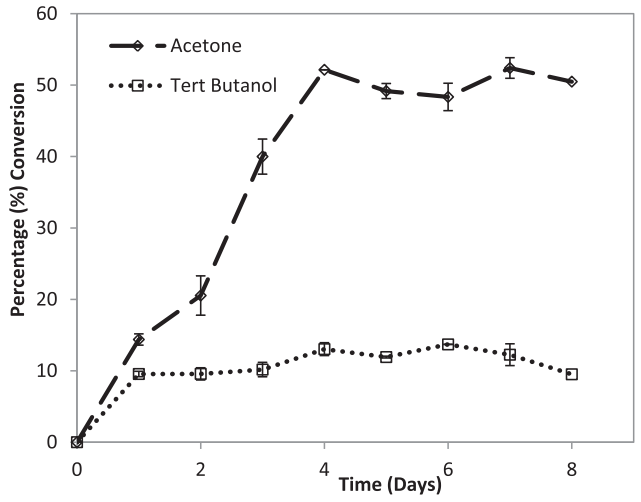


**Fig. 4.** Lipase-catalysed esterification of rutin with decanoic acid over a period of eight days at 55 °C, 150 rpm with 15 mg/mL enzyme load and 1:4 fatty acid:rutin molar ratio without setting water activity. Bars represent standard deviation (n = 2).

version (28.15%) was found at 15 mg/mL enzyme load (Fig. 5A). This marginal difference in the enzyme load requirement in two different solvent systems could be associated to the water liberated during esterification to act as limiting factor on enzyme catalysis that further depends on the solvent polarity used in the reaction system.

### 3.2.2. Effect of molar ratio

Considering the varying effect of enzyme load in different solvent systems on rutin conversion, the molar ratio of the substrates was verified for each solvent system. Since higher concentrations of acyl donor are generally preferred for esterification in organic media, decanoic acid level was increased by keeping rutin concentration constant. In acetone solvent system, low percentage conversion with increase in molar ratio up to 3 was noted (Fig. 5B), after which the conversion increased to a maximum of 55.5% at a molar ratio of 5. However further increase in molar ratio resulted in the dip in conversion to 46.4% (Fig. 5B).In *tert*-butanol, increasing the decanoic acid to rutin ratio from 1 to 4 increased the percentage conversion from 5.53% to 34.25% respectively (Fig. 5B). However, further increase in the molar ratio decreased the ester formation to 14.74%.This is consistent with the results obtained by Wang et al. (2013), who noted that increasing the molar ratio increased ethyl cinnamate production till certain point, after which further increase in acyl donor concentration decreased the production significantly. Though Kontogianni et al. (2001) suggested no inhibition by higher concentrations of decanoic acid during esterification with naringin, some recent studies suggest a possibility of substrate inhibition of *Candida antartica* lipase at higher acyl donor concentrations(Zhu et al., 2014). This indicates the need for a more detailed study on the kinetics of *Candida antartica* lipase for each reaction medium.

When the purified ester was subjected to ESI-MS analysis, spectral profile showed the acylation of single decanoic acid with one molecule of rutin. Interestingly, the degree of acylation did not increase even at higher molar ratio of acyl donor to rutin in both solvents. This is in contrast to the results obtained by Chebil et al. (2007a), where increasing the concentration of vinyl acetate increased the synthesis of di- and tri-acetates of quercetin, with *Pseudomonas* sp. Lipase. This demonstrates the regioselective nature of lipase used in this study. The varying molar ratio optimum in acetone and *tert*-butanol was due to the slight difference in polarity between the two solvents used. As a result, the relatively polar *tert*-butanol shows optimum at lower ratio, due to its inability to dissolve higher concentration of non-polar fatty acid.

### 3.2.3. Effect of reaction temperature

The reaction temperature is generally a significant factor that is responsible for the solubilities of the reactants and products, thermal denaturation of enzymes and also the viscosity of the reaction mixture (Chebil et al., 2006). As immobilised enzyme show higher thermal stability and different optimum temperature for esterification reaction than free enzymes, it is necessary to test the effects of varying temperature on the yield of rutin ester. Hence, for analysing the temperature effects on the esterification of rutin with decanoic acid, reactions were carried out in the range of 25–60 °C (Fig. 5C) in acetone and *tert*-butanol.

The ester formation was found to increase linearly with increase in temperature from 25 to 55 °C for both solvents (Fig 5C). This is consistent with the results obtained by Badgujar and Bhanage (2004), who established an optimum of 55 °C for the biotransformation of geraniol with vinyl acetate. This could be attributed to the reduction in density and viscosity of the reaction mixtures at elevated temperatures leading to an increase in the conversion at higher temperatures (Wang et al., 2015). From our data it can be noted that with further increase of temperature beyond 55 °C, the conversion of rutin ester in acetone system reduced significantly (Fig. 5C). This could be due to the thermal inactivation of enzyme at higher temperature as suggested by Li et al. (2015). However, *tert*-butanol system showed the maximum conversion at 60 °C, indicating the thermal stability of the immobilised *Candida antartica* lipase. These results indicate the importance of high temperature in such reactions unlike several others. For instance, the conversion was only 5.43% at a temperature of 35 °C, while the conversion increased to 55.44% with further increase in temperature up to 55 °C in acetone (Fig. 5C).

## 3.3. Effect of water activity

In case of biocatalysis in non-aqueous media, some amount of water is required to maintain enzyme stability, as the water monolayer around enzyme reduces the protein rigidity in enzyme resulting in exposed active sites (Duan et al., 2006). On the other hand, excess water can lead to unfavourable equilibrium shift to hydrolysis reaction (Duan et al., 2006; Humeau et al., 1995; Yang, Dordick, & Garde, 1988). Therefore, it is crucial to study the influence of water content in the medium that is usually quantified as water activity (Ma et al., 2002). The optimum water activity of the system depends on the medium composition. In addition, the water activity of free enzyme tends to differ from that of immobilised enzyme that further depends on the type of immobilisation support. Although many reports are available on the water activity in non-aqueous media, dependence of water activity requirement on polarity of medium used is seldom studied. Hence, it is crucial to examine the molecular events occurring during the interaction between enzyme molecules, its micro-aqueous environment and surrounding organic media. Hence, the enzyme and the reaction components were allowed to equilibrate to specified water activity separately before synthesis reaction.

As the optimum levels of reaction variables varied with the type of solvent, the dependence of product formation over a range of water activity was tested for the two solvents. The effect of three different water activities on the esterification of rutin with decanoic acid in acetone and *tert*-butanol was studied (Fig. 6). At a water activity of 0.07, the conversion in acetone showed a maximum of 53.96%, while *tert*-butanol showed a conversion of 16.08% (Fig. 6). This is in contrast with the results obtained by Kontogianni et al. (2003), who noted a maximum of 50% conversion at $a_w$ less

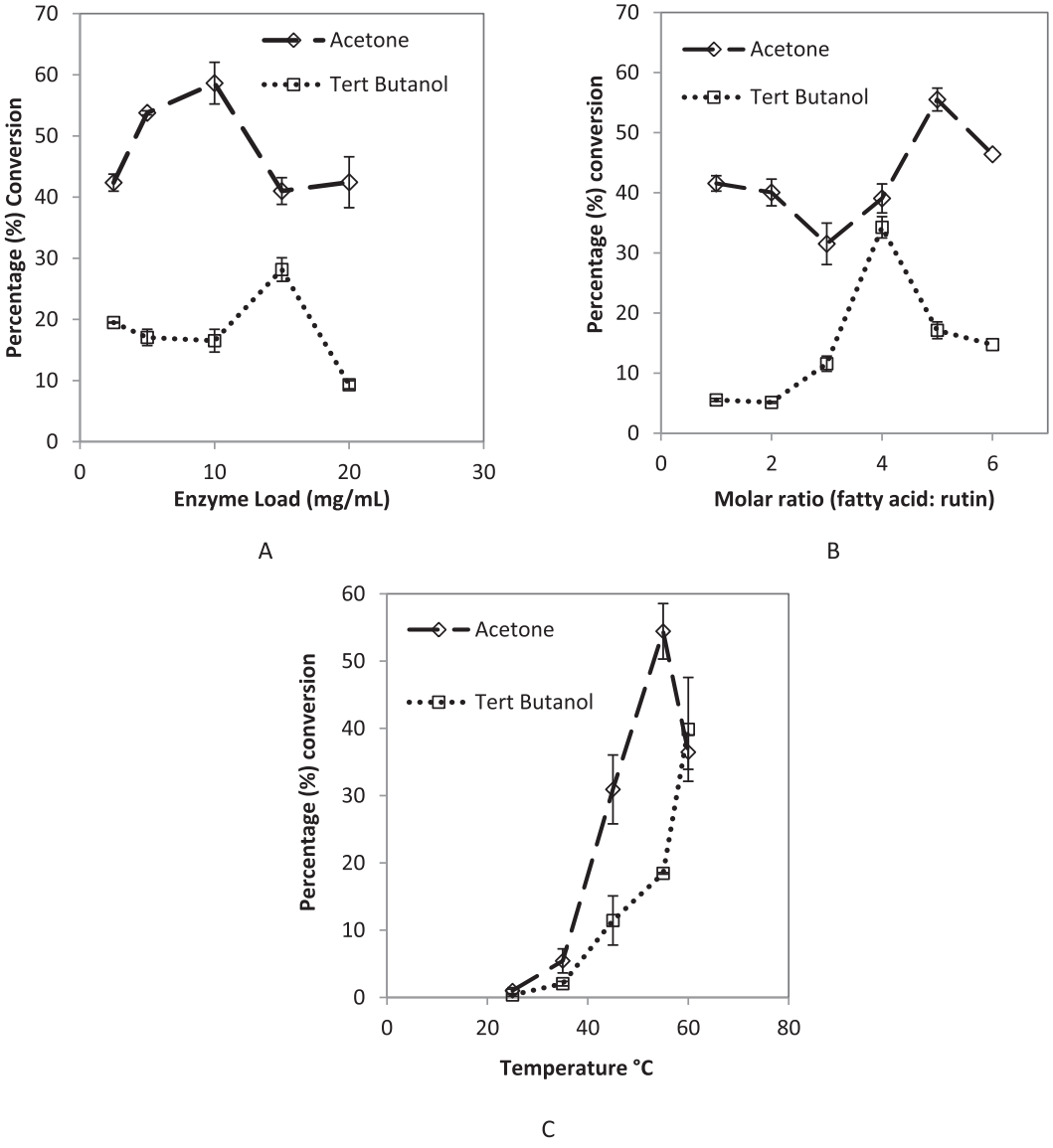


**Fig. 5.** Effect of process parameters on the synthesis of rutin decanoic acid ester (A) Enzyme load (B) Molar ratio (C) Temperature.

than 0.1, when naringin was esterified with decanoic acid in *tert*-butanol using the same immobilised lipase. This indicates the importance of the type of substrate and its influence on the optimum water activity. Thus it becomes crucial to study the influence of each process parameter and water activity that tends to vary depending on the reaction medium components. The conversion was reduced to 11.48% and 5.63% at a water activity of 0.7 for both acetone and *tert*-butanol system respectively. From these results it can be surmised that the $a_w$ of the system prior to setting of water activity was closer to $a_w$ 0.07. The mild difference in the polarity between acetone and *tert*-butanol was the reason for the difference in the optimum water activity for synthesis of rutin ester (Ducret, Trani, & Lortie, 1998). Though both solvents performed well at lower water activity, a thin monolayer of water is necessary to retain the enzyme activity (Sorour, Karboune, Saint-Louis, & Kermasha, 2012). Hence, acetone showed a higher activity at lowest $a_w$ of 0.07 as the stripping of water monolayer by acetone is relatively low. However, due to the polar nature of *tert*-butanol, solubilisation of the bound water monolayer occurs (Zaks & Klibanov, 1988), leading to low reaction rate at a lower activity of 0.07, indicating the need for more water molecules at the enzyme micro environment. This is further confirmed from our results, where *tert*-butanol showed highest conversion at a relatively higher water activity of 0.23. From these results it can be understood that optimum $a_w$ varies with the reaction medium hydrophobicity, which in turn is affected by the type of solvent used. Though setting of water activity prior to synthesis reaction led to marginal increase in the product conversion, the water released during the progress of esterification, changes the water activity of the system ultimately affecting the product formation. Hence, it is crucial to remove the liberated water to improve the synthesis of rutin fatty ester.

### 3.4. Effect of molecular sieves addition

In case of lipase catalysed hydrolysis reactions, reaction generally occurs in two steps. Initial step involves the formation of covalently modified acyl enzyme intermediate. This intermediate is further attacked by water (hydrolysis) or by another nucleophile (esterification) depending on the reaction medium (Ma et al., 2002). Thus, it becomes critical to maintain optimal concentration of water throughout the reaction for maximum ester synthesis.

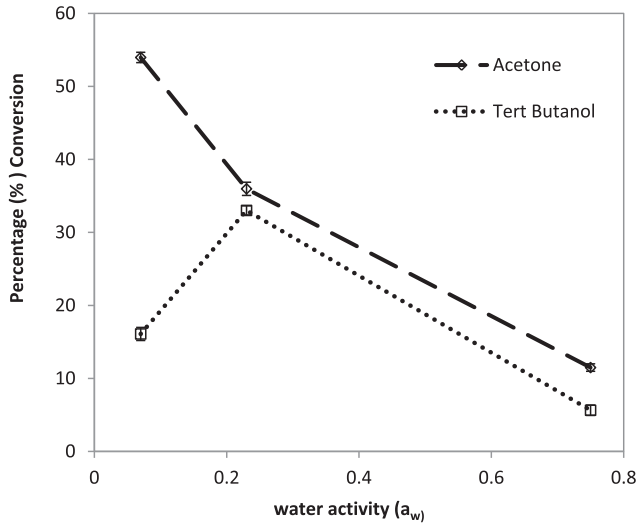


**Fig. 6.** Effect of water activity on the lipase-catalysed esterification of rutin with decanoic acid for 96 h with 15 mg/mL enzyme load and 1:4 rutin: fatty acid molar ratio at 55 °C and 150 rpm. Bars represent standard deviation (n = 2).

However, for every single esterification between rutin and decanoic acid, one molecule of water is released. When sufficient esterification reaction occurs, there is a build-up of the by-product water that might shift the reaction equilibrium towards hydrolysis. They further affect the initial reaction rate leading to the decrease in the overall conversion. Hence, to eliminate this problem of by-product effect on the product formation, addition of molecular sieves was proposed. Molecular sieves are generally made of zeolites and they are used to absorb or separate molecules (Duan et al., 2006). The adsorbed molecules are trapped and the pore size of these trap determine the efficiency. For instance, 3 Å molecular sieves have a critical diameter less than 3 Å and hence water molecules get adsorbed. In the current study, 3 Å molecular sieves were added to the reaction mixture at a concentration of 150 g/L.

The lipase catalysed esterification with and without molecular sieves is represented in Fig. 7. It can be noted that there was a significant increase in conversion (from 14.4% to 62.1%) in *tert*-butanol solvent system in the presence of molecular sieves (Fig. 7), which is consistent with the results obtained by Duan et al. (2006) when rutin was esterified with stearic acid. Such an increase in the conversion could be due to the adsorption of water released during the initial reactions, maintaining the equilibrium towards esterification. Nevertheless, acetone failed to show any increase in conversion in presence of molecular sieves, instead showed a profound decrease in the conversion (Fig. 7). This could be due to the participation of acetone in aldol condensation reaction with molecular sieves leading to self condense (Flego & Perego, 2000). Another possible reason for this decreased conversion in acetone could be due to the loss of lipase activity, when the hydration layer around the enzyme is removed by molecular sieves (Sharma, Dogra, Chauhan, & Kanwar, 2014) resulting in degradation reactions. Also, the by-product water did not seem to affect the reaction rate in case of acetone, as the conversion of rutin ester was consistently high even without molecular sieve addition indicating the importance of solvent polarity in such reactions.

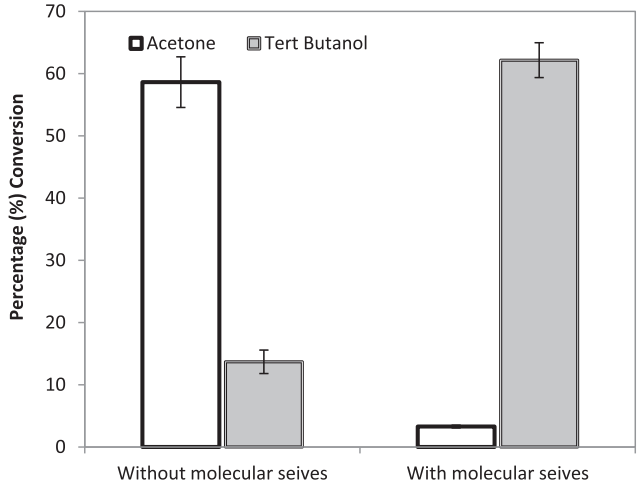


**Fig. 7.** Effect of addition of molecular sieves on the lipase-catalysed esterification of rutin with decanoic acid for 96 h with 15 mg/mL enzyme load and 1:4 rutin: fatty acid molar ratio at 55 °C and 150 rpm. Bars represent standard deviation (n = 2).

Thus, on validation of the optimum process parameters, acetone system gave a conversion of 60.74%, when the reaction system consisted of 10 mg/mL enzyme with 1:4 M ratios of rutin and decanoic acid incubated at 55 °C for 96 h, without setting the water activity and devoid of molecular sieves (Table 1). However, in case of *tert*-butanol prior adjustment of water activity to 0.23, along with molecular sieve addition of 150 mg/mL increased the conversion to 65.73% at 15 mg/mL enzyme load, 1:5 M ratios of rutin and decanoic acid incubated at 60 °C for 96 h. From this it can be inferred that maintaining optimal water activity becomes crucial as the reaction systems becomes less hydrophobic. Hence the removal of released water by using appropriate amount molecular sieves is crucial in those systems.

## 4. Conclusions

From the above results it can be concluded that altering the process parameters can lead to increase in the production of rutin fatty ester. Process parameters like temperature, enzyme load and molar ratio of substrates were found to be affected by the relatively minor difference in polarity of the solvent system. This was attributed to the significant role played by minor concentration of water in enzyme catalysis. This profound effect of water on esterification was more clearly understood by the difference in optimal water activity of two closely polar solvents. Hence, it can be concluded that a more polar solvent like *tert*-butanol require a relatively higher water activity for maximum product formation than acetone. Attempts to increase the conversion by removal of by-product water lead to a twofold increase in the product formation in *tert*-butanol system. However, the generated water failed to have higher effects in acetone, which could be attributed to its relatively less polar nature. Hence, the optimum conditions for acetone involved no manipulation of water activity resulting in

**Table 1**
Optimum conditions for maximum ester synthesis in acetone and *tert*-butanol solvent systems.

| Process parameters | Optimum conditions | |
|---|---|---|
| | Acetone (log*P* −0.23) | *tert*-Butanol (log*P* 0.6) |
| | 60.74% conversion | 65.73% conversion |
| Enzyme load | 10 mg/mL | 15 mg/mL |
| Molar ratio (rutin:fatty acid) | 1:5 | 1:4 |
| Temperature | 55 °C | 60 °C |
| Water activity | 0.07 | 0.23 |
| Molecular sieves | Nil | 150 mg/mL |

60.74% conversion, while *tert*-butanol system gave a maximum of 65.73% only on altering water activity and removal of by-product. Perhaps, in case of reaction systems having lower hydrophobicity (more polar solvents) slightest addition of water leads to switch over of reaction from synthesis to hydrolysis. Thus a precise control of water activity close to its optimal value during the esterification reaction, by incorporating molecular sieves in the reaction system is extremely important while using solvents having higher log *P* values.

## Conflict of interest

The authors have no conflict of interest to declare.

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