Biochemical Engineering Journal 47 (2009) 93–99

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Effect of various additives on enzymatic hydrolysis of castor oil

Virendra K. Rathod, Anirudha B. Pandit*

Chemical Engineering Department, Institute of Chemical Technology, Matunga, Mumbai-40019, India

ARTICLE INFO

Article history:
Received 22 February 2009
Received in revised form 17 June 2009
Accepted 7 July 2009

Keywords:
Enzyme
Castor oil hydrolysis
Stirred reactor
Optimization
Solvent
Glycerol

ABSTRACT

Hydrolysis of castor oil using lipase enzyme is carried out in a batch reactor at room temperature (35–40 °C). In order to reduce the cost of enzyme catalyzed reaction, water in oil emulsion and a 3:1 ratio of oil to water is selected. The concentration of enzyme in the reaction mixture is optimized. The effect of various additives like solvent and salt which can enhance the rate of reaction is studied. It is found that the glycerol has no effect on the hydrolysis of oil. The reusability of the lipase enzyme has also been tested. The yield of enzymatic hydrolysis of castor oil is compared with those of coconut oil and olive oil.

1. Introduction

Ricinoleic acid is usually produced by the saponification followed by acidification. Although the reaction conditions are comparatively mild as compared to the high-pressure and high-temperature hydrolysis for other oils, ricinoleic acid produced by this process of castor oil has a characteristic unacceptable odour and coloration. Also, this process (saponificaton followed by acidification) gives a high quantity of the by-product, Na_2SO_4, which is difficult to dispose off. Hydrolysis of oil using enzyme is an alternative for the production of fatty acid to overcome these drawbacks as it operates at moderate temperatures (around 35–40 °C) and to produce better quality products. However, the major drawback of enzyme catalyzed process is the slow rate of the reaction and the higher cost of the enzyme. As, the enzymatic reactions are too slow and although enzymatic process requires less magnitude of energy rates, it requires this energy over an extended length of time, because of which the overall accumulated cost of the process due to the utilization of large amount of utilities is higher. Because of these drawbacks, enzymatic process is not industrially readily suitable. Recently, due to reduction in the cost of enzyme and possibility of reusing its immobilized form, the importance of enzymatic splitting of fat has a renewed interest for various catalyzed reactions [1]. Usually, lipase catalyzed enzymatic hydrolysis has been carried out in oil in water emulsions, which require rather high quantities of aqueous enzyme solution limiting the industrial scale operation due to very high operating costs and the subsequent loss of the enzyme. It is reported that the enzymatic hydrolysis of castor oil when performed in water in oil (3:1 oil water ratio) type of emulsion gives significantly higher yield as compared to oil in water type of emulsion system [2]. Hence in the present study, 3:1 oil to water ratio was used to study the enzymatic hydrolysis of castor oil using lipase enzyme at room temperature (35–40 °C). Also, it is reported that the various process parameters such as agitation, homogenization, addition of proline, solvent use for viscosity reduction can enhance the percentage hydrolysis of castor oil using immobilized enzyme by 5–6% only [2]. It has been also reported that addition of salt and solvent improves the rate of hydrolysis of oil [3–8]. However, all these studies of enzymatic hydrolysis have been carried out using different oils but not castor oil. Hence, in the present study the effect of all these parameters i.e. salt and solvent on the rate of hydrolysis of castor oil has been studied using free enzyme solutions. Pertaining to interfacial nature of lipase enzyme interfacial concentration effect has been taken under consideration. Comparative study of the rate of hydrolysis of castor oil with olive oil and coconut oil is also carried out. Also, it is reported that the reduction in the hydrolysis of oil using enzyme is due to product inhibition [2,9]. Hence the effect of glycerol concentration on the rate of castor oil hydrolysis was studied. The possibility of recycle of free enzyme has also been explored.

* Corresponding author. Tel.: +91 22 24145616; fax: +91 22 24145614.
E-mail address: abp@udct.org (A.B. Pandit).

1369-703X/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2009.07.008

2. Materials and methods

2.1. Enzymes and chemicals

The substrates, tributyrin and castor oil were purchased from Himedia Laboratories Ltd. (Mumbai, India) and IPCA Chemicals and Cosmetics Ltd. (Mumbai, India), respectively. The enzyme lipase (lipolase) was kindly supplied as a gift sample by Novo Nordisk Ltd. (Bagsvaerd, Denmark). The biological source of the enzyme was a genetically engineered species of *Aspergillus oryzae*. As per the specifications provided by the manufacturers the enzyme promoted the hydrolysis of a wide variety of triglycerides with 1,3-specificity. All the other chemicals used were of reagent grade.

2.2. Experimental methods

2.2.1. Lipase assay with tributyrin as a substrate and protein content

Lipase assay was performed with tributyrin as a substrate. Tributyrin (1 ml) was incubated with the conjugated lipase (1 ml) in the presence of phosphate buffer (pH 7) for 10 min [2]. At the end of the incubation the reaction was terminated by the addition of 20 ml methanol and the contents were titrated against NaOH (2 M, in methanol) using phenolphthalein as an indicator. The blank contained the same constituents as the test except the enzyme.

One unit of lipase activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol of ester bond per minute under assay conditions.

The protein content of the enzyme solution has been analyzed using modified Folin Lowry method [10].

2.2.2. Determination of the fatty acid concentration

The concentration of fatty acid was determined by titrometric method as discussed in earlier work [2].

2.2.3. Enzyme preparation

The enzyme used for the hydrolysis of castor oil was lipolase. The enzyme was dissolved in 0.2 M sodium phosphate buffer solution of pH 7. The enzyme solution was kept overnight to settle the binder added in the solid enzyme. The next day the enzyme solution was centrifuged and the clear enzyme solution was used for the experiments after finding its activity.

2.2.4. Hydrolysis of castor oil

Hydrolysis of castor oil was carried out in 100 ml stoppered conical flask. The required amount of castor oil and free enzyme solution of known concentration were added into the conical flask. The mixture was stirred using magnetic stirrer under ambient condition. After the desired time the reaction mixture was centrifuged at about 8000 rpm for 20 min. The two layers formed after the centrifugation were separated from each other. The aqueous phase contacting the enzyme was separated carefully with the help of 5 ml micropipette to determine the activity of the solution. The non-aqueous phase was used to determine the percentage hydrolysis of oil. In order to find initial fatty acid content of the oil, the initial acid value of the oil was also found out using the same method. In order to check the reproducibility of the experiment, each experiment was performed thrice and average values have been reported. A schematic representation of batch castor oil hydrolysis process is given in Fig. 1. The experimental method used to study the effect of various parameters has been discussed as follows.

2.2.5. Effect of enzyme concentration

The enzyme concentration in the solution plays an important role in the hydrolysis of oil as it influences the rate of hydrolysis reaction. Thus, to find out the optimum enzyme concentration for the hydrolysis reaction and to study the effect of enzyme concentration on the rate of hydrolysis reaction, few batch experiments were carried out using different enzyme concentrations in the reaction mixture keeping all other conditions same.

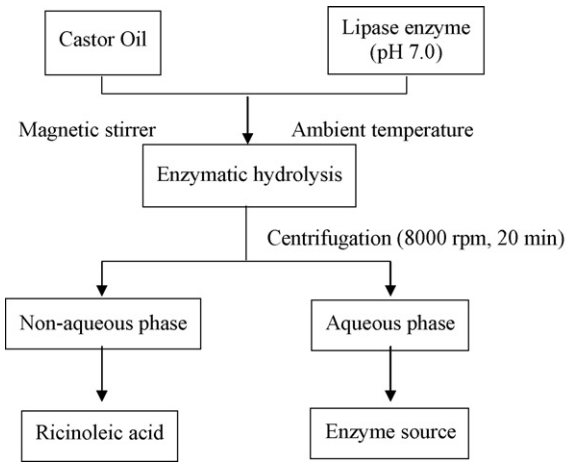

Fig. 1. Schematic representation of batch castor oil hydrolysis process.

15 g of castor oil was taken in a conical flask and 5 ml of enzyme solution of known concentration was added to the castor oil to keep the ratio of oil to enzyme as 3:1. The ratio (3:1) was optimized in earlier work using immobilized enzyme [2]. The mixture was stirred using magnetic stirrer for 1 h. After every 15 min, samples were taken and it was then centrifuged (Remi centrifuge 15,000 × *g*, for 20 min, room temperature) by rotating at 8000 rpm to separate oil and enzyme solution phase. The oil was used to determine the acid value and thus the percentage hydrolysis of the reaction.

The different concentrations of enzyme solution tested were in the range of 0.2–1.2% by weight of enzyme of which details are mentioned in Table 1. All the experiments were carried out at ambient temperature (35–40 °C) for a specific time.

2.2.6. Effect of solvent

Solvents are usually used to solubilize the hardened fats and oil in the hydrolysis reaction especially when carried out at temperature, below the melting point. Solvents help to improve the rate of the hydrolysis reaction by reducing the viscosity of oil and thereby increasing the ease of emulsification and increase in interfacial area promoting the reaction. It has been reported that lipase shows improved activity in the presence of different organic solvents [8] possibly by dissolving lipids in the original enzymes. Solvents such as, short-chain alcohols [11], iso-octane [12], hexane [13] have been successfully used in enzymatic reactions to get high hydrolysis yields. Kulkarni and Pandit [14], have reported the effect of different solvents like iso-octane, n-hexane, n-heptane and diethyl ether using oil in water type of emulsion. In the present study, the effect of these solvents using water in oil type of emulsion has been studied. 3 g of castor oil and 1 ml of 1% free enzyme solution were magnetically stirred along with known amount of

Table 1
Details of enzyme solution.

Sr. No.	Enzyme concentration (%)	Activity (units/ml)	Protein content (mg/ml)
1	0.2	5.3	0.14
2	0.4	7.5	0.21
3	0.6	8.0	0.32
4	0.8	8.7	0.39
5	1.0	10	0.47
6	1.2	10.3	0.54

different solvents ranging from 0.5 ml to 3 ml in a 60 ml sample bottle for 1 h at room temperature. After 1 h the entire reaction mixture was titrated to determine the acid value. Different solvents studied were hexane, iso-octane, acetone and methanol.

2.2.7. Effect of salt

It has been reported that calcium and sodium ions are widely used in the hydrolysis of fats and oils studies and as an important activity enhancer for various enzymes [3–7]. Wang et al. [4] and Fu et al. [5] showed that the yield of hydrolysis reaction increases 1.4 times than that of additive free lipase reaction when lipase from *Aspergillus* sp. was used in the hydrolysis of olive oil. It was also suggested that calcium ions may remove the free fatty acids formed during the hydrolysis reaction as insoluble soap from the interface [15]. Also, it has been reported that the decrease in the rate of hydrolysis is due to product inhibition [2]. Thus, to study the effect of different salts (i.e. KCl, $CaCl_2$ and NaCl) on the hydrolysis reaction, few batch experiments were carried out. 15 g of castor oil and 5 ml of 1% enzyme concentration solution with known amount of salt are magnetically stirred in a conical flask using magnetic stirrer for 1 h at room temperature. After 1 h the reaction mixture was centrifuged and non-aqueous phase was titrated to find the acid value. The molar concentrations of salt studied were 0.006, 0.01, 0.04 and 0.08 in the aqueous phase containing enzyme.

2.2.8. Effect of glycerol concentration

Glycerol is one of the products of the hydrolysis of oil. It has been reported that removal of the glycerol increases the rate of hydrolysis reaction [9]. Thus, in order to study the effect of glycerol concentration in the reaction mixture, various schemes studied were as follows.

2.2.8.1. Scheme I: effect of aqueous phase addition. In order to vary the concentration of glycerol formed during the reaction, two different sets of experiments were performed at ambient temperature (i.e. 35–40 °C).

In first set 15 g of castor oil and 5 ml of enzyme solution were stirred in a conical flask. After each hour, samples were removed to determine the acid value of the oil till 6 h and reaction was continued up to 25 h.

In second set, 15 g of oil, 0.5 g of enzyme and 2 ml of buffer solution were stirred in a conical flask. After each hour, samples were removed to determine the acid value and 1 ml of buffer solution was added every hour till 3 h making the total aqueous phase to 5 ml (same as set I). After 3 h, samples were removed to check the acid value till 6 h (from the start) while remaining procedure is same as described in set I. A schematic representation of aqueous phase addition for hydrolysis of castor oil is given in Fig. 2.

2.2.8.2. Scheme II: effect of glycerol addition. The concentration of glycerol was changed by direct addition of glycerol at the start of the reaction. 15 g of castor oil and 5 ml of 1% enzyme solution were added in a conical flask. In this mixture a known amount of glycerol was also added and the complete reaction mixture was stirred using magnetic stirrer for 1 h . After 1 h the reaction mixture was centrifuged to determine the yield (percentage hydrolysis) of the reaction. The different amounts of the glycerol added at the start of the reaction were 0.5 ml and 1.0 ml. An additional experiment was also performed without adding glycerol with the same quantity of oil and 1% enzyme solution which was used earlier in the presence of glycerol. This was to check the possible deactivation of the enzyme due to glycerol.

2.2.8.3. Scheme III: effect of glycerol removal. Puthli et al. [2] showed that the removal of product fatty acid from the reaction mixture improves the overall rate of castor oil hydrolysis. Thus, hydrolysis of oil being an equilibrium controlled reaction there is also a possibility of improvement in the rate of castor oil hydrolysis by removing the glycerol formed from the reaction mixture. The removal of glycerol formed, from the reaction is easy as compared to the separation of fatty acid. Thus to study the effect of glycerol removal, two experiments were performed.

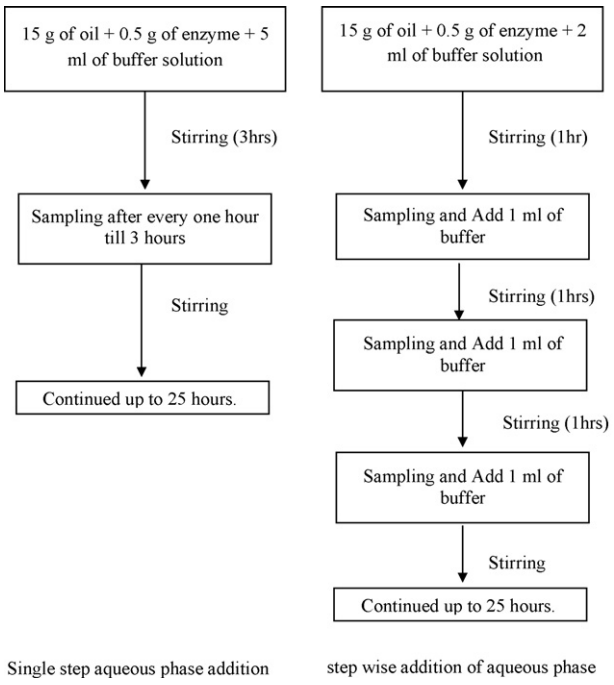

Fig. 2. Schematic representation of aqueous phase addition for hydrolysis of castor oil.

Batch I: 15 g of oil and 5 ml of 1% enzyme solution were added in a conical flask and stirred using magnetic stirrer at ambient temperature. Samples were collected after 1 h to find out the progress of the reaction. The reaction was allowed to continue till 3 h.

Batch II: 15 g of oil and 5 ml of 1% enzyme solution were stirred in a conical flask at ambient temperature. After 1 h the reaction mixture was centrifuged and separated carefully to avoid the traces of the aqueous phase and non-aqueous phase. The extent of the reaction mixture was measured by analyzing the non-aqueous phase. The non-aqueous phase was weighed to find the loss during the centrifugation. The weighed amount of partially hydrolyzed oil was placed in a separate conical flask and the required amount of the freshly prepared 1% enzyme solution was added so that the ratio of non-aqueous to aqueous phase remains the same i.e. 3:1. Again the reaction mixture was stirred for another 1 h and repeat the procedure i.e. centrifuge, separation weighing as discussed earlier was repeated. The partially hydrolyzed oil (i.e. oil hydrolyzed for 2 h) was again separated and further subjected to fresh hydrolysis using freshly prepared 1% enzyme solution maintaining the ratio of non-aqueous to aqueous phase 3:1. The removal of aqueous phase after every hour also removes the glycerol formed and hence in the subsequent hydrolysis the possibly inhibitory effect of glycerol can be eliminated.

2.2.9. Recovery of enzyme

Major drawback of the enzymatic hydrolysis process is the high cost of the enzyme which makes the enzymatic hydrolysis process uneconomical. The cost of the enzymatic process can be reduced by the use of immobilized enzyme as it can be recycled more times [2]. In the present study, the possibility of free enzyme to recycle for the second hydrolysis reaction was explored. Experiment was performed with 30 g of castor oil and 10 ml of 1% enzyme solution

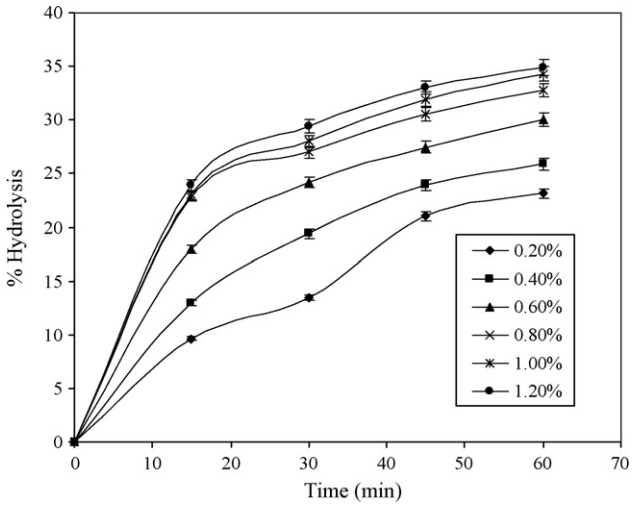

Fig. 3. Effect of enzyme concentration on hydrolysis of castor oil.

(activity 10.3 units/ml) in a stoppered conical flask. The mixture was stirred using magnetic stirrer for 1 h. After 1 h, aqueous phase and non-aqueous phase were separated using centrifuge. The aqueous phase which contains enzyme as well as very small amount of glycerol formed during the hydrolysis reaction is used for the hydrolysis of fresh oil. This process was repeated for four cycles. The amount of enzyme obtained after separation was measured and fresh oil was added to make the oil to enzyme solution ratio of 3:1 as before.

2.2.10. Comparison of different oils

Linfield et al. [3] reported that lipase gives higher rate of hydrolysis reaction for olive oil as compared to that for coconut oil under same experimental conditions. Khor et al. [13] also compared the hydrolysis of soybean, corn, palm and peanut oil and found slower rate of hydrolysis of peanut oil than the other three oils. Kulkarni [16] observed that the rate of hydrolysis of castor oil was approximately twice the rate of hydrolysis of coconut oil and ground nut oil in the initial period of the reaction with lipolase enzyme using oil in water type emulsion system. In the present work, hydrolysis capacity of lipolase enzyme for castor oil, coconut and olive oils was compared using water in oil type emulsion system. Experiments were carried out separately for each oil. 15 g of oil and 5 ml of 1% enzyme solution were taken in a conical flask. The reaction mixture was stirred using magnetic stirrer at ambient temperature. The samples were removed to check the progress of the hydrolysis reaction at an interval of 15 min, 30 min, 60 min, 6 h, 12 h and 24 h. This was done to test the hypothesis that the structural (composition) differences in the different oils affect the hydrolysis rates.

3. Results and discussion

3.1. Effect of enzyme concentration

Fig. 3 shows the effect of enzyme concentration on the rate of reaction. It has been found that with an increase in the concentration of the enzyme in the reaction mixture, the yield of reaction increases. Tsai et al. [17] have also reported that the increase in the rate of hydrolysis and lipase loads was linear in the hydrolysis of olive oil for the enzyme concentration lower than 0.2%. But in the present study the yield of hydrolysis does not increase linearly with the concentration of the enzyme. The percentage hydrolysis obtained for the enzyme concentrations of 0.2%, 0.4%, 0.6% and 0.8% was 25.14, 27.87, 32.60 and 34.78 respectively after 1 h. Beyond 0.8% enzyme concentration the increase in the rate of hydrolysis is marginal and it is almost the same for 1% and 1.2% of the enzyme solution concentration. The hydrolysis obtained after 1 h for 1% enzyme concentration and 1.2% enzyme concentration was 36.3% and 36.9% respectively. It has been found that the rate of hydrolysis increases very slowly up to 1% enzyme concentration after that it remains almost the same. The reason attributed for the difference in trends obtained by Tsai et al. [17] is that the concentration of enzyme used by Tsai et al. [17] was very low as compared to the present study and possibly lies in the linear region of the curve of increasing hydrolysis. As lipase catalyzed reactions take place on the interface, the amount of enzyme at the interface is very important. It is possible that after 1% enzyme concentration the oil–enzyme solution interface generated under current experimental condition gets saturated with enzyme i.e. formation of monolayer at the interface and hence further increase in the enzyme concentration would not show any change in the rate of hydrolysis reaction. Rooney and Weatherly [9] have also reported that the after optimum concentration there was no change in the rate of hydrolysis for sunflower oil under similar experimental condition. Thus, rather than using very high concentration of enzyme in the reaction mixture, optimum concentration of enzyme should be used which will help to reduce the cost of enzymatic process. Thus, in all the further study 1% enzyme concentration has been considered as an optimum concentration.

3.2. Effect of solvent

The effect of various solvents used on the yield of hydrolysis of castor oil is shown in Fig. 4. It is clear from the figure that except iso-octane all other solvent used inhibited the lipase activity over the tested solvent amount. However, it has been found that with an increase in the amount of iso-octane, marginal change in the extent of reaction has occurred. The yield obtained in the presence of hexane shows a marginal change up to 25% solvent content. With an increase in the content of hexane to 50% and 75% of the substrate (oil), the yield of hydrolysis reaction decreases marginally by 2.5% and 5%. On the other hand, methanol and acetone show a drastic reduction in the yield of hydrolysis with an increase in the solvent content. The reduction in the yield is more for methanol as compared to acetone. In the presence of methanol and acetone, the reduction in yield was 78% and 56% respectively for only 25% of solvent content. While it has been found that enzyme losses all its activity when 50% solvent content for both methanol and acetone was used. The results obtained are consistent with those from the reported data [5,18]. Fu et al. [5], reported that iso-octane has no inhibitory action and showed that 90% of hardened oil which

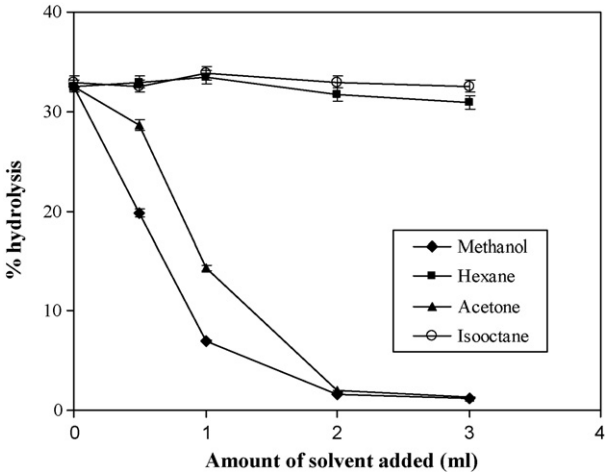

Fig. 4. Effect of solvent on hydrolysis of castor oil.

Table 2
Effect of salt on the hydrolysis of castor oil.

Salt conc. (mol/ml)	% Hydrolysis		
	KCl	$CaCl_2$	NaCl
0	45.63	42.7	42.7
0.006	42.84	41.96	33.71
0.01	28.97	37.54	31.45
0.04	25.5	5.88	33.45
0.08	28.31	2.52	34.5

dissolved in iso-octane was enzymatically hydrolyzed when lipase from *Aspergillus* sp. was used. Kim et al. [18] also have reported that lipase shows higher activity for olive oil hydrolysis using lipase *Candida ruogsa* in the presence of iso-octane. This may be due to the dissolution effect, reducing the effective viscosity of the olive oil resulting into better emulsification. Linfield et al. [3] reported significant reduction in the yield of hydrolysis of tallow by *Candida rugosa* by adding n-hexane. Similarly, it has been reported that iso-octane gives higher yield for hydrolysis of castor oil by lipolase enzyme using water in oil type of system [16]. In the present study, there is no significant effect on the rate of hydrolysis of castor oil with the same enzyme. The reason attributed to the fact the percentage of solvent used in present system was comparatively very high as compared to the earlier studies.

The possible reasons for the different extent of hydrolysis for various solvents with the same reaction system and conditions can be described as follows. As the enzymes are mainly soluble in water and not soluble in oils, the reaction take place mainly at the oil–water interface. Thus, the solubility of oil in solvent is one of the important criteria which affect the yield of the reaction. Amongst the solvent used, iso-octane is the only solvent which is slightly soluble in water while acetone and methanol are highly soluble in water and are incapable of dissolving any oil. The slight solubility of iso-octane in water introduces additional oil interface for enzyme to act. Another possible reason for this behavior is the interaction between the polar groups of the active sites of the enzyme and the organic solvents [5,19]. Methanol being highly polar solvent followed by acetone; deactivates the enzyme very fast. Thus, with an increase in the amount of these solvents yield reduces drastically.

3.3. Effect of salt

Table 2 illustrates the effect of inorganic salts on the castor oil hydrolysis yield. It is clear from the data that almost all the salt used in this work deactivates the enzyme with an increase in the concentration of the salt. However, the yield of the fatty acid product for KCl, $CaCl_2$ and NaCl are different for same salt concentration. It has been observed that the extent of hydrolysis decreases with an increase in the concentration of the salts KCl and NaCl till the concentration of 0.01 mol/ml and 0.006 mol/ml respectively. Beyond these concentrations, the conversion of reaction remains almost the same for KCl and NaCl (Table 2). The yield of hydrolysis obtained with KCl at the concentration of 0.01 mol/ml was reduced by 36% as compared to 22% for NaCl at the concentration of 0.006 mol/ml. However, the inhibitory effect of calcium ion increases with an increase in the concentration from 0.01 mol/ml to 0.08 mol/ml. This result obtained is in contradiction to previous study [4–7]. This can be attributed to the different substrates and different sources of the lipase used in the present study. It has been reported that with the fungal lipase the calcium ions have no effect on hydrolysis [20] while with the yeast lipase calcium ion have an inhibitory effect on the hydrolysis of olive oil when lipase from *C. rugosa* was used [3]. Khor et al. [13] have also showed an inhibitory effect of calcium ion on the hydrolysis of palm oil using *C. rugosa* with increasing the concentration of the calcium ions. It has been observed that

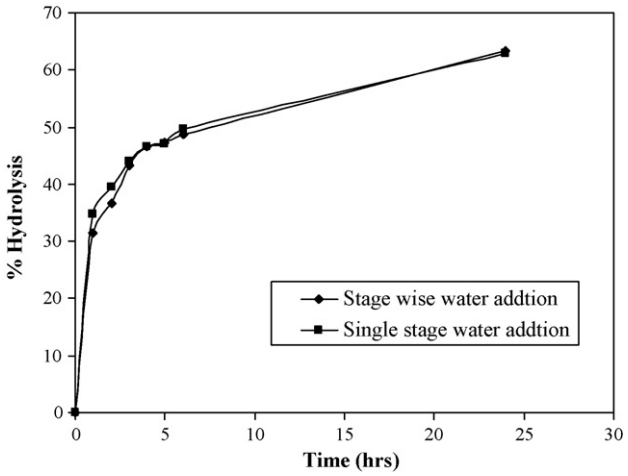

Fig. 5. Effect of water addition on hydrolysis of castor oil.

calcium ion has no effect on the rate of hydrolysis in water in oil type of system, in contrast to its positive effect observed in the oil in water system [4]. They reported that this phenomenon is due to the removal of fatty acid from the interface into the vigorously stirred oil mixture, as the solubility of long chain fatty acid in oil is relatively higher than in [21]. However, in the present study, calcium ion gives an inhibitory effect for the same system (water in oil type). This can again be explained in terms of differences in the substrate and enzyme used in the present study. The fatty acid product i.e. ricinoleic obtained has a high affinity for the enzyme and even after high stirring and solubility in water it will not be removed easily from enzyme oil interface.

3.4. Effect of glycerol concentration

Three different schemes were studied to see the effect of glycerol concentration. The result obtained with all the three schemes is as follows.

3.4.1. Effect of periodic aqueous phase addition

Data in Fig. 5 show the effect of adding water in a step wise manner and in a single step during the course of the reaction. There is no significant change observed in the rate of hydrolysis for single step and step wise addition of aqueous phase which showed that there was no effect of glycerol concentration on the rate of hydrolysis of the castor oil. However, after 1 h the hydrolysis of castor oil for the single step addition was higher (i.e. 36.78%) than step wise buffer addition (33.44%). The reason attributed to the fact that for single step addition the oil to enzyme ratio was 3 while it was 7.5 for step wise addition after 1 h. This also proves that lower the ratio of oil to enzyme solution higher is the extent of hydrolysis.

3.4.2. Effect of glycerol addition

Table 3 shows the effect of glycerol content at the start of the reaction on the yield of hydrolysis after 1 h. It shows that there is almost no change in the conversion of castor oil hydrolysis with an increase in the glycerol content in the reaction mixture from 12.5% to 25% (by weight of aqueous phase) at start of the reaction mixture.

Table 3
Effect of addition of glycerol on % hydrolysis of castor oil.

Amount of glycerol added (ml)	% Hydrolysis
0	34.7
0.5	35.2
1.0	33.9

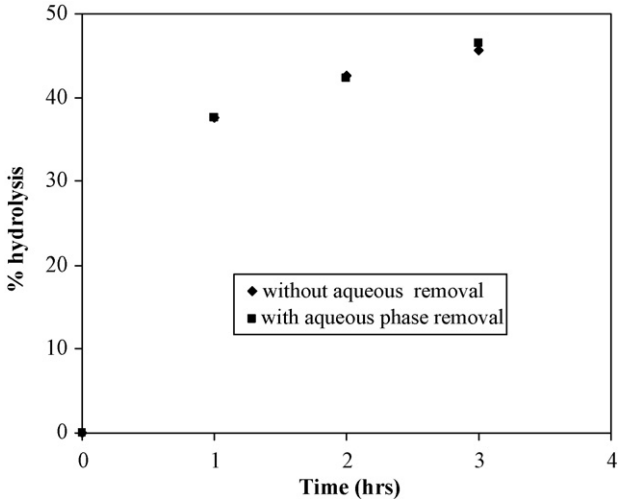

Fig. 6. Effect of glycerol removal on hydrolysis of castor oil.

The concentration of the glycerol added at the start of the reaction was very high as compared to the stoichiometric amount of glycerol formed for the 1 h hydrolysis of castor oil. This clearly shows that the glycerol has no inhibitory effect on the rate of hydrolysis of the reaction mixture for 1 h.

3.4.3. *Effect of glycerol removal*

The effect of glycerol removal on the rate of hydrolysis of castor oil has been depicted in Fig. 6. It was observed that yield obtained after the removal of enzyme solution and using fresh enzyme solution after 1 h is almost the same with the conversion obtained without the removal of enzyme solution i.e. glycerol. This indicates that even after removing the enzyme solution and using fresh enzyme after each hour there is no increase in the rate of hydrolysis of castor oil. This supports the earlier described effect of glycerol concentration i.e. water and glycerol addition and also confirms that there is no inhibition of the rate of the reaction due to the presence of glycerol. However, it has been reported that the removal of glycerol after reaction equilibrium increases the rate of hydrolysis of sunflower oil using *C. rugosa* enzyme [9]. The possible reason for this variation in the results is due to difference in the system used. These results are also comparable with the data reported by [2,16]. Kulkarni [16] has reported that there is no effect of glycerol on the rate of hydrolysis of castor oil for oil in water type of emulsion system.

Thus, from all these three effects it has been confirmed that the reduction in the rate of hydrolysis of castor oil after 1 h is due to the product fatty acid accumulating at the interface displacing the enzyme, preventing the continued action of the enzyme and not the glycerol. Replacing the old enzyme solution by fresh enzyme also does remove the adsorbed fatty acid and the fresh enzyme is still ineffective in forwarding the hydrolysis.

3.5. *Recovery of the enzyme*

The result obtained for the recovery of the enzyme solution has been shown in Table 4. It has been observed that enzyme yield of hydrolysis of castor oil decreases after each cycle. The reduction in the percentage hydrolysis after 1, 2 and 3 cycles was 21.3%, 43.3% and 70% respectively. It is observed that the enzyme losses 30% and 55% activity after first and second reuse, while the reduction in the enzyme activity is almost 80% after third use. The reason attributed for this large reduction in the percentage hydrolysis is due to the loss of enzyme during centrifugation and separation as enzyme remains at the interface.

Table 4
Reusability of the free enzyme.

% Hydrolysis	No. of reuse	Activity (units/ml)
42.50	0	10.3
33.5	2	7.7
24.10	3	5.6
12.75	4	2.3

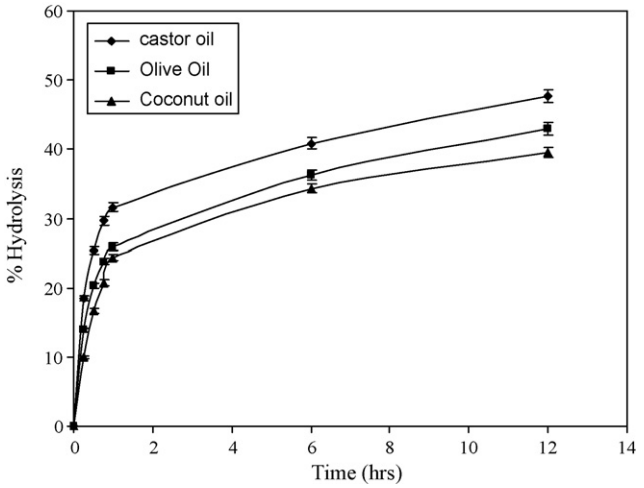

Fig. 7. Comparison of coconut and olive oils with castor oil.

3.6. *Comparison of different oils*

Fig. 7 gives the yield of hydrolysis obtained for different oils using 1% enzyme solution using similar properties. It shows that the castor oil gives higher yield as compared to other oil. After 1 h the yield of hydrolysis of castor was 48% as compared to 43% and 39.5% of olive oil and coconut oil respectively. But for all the oils studied the rate of hydrolysis decreases after 1 h. These results agree well with the previous publications [3,5]. It has been reported that the difference in the yield of hydrolysis for the different oils is due to oil impurities in the olive oil [22] or due to the physical structure of oil [13]. Fu et al. [5] have reported higher rate of hydrolysis for olive oil, followed by soyabean oil, mink fat, lard and coconut oil with the lipase *Aspergillus* species. They have also reported that if more is the unsaturated fatty acid content in the oil, faster will be the rate of hydrolysis of oil. In the present study castor oil is highly unsaturated oil followed by olive oil and coconut oil (Table 5) as a result, castor oil gives higher rate of hydrolysis as compared to the olive oil and coconut oil, confirming the trends proposed by Fu et al. [5].

Table 5
Effect of component of oil and fats on hydrolysis.

	Substrate	Castor oil	Olive oil	Coconut oil
Saturated fatty acid (%)	C12:0	–	–	43.8
	C14:0	–	–	16.2
	C16:0	–	13.8	18.3
	C18:0	2	2.3	5.7
	Total	2	16.1	84
Unsaturated fatty acid (%)	C16:1		–	–
	C18:1	93	64.7	13.5
	C18:2	4	17.7	2.3
	Total	97	82.4	15.8
% Hydrolysis (h)		31.61	25.8	24.33

4. Conclusions

- Rate of hydrolysis of oil increases exponentially up to 1 h after that it increases very gradually.
- Rate of hydrolysis increases with the enzyme concentration and has only marginal effect after an optimum enzyme concentration (i.e. 1%).
- Solvents i.e. iso-octane has no effect on the rate of hydrolysis of castor oil while methanol and acetone deactivate enzyme, reducing the extent of hydrolysis.
- Salt has a negative effect on the rate of hydrolysis using free enzyme solution.
- There is no effect of glycerol on the rate of hydrolysis of castor oil.
- Castor oil gives higher yield as compared to olive and coconut oils possibly due to degree of unsaturation.
- The additives cannot be used to enhance the rate of reaction as it has either no significant or negative effect on the rate of the reaction.

References

[1] I.M. Noor, M. Hasan, K.B. Ramchandran, Effect of operating variables on the hydrolysis rate of palm by lipase, Process Biochemistry 39 (2003) 13–20.
[2] M.S. Puthli, V.K. Rathod, A.B. Pandit, Enzymatic hydrolysis of castor oil: process intensification studies, Biochemical Engineering Journal 31 (1) (2006) 31–41.
[3] W.M. Linfield, D.J. O'Brien, S. Serota, R.A. Barauskas, Lipid–lipase Interactions. 1. Fat splitting with lipase from *Candida rugosa*, Journal of the American Oil Chemists Society 61 (1984) 1067–1071.
[4] Y.T. Wang, J.Y. Sheu, F.F. Wang, J.F. Shaw, Lipase-catalyzed oil hydrolysis in the absence of added emulsifier, Biotechnology and Bioengineering 31 (1988) 628–633.
[5] X. Fu, X. Zhu, K. Gao, J. Duan, Oil and fat hydrolysis with lipase form *Aspergillus* sp., Journal of the American Oil Chemists Society 72 (1995) 527–531.
[6] J. Xia, X. Chen, I.A. Nnanna, Activity and stability of penicillium cyclopium lipase surfactant and detergent solutions, Journal of the American Oil Chemists Society 73 (1996) 115–120.
[7] D. Han, J.S. Rhee, Characteristics of lipase-catalyzed hydrolysis of olive in AOT-isooctane reversed micelles, Biotechnology and Bioengineering XXVIII (1986) 1250–1255.
[8] C. Torres, C. Otero, Influence of the organic solvents on the activity in water and the conformation of *Candida rugosa* lipase: description of a lipase – activating pre-treatment, Enzyme and Microbial Technology 19 (1996) 594–600.
[9] D. Rooney, L.R. Weatherley, The effect of reaction conditions upon lipase catalyzed hydrolysis of high oleate sunflower oil in a stirred liquid–liquid reactor, Process Biochemistry 36 (2001) 947–953.
[10] O.H. Lowry, N.J. Rosebroug, A.L. Parr, Protein measurement with Folin phenol reagent, Journal of Biological Chemistry 193 (1951) 65–75.
[11] S. Chamorro, J.M. Sanchez-Montero, A.R. Alcantara, J.V. Sinisterra, Treatment of *Candida rugosa* lipase with short-chain polar organic solvents enhances its hydrolytic and synthetic activities, Biotechnology Letters 20 (1998) 499–505.
[12] D. Yang, J.S. Rhee, Continuous hydrolysis of olive oil by immobilized lipase in organic solvent, Biotechnology and Bioengineering 40 (1992) 748–752.
[13] H.T. Khor, N.H. Tan, C.L. Chua, Lipase catalyzed hydrolysis of palm oil, Journal of the American Oil Chemists Society 63 (1986) 538–540.
[14] S.R. Kulkarni, A.B. Pandit, Enzymatic hydrolysis of castor: an approach for rate enhancement and enzyme economy, Indian Journal of Biotechnology 4 (2005) 241–245.
[15] K.M. Shkahani, in: G. Reed (Ed.), Lipases and Esterases: Enzyme in Food Processing, Academic Press, New York, 1975, p. 181.
[16] S.R. Kulkarni, Studies in enzyme modification, Ph.D. (Tech) Thesis, Mumbai University, Mumbai, India, 2001.
[17] S.W. Tsai, G.H. Wu, C.L. Chiang, Kinetics of enzymatic hydrolysis of olive oil in biphasic organic aqueous systems, Biotechnology and Bioengineering 38 (1991) 761–766.
[18] K.H. Kim, D.Y. Kwon, J.S. Rhee, Effects of organic solvents on lipase for fat splitting, Lipids 19 (1984) 975–977.
[19] F.X. Malcata, H.R. Reyes, H.S. Garcia, C.G.B. Hill, C.H. Amudaon, Kinetics and mechanisms of reactions catalyzed by immobilized lipases, Enzyme and Microbial Technology 14 (1992) 426–446.
[20] H.W. Knoche, T.L. Horner, Some characteristics of a lipase preparation from the uredospores of *Puccinia graminis tritici*, Plant Physiology 436 (3) (1970) 401–405.
[21] J.S. Patton, B. Stone, C. Papa, R. Abramowits, S.H. Yalkowsky, Solubility of fatty acids and other hydrophobic molecules in liquid trioleoylglycerol, Journal of Lipid Research 25 (1984) 189–197.
[22] W.M. Linfield, R.A. Barauskas, L. Sivieri, S. Serota, R.W. Stevenson, Enzymatic fat hydrolysis and synthesis, Journal of the American Oil Chemists Society 61 (1983) 191–195.