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Monitoring the *Rhizopus oryzae* lipase catalyzed hydrolysis of castor oil by ATR-FTIR spectroscopy

Abid Ali Khaskheli[a], Farah N. Talpur[a,*], Muhammad Aqeel Ashraf[b], Aysun Cebeci[c], Sana Jawaid[a], Hassan Imran Afridi[a]

[a] National Center of Excellence in Analytical Chemistry, University of Sindh, 76080 Jamshoro, Pakistan
[b] Department of Geology University of Malaya, Kuala Lumpur, Malaysia
[c] Department of Food Engineering, Abdullah Gul University, Kayseri, Turkey

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ABSTRACT

A rapid and environmental friendly Attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopic method was developed for monitoring the *Rhizopus oryzae* lipase (ROL) catalyzed hydrolysis of castor oil in oil-in-water emulsion system. A calibration curve was constructed using partial least square (PLS) model by gravimetric addition of oleic acid (10–50%) in castor oil to detect the carbonyl absorption of free fatty acids (FFA) in the region (1690–1730 cm^{-1}). The correlation co-efficient (R^2) and root mean square error of calibration (RMSEC) by PLS model were found to be 0.999 and 0.316, respectively. ROL was found to be an efficient biocatalyst to produce free fatty acids (FFA) from castor oil. Factors affecting the rate of hydrolysis such as enzyme concentration (0.01%, w/v), pH (7), temperature (37 °C), oil–water ratio (1:4) and reaction time (12 h) were optimized. Under all set of conditions the ROL effectively hydrolyzed castor oil up to 90% yield of fatty acids. The methodology is fairly environmental friendly in both cases, i.e. using lipase for hydrolysis of castor oil and analyzing the product through FTIR spectroscopy.

1. Introduction

Hydrolysis of castor oil yields a mixture of fatty acids but the major proportion of the mixture (80–90%) is constituted by ricinoleic acid (cis-12-hydroxy-9-octadecenoic acid), which is an important raw material for many chemical industries. Physically, castor oil is a viscous, pale yellow in colour, clear liquid at room temperature (27 °C) and showed no solid fat content at 0 °C. The oil is high in viscosity which this is unusual for a natural vegetable oil; this behavior is due to hydrogen bonding of its hydroxyl groups. Different derivatives of ricinoleic acid, such as ricinoleyl alcohol, methyl ricinoleate, azelaic acid and ricinoleic acid are used in making plasticizers, emulsifiers, soaps and detergent formulation [1]. Castor oil and ricinoleic have also been used as substrates for the production of conjugated linoleic acid (CLA) a potentially anticarcinogenic, antiobestic, antiatheroscleritic and a number of other therapeutic qualities bearing fatty acid [2].

Industrially castor oil hydrolysis is carried out at high-temperature (250–360 °C) and high-pressure (5000 kPa) for manufacturing fatty acids which is not suitable for castor oil hydrolysis because of the intermolecular esterification of ricinoleic acid, that results in the formation of estolides [3,4]. So an enzymatic approach avoids these limitations by using mild hydrolysis conditions due to lower activation energy with high catalytic efficiency, giving high product purity without altering the fatty acid composition of castor oil [5]. Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface and do not hydrolyze dissolved substrates in the bulk fluid [6]. Microbial lipases like fungal and bacterial lipases have been used for the lipolysis of castor oil, such as lipase *Avena sativa*, *Pseudomonas aeruginosa KKA-5, Aspergillus oryza, Candida rugosa* and *Pseudomonas cepacia* [7]. *C. rugosa* lipase is used industrially for hydrolysis of linseed and castor oil for production of ricinloeic acid and poly unsaturated fatty acids PUFAs [8]. Yet no study has been carried out on the hydrolysis of castor oil by *Rhizopus oryzae* lipase; so one of the aims of this study was to explore the lipolytic ability of *R. oryzae* using castor oil as a substrate for valuable product (ricinoleic acid) formation.

Infrared spectroscopy has been used as a tool for the analysis of lipase catalyzed hydrolysis of different vegetable oils in reverse micelles [9]. ATR-FTIR was applied for monitoring lipase activity

* Corresponding author. Tel.: +92 222 772065; fax: +92 22 9213431.
E-mail address: fnaz_1talpur@yahoo.com (F.N. Talpur).

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on surface attached substrate films [10]. In order to prove the *Jatropha curcas* seed oil hydrolysis, FTIR spectroscopy supported the FFA% by showing the main peaks and their functional groups of the *Jatropha curcas* seed oil [11]. Enzymatic triacylglycerol degradation involves hydrolysis of ester linkages resulting in the release of free fatty acids. Many studies have been carried out using the application of ATR-FTIR spectroscopy for the determination of free fatty acids in vegetable oils [12,13]. The most common method employed for lipase assisted hydrolysis product determination is titration. But the method is laborious, involve toxic substances and chances of error are likely in the determination of end point when used for dark colored oils [14]. Other techniques used for the detection of hydrolytic activity of lipases are chromatography, microscopy, radioactivity and turbidimetry [15]. Each technique has some limitations either involves derivatization of product or laborious to perform daily operations for product monitoring. When comparing FTIR spectroscopy with above prescribed methods, it is fast, non-destructive and sensitive that can be used for routine analysis of lipolytic activity by measuring FFA content of hydrolysis mixture.

2. Materials and methods

2.1. Materials

Castor oil was obtained from local market of Hyderabad Sindh. Lipase *R. oryzae*, tributyrin and oleic acid (99%) were purchased from Sigma (St. Louis, MO, USA). Ethanol (analytical grade) Tween-80 and potassium hydroxide were purchased from Fluka (Germany). All other chemicals used were of analytical grade.

2.2. Lipase activity determination

Tributyrin was used as a substrate for lipase activity determination. A fine emulsion of tributyrin with water was obtained by sonicating the mixture, to which buffered enzyme was added. The mixture was incubated for 10 min and the product was analyzed for the quantity of FFA formed. Activity of lipase was expressed as the amount of lipase required to release one μmole of fatty acids per min under optimized conditions.

2.3. Castor oil lipolysis protocol

One gram of castor oil and 0.1% Tween-80 sonicated with 10 ml phosphate buffer solution (0.1 M, pH 6–8) in a 20 ml test tube for 15 min. After the formation of stable emulsion, 0.5 ml of lipase solution (U, i.e. 1 mg of enzyme) in phosphate buffer (pH 7) was added to the mixture. The hydrolysis reaction was carried in a mechanical shaker (Gallenhamp) by gentle shaking (120 rpm) for different time intervals (4–30 h) at temperature 37 °C. All the experiments were carried out in triplicates.

2.4. Preparation of FTIR calibration standards

A set of 5 calibration standards were prepared in the range 10–50% by gravimetric addition of oleic acid to castor oil containing (FFA 0.2%) previously determined by the standard method [16].

2.5. Instrumentation

A Thermo Nicolet 5700 FTIR spectrometer (Thermo Nicolet Analytical Instruments, Madison, WI) was used to carry out IR analysis, equipped with a pyroelectric deuterated triglycine sulphate (DTGS) detector. An ATR accessory with a removable ZnSe crystal was mounted in the sample compartment. The FTIR controlled by OMNIC software (version 7.2) and analysis was carried out in the range between 4000 and 650 cm^{-1} by addition of 32 scans at a resolution of 4 cm^{-1}. The fresh background spectrum recorded from the bare ATR crystal was subtracted from each standard or sample spectrum. Before taking each spectrum, the ATR crystal was carefully cleaned with propanol to remove any lipo- or hydrophilic residues of the previous sample and the residual solvent was evaporated using a stream of nitrogen gas.

2.6. FTIR analysis of hydrolysis mixture

After completion of hydrolysis reaction, the mixture was centrifuged at 8000 rpm (5152 × *g*) to separate the water and oil phases. 100 μl of castor oil was taken from the upper oil layer that was placed over ATR crystal and the FFA% content were determined from calibration curve obtained using PLS model.

2.7. Chemometric analysis

Partial least square chemometric analysis was carried out using the Turbo Quant (TQ) analyst 7.2 software package from Nicolet (Madison, WI, USA). The spectra of FFA calibration in combination with reference FFA values were used by the software to develop PLS calibration in the range from1730 to 1690 cm^{-1}. To assess the capability of the model to fit the calibration data and to calculate the deviation of the model; root mean square error of calibration (RMSEC) root mean square error of cross-validation (RMSECV) and root mean square error of prediction (RMSEP) were used [14].

3. Results and discussion

3.1. FTIR analysis of lipolytic activity

When a molecular structure is modified in an enzymatic reaction, the infrared spectrum is altered and changes in infrared absorption can be followed to monitor the progress of the reaction [17].

During the course of vegetable oil hydrolysis main changes occur in the IR spectrum at carbonyl C=O region, a decrease in intensity around 1746 cm^{-1} as a result of the decrease in fatty acid ester concentration and increase in intensity around 1711 cm^{-1} due to the formation of free fatty acids is indicative of lipolysis [18]. FTIR spectroscopy can successfully be applied to the investigation of lipase catalyzed hydrolysis of vegetable oils by measuring the amount of liberated fatty acids [9]. In order to prove the castor oil hydrolysis, FTIR spectroscopy supported the FFA% by showing the main peaks and their functional groups of the castor oil; Fig. 1 shows the IR spectra of castor oil before and after lipolysis. Spectra (a) indicates that there is no carbonyl (C=O) FFA band at 1711 cm^{-1} only C=O ester bands 1743 and1162 cm^{-1} are present before hydrolysis. Appearance of stretching C=O FFA band at 1711 cm^{-1}, stretching asymmetric C–O band at 1281 cm^{-1}, while at 1413 and 941 cm^{-1} bending vibration of O–H bands in spectra (a) show the hydrolysis of castor oil and also decrease of intensity of ester bands (1743 and 1162 cm^{-1}) in spectra (b) clearly indicates the lipolytic activity (FFA 90%) of ROL at optimized reaction conditions. Peaks at 2924–2854 cm^{-1} indicated the CH_2 and CH_3 scissoring while the peak at 723 cm^{-1} showed absorption of (C–H) group vibration [11,19,20].

3.2. Quantification of free fatty acids

For the quantification of the reaction, we mainly looked at the spectral changes occurring at the region 1730–1690 cm^{-1}. PLS model was developed to calculate the FFA% [14] liberated during the course of lipolysis of castor oil. Fig. 2 represents the standard

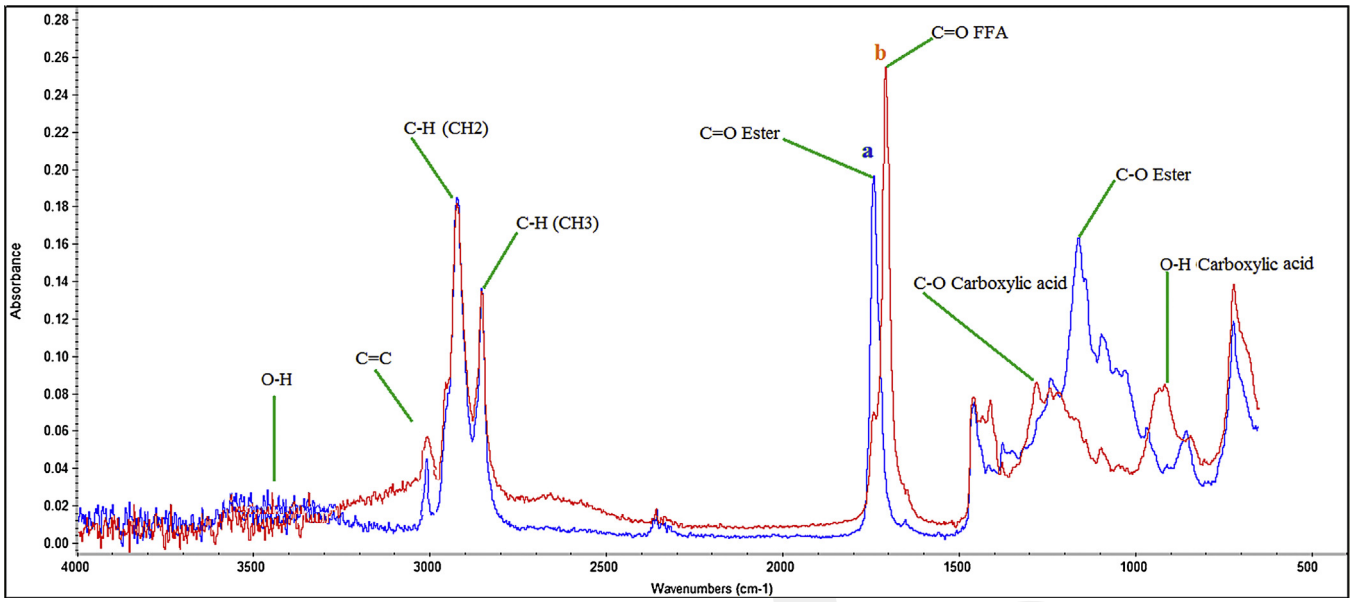

Fig. 1. (a) Castor oil ATR-FTIR spectra before hydrolysis and (b) castor oil ATR-FTIR spectra after hydrolysis at all optimized conditions: oil–water ratio, 1:04; lipase concentration 0.01% (w/v); pH, 7; stirring speed, 120 rpm; incubation time 12 h; temperature 37 °C.

calibration curve prepared in the range of 1730–1690 cm^{-1}. The number of factors (3) used in the PLS model were automatically selected by the TQ analyst software. An excellent correlation coefficient (R^2 0.999) value was obtained and the low RMSECV value (0.316) indicate the robustness and suitability of the method for the determination of lipase activity. Although the lipase-catalyzed hydrolysis of triglycerides is rather complex due to the formation of different reaction products (monoglycerides, fatty acids, and eventually glycerol), the presence of isosbestic point in the spectrum simplifies the quantitative analysis remarkably. For this, one can select the C=O stretching band of the formed free fatty acids at 1711 cm^{-1} because changes here are comparatively large during the reaction and hence standard curves can easily be made with commercially available fatty acids [9]. It is therefore possible to determine the amount of fatty acids released during the hydrolysis reaction.

3.3. Statistical validation of the method

Validation of the calibration model was performed using cross validation to evaluate the general extrapolative performance followed by analysis of a separate validation set. FFA content was initially determined by the standard AOCS titration method [21] followed by the proposed ATR-FTIR method applied to the same

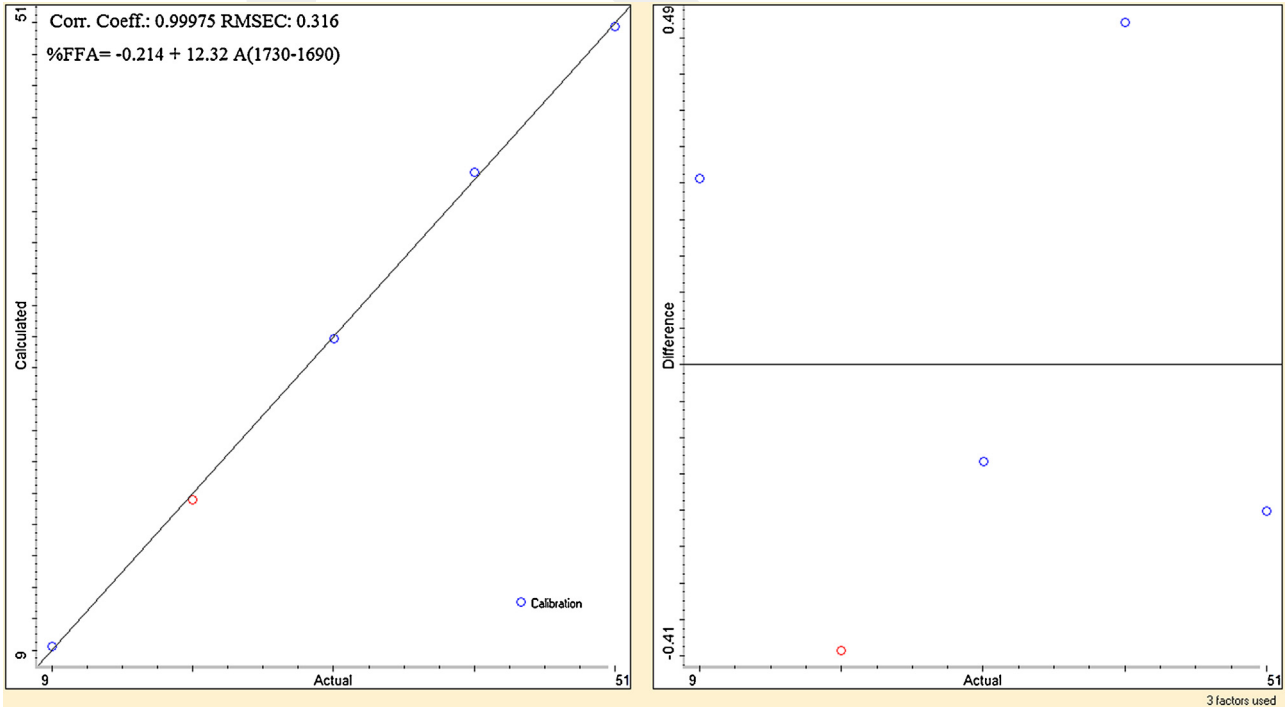

Fig. 2. PLS relationship plot of actual vs. predicted FFA values with their differences.

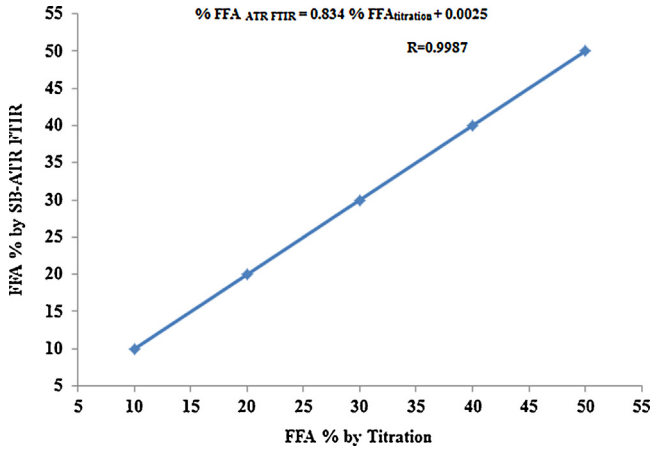

Fig. 3. Calibration plot of predicted FFA obtained by ATR-FTIR analysis vs. titration method.

samples. Fig. 3 graphically illustrates that the relationship between the mean values of the duplicate FTIR and titrimetric analyses.

Obtained from the PLS calibration model is linear along with regression equation. Excellent predictions were achieved in this calibration with a standard error (SE) of 0.019% FFA units and a standard deviation (SD) of 0.156 FFA units. Comparing the data in terms of mean difference (MDr = -0.025_{FTIR}, $-0.042_{titration}$) and standard deviation of difference (SDDr = 0.013_{FTIR}, $0.036_{titration}$) for reproducibility between duplicates of the titration and FTIR results gives comparable mean differences. As such, the overall reproducibility of the FTIR method is better than that of the chemical method [13]. In terms of accuracy, the FTIR results were 0.018% FFA units higher than the titration method with standard deviation of the differences SDDa of 0.014 FFA units.

3.4. Optimization of factors effecting castor oil hydrolysis

3.4.1. Effect of oil–water ratio on lipolysis

Enzymatic hydrolysis of oils takes place at water oil interface because triglycerides are insoluble in aqueous phase [6] thus to achieve better hydrolysis the oil–water ratio was optimized. As shown in Fig. 4a, the FFA% yield is low at high oil–water ratios 1:1and 1:2, it is because the interfacial area between two phases during mixing is low; as lipase acts at interface, the rate of reaction remains low. FFA competes with lipase to occupy the interface and in higher ratios, replace it with interface to a high extent. Consequently, the contact between lipase and castor oil decreases and it results in a decreased rate of hydrolysis [22]. However, the best ratio of oil–water was found to be 1:4 where 89% FFA yield resulted. Water is a direct participant in hydrolysis reaction, so variance of water content in the reaction affects rate of hydrolysis. For further experiments the oil–water ratio was kept 1:4.

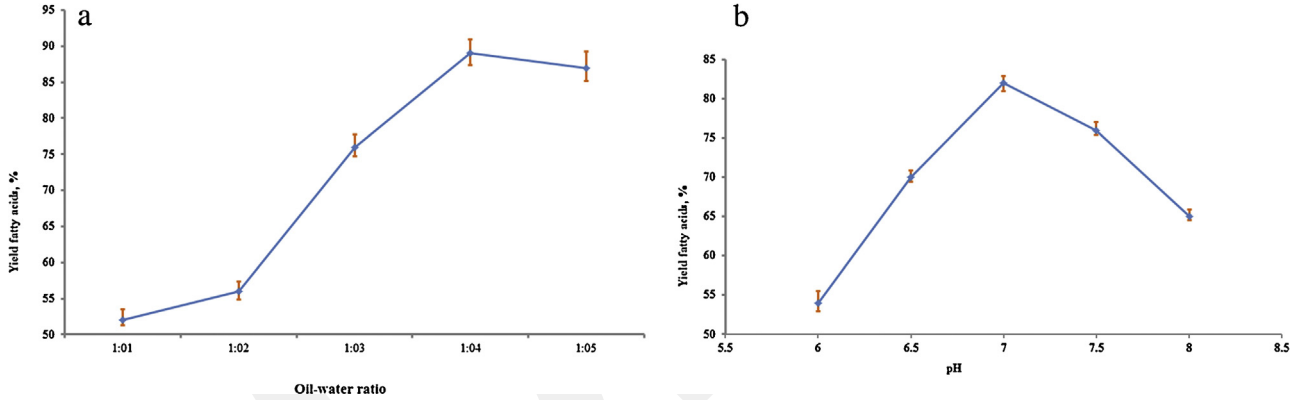

Fig. 4. (a) Effect of oil–water ratio on lipolysis of castor oil (hydrolysis conditions: pH, 6; lipase concentration 0.02% (w/v); stirring speed, 120 rpm; temperature 35 °C; incubation time 15 h). (b) Impact of pH on the rate of castor oil hydrolysis (hydrolysis conditions: oil–water ratio, 1:04; lipase concentration 0.02% (w/v); stirring speed, 120 rpm; temperature 35 °C; incubation time 15 h).

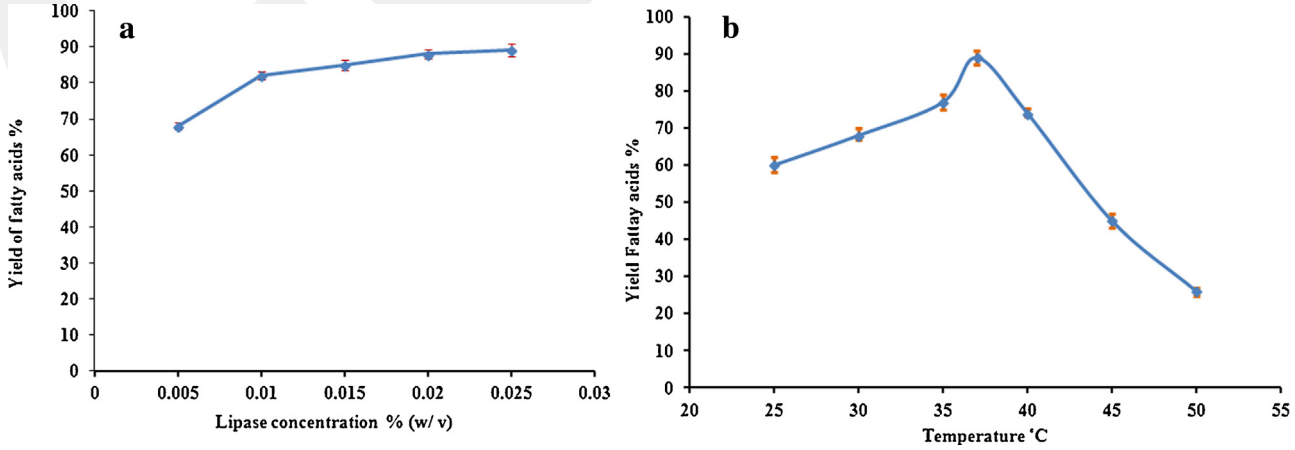

Fig. 5. (a) Effect of lipase concentration on FFA% yield (hydrolysis conditions: oil–water ratio, 1:04; pH, 7; stirring speed, 120 rpm; temperature 35 °C; incubation time 15 h). (b) Impact of temperature on the rate of castor oil lipolysis (hydrolysis conditions: oil–water ratio, 1:04; pH, 7; lipase concentration 0.01% (w/v); stirring speed, 120 rpm; incubation time 12 h).

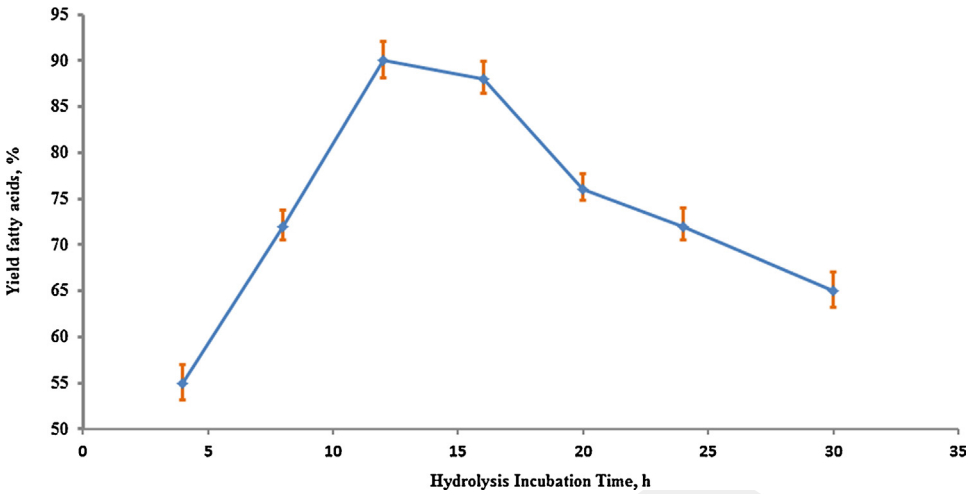

Fig. 6. Effect of incubation time on castor oil hydrolysis (hydrolysis conditions: oil–water ratio, 1:04; pH, 7; lipase concentration 0.01% (w/v); stirring speed, 120 rpm; temperature 37 °C).

3.4.2. Influence of pH on castor oil hydrolysis

The pH affects the stability, structure and function of many globular proteins because of their ability to influence electrostatic interactions, the extent of hydrolysis is different at different pH values [23]. According to the specifications provided by supplier the optimum pH for ROL is 7.2 (highly active from pH 6.5–7.5), Fig. 4b shows the pH profile of castor oil enzymatic hydrolysis. There is a gradual increase in hydrolysis from pH 6 to 6.5 and highest yield of FFA was produced at pH 7, after that the hydrolysis rate decreased in alkaline pH 7.5–8. The decline in enzyme activity after the optimum pH (7) was due to a reversible reaction that involved the ionization or deionization of acidic or basic groups in the active center of the enzyme protein [22]. The optimum pH of tributyrin hydrolysis using ROL as catalyst was also at pH 7 [24]. Further experiments were carried out at pH 7.

3.4.3. Effect of enzyme dose on castor oil hydrolysis

Fig. 5a displays the yield of FFA produced at various enzyme concentrations (0.005–0.025%, w/v), a fast increase in FFA (30%) was noted when lipase dose increased from 0.005 to 0.01% (w/v), total yield of FFA reached 82% at 12 h of incubation. After that increasing ROL concentration had no any profound effect on FFA% yield, so 0.01% (w/v) of lipase was optimized for further study. Due to high cost of lipase, it is advantageous to carryout lipolysis at 0.01% (w/v) of enzyme concentration. *Aspergillus niger* lipase produced 56% FFA after 72 h of incubation at pH 7 at 50 U enzyme dose, other fungal lipases such as, *Rhizopus delemar* and *Humicola lanuginose* were used to produce fatty acids from castor oil but no appreciable hydrolysis was noted [25,26].

3.4.4. Effect of temperature on castor oil hydrolysis

It is known that the variation of temperature significantly effect on the rate of enzymatic reactions. So keeping this point in mind the temperature of castor oil lipolysis was studied between 25 and 50 °C. Fig. 5b indicates the temperature profile of the lipolysis, initially there was gradual increase in FFA content of mixture from 25 to 35 °C and reached optimum at 37 °C, although the optimum temperature of ROL according to specifications is 40 °C. Further increase in temperature value did not assisted to increase the yield of FFA; this might be due to the partial denaturation of lipase resulting deactivation of active sites [27]. The optimum temperature of castor oil hydrolysis was also 37 °C using lipase *Pseudomonas gessardii* as a catalyst [28]. For further experiments 37 °C was kept constant.

3.4.5. Effect of incubation time on castor oil hydrolysis

The incubation time was kept from 4 to 30 h, as can be seen in Fig. 6, maximum castor oil hydrolysis (FFA 88–90%) was achieved between 12 and 16 h incubation. Further incubation time did not caused a positive effect on FFA% yield, it is because higher FFA content inhibit the lipase activity. This can be explained as in the initial stage of lipolysis there are more interactions between castor oil and enzyme so the rate of hydrolysis increase linearly with time. However, after a certain time the FFA content is higher than the substrate concentration resulting less interaction between enzyme and oil so decrease in FFA yield is obvious after optimum incubation time [23,29]. Therefore, 12 h incubation time period was optimum to carryout hydrolysis of castor oil using ROL.

4. Conclusions

The enzymatic hydrolysis of castor oil using lipase *R. oryzae* was successfully carried out at mild reaction conditions and the maximum transformation of castor oil (FFA 90%) was achieved at only 0.1% (w/v) of enzyme concentration at 12 h of incubation period. The developed PLS model showed better performance in terms of correlation coefficient values (0.999). Therefore, the ATR-FTIR method using PLS model can represent a good alternative to the standard methods applied to assess lipase catalyzed hydrolysis of vegetable oils. Without consumption of any organic solvent which will not only reduce the cost of solvent but also avoid the disposal issues and environmental problems associated with chemical methods of analysis.

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