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EFFECTS OF PROCESS VARIABLES AND ADDITIVES ON MUSTARD OIL HYDROLYSIS BY PORCINE PANCREAS LIPASE

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Abstract - Selective hydrolysis of brown mustard oil (from *Brassica juncea*) with regioselective porcine pancreas lipase was studied in this work. Buffer and oil phase were considered as the continuous and dispersed phases, respectively. Effects of speed of agitation, pH of the buffer phase, temperature, buffer-oil ratio and enzyme concentration on hydrolysis were observed. The best combination of process variables was: 900 rpm, pH 9, 35 ºC, buffer-oil ratio of 1:1 and enzyme concentration of 10 mg/g oil. These standard conditions led to 50% hydrolysis and selective production of 55% erucic acid in 6 h. Cations like Mg^{2+} and Ca^{2+} increased hydrolysis, but Cu^{2+} strongly inhibited it. Organic solvents decreased hydrolysis, though the decrease was minimum for isooctane. A mixed surfactant comprising of Span 80 and Tween 80 increased erucic acid production by 57% at a buffer-oil ratio of 0.2:1.
Keywords: Mustard oil; Porcine pancreas lipase; Hydrolysis; Erucic acid; Surfactant.

INTRODUCTION

Mustard oil of different origins contains erucic acid, mainly at the 1 and 3 positions of its triacylglycerol structure (18–51% of the total fatty acids) (Mazza, 1998; Myher *et al*., 1979). This acid is harmful to human beings (West *et al*., 2002). Its allowable limit for human consumption is quite low (2% in edible oil) according to a regulation of the Central Council for Food Standards of India (2008). In the USA, the allowable limit of erucic acid in edible (canola) oil is 2% (U. S. Department of Health and Human Services, 2011). So, mustard oil should not be used as an edible oil.

Erucic acid and its derivatives can be used to produce different commercially important products like biodiesel, emulsifiers, high grade lubricants, high grade engineering plastics, pour point depressants, corrosion inhibitors etc. (USDA, 1996; Kaimal *et al*., 1993). Mustard oil also contains linoleic and linolenic acid. These acids are considered to be essential fatty acids for human beings (Osbourn, 2009). Besides, these two acids are key ingredients in personal care products and cosmetics (Rosen, 2005). In rheumatoid arthritis, γ-linolenic acid is quite useful (Soeken, 2003). So, mustard oil can act as a source of erucic acid and other fatty acids for industrial applications.

Various methods are available for oil hydrolysis to produce fatty acids. Alkaline hydrolysis employs mild reaction conditions (70–100 ºC), but the product acquires unwanted odour and colour. Continuous processes use high pressure (~5000 kPa) and temperature (250–360 ºC), leading to possible denaturation of the product, i.e., fatty acid (Majid and Hossain, 1980). Some other processes like low-temperature crystallization (Vargas-Lopez *et al*., 1999), 'silicalite' adsorption, aqueous surfactant

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separation (Sonntag, 1991) and chromatography (Wilson and Sargent, 2001) have also been used for production of erucic acid, but conversions were low in these methods.

Lipase enzyme can act as biocatalyst in the hydrolysis of vegetable oil and it possesses chemoselectivity, regioselectivity and stereoselectivity (Lerin *et al*., 2011; Tan *et al*., 2004; Saxena *et al*., 2003). At oil-water interface, it hydrolyzes carboxyl ester bond to release fatty acids and organic alcohols (Pereira *et al*., 2003; Leal *et al*., 2002; Kamimura *et al*., 1999; Merçon *et al*., 1997). In comparison with the aforementioned processes, lipase catalyzed hydrolysis has distinct advantages of excellent product purity and mild process conditions (normal pressure and nearly ambient temperature). Non-commercial lipases from *Bacillus stearothermophilu*s SB-1 and *Burkholderia cepacia* RGB-10 (Bradoo *et al*., 2002), *Pseudomonas mendocina* PK-12CS (Jinwal *et al*., 2003), *Pseudomonas aeruginosa* BN-1 (Syed *et al*., 2010), *Acinetobacter johnsonii* LP28 (Wang *et al*., 2011) and *Pseudomonas aeruginosa* PseA (Gaur and Khare, 2011) were used to catalyze the hydrolysis of mustard oil. Lipases from *Geotrichum candidum* and *Candida rugosa* released erucic acid more slowly than 20-carbon and 18-carbon fatty acids and so were not suitable (Mcneill and Sonnet, 1995). Regioselective lipase hydrolyzed ester bonds at the 1 and 3 positions much faster (50–100 times) than the bond at the 2-position and this could concentrate erucic acid in the free fatty acid (FFA) fraction (Brockerhoff, 1973). Regioselective porcine pancreas and *Rhizopus arrhizus* lipase performed quite well in concentrating erucic acid in the FFA fraction from varieties of mustard oil (Mukherjee and Kiewitt, 1996). In this study, porcine pancreas lipase was selected for its low price. Brown mustard oil from *Brassica juncea* was chosen as the substrate for hydrolysis.

Weak interaction forces stabilizing the secondary, tertiary and quaternary structure of enzymes are affected by changes in different process variables (temperature, pH etc.) and various additives. Alteration of such forces leads enzymes to attain less biologically active configurations; thus, these variables can have significant effects on enzyme activity (Bailey and Ollis, 1986). On increasing the temperature, the kinetic energy of the substrate and enzyme increases and these collide with other molecules more frequently. Consequently, the rate of reaction increases (Dee and Stoker, 2009). When the temperature surpasses a certain value, the increased energy alters the molecular conformation of the enzyme as the hydrogen bonds stabilizing its secondary, tertiary and quaternary structures are broken (Maidina *et al*., 2008; Solomon *et al*., 2004; Uhlig and Linsmaier-Bednar, 1998). This impedes its catalytic action. Besides, pH affects the charge of the acidic and basic amino acid residues located in the active site of enzyme. So, even small changes in pH can affect the ionic bonds stabilizing its structure and thus change its conformation and activity (Dee and Stoker, 2009; Solomon *et al*., 2004). Buffer composition and the degree of enzyme stabilization by the substrate are also important factors in determining enzyme activity (Adams *et al*., 2001). Kaimal *et al*. (1993) used buffers of pH 7, 8 and 9 instead of water as the hydrolyzing medium and found that hydrolysis as well as the amount of erucic acid remained the same. The buffer-oil ratio affects the extent of interfacial area where lipase catalyzed hydrolysis takes place. Speed of agitation affects the hydrolysis reaction by affecting mass transfer between buffer and the oil phase and by denaturation of the lipase due to fluid shear (Puthli *et al*., 2006). The enzyme concentration also affects the lipase catalyzed process considerably as its change affects the amount of lipase active site (Straathof, 2003). On the basis of these earlier studies, the effects of process parameters like speed of agitation, pH, temperature, buffer-oil ratio and enzyme concentration were determined in the present study.

The effects of salts on lipase catalyzed oil hydrolysis have been examined in a few studies (Shu *et al*., 2007; Sharon *et al*., 1998), as well as the effects of surfactants (Goswami *et al*., 2010; Yamamoto and Fujiwara, 1988) and different organic solvents (Puthli *et al*., 2006; Kulkarni and Pandit, 2005). The current study tested whether salt, solvent and surfactant could have an enhancing effect on lipase catalyzed mustard oil hydrolysis.

EXPERIMENTAL SECTION

Materials

The enzyme porcine pancreas lipase (type II, activity of 100–400 units/mg solid, where one unit activity means production of 1 μmole fatty acid/h) was obtained from Sigma-Aldrich Co., Germany, and was used without further purification. Oriental brown mustard oil (from *Brassica juncea*) was purchased in the local market in Kharagpur, India and was used without any further purification. Sodium chloride, calcium chloride, magnesium chloride, barium chloride, cupric chloride and ferric chloride, pentane, hexane, isooctane, butanol and

DMSO (dimethyl sulphoxide) were purchased from Merck India Ltd. Acetone, methanol, potassium hydroxide, titrisol buffer (boric acid/potassium chloride/sodium hydroxide) of pH 9, SDS (sodium dodecyl sulphate) and Tween 80 (polyoxyethylene sorbitan monooleate) were also obtained from Merck India Ltd. Aluminium chloride and heptane were procured from S-D-Fine Chem Ltd., India. Hexanol was procured from BDH Chemicals Ltd., England. Ethanol was procured from Jiangsu Huaxi International Trade Co. Ltd., China. Pure erucic acid (90%) was a kind gift from Godrej Industries Pvt. Ltd., India. Tris (hydroxymethyl) aminomethane was obtained from Himedia Laboratories Pvt. Ltd., India. Maleic anhydride, CPC (cetyl pyridinium chloride), Triton X-100 (octylphenoxy polyethoxy ethanol), boron trifluoride and disodium hydrogen phosphate were purchased from SRL Ltd., India. CTAB (cetyl trimethyl ammonium bromide) and Span 80 (sorbitan monooleate) were procured from Loba Chemie Pvt. Ltd., India.

Experimental Set Up

A cylindrical glass reactor (Remco, India) of inner diameter 0.06 m and length 0.12 m was used in batch mode. The reaction mixture was stirred using a mechanical stirrer (Remi, India) attached with a 4-bladed paddle type glass impeller of 0.02 m diameter. The ratio of reactor diameter and impeller diameter was fixed at 1:3 such that the effect of turbulence became constant under different conditions. The distance between the lowest point of the impeller and the bottom of reactor was 0.02 m, i.e., equal to the impeller diameter. A thermostatic water bath (Thermocon, India) was used to keep the temperature constant (±1 ºC). The experimental set up is represented pictorially in Fig. 1. All the experiments were performed under atmospheric pressure.

Procedure

Initially, tris–maleate buffers of different pH (6, 7 and 8) were prepared following standard procedure (Gomori, 1955). Titrisol buffer of pH 9 was used. A buffer of pH 10 was prepared by mixing 0.1 M disodium hydrogen phosphate with 0.1 M NaOH in the appropriate proportions. In each experiment, a measured weight of mustard oil was initially added to the reactor and heated to the reaction temperature. Then, a certain weight of buffer solution containing a measured weight of lipase was added to the oil in order to maintain the desired buffer-oil ratio and enzyme concentration (g/g oil basis). At fixed temperature, pH, buffer-oil ratio and enzyme concentration, the mixture was stirred at a particular speed of agitation. The speed of agitation was varied from 500 to 1100 rpm (0.52 to 1.15 m/sec of impeller tip velocity) and a standard speed was selected. Next, the temperature was varied from 30 to 45 ºC at standard speed of agitation and other variables fixed at previously set values to determine the standard temperature. The pH was then varied from 7 to 10 at standard speed, temperature and previously fixed buffer-oil ratio and enzyme concentration to find the standard pH. The buffer-oil ratio was then varied from 1:1 to 5:1 at standard speed, temperature, pH and fixed enzyme concentration to determine the standard buffer-oil ratio. Next, enzyme concentration was varied from 2 to 14 mg/g oil at standard speed of agitation, temperature, pH and buffer-oil ratio. Each experiment was terminated after 6 h by adding a certain volume of 1:1 (v/v) acetone–ethanol mixture. The oil phase was then separated from the aqueous phase and was used for analysis. At the standard set of process variables, certain concentrations of different salts, solvents and surfactants were added separately to the reaction mixture to observe the effect of each additive.

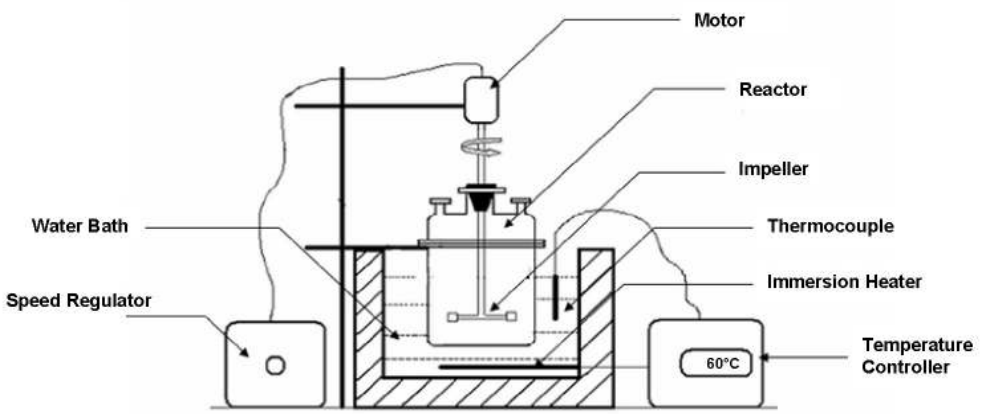

Figure 1: Schematic diagram of the experimental set up.

Analyses

Preparation of Methyl Erucate

A sample from the oil phase was withdrawn and mixed with boron trifluoride–methanol (14% v/v) in a 15 mL screw-capped vial. Then the mixture was heated in a water bath at 55 ºC for 1.5 h with stirring for 10 seconds at 15–20 minutes intervals. After that, two phases were separated. The sample was collected from the top layer and used for analysis by gas chromatography on a capillary column (O'Fallon *et al*., 2007).

Capillary Gas Chromatography

A 0.2 µL sample containing methyl erucate was injected into a gas chromatograph (Chemito GC 8610) with a SGE forte GC capillary column (BPX 70, 25 m × 0.53 mm × 0.5 µm). The column was preheated at 230 ºC for half an hour prior to injection of each sample. At the beginning, the oven temperature was 60 ºC; it was increased at 10 ºC/min to 150 ºC, and then increased at 5 ºC/min to the final temperature of 230 ºC. The temperatures of the injector and detector ports were 240 ºC and 280 ºC, respectively. A split flow (10:1) was used and the capillary pressure was 0.4 bar.

$$\text{Percentage of total erucic acid formed (\%)} = \frac{\text{Net amount of erucic acid in product (g)} \times 100}{\text{Net amount of erucic acid in oil (g)}}$$

It was found that the percentage of erucic acid in the fatty acid profile of brown mustard oil from *Brassica juncea* was 49% (Oram *et al*., 1999).

Determination of the Saponification Value of Mustard Oil

Initially, 4 g of mustard oil and 50 mL of 0.5 M ethanolic KOH solution were added into a round bottom flask and the mixture was refluxed for 1 h. The resulting solution was titrated against a standard oxalic acid solution. The same experiment was carried out again without mustard oil. The saponification value was calculated from the titer values using the following formula (Paquot and Hautfenne, 1987):

$$\text{S.V.} = \frac{56.1 \times N_1 \times (V_1 - V_0)}{m_1}$$

where, S. V. was the saponification value, N_1 was the strength of the standard oxalic acid (N), V_0 and V_1 were the volumes of standard oxalic acid solution required to neutralize the blank solution (mL) and the test sample solution (mL), respectively; and m_1 was the mass of mustard oil (g).

Determination of the Acid Value

An aliquot of the sample from the oil phase was added to 100 mL of neutralized ethanol–toluene (1:1, v/v) mixture. Next, it was titrated against standardized potassium hydroxide solution with phenolphthalein as indicator. With the help of the titer values, the acid value was calculated using the following formula, (Paquot and Hautfenne, 1987):

$$\text{A.V.} = \frac{56.1 \times N_2 \times V_2}{m_2}$$

where A.V. was the acid value, V_2 was the volume of standardized KOH solution required to neutralize the test sample solution (mL), N_2 and m_2 were the strength of the standard oxalic acid (N) and mass of the sample from the oil layer (g), respectively.

On the basis of these two parameters, the percentage of hydrolysis was calculated using the following formula (Virto *et al*., 1991):

$$\text{Percentage hydrolysis} = \frac{\text{Acid Value} \times 100}{\text{Saponification Value}}$$

RESULTS AND DISCUSSION

Effects of different process variables on porcine pancreas lipase catalyzed mustard oil hydrolysis are described in this section.

Effect of Speed of Agitation

Lipase is adsorbed at the oil–water interface with its simultaneous depletion from the bulk aqueous phase. Then, a special fit between the respective geometries of the lipase active site and aggregates of substrate occurs, leading to a large activation effect. This clearly signifies that the lipase catalyzed reaction rate increases with increasing interfacial area (Sadana, 1991; Verger, 1980).

On increasing the speed of agitation, the number of smaller droplets increases in the dispersed phase (oil), leading to enhancement of the interfacial area. As a result, a higher number of lipase molecules

leave the buffer phase and start to split the interfacial triacylglycerol. This increases the extent of hydrolysis. Mechanical agitation leads to exposure of the lipase to shear stress and unfolding and surface denaturation can occur, leading to its deactivation. This decreases the extent of hydrolysis. At low speed, the effect of enhancement of the interfacial area on hydrolysis is greater than the effect of lipase deactivation. Consequently, hydrolysis increases with increasing speed. At a certain speed, hydrolysis becomes maximum and this speed is termed the standard speed of agitation. Above this speed, the effect of deactivation of lipase surpasses the effect of interfacial area enhancement and, as a result, hydrolysis decreases (Sadana, 1991).

The effect of speed of agitation on hydrolysis, as well as the extent of production of erucic acid is presented in Fig. 2. This figure clearly shows that the standard speed of agitation is 900 rpm (impeller tip velocity of 0.95 m/sec), corresponding to maximum values of 'percentage hydrolysis' and 'percentage of total erucic acid formed'.

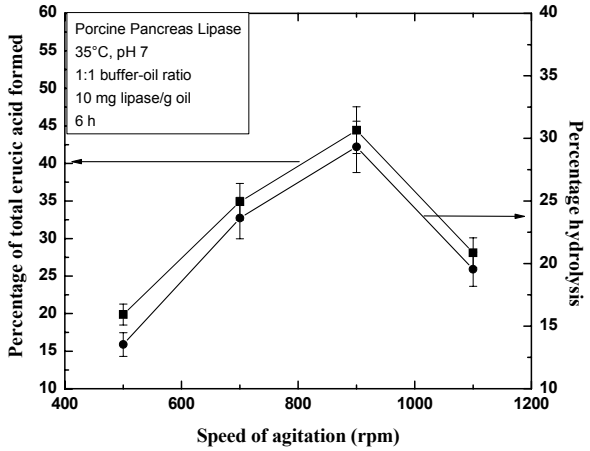

Figure 2: Variation of 'percentage of total erucic acid formed' and 'percentage hydrolysis' with speed of agitation.

Effect of pH of the Buffer Phase

There are several reasons for the dependence of lipase catalysis on the pH of the buffer medium. A change in pH results in conformational changes of lipase by a change of strain on the 'lid' covering the active site. In this way, pH controls the opening or closing of the catalytic centre for substrate binding (Benjamin and Pandey, 1998). Besides, a change in pH changes the substrate concentration at the interface, ionization of free substrate and ionization of the lipase–substrate complex. Extreme values of pH, i.e., very high or very low pH lead to the irreversible denaturation of lipase and breakdown of substrates. As a result, the concentration of substrate decreases and breakdown products often inhibit lipase activity, leading to low extent of hydrolysis (Tipton and Dixon, 1979; Verger *et al*., 1973).

As lipase action often involves acid and base type catalytic actions, ionizable amino acid residues containing a partial charge are an important part of the active site of lipase. While one type of residue almost fully combines with hydrogen ions at the standard pH, the other type of residue remains free from protonation by hydrogen ions. As the active site exists only in one particular ionization state, pH quantitatively controls the state of the active site in lipase. At the standard pH, lipase has the most active catalytic site (Kuo and Gardner, 2002; Lindley, 1954).

In an earlier study, the standard pH for porcine pancreas lipase was found to shift from 7 to 8.8 with increasing chain length of the resultant fatty acids (Whitaker, 1993). So, the range of pH was selected as 7 to 10 in this study. The effect of variation of the pH on 'percentage hydrolysis' and 'percentage of total erucic acid formed' is shown in Fig. 3. This figure clearly shows that, with increasing pH, hydrolysis and simultaneously the extent of erucic acid formation reach a maximum at pH 9 and then decrease. So, the standard pH is 9.

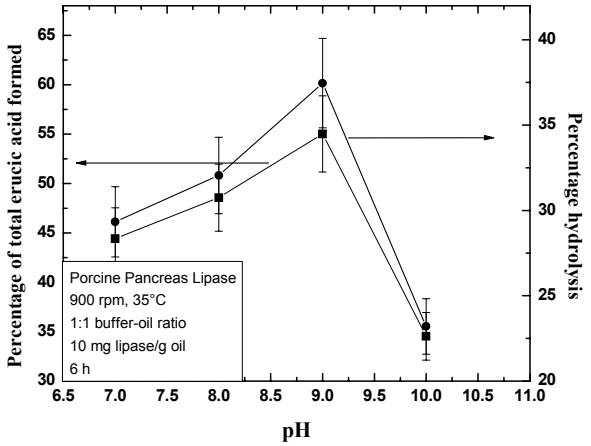

Figure 3: Variation of 'percentage of total erucic acid formed' and 'percentage hydrolysis' with pH.

Effect of Temperature

The effect of temperature on the enzyme catalyzed reaction is smaller than its effect on the uncatalyzed reaction. Important factors like protein denaturation, protein ionization state, and solubilities of substrates in solution are also affected by temperature (Zeffren and Hall, 1973). Temperature

controls the substrate concentration at the oil–water interface to a certain extent (Verger *et al*., 1973). The increase of temperature affects the rates of two independent processes, namely, lipase catalyzed hydrolysis and deactivation of lipase (Maidina *et al*., 2008; Uhlig and Linsmaier-Bednar, 1998). With increasing temperature, the mobility of the enzyme (lipase) molecules increases. Besides, the enzyme (lipase) molecules acquire sufficient energy to overcome the weak interactions holding the globular protein structure together. This leads to its deactivation (Bailey and Ollis, 1986). In the lower temperature range, the rate of thermal deactivation is nominal and the net extent of hydrolysis increases with the increase in temperature. At a particular temperature, overall hydrolysis becomes maximum and this is the standard temperature (Laidler and Peterman, 1979).

Mcneill and Sonnet (1995) found that the hydrolysis of high erucic acid rapeseed oil with *Candida rugosa* lipase at 10, 15 and 20 ºC resulted in a cloudy mixture, whereas there was no cloudiness at 35 ºC. They also found that the final concentration of erucic acid increased with increasing temperature and was strongly temperature dependent. So, the range of reaction temperature was chosen as 30 to 45 ºC in our study. The effect of variation of temperature on overall hydrolysis, as well as erucic acid formation, is shown in Fig. 4. From this figure, it is observed that, with increasing temperature, the extent of hydrolysis and production of erucic acid reach a maximum and then decrease. The standard temperature corresponding to maximum hydrolysis, as well as maximum extent of erucic acid formation, was found to be 35 ºC. This finding is supported by an earlier study (Bagi *et al*., 1997) where the optimum temperature of porcine pancreas lipase at pH 8.9 was found to be 35 ºC. In the present study, the increase in rate of reaction was higher than the denaturation of lipase at a temperature less than 35 ºC; as a result, the net rate of hydrolysis increased. But, above this standard temperature, denaturation of lipase surpassed the effect of rate of reaction increase and, consequently, the overall rate of hydrolysis started decreasing.

Effect of Buffer-Oil Ratio

Porcine pancreas lipase has more hydrophobic amino acid residues on the surface
and so remains stable in a non-polar medium. The structure of this lipase is destabilized by increasing amounts of water, whereas buffer stabilizes it (Zaks and Klibanov, 1984). In lipase catalyzed hydrolysis, the buffer-oil ratio plays an important role by directly controlling the interfacial area. Piazza and Farrell (1991) found that castor oil hydrolysis by ground oat lipase releasing ricinoleic acid was highest when 50% of the emulsion was castor oil. For higher proportions (>50%) of castor oil, ricinoleic acid production decreased more sharply than in the case of lower proportions (<50%) of castor oil; i.e., a higher proportion of water gave better results. Based on their finding, the buffer concentration was never used below 1 g/g oil in the present study. Kulkarni (2001) found that aqueous and non-aqueous phases at a 1:1 ratio led to a higher extent of oil hydrolysis than other ratios. The range of buffer-oil ratios was chosen as 1:1 to 5:1 in this study.

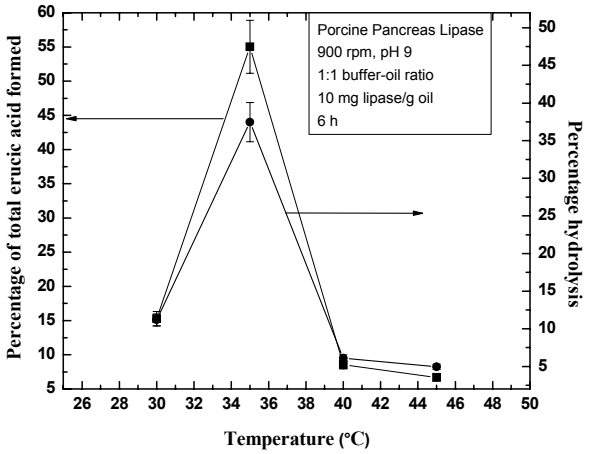

Figure 4: Variation of 'percentage of total erucic acid formed' and 'percentage hydrolysis' with temperature.

Figure 5 shows the effect of buffer-oil ratio on 'percentage hydrolysis' and 'percentage of total erucic acid formed'. This figure clearly shows that a buffer-oil ratio of 1:1 leads to the highest extent of hydrolysis and erucic acid formation. At high buffer-oil ratio (2:1 to 5:1), a large interfacial area is created during mixing. This leads to high initial rate of hydrolysis. Porcine pancreas lipase selectively cleaved ester bonds at the 1 and 3 position of triacylglycerol to produce fatty acid and 2-monoacylglycerol. These compounds have higher surface affinity than lipase and so replace it from the interface at a high rate (Reis *et al*., 2009). As a result, contact between lipase and mustard oil decreases and hence the extent of hydrolysis remains low for these higher buffer-oil ratios. Besides, for those higher buffer-oil ratios, substrate is diluted at the interface and interacts with lipase to a low extent. High concentration of free fatty acid resulted in high

concentrations of ionized carboxylic acid groups, which acidified the microaqueous phase surrounding lipase and resulted in desorption of water from the interface. These changes adversely affected lipase activity. On desorption from the interface, short and medium chain fatty acids dissolved partially in water, leading to limited accessibility of the substrate to water and hydrolysis further decreased (Kuo and Gardner, 2002).

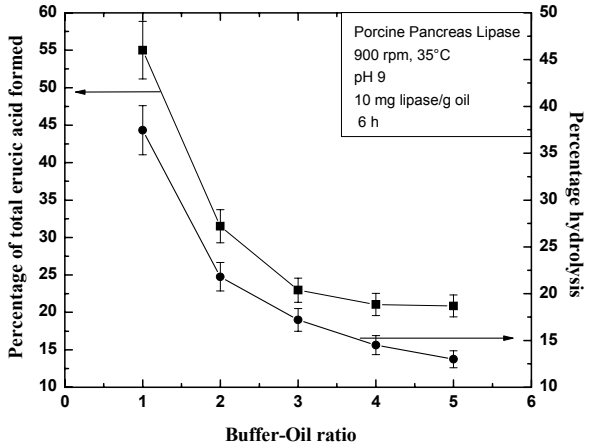

Figure 5: Variation of 'percentage of total erucic acid formed' and 'percentage hydrolysis' with the buffer-oil ratio.

In the case of a 1:1 buffer-oil ratio, the interfacial area was not so high and subsequent product inhibition remained low, resulting in a moderate rate of hydrolysis. Besides, sufficient interaction between lipase and substrate occurred. These result in maximum values of 'percentage hydrolysis' and 'percentage of total erucic acid formed'.

Effect of Enzyme Concentration

The enzyme concentration has a strong impact on the catalytic process (Straathof, 2003). On increasing the lipase concentration, lipase goes from the aqueous phase to the interface at an increasing rate; its interaction with the substrate increases, leading to enhanced hydrolysis. When the lipase concentration is sufficiently high to saturate the available interface, the extent of hydrolysis becomes constant and does not increase further on increasing lipase concentration (Desnuelle, 1961).

Some initial studies were performed with a lipase concentration of 1 mg/g oil, but the extent of hydrolysis was very low such that it was tough to discriminate between the hydrolysis obtained at different pHs or temperatures. So, deliberately, 10 mg lipase/g oil was chosen for carrying out initial studies. For standardization purposes, the range of enzyme concentration was chosen as 2 to 14 mg/g oil. Fig. 6 presents the variation of 'percentage hydrolysis' and 'percentage of total erucic acid formed' with enzyme concentration. This figure shows that, with increasing enzyme concentration, the extent of hydrolysis and erucic acid production increases constantly up to 10 mg/g oil of enzyme concentration and then attains a constant value (37.46% hydrolysis and 55% erucic acid production). So, an enzyme concentration of 10 mg/g oil was considered as standard.

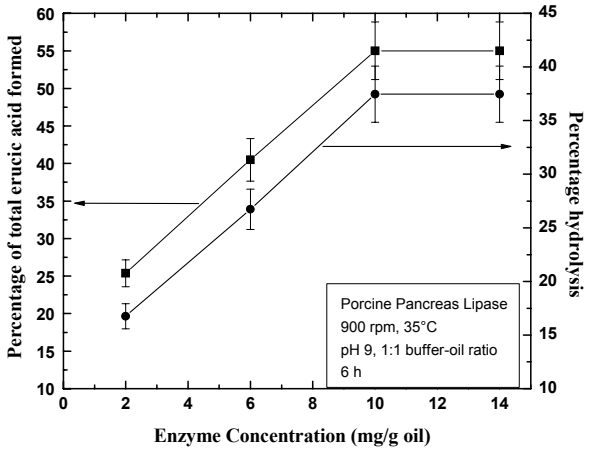

Figure 6: Variation of 'percentage of total erucic acid formed' and 'percentage hydrolysis' with the enzyme concentration.

Finally, the standard process conditions were: 900 rpm, pH 9, 35 °C, buffer-oil ratio of 1:1 and enzyme concentration of 10 g/g oil, leading to 'percentage hydrolysis' of 37.46% and 'percentage of total erucic acid formed' of 55% in 6 h.

Effects of Salts

Fatty acids are more surface active than lipase and so replace it from the oil–water interface significantly. This decreases the extent of hydrolysis (Reis *et al*., 2009). Besides, the formation of the fatty acid–lipase complex is considered to be the major factor in the product inhibition of triacylglycerol hydrolysis (Bengtsson and Olivecrona, 1980). Cations of inorganic salts form salts with fatty acids and thus remove them from the oil–water interface. As a result, the availability of the interfacial area towards lipase increases, fatty acid–lipase complex formation remains low, and hydrolysis increases.

Table 1 describes the effects of the salts (0.01 M in buffer) on hydrolysis under standard process conditions. In Table 1, the term 'None' represents

the experiment under standard process conditions in the absence of any salt; this results in 37.46% hydrolysis in 6 h. This table shows that addition of Na^+ ion (Group IA) enhanced hydrolysis to 45%, whereas only Cu^{2+} ion (Group IB) showed strong inhibition, leading to 7.43% hydrolysis.

Table 1: Effects of different salts on the hydrolysis of mustard oil under standard process conditions

Salt (0.01 M in buffer)	Percentage Hydrolysis
None	37.46
NaCl	45.09
$CuCl_2$	7.43
$CaCl_2$	65.36
$MgCl_2$	84.35
$BaCl_2$	45.54
$AlCl_3$	46.91
$FeCl_3$	43.40

Ions from Group IIA metals like Mg^{2+} and Ca^{2+} led to large increases in hydrolysis (84.35 and 65.36% for Mg^{2+} and Ca^{2+} respectively). Such divalent cations can react with two fatty acid molecules to form di-salts, unlike monovalent cations. Consequently, divalent cations (Group II) were more effective than monovalent cations (Group I) in separating fatty acid and reutilizing lipase. Again, cations from Group IIA formed planar salts, leading to less steric effects (Sharon *et al*., 1998). Here, Mg^{2+} was found to be more active than Ca^{2+} ion. Ba^{2+} of Group IIA led to a comparatively lower extent of hydrolysis (45.54%). Al^{3+} of Group IIIA and Fe^{3+} of group VIII showed performance similar to Ba^{2+} as these ions also formed di-salts.

Effects of Organic Solvents

Table 2 shows the effects of various organic solvents (0.5 g/g oil) on hydrolysis under standard process conditions. In this table, the term 'None' in the "Organic Solvent" column represents the experiment under standard process conditions without organic solvent. This experiment used only buffer solution containing lipase and led to 37.46% hydrolysis in 6 h. Table 2 shows that all the organic solvents led to a decrease in hydrolysis. The best performance was shown by isooctane, which only decreases hydrolysis a little (37.46% to 36.50%). Hexane and heptane also perform better than other solvents, resulting in 33.83% and 30.50%, respectively. But solvents like pentane, butanol, hexanol and DMSO drastically deactivate lipase, leading to 9.11, 15.51, 13.90 and 12.45% hydrolysis, respectively. The reason is that the lid on active site of lipase opens only in the presence of an oil–water interface as a result of which substrate (oil) molecule can access active site of lipase (Brozozowski *et al*., 1991). But, as lipase is insoluble in organic solvent, the presence of such a solvent hinders lipase reaching the oil–water interface due to diffusional limitations. Consequently, a sufficient number of active sites of lipase cannot open up and finally, hydrolysis decreases. Besides, the presence of organic solvent decreases the conformational mobility of enzymes like lipase and destabilizes the transition state during reaction (Klibanov, 1997). All these factors decrease hydrolysis.

Table 2: Effects of different organic solvents on the hydrolysis of mustard oil under standard process conditions

Organic Solvent (0.5 g/g oil)	Percentage Hydrolysis
None	37.46
Hexane	33.83
Isooctane	36.50
Heptane	30.50
Pentane	9.11
Butanol	15.51
Hexanol	13.90
DMSO	12.45

Effects of Surfactants

Table 3 shows the effect of different surfactants on the hydrolysis of mustard oil catalyzed by porcine pancreas lipase in the presence of 10 mg lipase/g oil at 900 rpm and 35 ºC. In the absence of surfactant, 55% erucic acid is produced in 6 h. Though in earlier studies (Antonov *et al*., 1988; Verger *et al*., 1970) it has been reported that a very low concentration of SDS increases activity of porcine pancreas lipase, it did not increase activity of the same lipase in this study. Cationic surfactants like CTAB also significantly decrease conversion. The nonionic surfactant Span 80 increased the hydrolysis of castor oil in an earlier study (Goswami *et al*., 2010), but it decreased erucic acid production in this study. Other nonionic surfactants like Triton X-100 and Tween 80 lead to a large decrease in erucic acid production. All the surfactants decrease the production of erucic acid to a great extent. Only Span 80 (0.01 M) shows a considerable amount of erucic acid production (30% in 6 h) in a water-in-oil emulsion with a buffer-oil ratio of 0.2:1.

Table 3: Effects of different surfactants on the production of erucic acid

No.	pH	Surfactant (Concentration in buffer)	Buffer concentration (g/g oil)	Response (Percentage of total erucic acid formed)
-	9	–	1	55.02
1	9	SDS (0.004 M)	3	6.04
2	9	SDS (0.01 M)	3	7.77
3	9	SDS (0.02 M)	3	6.64
4	9	Span 80 (0.01 M)	3	11.44
5	9	Span 80 (0.02 M)	3	6.64
6	7	SDS (0.01 M)	1	2.64
7	7	Span 80 (0.01 M)	1	14.00
8	7	CTAB (0.01 M)	1	14.00
9	7	Triton X-100 (0.01 M)	1	3.80
10	7	CPC (0.01 M)	1	18.10
11	7	Tween 80 (0.01 M)	1	8.76
12	7	Span 80 (0.01 M)	0.2	30.13

As no single surfactant was found to be effective, a mixed surfactant system consisting of Span 80 and Tween 80 was tested for possible enhancement. In castor oil hydrolysis, a quite low concentration of Span 80 (0.006 M) was found to be optimum (Goswami *et al*., 2010). Naturally, its chosen concentrations were also low (0.001, 0.003 and 0.005 M) in the present study. A very low concentration of Tween 80 stimulated lipase at 1/100 to 1/10,000 of the CMC in an earlier study (Li *et al*., 1986). As the CMC of Tween 80 is very low (0.038 M), a low concentration (0.0015 M or 1/25 of its CMC) was chosen in the present study. Table 4 shows the effect of mixed surfactant consisting of nonionic Span 80 (dissolved in the oil phase) and Tween 80 (dissolved in the buffer phase) under constant conditions of 10 mg lipase/g oil, buffer-oil ratio of 0.2:1, 900 rpm, pH as 9 and 35 ºC. A combination of Span 80 (0.005 M in the oil) and Tween 80 (0.0015 M in the buffer) increases selective production of erucic acid from 27.87% (hydrolysis without surfactant) to 43.71%, i.e., overall 57% with respect to hydrolysis without surfactant in 6 h.

Table 4: Effects of various combinations of mixed surfactants on the production of erucic acid

No.	Concentration of Span 80 (M in oil)	Concentration of Tween 80 (M in buffer)	Response (Percentage of total erucic acid formed)
1	0	0	27.87
2	0.001	0.0015	19.77
3	0.003	0.0015	24.77
4	0.005	0.0015	43.71

CONCLUSIONS

In this study, the moderate value of the standard speed of agitation (900 rpm) shows that deactivation of porcine pancreas lipase becomes significant at comparatively low shear stress. The standard pH of this lipase was found to be basic (9). The change in hydrolysis as well as erucic acid production is very sharp with the change in temperature around the standard temperature of 35 ºC. Temperature affects hydrolysis most significantly. With buffer as the dispersion medium, increasing the amount of buffer actually decreases the extent of hydrolysis rapidly. The hydrolysis attains its maximum when the amount of buffer and oil is the same (buffer-oil ratio of 1:1). As porcine pancreas lipase is of quite low activity, a comparatively high lipase concentration (10 mg/g oil) is found to be standard.

Metal ions from Group II like Mg^{2+} and Ca^{2+} increase hydrolysis significantly, probably due to formation of di-salts with fatty acid molecules. Ions like Ba^{2+} (Group II), Al^{3+} (Group III) and Fe^{3+} (Group VIII) increase hydrolysis moderately. This is probably due to some kind of inhibition by these ions as these also form di-salts with fatty acid molecules. Only Cu^{2+} ion strongly inhibits hydrolysis. All the tested organic solvents inhibit hydrolysis, presumably because these solvents hinder diffusion of lipase from the bulk aqueous phase to the oil–water interface and also deactivate lipase. Only isooctane shows small inhibition, whereas pentane, butanol, hexanol and DMSO lead to strong inhibition. No single surfactant can increase hydrolysis. A mixed surfactant system composed of nonionic Span 80 and Tween 80 increased erucic acid production significantly when oil was used as the dispersion medium (buffer-oil ratio of 0.2:1).

NOMENCLATURE

A. V.	Acid value	
S. V.	Saponification Value	
m_1	mass of mustard oil for determination of the saponification value	g
m_2	mass of sample taken from the oil layer to determine the acid value	g
N_1	Strength of standard oxalic acid used for the determination of the saponification value	Normality (N)
N_2	Strength of standard oxalic acid used for the determination of the acid value	Normality (N)
V_0	volume of standard oxalic acid solution required to neutralize the blank solution	mL
V_1	volume of standard oxalic acid solution required to neutralize the test sample solution	mL
V_2	volume of standardized KOH solution required to neutralize the test sample solution	mL

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