Kinetic Study of Ethyl Hexanoate Synthesis Using Surface Coated Lipase from Candida Rugosa

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ABSTRACT: *Kinetics of lipase-catalyzed esterification of hexanoic acid and ethyl alcohol using the solvent-free system, surface coated lipase from Candida rugosa, had been studied. The effect of various parameters such as reaction time, reaction temperature, reaction kinetics, water removal and feasibility of solvent-free system had been focused. Candida Rugosa lipase was more effective than other lipases when ethyl hexanoate was synthesized in n-hexane. The highest esterification yield after 72 h (93 %) was achieved at a pH of 5.2 and the esterification yield was reduced to 73% at pH 4.0. The values of the apparent kinetic parameters were computed as* V_{max} *= 0.146 µmol/min/mg enzyme;* $K_{M, Acid}$ *= 0.296 M;* $K_{M, Alcohol}$ *= 0.1388 M;* $K_{i, Acid}$ *= 0.40 M; and* $K_{i, Alcohol}$ *= 0.309 M. The reaction rate could be described in terms of the Michaelis–Menten equation with a Ping-Pong Bi-Bi mechanism and competitive inhibition by both the substrates.*

KEYWORDS: *Candida rugosa; Ethyl hexanoate; Surface coated lipase; Transesterification; Kinetic studies.*

INTRODUCTION

Fatty acids of short chains and alcohol esters were important components of natural flavors used in the food and flavor industry. Flavor and fragrance ingredients were one of the intentional additives used by the cosmetic, food, beverage, pharmaceuticals and personal care industries. Worldwide demand for flavor and fragrances, as well as essential oils and other natural extracts, was expected to increase from 4.3% per year to $23.5 billion in 2014 [1-3]. Currently, most of the flavor and fragrance components were produced by traditional methods, which include synthesis by chemical means or extraction from natural sources. The cost for extraction procedures of these valuable esters makes the process commercially unviable [4-6]. The concept of a natural flavor made by enzymatic synthesis with the use of lipases and natural substrate components is a prominent alternative to such methods. On the other hand, the use of chemical methods leads to the formation of some undesired side products and color impurities which thus had limited application in the food and beverage

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industries. Enzymatic catalysis appears to be a useful methodology for the synthesis of those chiral flavors or fragrance molecules which would be otherwise difficult using conventional chemical synthetic route [7].

Biotechnological processes were more expensive than chemical processes but eco-friendly, since the use of inorganic acids (employed as catalysts in chemical syntheses) could be avoided and the immobilized enzymes were reused, thereby, minimizing the reaction residues. The most used reaction media in enzymatic syntheses were organic solvents such as hexane or heptane, which could be recovered for reuse.

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) were one of the most important classes of hydrolytic enzymes, which were served as catalysts for both, hydrolysis and synthesis of esters. Microbial lipases were widely diversified in their properties and substrate specificity, which made them attractive tools for industrial applications [8]. Lipases display catalytic activity towards a large variety of alcohols and acids in reactions involving ester synthesis [9].

Lipases were known for their broad substrate specificity and were, therefore, had a wide range of applications, both in research laboratories and in industry. The use of lipases in organic media had expanded dramatically since the mid-1980s which had facilitated the efficient use of lipases in esterification and transesterification reactions, in addition to the traditional hydrolysis reactions. An important reason for this development could be that it was easier to achieve higher activity and stability of lipases in organic media than most other enzymes. Previous studies indicated in the literature had mainly focused on the use of lipases in organic reaction media, with organic solvents. These could be ‘‘normal’’, non-ionic solvents or ionic liquids. Furthermore, solvent-free mixtures, in which the substrates also act as the solvent, were attractive for many applications. Other reaction media which were not covered in great detail were supercritical fluids. All these media had the common feature that the water content is low, and similar methodologies could be employed to prepare enzymes.

Coating the enzymes with surfactants was advantageous due to its ease in preparation procedure and good solubility in a wide range of organic solvents [10,11]. Therefore, surfactant coated enzymes had been regarded as very competitive and promising biocatalysts in organic media in comparison to enzymes modified with polyethylene glycols (PEG) [12] or entrapped in reverse micelles [13].

In the present investigation, efforts were made to synthesize ethyl hexanoate using surface-coated lipase in the organic solvent system. Several lipases had been screened initially for examining the efficacy of the reaction in an organic solvent. Further, the influence of various reaction parameters had been studied. Subsequently, a kinetic study had been performed to determine the M-M kinetic parameters.

EXPERIMENTAL SECTION

Materials

Enzymes

The lipase from *Candida rugosa* (CRL) Type-VII and porcine pancreatic lipase (PPL) Type-II were procured from Aldrich Chemicals (Milwaukee, WI, USA) and Sigma (St. Louis, MO, USA) respectively. Lipase *Mucor javanicus* was from Amano Pharmaceuticals Co. Ltd (Nagoya, Japan).

Chemicals

Butyric, valeric, hexanoic, octanoic, octanol, ethyl butyrate, ethyl caprate (ethyl decanoate) were from Aldrich Chemicals (Milwaukee, WI, USA). Ethyl stearate was from Sigma Chemicals Co. (St. Louis, MO, USA). Tributyrin (Glycerol tributyrate) was from Merck (Darmstadt, Germany). Methanol, ethanol, butanol, *n*-hexane, *n*-heptane, *n*-octane, isooctane, petroleum ether (40-60° and 60-80°) and molecular sieves (3°A) were from Sd fine-Chem Ltd. (Mumbai, India). Phenolphthalein and sodium hydroxide, chloroform, dichloromethane, tetrahydrofuran were from SISCO Research Laboratories Pvt Ltd. (Mumbai, India). Ethyl methyl ketone was from Loba Chemie Pvt Ltd. (Mumbai, India). Dioxane was from Ranbaxy Fine Chemicals Ltd. (New Delhi, India). All substrates were dried over molecular sieves before use. The solvents were distilled before use.

Methodology

Hydrolytic activity of lipase

Lipase activity was measured by estimating the liberated butyric acid by titrating against 0.04 N

sodium hydroxide using the pH-stat method of Tietz and Fereick (1966) [14]. Tributyrin emulsion (substrate) was prepared in phosphate buffer (0.01 M, pH 7.0) at a concentration of 0.34 M (10 mL tributyrin in 90 mL buffer). Sodium benzoate (0.2 % w/v) was used as a preservative and gum acacia (0.5 % w/v) was used as an emulsifying agent. Tributyrin (4 mL) and phosphate buffer (8 mL) mixture was incubated with the enzyme for 30 minutes at 37°C and agitated at 150 rpm. A blank was also run without the enzyme under similar conditions. Samples were assayed by adjusting the pH of the sample to 9.5 using 0.04 N NaOH [15]. The hydrolytic activity values were calculated using the following Equation (1):

$$\text{Enzyme activity} = \frac{[\text{Blank} - \text{Sample}] \times \text{Normaliy(N)} \times 100}{\text{Enzyme(mg)} \times \text{inc.time(Minutes)}} = \ldots\text{Units} \quad (1)$$

The difference in volume of NaOH consumed (mL) between the blank and the samples after incubation time was used to measure the butyric acid released by hydrolysis. One unit of hydrolytic activity has been defined as one μmol of butyric acid released per minute per mg enzyme. The hydrolytic activity of *Candida rugosa* was 32,000 U/g; Porcine Pancreas was 40,000 U/g of enzyme and the hydrolytic activities of *Mucor javanicus* was 11,000 U/g of the enzyme, respectively. All the experiments were performed in duplicate and the average of the results were reported.

Preparation of surfactant - coated lipase

The surfactant-coated lipase was prepared according to the methods given elsewhere [16]. Five hundred milligram of lipase and five hundred milligrams of surfactant was mixed with 500 mL of 0.1M phosphate buffer solution (pH 7.0) and sonicated for 20 minutes. The mixture was stored at 4ºC for 24 h. Later, the solution was centrifuged and the translucent solution was lyophilized (-40ºC) for 2 h. A white powder was obtained.

Protein content determination

The amount of protein was determined by titration with trinitrobezene sulfonate (TNBS) of the amino acids produced after hydrolysis of the modified enzyme. A 500 mg of modified lipase was suspended in 2 mL of 3M HCl and the sample was glass sealed and heated at 80ºC for 5h., cooled and passed through cotton wool. The soluble fraction was collected and reacted with TNBS. The extent of protein modification was determined by comparing the number of amino groups reacted with TNBS in the modified and unmodified form [17,18].

Activity Measurements

The reaction of the esterification of ethanol and hexanoic acid was carried out in 5 mL total volume of AOT/isooctane microemulsion with *Candida rugosa* lipase. The reaction was performed at a temperature of 37°C, with a molar hydration ration w_o = [H_2O]/[AOT] = 4, while the pH of the dispersed aqueous phase was fixed at 6.0. Initial reaction rates were determined in closed vials placed in a thermostatic bath at 37°C. Aliquots were withdrawn at selected time intervals and analyzed for fatty acid content. The rate of esterification was determined by spectrophotometric method [19]. The depletion of fatty acid was typically monitored as follows: 0.1 mL samples from the reaction mixture were added to screw-cap test tubes containing 4.7mL of isooctane, 0.2 mL chloroform and 1mL of cupric acetate in pyridine (5 % w/v pH 6.0). After centrifugation at 1500 rpm for 3 min, the upper organic phase (the free acid present) was determined by measuring the absorbance at 715 nm. Curves of product concentration as a function of time over several minutes were linear, thus the initial slopes could be determined by the linear regression method.

Esterification Reaction

Esterification reactions were carried out in a closed conical flask (100 mL) with a 10 mL working volume of organic solvent. Freshly prepared substrates (alcohol and acid), were dissolved in organic solvent with or without buffer saturation and an appropriate amount of modified enzyme was added to initiate the reaction. The flasks were incubated at 37°C in an orbital shaker (Lab-line, Melrose Park, IL) at 150 rpm for 96 h of incubation time unless otherwise stated.

Buffer saturation of solvent

Solvents such as dioxane, tetrahydrofurone, ethyl methyl ketone, cyclohexane, chloroform, hexane, heptane, and isooctane were saturated with buffer (0.1 M phosphate buffer, pH 7.0) at a ratio of 10:1 [V/V] (solvent: buffer). The buffer saturated organic solvent was used directly for the synthesis of esters.

Transesterification Reaction for Synthesis of ethyl hexanoate

Transesterification reactions were carried out in closed conical flasks (100 mL) with ethyl caprate and hexanoic acid (acidolysis), as the substrates (equimolar concentration) in 10 mL *n*-hexane, and the surface coated lipase from *Candida rugosa* enzyme was added to initiate the reaction. Flasks were incubated at 37°C for 96 h of incubation time at 150 rpm on a rotary shaker (Remi Instruments, Model No CIS –24, Mumbai, India).

ANALYSIS

Titration

Aliquots of the reaction mixture were withdrawn periodically and assayed by titrating the samples against sodium hydroxide (0.04 to 0.1 N) to determine the residual acid content using phenolphthalein as the indicator and methanol as a quenching agent. Acid consumed in the reaction was calculated from the difference in the values of blank and test samples. In the case of ethyl hexanoate, the product concentration was determined by titrimetry and Gas Chromatography (GC). In all other cases, the product concentration was estimated using GC.

Gas Chromatography (GC)

Esters of ethyl hexanoate were analyzed using GC (Shimadzu GC 14 B), equipped with FID detector and Carbowax 20M column (3 m length, 3.175 ID). The column temperature was maintained (isothermal) at 100°C for the analysis. Injection port and detector temperatures were maintained at 200 and 250°C respectively. Nitrogen was the carrier gas (30 mL/minute). Peak areas were computed using Chromatopac (CR6A, Shimadzu, Japan) integrator. A sample volume of 0.1 mL was mixed with 2 mL hexane containing 2-4 mg internal standard, and 1µl sample from this mixture was injected to the column. *n*-Octanol was used as the internal standard for the direct esterification reactions. Ethyl stearate was used as the internal standard for the quantification of transesterification reactions.

Gas Chromatography-Mass Spectrometry (GC-MS)

The samples were analyzed using a Shimadzu 17 A-GC chromatograph equipped with a QP – 5000 (Quadrapole) mass spectrometer. A sample volume of 0.1mL ethyl hexanoate was mixed with 2 mL hexane and 0.5 µl sample was injected to Capillary column (DBwax, 30 m x 0.2 mm ID, film thickness 0.25 µm). Helium was the carrier gas at a flow rate of 2 mL per minute. Injection port and detector temperatures were maintained at 200 and 250°C respectively. The column temperature was initially kept at 65°C for 5 min and then increased to 200°C at a rate of 10°C per minute and at that temperature the column was maintained for 10 min. The splitting ratio was 1:50; and the ionization voltage was 70eV. The compounds were identified by matching their fragmentation pattern in mass spectra with those of NIST library and standard.

Determination of kinetic constants

Reactions were carried up to 3h with periodic sampling (1–3 h). The initial rates were calculated from the linear portions of the plots of product concentration *versus* reaction time by fitting the time course of the reaction to a linear function by regression, and determining the slope of the tangent to the curve. The effects of alcohol and acid or ester concentration on the reaction rate were investigated by esterification or transesterification of various concentrations of acid (ranging from 0.02 M to 0.8 M) with various initial concentrations of alcohol or ester (0.05 M to 0.8 M) and *vice versa*. The initial reaction rates obtained were fitted to Michaelis-Menten kinetics with Ping-Pong Bi-Bi mechanism by nonlinear regression, [20] using Microsoft Excel software (version 5.0; Microsoft Corporation, Redmond, WA).

Determination of equilibrium constants (Ko)

The equilibrium constants were determined using Equations (2) and (3)

[A]. In Esterification,

$$K_o = \frac{[P]\times[a_w]}{[SAL]\times[SAC]} \quad (2)$$

Where, *[P]* = ester formed [M], *[a_w]* = the thermodynamic water activity; *[SAL]* = Alcohol at equilibrium [M]; *[SAC]* = acid at equilibrium [M] and *[K_o]* = the equilibrium constant [M].

[B]. In Transesterification (Equation (3)),

$$K_o = \frac{[PES]\times[a_w]}{[SES]\times[SAC]} \quad (3)$$

Where, *[PES]* = the product ester [M]; *[aw]* = thermodynamic water activity; *[SES]* = substrate ester [M]; *[SAC]* = acid substrate [M].

RESULTS AND DISCUSSION

Effect of lipase source

The higher cost of the enzymes plays a major role in obstructing the scale-up of bioprocesses for the synthesis of flavors to commercial scale. Hence, for the production of low-cost biocatalysts, it would be essential to optimize the process. However, free lipases are used only for preliminary studies for characterization and performance evaluation of the biocatalyst, but not for commercial application. This might be due to the characteristics of bioconversion [21].

Preparation of Surface-Coated Lipase (SCL) was conducted using span 85 surfactant and five lipases. The yield of SCL ranged from 21-28 %. The SCL obtained is of white color. All SCL contained about 10% protein. The surface-coated lipase was insoluble or sparingly soluble in the organic solvent range tested. The esterification of ethanol and hexanoic acid was carried out by the addition of SCL in *n*-hexane with comparable protein content (CRL**:** 0.092 g/L; *Mucor* sp**:** 0.091 g/L; P*seudomonas* sp**:** 0.089 g/L; *A. niger***:** 0.09 g/L and porcine pancreatic lipase (PPL)**:** 0.09 g/L). Of the various lipase sources examined in the synthesis of ethyl hexanoate, surface-coated lipase from *Candida rugosa* and *Mucor* sp. showed highest esterification activity of 93 and 92 % respectively, whereas lipases from other sources were able to perform esterification reaction in the range of 30-50 %. These results indicated that CRL and *mucor* sp. were highly selective towards the synthesis of short chain esters. It had been already reported that the surface-coated CRL showed the highest selectivity for resolution of menthol [22]. Further, the efficiency of the surface-coated lipase was studied while comparing to its native counterpart. When the esterification reaction was performed with both the lipases, surface-coated lipase showed higher activity than free lipase (Fig 1). The esterification yields were almost identical during the first 5h of reaction, which was more than 95% using surface-coated lipase. However, in the case of free lipase, 65% of esterification was achieved under identical conditions. With this observation, it could be concluded that the lipase coated with span 85 was protected from

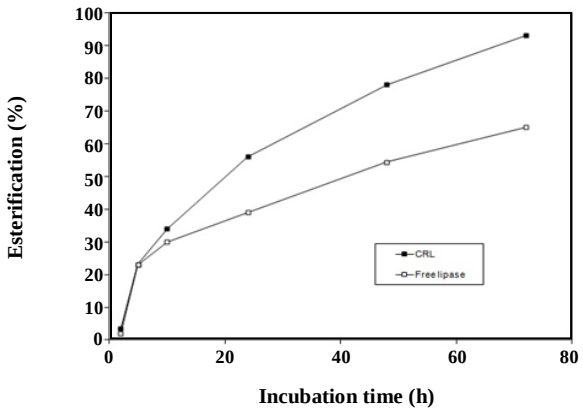

Fig. 1:: Comparison of free and surface coated lipase for the synthesis of ethyl hexanoate at 0.05 M substrate concentration at 40°C for 72 h of incubation time.

denaturation in organic solvents and therefore exhibited the highest catalytic activity compared to its native form. These results were consistent with results reported in the literature [23]. The increased stability of surface-coated lipases was also reported by *Basheer et al.* [24].

Effect of surfactant source

Various commercial surfactants were employed to produce SCL containing *Candida rugosa* lipase. The surfactants used in these experiments were: (i) Nonionic: Span 85, Tween 85, OMG (N-octanoyl –N-methyl-d-glucamine). (ii). Cationic**:** CTAB (cetyl trimethyl ammonium bromide). (iii). Anionic**:** AOT (Bis- (2-ethylhexyl) sulfosuccinate sodium salt). The yields and protein content of SCLs, as well as the esterification yields, were presented in Table 1, which indicated that the esterification yield depends on the nature of surfactant used to coat the lipase. Span 85 produced the highest esterification yield of 93 % after 72h. The Tween 85 and CTAB were able to coat surface layer of lipase, but protein content was found to be low, which in turn resulted in poorest esterification yields (12 and 6.7 %) whereas AOT and OMG were unable to coat the surface of lipase. These results clearly demonstrated that the charged surfactant interacts through attractive and repulsive forces with the electric charges present on the enzyme surface and this factor drives or alters the stereo configuration of the enzyme and results in the decrease of lipase activity. Therefore, the weak interaction between the non-ionic surfactant and the lipase-like Van der Waals forces, favor the maintenance of the stereo configuration

Table 1: Effect of different types of surfactants on the preparation of SCL and esterification yields.

Surfactant	SCL yield(%)	Protein content (%)	Ester yield(%, 72 h)
Span 85	25.45	12.56	93
Tween 85	13.45	6.78	12
AOT	0.1	0	0
OMG	0.2	0	0
CTAB	19.45	10.34	6.7

Table 2: Effect of pH on the preparation of surfactant coated lipase and the esterification yield.

pH	SCL yield (%)	Protein Content (%)	Esterification Yield (%, 72 h)
4.0	23.45	11.05	72
5.2	*25.45*	*12.56*	*93*
7.6	28.56	14.32	78.76
8.0	32.12	13.67	56.66
10.0	22.23	5.67	13.56

of the lipase of catalytic performance [23], thus producing the highest yields.

Effect of aqueous pH on the preparation of SCL

The pH of the micro-aqueous layer inside the SCL played a major role in controlling the lipase activity. Experiments were conducted at different pH ranges between 4.0-10.0 for the preparation of SCL and compared with esterification yield. The results were shown in Table 2. A wide distribution in SCL yield from 22 to 32 % at various pH values was found. This range in SCL yield could be attributed to the change in net charges possessed by the lipase. Protein content ranged from 11.05 to 13.65 %. However, at pH 10.0, the protein content was found to be very low. The highest esterification yield after 72 h (93 %) was achieved at a pH of 5.2 and the esterification, yield was reduced to 73% at pH 4.0.

Effect of lipase/surfactant ratio

The ratio of lipase/surfactant produced a remarkable effect on the yield of SCL, protein recovery and esterification yield. The yield of SCL was only 19.87% for a lipase/surfactant ratio of 4 whereas, at a ratio of 0.5, the yield increased to 34.67% (Table 3). The yield of SCL increased as the lipase/surfactant ratio was decreased. Protein content, in contrast, increased with the lipase/ surfactant ratio. This might be attributed to the formation of thicker surfactant loadings. Larger particles increase the yield of SCL.

A series of experiments were performed to determine the factors affecting the esterification efficiency with the surface-coated lipase (span 85) at a ratio of 2/1 (lipase to surfactant) at 5.6 pH using *Candida rugosa* lipase.

Effect of Organic Solvent

The nature of organic solvent affects the enzymatic synthesis by causing inactivation/inhibition by directly interacting with the enzyme or with the diffusible substrates/product, and by direct interaction with the water layer at the vicinity of the enzyme [26]. *Laane et al.*, [27] had proposed the log *P* (logarithm of the partition coefficient of the solvent between the octanol-water two-phase system) of an organic solvent for its selection in biocatalytic reactions. Esterification results of ethanol and hexanoic acid using surface-coated lipase from *Candida cylindracea* in different solvents at 37°C for 96 h of incubation time was compared.

A progressive increase in percent esterification had been observed with an increase in log P of the solvent. In more hydrophilic solvents such as dioxane, acetonitrile, tetrahydrofuran, and diethyl ether, esterification yields drastically decreased (< 6%). This downtrend could be attributed to the dehydration of enzyme owing to high

Table 3: Effect of Lipase to surfactant ratio [L/S].

L/S ratio (w/w)	SCL yield (%)	Protein Content (%)	Esterification yield (%)
0.5	34.67	7.8	70
1	30	10.23	76
2	25.45	12.56	93
4	19.87	13.45	95

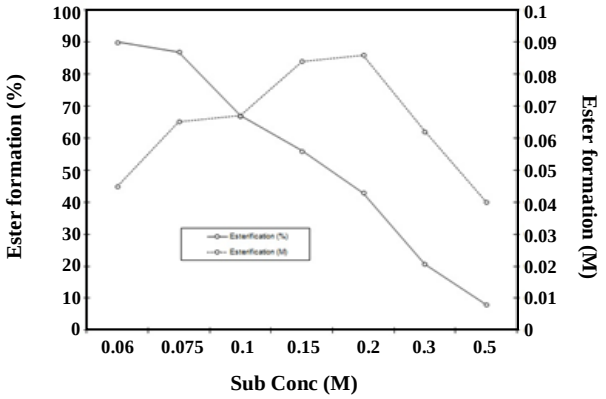

Fig. 2: Effect of substrate concentration on the synthesis of ethyl hexanoate using surface coated lipase from CRL at 10 g/L, 40°C and 72 h of incubation time.

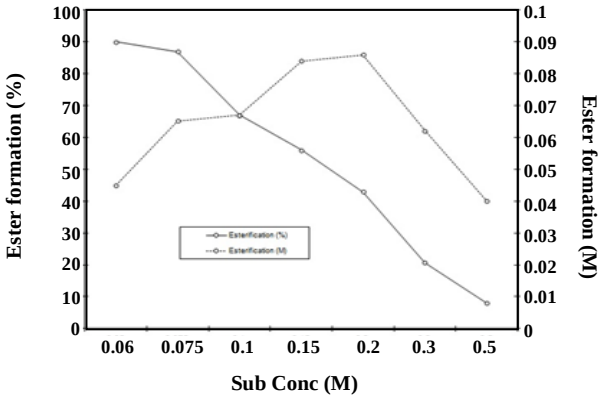

Fig. 3: Effect of enzyme concentration on the synthesis of ethyl hexanoate using surface coated lipase from CRL at 0.5 M substrate concentration, 40°C and 72 h of incubation time.

polar nature of solvents stripping the surrounding micro-aqueous layer. In hexane, esterification was very effective and reached to a maximum of more than 90 %. While in other non-polar solvents, the esterification yields ranged between 45 to 90 %. This suggested that biocatalysis with polar substrate might be impeded in relatively non-polar organic media by the lack of substrate solubility [27]. These results suggested that non-polar solvents were more supportive of biocatalysis. However, there was no general trend of higher activity at higher log P value.

Effect of Substrate and Enzyme Concentrations

The effect of substrate and enzyme concentrations on the synthesis of ethyl hexanoate was depicted in Fig. 2 and Fig. 3 respectively.

In these set of experiments, stiochiometric proportions of alcohol and acid were employed in *n*-hexane at 100 mg SCL (10 % protein). Esterification was found to decrease with increasing substrate concentration and higher substrate concentrations led to lower yields. The maximum percent ester (90 %) was noticed at 0.05 M substrate concentration and esterification yields dropped drastically to 6 % at 0.5 M substrate concentration. The lower esterification at higher substrate concentrations could be attributed to the accumulation of water during the progress of the reaction, which favors backward reaction (hydrolysis) or the possible substrate inhibitions. In this context, alcohols were reported to be dead-end inhibitors of lipases [26,27] and acids might cause acidification of the micro-aqueous interface leading to enzyme inactivation. However, the acidification of the micro-aqueous interface might be less pronounced in the present case, since hexanoic acid is more hydrophobic than the other low molecular weight acids like acetic and propionic acids, and would accumulate to a lesser extent in the micro-aqueous interface. When observed in terms of ester concentration, an increase in ester concentration was observed up to 0.2 M substrate (where a maximum ester product was obtained), after which ester concentration remained nearly constant. The increase in ester concentration was 0.045 at 0.05 M substrate concentration to 0.086 M at 0.2 M substrate concentration. With a further increase in substrate concentration, the ester concentration reduced to 0.01 M

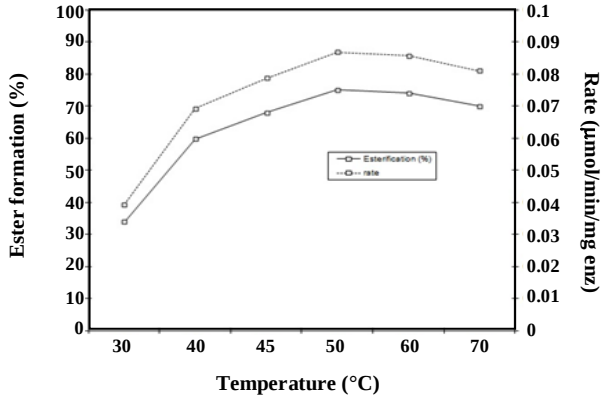

Fig. 4: Effect of Temperature on the synthesis of ethyl hexanoate using surface coated lipase from CRL at 20 g/L, 0.5 M substrate concentration and 72 h of incubation time.

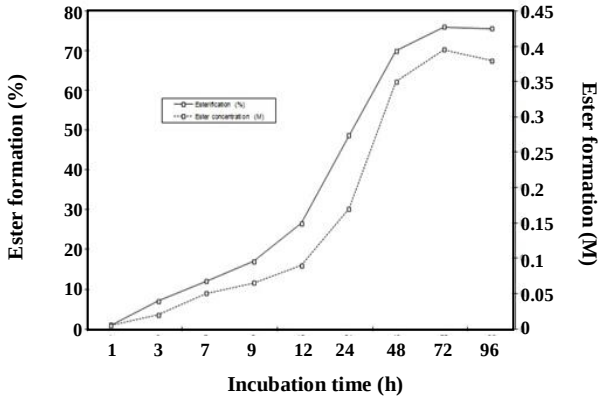

Fig. 5: Effect of incubation time on the synthesis of ethyl hexanoate using surface coated lipase from CRL at 20 g/l, 50°C and 0.5 M substrate concentration.

at 0.5 M substrate concentration. Thus, both the percent esterification and ester formation were severely affected at higher substrate levels (Fig. 2). In order to increase the ester formation, the enzyme in the reaction mixture had been increased. The results were depicted in Fig. 4. As the SCL concentration increased in the media, the esterification yields increased from 20 % at 10 g/L to 76 % at 30 g/L. However, the rate of reaction consistently increased with an increase in enzyme loading from 0.239 at 10g/L to 0.346 at 20 g/l enzyme concentration, and later, it showed a downtrend at higher enzyme concentrations (0.293 µ mol/min/mg enzyme at 30 g/L enzyme concentration). Thus, from the above results, it could be concluded that an increase in enzyme concentration beyond optimum levels was not beneficial in terms of ester concentration. The possible reason for this was that the extra enzyme molecules (active sites) were not exposed to the substrate, and were only present inside the bulk of the reaction mixture with zero involvement [28]. Therefore, further experiments were carried out using the 0.5 M substrate concentration and 20 g/L enzyme concentration for 72 h of incubation time.

Effect of Temperature

The optimum temperature for a given enzymatic synthesis depends on the several physical parameters of enzyme, nature, and conditions of reaction media [30]. The effect of temperature on the behavior of the enzyme was studied at a substrate concentration of 0.5 M (1:1 ratio of ethanol and hexanoic acid) using 20 g/L enzyme concentration in hexane. From Fig. 4 it was evident that, as the temperature in the reaction increased, esterification increased linearly up to 50°C (75 %) and a further increase in temperature resulted in the decrease in esterification yields (70 % at 70°C). The ester concentration was maximum at 50°C (0.434 M) and decreased to 0.405 M at 70°C. These results indicated that the temperature had a profound effect on biocatalyst. The marginal drop in esterification yields at high temperatures could be attributed to the inactivation of the enzyme at this temperature. However, there were reports in which lipases sustained even at a temperature of 90°C, in organic solvents [32]. Therefore, the stability of lipases at higher temperatures was related to the reaction conditions in which they were used. Choosing 50°C as the optimum, the influence of the molar ratio was investigated.

Effect of incubation time

The extent of esterification was studied using 0.5 M substrate concentration and 20 g/L enzyme concentration at 50°C as a function of incubation time. The results are shown in Fig. 5 indicated a sigmoid curve. The esterification was slightly linear up to first 12 h of incubation time. A drastic increase in ester formation was observed between 12 to 72 h of incubation time. This effect could be attributed to the released by-product (water), which could accelerate the enzyme by providing the sufficient micro- aqueous phase that would stimulate the rate of formation of the product. Such an effect of acceleration due to a by-product had been reported [33]. Beyond 72 h of incubation time, saturation in ester yields

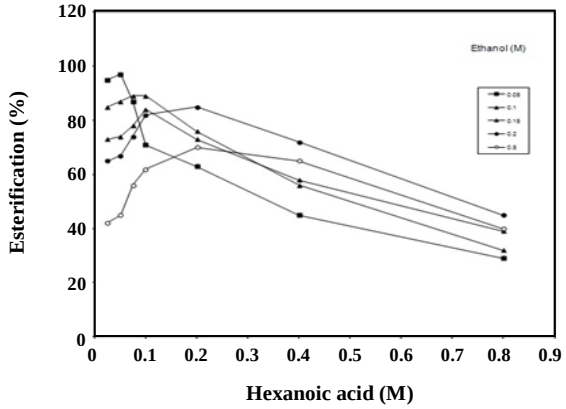

Fig. 6: The percent esterification at various ethanol concentrations as a function of hexanoic acid at 20 g/L surface coated lipase, 50°C and 72 h of incubation time.

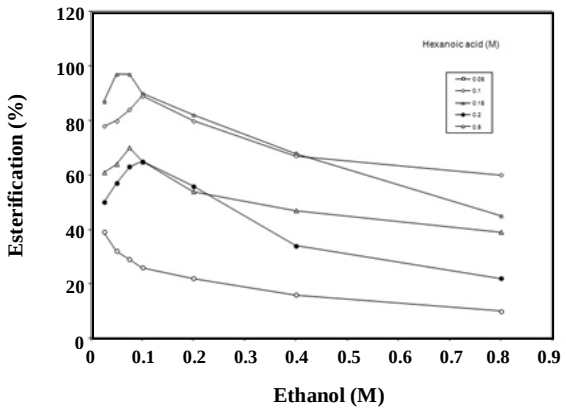

Fig. 7: The percent esterification at various hexanoic acid concentrations as a function of ethanol at 20 g/L surface coated lipase, 50°C and 72 h of incubation time.

was observed up to 120 h of incubation time. The maximum esterification (76 %) and ester concentration (0.395 M) were attained at 72 h of incubation time.

Effect of Substrate molar ratio

The equilibrium of the reaction could be pushed in the forward direction by increasing the nucleophile concentration [34]. However, high alcohol concentration might slow the rate of reactions. Therefore, it is necessary to optimize the actual excess nucleophile concentration to be employed in a given reaction. The effect of acid to alcohol and alcohol to acid molar ratio were investigated. Fig. 6 aqaqx depicts the esterification at fixed ethanol concentrations (0.05, 0.1, 0.15, 0.2 and 0.5 M) as a function of hexanoic acid (from 0.025 to 0.8 M) at 50^0C for 72 h of incubation time. The results indicated that for any given ratios of alcohol to acid, there was a decrease in percent esterification throughout the concentration ranges studied. Esterification drastically decreased at 0.8 M hexanoic acid for any given alcohol concentration employed. These results suggested an acid inhibition in the reaction. Similarly, the esterification at fixed concentrations of hexanoic acid was studied as a function of ethanol concentration (0.025 M to 0.8 M). A similar effect was observed upon increasing the ethanol concentration in the reaction. Esterification increased up to 1:1 ratio of both the substrates and reduced constantly with increasing ethanol concentration (Fig. 7). The inhibition was much higher at higher substrate concentrations.

Hence, no beneficial effects were observed beyond 1:1 molar ratio of alcohol to acid and 1:1 molar ratio of acid to alcohol and in fact, enzyme activity had gradually reduced upon increasing either of these ratios. These results suggested the inhibitory effect of acid and alcohol. Several observations on alcohol inhibition had been reported for lipase catalysis. Synthesis of ethyl esters had been affected by increasing ethanol concentration [35-37]. The inhibitory effect of isoamyl alcohol had been studied in a transesterification reaction [38]. In the present study, the inhibitory effect of ethanol and hexanoic acid in the synthesis of ethyl hexanoate had been observed.

Kinetic study

The important data required for the development of a suitable reactor was the kinetic information on the product formation and influence of changes in the system. Kinetic models, rate expressions and the reaction mechanism had been reported for the lipase-catalyzed reactions [39,40]. Several authors had reported that lipase-catalyzed esterification in organic solvents could be described by the Ping-Pong Bi-Bi model with substrate inhibition [29,37,38,41]. In these kinetic studies, the important aspects were reversibility of reaction, substrate inhibition, and presence of mass transfer limitations.

Determination of Kinetic parameters

Kinetic study for the synthesis of ethyl hexanoate had been carried out at different substrate concentrations. Based on the results from the studies of the effect of substrate molar ratio, it was found that

at a higher molar ratio of alcohol and acid concentrations, the reaction ratewas found to be decreased. The kinetic constants of the esterification reaction were determined by a reaction rate analysis

The kinetic constants V_{max} and K_m were estimated by plotting the values of the reaction rate *versus* substrate concentration. From these plots, the double reciprocal initial rates of esterification *versus* reciprocal substrate concentration had been constructed. These plots indicated a set of parallel lines at low substrate concentrations. As the substrate concentrations increased, the slope increased and the value of intercept [$1/V_{max}$] decreased. The values of the apparent kinetic parameters were computed as: V_{max}= 0.146 µmol/min/mg enzyme; $K_{M,\ Acid}$ = 0.296 M; $K_{M,\ Alcohol}$ = 0.1388 M; $K_{i,\ Acid}$ = 0.40 M; and $K_{i,\ Alcohol}$ = 0.309 M. These results suggested an assumed Ping-Pong Bi-Bi mechanism with typical competitive inhibition by both the substrates [42,43]. The shapes of the curves indicated the inhibition at higher concentrations of ethanol and also the inhibition was much higher at lower hexanoic concentration. These results suggested that the binding of hexanoic acid (K_m = 0.296 M) could be less stronger than the binding of ethanol (Km = 0.1388 M) to the lipase. The lack of binding of hexanoic acid to the acyl-enzyme complex may be an effect of steric interaction by the carbonyl group [41]. This kind of inhibition had been reported earlier in the acyl transfer reaction catalyzed by lipase B from *Candida antarctica* in organic solvent systems with competitive inhibition by alcohol [42].

Candida antarctica lipase B has also been displayed on the yeast cell surface using the α-agglutinin and Flo1p (FS) anchor systems in *Pichia pastoris* as a biocatalyst for the production of ethyl hexanoate [44]. Recently, a comparison in the production of 12 flavor esters using CALB-displaying yeast whole cells with other studies has been reported. The results showed that this approach is promising for further large-scale production of flavor esters in nonaqueous media [45].

In general, the bi- substrate reactions catalyzed by the lipase followed a Ping-Pong Bi-Bi mechanism [46]. In this reaction sequence, using two substrates, there was probably an alternate release of two products (water and ester). With the binding of acid to the enzyme, first product water was released, followed by which alcohol bound to the enzyme complex and the second product (ester) was released.

CONCLUSIONS

The synthesis of ethyl hexanoate, SCL from *candida rugosa* and *Mucor* sp. showed highest esterification activity when compared to lipases from other sources. It could be concluded from these results that CRL and mucor sp. were very selective towards the synthesis of short chain esters. Lipase coated with span 85 was protected from denaturation in organic solvents. pH has a significant effect on ester synthesis. The yield of SCL increased as the lipase/surfactant ratio decreased. Protein content, in contrast, increased with the lipase/surfactant ratio, and thus, the esterification.

Esterification was found to decrease with increasing substrate concentration in both cases (SCL and microemulsions). Even an increase in enzyme concentration had no significant influence on percent esterification. Optimum reaction temperature found was 50ºC for SCL ester synthesis and 40ºC for microemulsions. The conclusion drawn from these results was that the optimum temperature of the lipase variation depends upon the conditions in which they were used. Kinetic study revealed that both ethanol and hexanoic acids were found to be inhibitory to the CRL enzyme in both the systems. However, ethanol was effecting more than the hexanoic acid. In both the systems, inhibitory pattern follows Ping-Pong Bi-Bi mechanism. Water removal is mandatory during the course of the reaction to enhance the yields. The synthesis of ethyl hexanoate in the solvent-free medium was feasible mainly because the SCL was protected from denaturation by ethanol.

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