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# Enhancement in synthesis of ethyl laurate catalyzed by fermase by combined effect of ultrasound and stage wise addition of ethanol

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ABSTRACT

The current work focuses on the intensification of fermase catalysed synthesis of ethyl laurate from ethanol and lauric acid in a solvent free condition with ultrasound and stage wise ethanol addition. The effect of molar ratio, stepwise addition of ethanol, catalyst loading, speed of agitation, temperature, ultrasound power and duty cycle was systematically studied. The optimal conditions were found as 1:2 lauric acid:ethanol mole ratio, 2% enzyme loading, 50 °C temperature, 40 min reaction time, 100 W power with 50% duty cycle. The highest conversion of 96.87% was obtained for stage wise addition of ethanol in presence of ultrasound and mechanical stirring. Thermodynamic study showed decrease in the value of enthalpy (ΔH), entropy (ΔS), free energy (ΔG) and activation energy (E) for ultrasound with stirring than that of ultrasound alone and conventional stirring. The thermodynamic study reveals that the combination of ultrasound and stirring is more effective as contrasted the ultrasound without stirring and mechanical stirring method. Application of ultrasound irradiation significantly decreased the time of reaction process when equated with mechanical stirring method. Under optimized conditions, the continuous use of fermase enzyme for repetitive cycles causes loss in percentage conversion of the reaction.

## 1. Introduction

Enzyme based synthesis of fatty acid ethyl esters provides greener route alternative to conventional chemical synthesis [1]. Short chain fatty acid esters show numerous applications in food, flavours, cosmetics, and pharmaceuticals while long chain fatty acid esters are used as biodiesel and as waxes in the oleo chemical industries [2]. Ethyl laurate is one of the short chain ethyl esters; that can be formed by esterification reaction of ethanol with lauric acid. Conventionally flavours have been obtained by the extraction from natural sources with low yield and therefore the high cost for commercial use. There are several reports available on synthesis of ethyl esters with inorganic catalyst at different temperature range (303 K–533 K), based upon the type of catalyst used [3–6]. These short chain flavours can also be produced by the use of strong alkali, acid and inorganic catalysts at high temperature [7,8]. However, these esters are not considered as "natural flavours" and use of chemical catalyst also generates waste. On the other hand, enzyme catalysed synthesis of esters is considered as natural flavours, with subsequent economic advantages [9]. Lipase mediated ester can also be synthesized in batch and continuous manner at low temperature and pressure [10–12]. The principle gain of immobilised enzyme suggests excessive catalytic activity even after various cycles of reusability and also a separation of catalyst is easy which reduces the manufacturing cost. However, strategies of enzymatic nature normally provide lower yields in comparison to the conventional chemical approaches. Therefore, the impact of low energy ultrasound has been extensively used in recent years to increase the rate of enzymatic schemes [13,14]. For enzymatic reaction conditions, ultrasound has been developed as a spontaneous source of homogenizing strength with significant applications. Ultrasound irradiation (UI) enhances the substrate dissolution and improves mass transfer in a system through cavitation as a consequence of the breakdown of bubbles which improves the enzymatic esterification reactions. When ultrasound propagates through the liquid medium, it induces the compression and rare fraction. The successive breakdown of cavitation bubbles may generate frequent physical effects such as micro jets, shock waves, shear forces and turbulence, which produce tremendous local heat and pressure in the reactor simultaneously which enhances the rate of mass transfer [15]. In the case of heterogeneous system, the quick collapse nearby the solid surface can result in high intensity shock waves which results in direct contact of enzyme surface with the substrate by decreasing the mass transfer limitations. In comparison to other traditional methods, ultrasound helps to improve the rate of mass transfer by physical turbulence in a solvent free condition [16,17]. As a popular

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biotechnological strategies, low frequency ultrasound has been used for a couple of enzymatic reactions, wastewater treatment and biofuels producing, with wonderful outcomes [18,19].

There are a few studies accounted on the enzyme catalysed synthesis of short chain ethyl esters [11,20–22]. Many researchers suggested the instability of protein in the presence of short chain alcohols such as ethanol and methanol. Shimada et al. examined the inactivation of immobilised *C. antarctica* lipase by ethanol in vegetable oil ethanolysis [23]. The lipase hindrance by alcohol was additionally explored by Ghamgui et al. in butyl oleate combination [24]. The polar substrate (alcohol) created denaturation of lipase, recommending a stepwise addition of ethanol to maintain a strategic distance from lipase deactivation because of the high initial amount of ethanol [25,26]. In earlier studies, the synthesis of ethyl laurate by hydrogel immobilised lipase of Bacillus coagulans MTCC-6375 gave 66% conversion in 15 h in presence of solvent nonane [27]. While Solarte et al. showed 95% yield of ethyl laurate in hexane by whole cell lipases in 24 h of reaction time [28]. These conventional synthesis of ethyl laurate reported major challenge for reduction of solvent and time requirement of reaction. Thus, recent studies are mostly focused on solvent free synthesis that is defined as the condition where reactant itself acts as a solvent, but in these systems, the rates of reactions are mass transfer limited [29–31]. In the previous study, we reported 92.46% conversion of ethyl laurate in 4 h and also demonstrated the Ordered Bi-Bi mechanism with the inhibitory effect of ethanol on Fermase [20]. Therefore, in order to reduce the reaction time and solvent inhibitory effect, a comprehensive study of ultrasound and stepwise addition of ethanol along with other parameters was performed for the solvent-free esterification of lauric acid and ethanol using fermase CALB 10000 as a catalyst.

## 2. Material and methods

### 2.1. Material

The commercial lipase Fermase CALB 10000 from *Candida Antartica* immobilised on polyacrylate beads (particle size 200–400 µm, bulk density of 0.453 g/cm$^3$, pore volume of 1.31 cm$^3$ g$^{-1}$) was supplied by Fermenta Biotech. (Mumbai, India). Molecular sieves (3 Å) and potassium hydroxide were purchased from Thomas Baker Pvt. Ltd., India. The lauric acid, hexane, and other chemicals were of analytical grade and purchased from Sigma–Aldrich (India). The phenolphthalein indicator and acetone were purchased from Himedia Laboratories Pvt. Ltd., Mumbai.

### 2.2. Enzymatic esterification reaction

The enzymatic esterification reaction was performed in a four baffled flat bottom reactor vessel of 50 mL capacity. The reactor was dipped in a preheated water bath and three blade turbine impeller with motor assembly was fixed to reactor for mixing of the reaction mixture. Here, the molar concentrations of the reactants can be expressed in volume as the pure ethanol having 17.12 M and pure lauric acid having 5.02 M were used directly without any solvent. In order to maintain the ratio of 2:1, ethanol (6.32 mol) to lauric acid (3.16 mol) i.e. 5.83 cm$^3$ of ethanol and 9.95 cm$^3$ of lauric acid, respectively were added into reactor resulting in a total volume of reaction as 15.77 cm$^3$. To initiate the reaction, the enzyme was further added to the reaction mixture. The samples were taken out from the reaction mixture at 10 min time intervals and titrated to evaluate percentage conversion of reaction. The different parameters such as temperature, enzyme loading, molar ratio were optimised.

### 2.3. Ultrasound assisted enzymatic esterification reaction

The fermase catalysed esterification reaction was carried out in an ultrasonic bath (Model 6.5l200H, Dakshin Pvt. Ltd., Mumbai, India) with a working frequency 25 kHz and 40 kHz and maximum input power of 210 W. The rectangular ultrasonic bath of 300 × 150 × 150 mm internal dimensions was provided with four transducers at the bottom of the bath. The ethanol and lauric acid were added into same three baffled flat bottom reactor vessel of 50 mL capacity used in the conventional reaction. The reactor vessel was positioned in the ultrasonic bath as described in the earlier literature [32]. The temperature of the reaction mixture was maintained at constant value by circulating water in bath. The percentage conversion of esterification was observed by determining the unconsumed acid content of the reaction by titrating the reaction samples with KOH (0.01 M) using phenolphthalein as an indicator. The ethanol was used as a quenching agent.

### 2.4. Lipase activity

Lipase activity was determined according to protocol described by Hari Krishna et al. [33]. A 85.95 mL of *n*-heptane stock solution was prepared which contained 1.35 mL of butyric acid (0.16 M) and 2.7 mL butanol (0.33 M). A known amount of enzyme was added to 3 mL of stock solution in the flask and incubated at 60 °C in the shaker for 1 h at 150 RPM. A flask without enzyme was considered as a blank that was used to calculate the relative activity of the enzyme. The mixture was quenched with 1 mL of methanol after 1 h and titrated with 0.02 M NaOH solution with phenolphthalein indicator. The activity of enzyme (A) is calculated by Eq. (1)

$$A = \frac{(V_0 - V) \times M \times 100}{W \times T} \quad (1)$$

Where V is the volume of sample, $V_0$ is the volume of blank, M is the molarity of NaOH, W is the amount of enzyme and T is the time (min) required for incubation. The activity of the untreated Fermase CALB 10000 lipase obtained was 8.32 U.

### 2.5. Determination of kinetic and thermodynamic parameters

In the current work, the pseudo first order kinetics was used to determine the kinetic parameters. The effect of temperature on the forward reaction rate constant, k, was evaluated by using Arrhenius equation [34]:

$$K = A\ exp\left[-\frac{E_a}{RT}\right] \quad (2)$$

and

$$lnk = \left[-\frac{E_a}{RT}\right] + lnA \quad (3)$$

Where k is the kinetic constant, R is the gas constant 8.314 (J mol$^{-1}$ K$^{-1}$), T is the absolute temperature (K), A is the Arrhenius pre-exponential factor (min$^{-1}$) and $E_a$ is the activation energy (kJ mol$^{-1}$). The Arrhenius plot correlates the logarithm of the kinetic constant of the reaction [ln (k)] with the inverse of temperature (1/T). By the use of Eyring equation of transition state theory (Eq. (3)) and some basic equations (Eqs. (4) and (5)), it is possible to estimate the enthalpy change (ΔH), the entropy change (ΔS) and Gibbs free energy change (ΔG) [35].

$$\ln\frac{k}{T} = \left[\frac{-\Delta H}{R}\right]\left[\frac{1}{T}\right] + \ln\left[\frac{k_B}{h}\right] + \frac{\Delta S}{R} \quad (4)$$

$$\Delta H = Ea - RT \quad (5)$$

$$\Delta G = \Delta H - T\Delta S \quad (6)$$

Where $k_B$ is the Boltzmann constant (J K$^{-1}$) and h is the Planck's constant (J S).