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# Green Synthesis of Isoamyl Acetate via Silica Immobilized Novel Thermophilic Lipase from *Bacillus aerius*[1]

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**Abstract**—Isoamyl acetate, a pear or banana flavor, is widely used in food, beverage, cosmetic, and pharmaceutical industries. In the present work, lipase from *Bacillus aerius* was immobilized on silica gel matrix in the presence of a cross-linking agent, glutaraldehyde, and its efficiency in synthesizing isoamyl acetate using esterification reaction was studied. The esterification of acetic acid and isoamyl alcohol by silica-bound lipase was studied as a function of time and temperatures. The incubation time of 10 h, temperature of 55°C, substrate molar ratio 1 : 1, and the amount of lipase as 1% were found to be optimal for the esterification reaction. The bound lipase catalyzed the esterification of acetic acid by isoamyl alcohol with the yield of about 68% under the optimized reaction conditions. The product was identified as isoamyl acetate using gas-liquid chromatography, nuclear magnetic resonance, and Fourier transform IR spectroscopy analysis by the presence of an ester group at the wavenumber of 1720.5 $cm^{-1}$.

*Keywords: lipase, immobilization, esterification, isoamyl acetate*

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## INTRODUCTION

Lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) is an efficient enzyme with a broad variety of applications in the food industry, fine chemistry, and pharmaceutical industry. It catalyzes the hydrolysis of triacylglycerol to glycerol and fatty acids [1]. Lipases are successfully used for the synthesis of esters. Flavor esters are synthesized by the reaction between short chain acids and alcohols.

Though esters are commonly produced using chemical catalysts (acidic or basic by their nature) there are certain limitations which restrict the usage of these catalysts, such as the necessity to remove inorganic salts, high temperature, and side reactions occurring along with the esterification [2]. Due to enzyme specificity, the enzymatic catalysis ensures formation of pure products. The cost of lipase production is one of the main obstacles for industrial application of lipases. Immobilization of lipases decreases the cost of production owing to their reusability, that makes them more potent and attractive for industrial applications, together with better thermal stability, activity in non-aqueous media, improved handling, recovery, and recycling the biocatalyst [3]. Previously, isoamyl acetate was synthesized using *Rhizomucor miehei* lipase immobilized on macroporous weak anionic exchange resin beads [4] and *Rhizopus oryzae* NRRL 3562 lipase immobilized on silica gel matrix [5].

Isoamyl acetate (3-methyl-1-butyl ethanoate) is often called the essence of pear or banana and found naturally in these fruits. It has a highly characteristic smell and is one of the most frequently used fragrance material in food, beverage, cosmetic, pharmaceutical industries, and household products. Due to its strong odor even at low concentrations and low toxicity, isoamyl acetate is well known as a chemical used for testing gas masks for tightness [6]. Isoamyl acetate is traditionally produced by extraction of banana flavor from natural sources, which results in difficulties with its supply. Though the fermentation was proposed as the alternative way, but, in the practice, this method turned out to be too scarce and expensive for commercial exploitation [7].

The enzyme catalysis in industrial applications requires the development of efficient processing methods and optimization of reaction conditions. In the present work, the extracellular thermoalkaliphilic purified lipase of *Bacillus aerius* has been immobilized by adsorption onto activated silica matrix using glutaraldehyde as a crosslinking agent. Immobilized enzyme was then used for synthesis of isoamyl acetate by esterification of acetic acid with isoamyl alcohol. The effects of incubation time, reaction temperature, relative molar concentration of reactants, and enzyme concentration on the rate of synthesis of isoamyl acetate were separately evaluated. The synthesized ester

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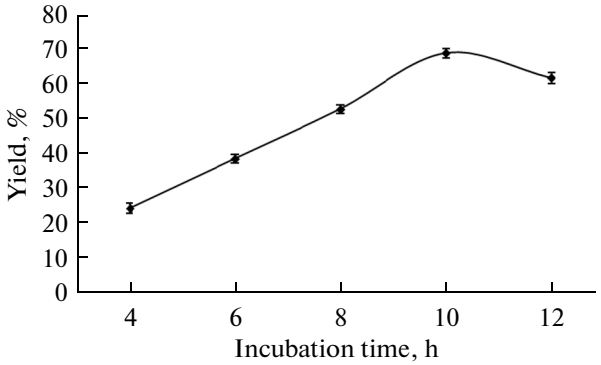


**Fig. 1.** Time-course of isoamyl acetate synthesis at 55°C. Reaction conditions: acetic acid/isoamyl alcohol ratio of 1 : 1 (mol/mol); 1% lipase by acid weight.

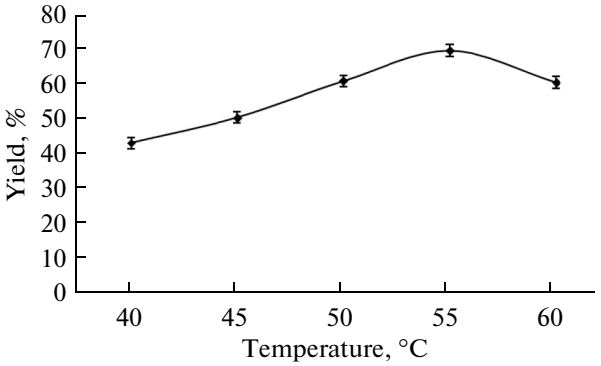


**Fig. 2.** Effect of temperature on the synthesis of isoamyl acetate. Reaction conditions: reaction time 10 h, acetic acid/isoamyl alcohol ratio of 1 : 1 (mol/mol), and 1% lipase by acid weight.

was characterized by FT-IR, NMR, and GLC techniques.

## RESULTS AND DISCUSSION

### *Immobilization of the Lipase onto Silica*

Lipase from *Bacillus aerius* gave maximum binding efficiency of 75.64% with 1.44 U/mg of enzyme activity on silica matrix when cross-linked with 4% of glutaraldehyde solution (v/v). The immobilization kinetics revealed that the lipase was optimally immobilized on silica matrix within 2 h of incubation. Previously, the binding efficiency of 75% was observed when lipase from *Bacillus coagulans* was immobilized on nylon-6 [8].

### *Effect of Incubation Time*

The study of the time course of an enzymatic reaction is a good indicator of enzyme performance and reaction progress. It can pinpoint the shortest or adequate time necessary to obtain a good yield and minimize the process cost. The yield of isoamyl acetate increased steeply with the time during early stages of the esterification reaction to attain the major part of the final conversion in 10 h (Fig. 1). At 10 h, approximately 68.55% of ester was produced. Thus, in the subsequent experiments, a reaction time of 10 h was considered the optimum for the ester synthesis at 55°C for the immobilized-lipase. Longer reaction time leads to the reduction of ester formation because of the esterification reaction reversal [9]. Kumar et al. found optimum incubation time of 12 h for the synthesis of ethyl propionate using lipase from *Bacillus coagulans* BTS-3 [10]. A reaction time of 3 h for immobilized lipase from *Bacillus coagulans* BTS-3 was considered optimum for synthesis of 4-nitrophenyl acetate [11]. In a previous study, immobilization of *Pseudomonas aeruginosa* lipase onto a synthetic poly-(AAc-*co*-HPMA-*cl*-EGDMA) hydrogel catalyzed the esterification of acrylic acid with methanol into methyl acrylate in a shorter period of 6 h at 55°C [12].

### *Effect of Reaction Temperature*

Changes in the reaction temperature can affect the activity and stability of the enzyme and thus the rate of the reaction. The maximum ester synthesis (68.38%) was observed at 55°C (Fig. 2) under the above reaction conditions. A rise in temperature increases the activity, however, at high temperatures enzyme becomes denatured, so that its activity decreases. Gogoi and Dutta have found a reaction temperature of 65°C to be optimal for the synthesis of isoamyl acetate using immobilized lipase from *Mucor miehei* [6]. A reaction temperature of 65°C was considered optimal for synthesis of 4-nitrophenyl acetate by immobilized *Bacillus coagulans* lipase [12]. In another study, the optimal temperature for the synthesis of ethyl ferulate using celite-bound commercial lipase was found to be 45°C [13].

### *Effect of Relative Molar Concentration of the Reactants*

In esterification reactions, the rate of reaction can be pushed forward either by using excess molar quantities of nucleophile (alcohol) or by removing product from the reaction mixture. The equimolar ratio of acetic acid and isoamyl alcohol in a solvent-free system was optimal to yield isoamyl acetate (68.38%) in the reaction (Fig. 3). Kumar et al. have found the reactants molar ratio of 1 : 3 (ethanol : propionic acid) to be optimumal for the synthesis of ethyl propionate in hexane [10]. The optimal synthesis of butyl acetate by lipase from *Bacillus coagulans* immobilized on Nylon-6 was achieved when the acid and alcohol were used in equimolar ratio (at 100 mM) in the reaction mixture [8]. Earlier Kumari et al. have reported that the molar ratio of 3 : 1 was optimal for the synthesis of isoamyl acetate using lipase from *Rhizopus oryzae* [5].

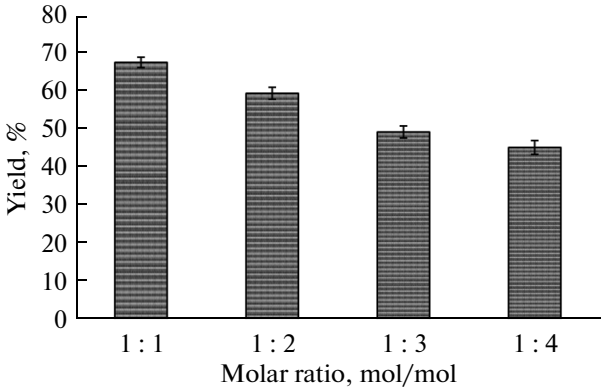


**Fig. 3.** The effect of acetic acid/isoamyl alcohol molar ratio on the esterification reaction. Reaction conditions: temperature 55°C, 1% lipase by acid weight, reaction time 10 h.

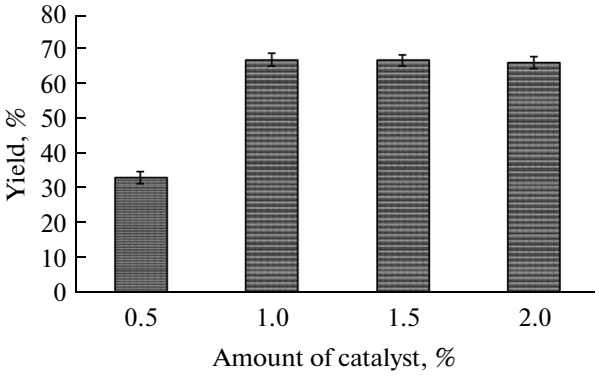


**Fig. 4.** Effect of lipase concentration on the synthesis of isoamyl acetate.

Reaction conditions: reaction time 10 h, temperature 55°C, acetic acid/isoamyl alcohol ratio of 1 : 1 (mol/mol).

### *Effect of the Biocatalyst Amount*

The amount of enzyme plays a crucial role in any biocatalytic process, especially for large scale production. The influence of this parameter on the reaction was therefore assessed to determine the minimal amount of the lipase required for achieving good yield of the product. We observed that with an increase of enzyme concentration the initial rate of esterification was increasing almost linearly at low lipase concentrations. Then the rate started to decrease slowly and after reaching the critical value it remained unchanged. The maximum yield of isoamyl acetate (68.38%) was obtained with 1% of immobilized lipase (Fig. 4). As reported for the synthesis of isoamyl acetate, 16% of immobilized lipase from *Rhizopus oryzae* was used to get maximal yield of the ester [5]. Recently it has been found that 5.78% of immobilized lipase from *Candida antarctica* was optimal for the synthesis of isoamyl acetate [7].

### *Reusability*

The residual activity of silica-bound lipase was calculated after every cycle to estimate the inactivation of the immobilized lipase. Silica-bound lipase retained almost 50% activity after five cycles (table). In a previous study, the lipase from *Bacillus coagulans* immobilized on a molecular sieve retained 58.5% of its original activity after five cycles of esterification [11]. The commercial lipase immobilized on silica gel matrix retained almost 52% activity after five cycles of reuse [14].

### *Spectral Data*

The structure of the target product isoamyl acetate was confirmed by the data of FT-IR spectra and $^1$H NMR spectrum. FT-IR spectrum showed the peaks at 1720.5 cm$^{-1}$ (–C=O stretching), 1246.9 cm$^{-1}$ (–C–O–C– stretching), and 2959.9 cm$^{-1}$ (–C–H stretching). The $^1$H NMR spectrum of the ester had peaks at 2.21 ppm due to methyl group protons confirming the presence of a –C=O group adjacent to these protons. Signals at 4.6, 1.52, and 1.81 ppm were due to protons of isoamyl group in the vicinity of the ester group. A doublet at 0.91 ppm was due to two protons in the same environment.

## EXPERIMENTAL

### *General*

Silica gel matrix (pore size 60–150 mesh), glutaraldehyde, *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl benzoate (*p*-NPB), *p*-nitrophenylformate (*p*-NPF), *p*-nitrophenyl palmitate (*p*-NPP), and acetic acid were purchased from Lancaster Synthesis, England; Tris buffer was from HIMEDIA Laboratory Ltd., Mumbai, India; and isoamyl alcohol was from MERCK, Mumbai, India. All chemicals were of analytical grade and were used as received.

### *Preparation of Lipase from Bacillus aerius*

The lipase producing bacteria were isolated from the soil and water samples of a hot-spring named Tattapani, Kullu, and sweet shops around Shimla. The thermophilic *Bacillus aerius* (identified at IMTECH,

Reusability of immobilized lipase in ester synthesis

| Cycle No. | Relative yield, % |
|---|---|
| 1 | 100 |
| 2 | 83.24 |
| 3 | 71.57 |
| 4 | 63.21 |
| 5 | 50.18 |
| 6 | 41.98 |

Chandigarh) was grown in the medium of the following composition (g/L): yeast extract, 2.0; peptone, 5.0; sodium chloride, 5.0; beef extract, 1.5; and ammonium chloride, 1.0, supplemented with cottonseed oil (10 mL/L) emulsified with 0.5% Gum arabic) at pH 8.5. The seed culture (7.5% inoculum) was transferred into 50 mL production medium (250-mL Erlenmeyer flask) and kept for 48 h under shaking conditions at 110 rpm at 55°C. The culture broth was centrifuged at 10000 rpm for 10 min at 4°C. The lipase activity was assayed both in the supernatant and pellet for determining extracellular and intracellular enzyme activity, respectively. The enzyme produced by thermophilic *Bacillus aerius* was purified to 9-fold with 7.2% recovery by ammonium sulfate precipitation and chromatography on a DEAE-cellulose column (size 12 × 2 cm) activated with 0.1 N NaOH and 0.1 N NaCl. Subsequently the column was equilibrated with 0.1 M Tris-HCl buffer (pH 9.5). The dialyzed sample (3 mL) was loaded on the column and eluted with 0.1 M Tris-HCl (pH 9.5) containing NaCl gradient of 0.3, 0.5, 0.7, and 1 M. According to the data of SDS-PAGE, the enzyme was obtained in a monomeric form with molecular weight of 33 kDa.

### *Determination of Lipase Activity*

The activity of free and silica-bound lipase was measured by a colorimetric method [15]. The reaction mixture (3 mL) contained 60 μL of *p*-nitrophenol palmitate (*p*-NPP) stock solution (20 mM *p*-NPP) prepared in isopropyl alcohol) and 40 μL lipase and Tris buffer (0.1 M, pH 9.5). The mixture was incubated at 55°C for 10 min on a water bath. The reaction was stopped by cooling the reaction mixture at −20°C for 10 min. The absorbance of p-nitrophenol released was measured at 410 nm. The enzyme activity was defined as the micromoles of *p*-nitrophenol released per minute on hydrolysis of *p*-NPP by 1 mL of free enzyme or by 1 mg of silica-bound enzyme (weight of matrix included) under standard assay conditions. The protein was assayed by a standard method [16].

### *Immobilization of Lipase onto Silica*

Two grams of silica gel matrix (60–150 mesh) was washed with 0.1 M Tris buffer (pH 7.0) and then centrifuged at 10000 rpm at 4°C for 10 min. The supernatant was discarded and the pellet was washed out 4–5 times with Tris buffer. The matrix was then kept at 4°C overnight in Tris buffer. Then 1–5% (v/v) glutaraldehyde solution was added to the matrix and the mixture was kept at 55°C under shaking for different time periods (1–5 h). The matrix was washed 3–4 times with Tris buffer (pH 7.0) to remove unbound glutaraldehyde. Four milliliter of lipase from *Bacillus aerius* (1.91 U/mL) was then incubated with the matrix for 1 h under shaking condition. The supernatant was discarded.

### *Esterification Reaction*

Isoamyl alcohol (1 M) and acetic acid (1 M) were taken in a screw-capped glass vial. To this mixture, the enzyme (*Bacilus aerius* lipase, ~1.44 U/mg) was added as a catalyst and the reaction mixture was incubated with constant shaking. The effect of reaction time (4, 6, 8, 10, and 12 h), temperature (40 to 60°C), acetic acid to isoamyl alcohol molar ratio (1 : 1 to 1 : 4) and immobilized lipase amount (1–4% of acid weight) on ester synthesis were investigated in a solvent free reaction system. The reaction mixture was washed with hot water to remove the reactants using separating funnel. The silica bound lipase of *Bacillus aerius* was used repetitively upto 6th cycle of esterification. After the first cycle of reaction, the immobilized lipase was recovered (by centrifuging and decanting the reaction mixture) and this biocatalyst was used to catalyze the fresh reaction.

The ester was quantified by FT-IR and NMR spectroscopy and GLC. FT-IR spectra were recorded on a Nicollet 5700 apparatus in KBr pellets. $^1$H NMR spectrum was registered on an Advance Bruker II-400 MHz in deuterated chloroform ($CDCl_3$) solution with TMS as an internal standard (0 ppm) and chemical shifts recorded in parts per million (δ, ppm). GLC apparatus Chrom WHP was equipped with a flame ionization detector and a column (10% SE-30, 2 m, mesh size 80–100, internal diameter 1/8 inches, maximum temperature limit 300°C; Netel Chromatograph, Thane, India); nitrogen was used as a carrier gas (30 $cm^3$/min); the injector was warmed to 250°C and the detector was set at 280°C. The quantification was accomplished using isoamyl acetate as internal standard. The ester yield was determined using GLC as follows:

$$\%\ yield = \frac{Weight\ of\ ester\ formed\ (g)}{Total\ weight\ of\ reaction\ mixture\ (g)} \times 100.$$

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