ISSN 1330-9862
(FTB-1993)

original scientific paper

Lipase Mediated Isoamyl Acetate Synthesis in Solvent-Free System Using Vinyl Acetate as Acyl Donor

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Received: November 6, 2007
Accepted: March 18, 2008

Summary

Synthesis of isoamyl acetate, a flavour ester extensively used in food industry, has been carried out in a solvent-free system. In the present study, an attempt has been made to enhance the isoamyl acetate synthesis yield by transesterification of isoamyl alcohol with vinyl acetate using immobilized *Rhizopus oryzae* NRRL 3562 lipase. In the present synthesis, substrates had no inhibitory effect on immobilized lipase. The effects of various reaction parameters on isoamyl acetate synthesis were studied and maximum conversion was achieved at 16 % (by mass per volume) of immobilized lipase, 40 °C and 200 rpm. Under these conditions, 8-hour reaction time was sufficient to reach a high ester conversion of 95 % with 0.5 mol/L of isoamyl alcohol. The structure of the transesterified product was confirmed by infrared and nuclear magnetic resonance spectroscopic studies. Immobilized lipase had K_m and v_{max} values of 306.53 mmol/L and 99 μmol/(h·g) respectively, for isoamyl acetate synthesis in a solvent-free system.

Key words: isoamyl acetate, immobilized lipase, *Rhizopus oryzae* NRRL 3562, solvent-free system, transesterification, vinyl acetate

Introduction

Isoamyl acetate is a short chain ester widely used in food and cosmetic industries due to its pleasant banana flavour. It is used as a solvent for some varnishes and nitrocellulose lacquers. Apart from that, being a honey bee pheromone, it can be used to attract large groups of honeybees to a small area. Natural flavour esters extracted from plant material are often in short supply and those produced by fermentation are too expensive for commercial use (*1,2*). Therefore, a selective enzyme mediated synthesis can be a good alternative as it is performed under moderate conditions, compared to chemical syntheses. Esters produced through biocatalysis can be considered close to 'natural' and can potentially satisfy the recent consumer demand (*3*).

Lipases have been employed for isoamyl acetate synthesis in organic solvents (*4–8*). However, there are certain problems related to the separation, toxicity and inflammability of the organic solvents. The solvent-free system has many advantages since the absence of solvents facilitates downstream processing, as fewer components are present in the reaction and the elimination of solvents from the production step makes the process cost effective and environmentally friendly (*9,10*). Although there are many reports about solvent-free isoamyl ester synthesis, the ester has not been obtained in sufficiently high yield. Novozym 435 lipase has been reported to catalyze the direct esterification of isoamyl alcohol and acetic acid with 80 % yield (*11*). More recently, Ghamgui *et al*. (*12*) have shown that a 64 % conversion

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of isoamyl acetate can be reached in a solvent-free system using immobilized *Staphylococcus simulans* lipase. However, inhibition of lipase activity by higher acetic acid concentration has also been reported in isoamyl acetate synthesis (*11*,*12*).

In the present study, vinyl acetate has been chosen as the acyl donor to diminish the inhibitory effect of acetic acid and consequently improve the conversion yield. Moreover, vinyl alcohol formed after transesterification reaction tautomerises to acetaldehyde, which makes the process irreversible (*13*). The aim of the present work is to study the process variables of isoamyl acetate synthesis in a solvent-free system using immobilized *R. oryzae* NRRL 3562 lipase.

Materials and Methods

Chemicals

Isoamyl alcohol (of AR grade) was purchased from Merck (Germany). Vinyl acetate (99 % pure) was obtained from HiMedia (India). All other solvents and reagents were either of HPLC grade or AR grade and were obtained from Merck (Germany).

Lipase production

All experiments were carried out using lipase from *R. oryzae* NRRL 3562. Lipase production from *R. oryzae* NRRL 3562 was performed in 250-mL Erlenmeyer flask using 4 g of wheat bran as a substrate, supplemented with 6 mL of modified Czapek-Dox medium containing (in g/L): KH_2PO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5, $NaNO_3$ 2.5, glucose 50 at pH=5 and 0.6 mL of coconut oil. The medium was sterilized and inoculated with 1 mL (10^6 spores/mL) of spore suspension, followed by incubation at 35 °C and 85 % relative humidity. After 5 days, the fermented biomass was soaked in 16 mL of water, followed by extraction through cheesecloth. Supernatant was collected by centrifugation at 10 000 rpm for 10 min and it was treated with ammonium sulphate (60 % saturation) at 4 °C. The precipitate was dissolved in 50 mmol/L phosphate buffer (pH=8) and dialyzed by keeping the enzyme in dialysis bag (12 kDa, HiMedia, India) overnight in 50 mmol/L phosphate buffer (pH=8) under constant stirring. The concentrated enzyme was lyophilized and used as crude lipase for subsequent immobilization.

Enzyme assay

Lipase assay was performed spectrophotometrically using *p*-nitrophenyl palmitate as a substrate (*14*). *p*-Nitrophenol was liberated from *p*-nitrophenyl palmitate by lipase-mediated hydrolysis. One unit (U) of lipase was defined as the amount of enzyme that liberates one micromole of *p*-nitrophenol per minute under the assay conditions.

Lipase immobilization

Lipase from *R. oryzae* NRRL 3562 was immobilized on silica activated with ethanolamine followed by cross-linking with glutaraldehyde, as described previously (*15*).

Reaction setup for transesterification

Ester synthesis was carried out in screw-capped vials containing 0.5 mol/L isoamyl alcohol in vinyl acetate to a total volume of 1 mL, where vinyl acetate acted as a solvent as well as an acyl donor. The reaction was initiated by the addition of 4 % (g of enzyme per mL of reaction mixture) of immobilized lipase. Samples were placed in an orbital shaker along with the respective controls (samples with no enzyme). Esterification reactions were carried out at 30 °C and 200 rpm, unless otherwise stated. All the experiments were performed in triplicate and the results were reported as the mean± standard deviation.

Sampling and analysis

Samples were taken from the reaction mixture at a specified time and centrifuged at 10 000 rpm for 5 min to remove the immobilized enzyme. Isoamyl acetate was analyzed by injecting the aliquots of the reaction mixture in a gas chromatograph (Agilent GC-6820) equipped with HP-5 capillary column (0.25 μm×0.25 mm×30 m) and flame ionization detector (FID). The column temperature was kept at 50 °C for 3 min, then raised to 100 °C and maintained at this temperature for 5 min. The temperatures of the injector and detector were set at 200 and 250 °C, respectively. Nitrogen was used as the carrier gas. The retention time of isoamyl alcohol and isoamyl acetate was 2.8 and 5.1 min, respectively.

Isolation and identification of the product

For isolation of the product, the immobilized enzyme was centrifuged and the remaining mass was extracted with diethyl ether. The organic phase was washed with $NaHCO_3$ followed by NaCl and was finally dried with anhydrous $CaCl_2$. The formation of isoamyl acetate was confirmed by ^1H nuclear magnetic resonance spectroscopy (Bruker Avance II 200 MHz NMR spectrometer) and Fourier transform infrared spectroscopy (FTIR, Nicolet Nexus 870, USA).

Results and Discussion

Effect of medium components on lipase stability

The individual effect of each component of the reaction mixture on the stability of immobilized *R. oryzae* NRRL 3562 lipase was observed while performing the stability tests in the presence of pure substrates and products. Moreover, the combined effect of all reactants and products on immobilized lipase was also studied by carrying out the stability test in the reaction mixture at various time intervals. The effect of vinyl acetate, isoamyl alcohol, isoamyl acetate and acetaldehyde on the lipase activity is shown in Fig. 1 for 48 h of the treatment. It was found that the immobilized *R. oryzae* NRRL 3562 lipase is stable in the presence of vinyl acetate, isoamyl alcohol and isoamyl acetate, while acetaldehyde has strong lipase inhibitory effect. Lipase inhibition by acetaldehyde is well documented in literature (*16*,*17*). Furthermore, it was also observed that the inhibitory effect of acetaldehyde was not significant in the reaction mixture (0.5 mol/L) and immobilized lipase remained stable

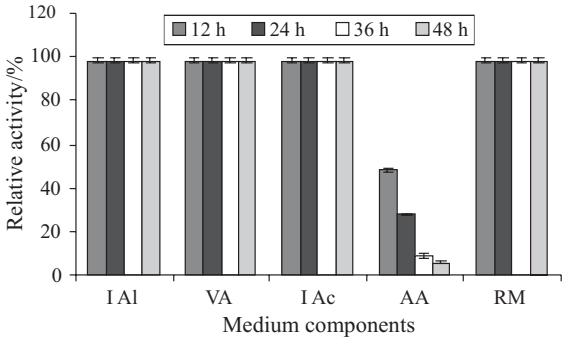

Fig. 1. Effect of medium components on immobilized *R. oryzae* NRRL 3562 lipase stability at 30 °C: isoamyl alcohol (I Al), vinyl acetate (VA), isoamyl acetate (I Ac), acetaldehyde (AA) and 0.5 mol/L reaction mixture (RM). Data are represented as mean±standard deviation of three replications

for 48 h. This can be attributed to the fact that acetaldehyde is volatilized due to its low boiling point.

Effect of initial addition of water

Zaks and Klibanov (*18*) and Katchalski-Katzir (*19*) showed that the activity of a biocatalyst depends on the amount of water adsorbed by the enzyme, as a certain quantity of water is required to maintain the three-dimensional structure of the enzyme. The effect of water content on enzymatic activity was examined by varying the volume fraction of water from 5 to 20 % of the total amount of reaction mixture and it was found that the conversion rate (in %) decreased with the addition of water (Fig. 2). Ghamgui *et al.* (*12*) found similar results for the synthesis of butyl oleate using *Rhizopus oryzae* lipase (*20*). Luhong *et al.* (*21*) and Iso *et al.* (*22*) also reported the decrease in ester synthesis with the addition of water using Novozym 435 and *Pseudomonas fluorescens* lipases, respectively. This decrease in conversion yield at higher water content suggests that the side-reaction, the hydrolysis of vinyl acetate, was favoured, which leads to the formation of acetic acid that has an inhibitory effect on the enzyme stability. Thus, it is also evident that water adsorbed in the substrates and enzymatic preparation was sufficient for the enzyme to exhibit the highest activity (*20*).

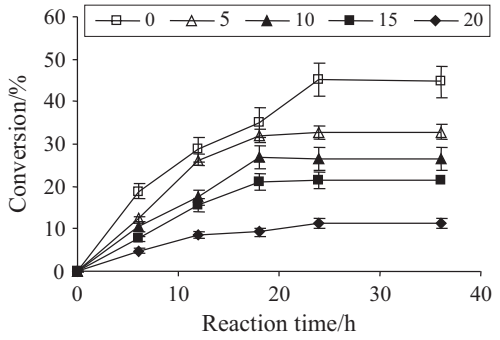

Fig. 2. Effect of the added water on isoamyl acetate synthesis. Reaction conditions: 4 % of immobilized *R. oryzae* NRRL 3562 lipase, 0.5 mol/L of isoamyl alcohol in vinyl acetate, 30 °C and 200 rpm. Data are represented as mean±standard deviation of three replications

Effect of agitation speed

External mass transfer limitations may be an important parameter as far as an immobilized enzyme system is concerned. To observe the effect of mass transfer limitations, experiments were carried out at different agitation speeds varying from 100–300 rpm. The conversion rate decreased slightly with the decrease in shaking speed below 200 rpm, while the conversions remained unaffected by further increase in shaking speed (data not shown). Ghamgui *et al.* (*12*) reported the maximum ester synthesis at 200 rpm and when a high rate of shaking was used, no further increase in synthesis rate was observed. Güvenç *et al.* (*11*) reported that isoamyl acetate conversion was unaffected by the increase of the rate of shaking.

Effect of reaction temperature

The effect of temperature is a very important parameter for ester synthesis because it may influence the reaction efficiency in contradictory ways. Increase in the temperature of a system can have a positive effect on the kinetic constant, as defined by the transition state theory. On the contrary, the treatment at high temperatures may lead to a thermal denaturation and thus inactivate the protein (*23*). The boiling temperature of the reaction mixture occurs around 70 °C, so experiments were performed in the temperature range from 30 to 65 °C using 0.5 mol/L substrate and 4 % of immobilized enzyme to examine the effect of temperature on the isoamyl acetate synthesis. It was observed that the reaction rate increased continuously with the increase in temperature (Fig. 3), so the enzyme was thermostable in this temperature range and the kinetic effect became predominant. There are several reports about constant increase of the reaction rate with the increase of temperature (*23,24*). However, the consecutive experiments were performed at 40 °C due to low boiling point of the reaction mixture and in order to minimize the thermal energy requirement.

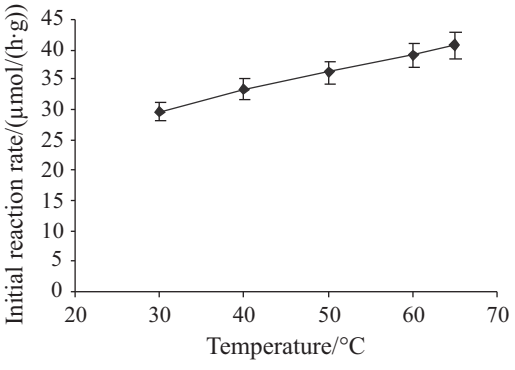

Fig. 3. Effect of reaction temperature on initial reaction rate of isoamyl acetate synthesis. Reaction conditions: 4 % of *R. oryzae* NRRL 3562 immobilized lipase, 0.5 mol/L of isoamyl alcohol in vinyl acetate and 200 rpm. Data are represented as mean±standard deviation of three replications

The apparent activation energy was calculated from Arrhenius plot using the data presented in Fig. 3. A straight line plot on a semi-logarithmic scale is known as an Arrhenius plot, where slope is equal to $-E/R$. In

the present reaction, the apparent activation energy was 7.35 kJ/mol for the temperature range investigated. Romero *et al*. (*23*) reported 11.3 kJ/mol activation energy for isoamyl acetate synthesis in hexane using Novozym 435.

Effect of enzyme amount

It has been reported that for an efficient and fast conversion, it is necessary to perform the ester synthesis with high amount of enzyme (*13,25,26*). In general, higher enzyme concentrations have been used for isoamyl acetate synthesis (*27,28*), except for one study (*7*). The effect of immobilized lipase amount was studied in the range of 4 to 20 % (by mass per volume) at 40 °C (Fig. 4). The conversion rate was found to increase with the increase of enzyme amount up to 16 % (by mass per volume), and the equilibrium was attained after 8 h with conversion of 95 %. However, the conversion rate remained unchanged by further increase of enzyme concentration beyond this level. The levelling-off behaviour of esterification at higher enzyme concentrations has also been reported earlier (*7,12,29,30*). This may be due to the agglomeration of immobilized lipase, which is consistent with previous results (*31,32*).

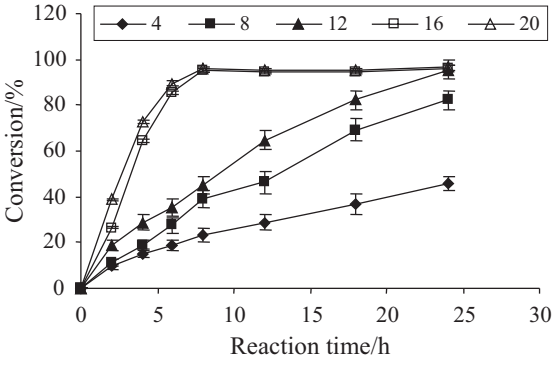

Fig. 4. Effect of immobilized *R. oryzae* NRRL 3562 lipase amount on isoamyl acetate synthesis. Reaction was carried out using 0.5 mol/L of isoamyl alcohol in vinyl acetate at 40 °C and 200 rpm. Data are represented as mean±standard deviation of three replications

Moreover, the reaction rate was found to decrease with the increase in enzyme amount beyond 16 % (Table 1). This can be explained by the fact that the enzyme molecules in excess are not accessible by the substrates and remain inside the bulk of enzyme particles without contributing significantly to the reaction (*29*).

Reusability of the biocatalyst

The major advantage of immobilized lipase lies in its reusability, thereby making the process cost effective. To evaluate the activity of the catalyst after each cycle, the enzyme was filtered, washed with hexane, dried at room temperature and used again for another cycle. It was observed that the conversion rate remained unchanged for three consecutive cycles; however, the conversion dropped to 51 % after 10 cycles of isoamyl acetate synthesis (Fig. 5). Krishna *et al*. (*7*) reported that lipase activity decreased drastically just after 2 cycles at 1:1 molar ratio of alcohol and acetic acid, while the stability of lipase was high (>80 % after 10 cycles) at low concentration of acetic acid. However, Ghamgui *et al*. (*12*) reported that *Staphylococcus simulans* lipase retained approx. 49 % of the synthesis activity after seven cycles of use. This could be attributed to enzyme leaking during repeated use or to the denaturation of the enzyme after many cycles of use.

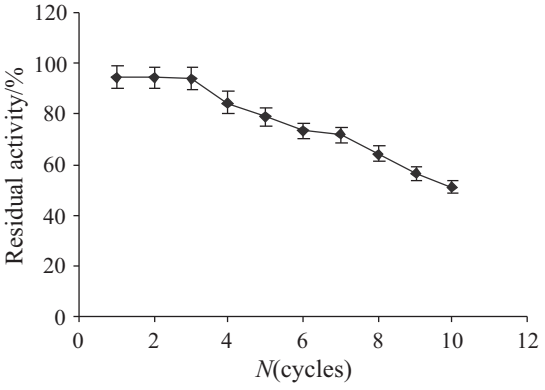

Fig. 5. Operational stability of immobilized *R. oryzae* NRRL 3562 lipase for the synthesis of isoamyl acetate. Reaction was carried out using 0.5 mol/L of isoamyl alcohol in vinyl acetate, 16 % of *R. oryzae* NRRL 3562 immobilized lipase at 40 °C and 200 rpm. Data are represented as mean±standard deviation of three replications

FTIR spectroscopic studies

An FTIR spectrum of the isolated product shown in Fig. 6 was analyzed when the absorption maxima were observed at the following positions: 2962, 1745 (C=O) and 1260 cm^{-1}. The absorption due to the OH group that was present in isoamyl alcohol was absent from the FTIR spectrum of the isolated product. Absence of the peak corresponding to the OH group also confirms the purity of the isolated product, implying that it is free from impurities like alcohol and moisture.

NMR spectroscopic studies

The purified product was analyzed by ^1H NMR spectroscopy (Fig. 7). The NMR spectrum of the product in $CDCl_3$ showed the following specific peaks expected for the product: three-hydrogen singlets at 2.0 ppm for

Table 1. Effect of the enzyme amount on the initial reaction rate of isoamyl acetate synthesis

$\frac{m}{V}$(enzyme)/%	4	8	12	16	20
Initial reaction rate/(μmol/(h·g))	36.38±2.69	43.80±1.60	50.33±2.95	62.45±2.43	48.7±3.01

Reaction was carried out using 0.5 mol/L of isoamyl alcohol in vinyl acetate at 40 °C and 200 rpm. Data are represented as mean± standard deviation of three replications

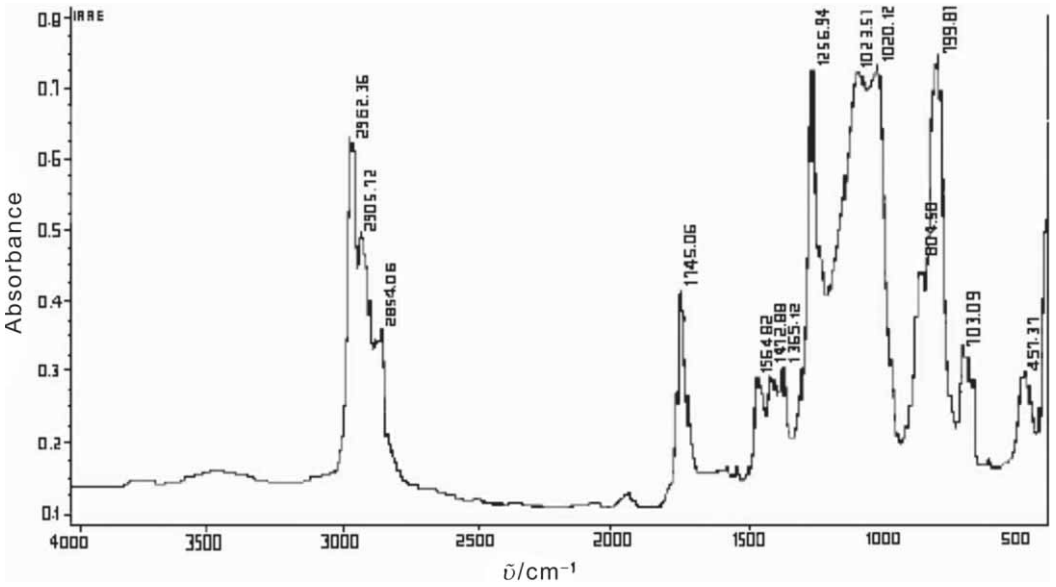

Fig. 6. FTIR spectra of isoamyl acetate

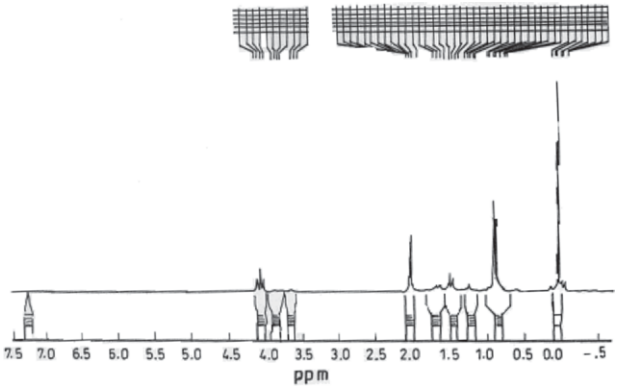

Fig. 7. NMR spectrum of isoamyl acetate (in $CDCl_3$)

the acetate methyl, and a two-hydrogen triplet at 4.0 ppm for the methylene attached to the oxygen. Therefore, from the spectroscopic analysis, the product was confirmed to be isoamyl acetate.

Conclusion

The study showed that efficient synthesis of isoamyl acetate is possible by lipase catalysis using vinyl acetate as the acyl donor. The present work is a comprehensive study on the reaction parameters influencing the enzymatic synthesis of isoamyl acetate in a solvent-free system. The immobilized *R. oryzae* NRRL 3562 lipase was employed to catalyze the transesterification reaction. The amount of enzyme and temperature were found to have an immense effect on isoamyl acetate synthesis. The conversion increased with increasing the temperature up to 65 °C, which was near the boiling point of the reaction mixture. About 95 % conversion was obtained using 16 % (by mass per volume) of immobilized *R. oryzae* NRRL 3562 lipase with 0.5 mol/L substrate concentration at 40 °C in 8 h, which was higher than that of the other previous reports. The high operational stability of immobilized lipase also indicates the efficiency of the process. From the present work, it can be inferred that with appropriate modifications of the reaction conditions, the commercial scale production of the monomeric flavour esters will be possible in a solvent-free medium.

Acknowledgement

The authors wish to acknowledge CSIR, Government of India, and the Department of Biotechnology, New Delhi, India for providing research fellowship to Annapurna Kumari and Paramita Mahapatra, respectively.

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