Journal of Industrial Microbiology & Biotechnology (2000) 25, 147–154

www.nature.com/jim

Enzymatic synthesis of isoamyl butyrate using immobilized *Rhizomucor miehei* lipase in non-aqueous media

S Hari Krishna, SG Prapulla and NG Karanth

Fermentation Technology and Bioengineering Department, Central Food Technological Research Institute, Mysore 570 013, India

Isoamyl butyrate, an important fruity flavor ester, was synthesized using *Rhizomucor miehei* lipase immobilized on a weak anion exchange resin (Lipozyme IM-20) by the esterification of isoamyl alcohol and butyric acid. The effects of various reaction parameters such as substrate and enzyme concentrations, substrate molar ratio, temperature and incubation time have been investigated. Yields above 90% were obtained with substrate concentrations as high as 2.0 M. No evidence of enzyme inhibition by butyric acid was present up to 1.0 M concentration. Acid inhibition and, to a small extent, alcohol inhibition were evident above 1.0 M substrate concentration. Conversions reached a saturation value by the end of 24–48 h of incubation due to the accumulation of the water of reaction. The equilibrium was successfully pushed forward towards esterification by removing the accumulated water using a molecular sieve. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 147–154.

Keywords: enzymatic synthesis; esterification; flavor ester; isoamyl butyrate; lipase; *Rhizomucor miehei*

Introduction

Currently, most flavor and fragrance chemicals are obtained by traditional methods that include extraction from natural sources or chemical synthesis [1]. With the current premium on the "natural" label, biotechnological methods have a high potential for application to the flavor and perfume industry. The ester concentrations and productivities obtained by fermentation methods are rather low [18,25,26]. Several authors have demonstrated a different strategy for ester synthesis that involves the use of enzymes [11,18].

It is well established that lipases (triacylglycerol hydrolases, EC 3.1.1.3), like many other enzymes, remain active in organic solvents. The growing interest in this area arises from the potential applications of these systems to carry out novel reactions for useful products. Recently, research work on the production of various esters by lipase catalysis has increased considerably. Esters of long-chain fatty acids and polyhydric alcohols like glycerol, sorbitol and other carbohydrates (called emulsifiers/surfactants) find wide applications in the food and pharmaceutical industries [8]. Esters of short-chain alcohols and long-chain fatty acids are valuable oleochemicals that may be used as lubricants, diesel fuel and antistatic reagents [22]. Esters of short-chain alcohols and short-chain fatty acids are extremely important aroma compounds [18]. Short-chain fatty acids, being more hydrophilic, lower the pH of the microaqueous layer that surrounds the enzyme which may lead to enzyme inactivation. This inactivation effect is more pronounced with acetic acid in comparison with propionic and butyric acids as substrates [26,37]. Short-chain alcohols tend to strip the essential water from the enzyme and act as dead-end inhibitors [5,6]. Branching at β- and γ-positions of alcohol/fatty acid has also been suggested to exert higher steric hindrance on enzyme activity [27,28].

Isoamyl butyrate is an important fruity flavor ester which has an annual demand of about 20,000 kg [36] and can serve as an excellent model system for the lipase-catalyzed esterification reaction. Lipase-catalyzed esterification and transesterification reactions have been performed in suitable organic solvents or under solvent-free conditions to produce esters of glycerol [8,14], aliphatic alcohols [12,13,35] and terpene alcohols [5]. A survey of the literature indicated that Lipozyme IM-20, a *Rhizomucor miehei* lipase immobilized onto macroporous anion exchange resin (Novo Nordisk, Bagsvaerd, Denmark), has been used in most of the research investigations. This enzyme has high capability for ester synthesis as well as broad substrate specificity ranging from low- to high-molecular-weight acids, alcohols to amines and amino acids with high yields of the corresponding esters (glycerides, amides and peptides).

Use of enzyme catalysis in industrial applications requires the development of efficient processing methods and optimization of reaction conditions. There are few reports on the feasibility of producing isoamyl butyrate using lipases from various sources [17,18,37]. However, a detailed study on the effect of various reaction parameters on lipase catalysis has not been done. Further, the need for high lipase and low substrate concentrations has been a significant impediment in previous studies from the application viewpoint. Considering the industrial importance of the ester, a better knowledge of significant factors influencing the esterification process should be relevant.

In the present communication, the results of a study on the effect of key reaction parameters on the lipase-catalyzed synthesis of isoamyl butyrate are presented. An immobilized lipase from *R. miehei* (Lipozyme IM-20) was employed.

Correspondence: Dr NG Karanth, Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore 570 013, India
Received 9 February 2000; accepted 24 June 2000

Materials and methods

Materials

Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3; Lipozyme IM-20, 25 BIU/g) from *R. miehei*, supported on macroporous anionic resin beads, was kindly provided by Novo Nordisk. Butyric acid, methanol, sodium hydroxide, molecular sieve (3 Å), *n*-hexane and other solvents used were all of analytical reagent grade and procured from S.D. Fine Chemicals Ltd. (Mumbai, India). Isoamyl alcohol (3-methyl-1-butanol) was purchased from Fluka AG (Buchs, Switzerland). The solvents were distilled prior to use.

Esterification method

Ester synthesis was carried out in 100 ml stoppered flasks with a working volume of 10 ml. The reaction mixture containing various concentrations of substrates and enzyme was incubated on an orbital shaker (Lab-Line Instruments Inc., USA) at 40°C and 150 rpm unless otherwise specified. *n*-Hexane was the reaction medium except for the solvent screening trials (where several other solvents were tested). Molecular sieve was added where indicated to remove the water of reaction. The initial reaction rates were calculated from the linear portions of the plots of product concentration vs. reaction time. The yield was calculated based on the conversion of butyric acid to the ester after a given time. Control experiments were also conducted without lipase under similar conditions.

Analysis and characterization

Aliquots of the reaction mixture were withdrawn periodically and assayed by both titrimetry and gas chromatography. Samples were titrated against sodium hydroxide (0.01 N) to find out the residual acid content using phenolphthalein indicator in the case of titrimetry. The percentage esterification and the moles of acid reacted were calculated from the values obtained for the blank and the test samples. To confirm ester formation, reaction samples were also analyzed using a gas chromatograph (GC 15-A; Shimadzu Corp., Kyoto, Japan) equipped with a Carbowax 20-M column (3 m length, 3.175 mm i.d.) and a flame ionization detector. Nitrogen was used as carrier gas with a flow rate of 30 ml/min. Column oven, injection port and detector temperatures were maintained at 100, 200 and 250°C, respectively. The percentage esterification determined by both GC analysis (which showed product formation) and titrimetry (which showed acid consumption) was in good agreement. The product was also characterized by recording the ^{1}H NMR spectra of the compound on a Brüker-DRX 500 NMR instrument (Brüker Physik AG, Karlsruhe-Forchheim, Germany) operating at 20°C.

Results and discussion

Selection of solvent

The polarity of the organic solvent employed is known to influence enzyme activity in biocatalysis. The polarity is quantitatively described by the value of log *P*, where *P* represents the partitioning of a given solvent between water and octanol in a two-phase system. It is generally agreed that log $P>4.0$ indicates higher enzyme activity and better ester synthesis [16], although a few exceptions have been reported. The results of the experiments on the effect of solvents on esterification efficiency, shown in Table 1, support this rule of thumb. Practically no ester synthesis occurred in THF (log $P=0.49$) or DMSO (log $P=-1.3$). The nature of the organic solvent is crucial in maintaining the essential water content required for the catalytic activity of the enzyme. More hydrophilic solvents such as THF, DMSO and dioxan strip essential water surrounding the enzyme and thus, distort its catalytic conformation leading to enzyme inactivation. Even water or buffer saturation of these solvents did not result in better yields (data not shown). Hydrophobic solvents such as hexane and heptane, on the other hand, preserve catalytic activity. Of the solvents studied, esterification was found to be highest in hexane. In addition to its suitability for biocatalysis, hexane is generally considered to be compatible with the processing of flavor and fragrance chemicals and other food materials [11].

Effect of substrate concentration

Substrate concentration had a significant effect on esterification (Figure 1). In this set of experiments, stoichiometric proportions of alcohol and acid were used in anhydrous hexane as the solvent. For an enzyme concentration of 5 g/l, the esterification yield steeply decreased from 92% (at 0.25 M) to 26% (at 1.0 M) with an increase in substrate concentration. To examine whether this may be due to inadequate enzyme level at higher substrate concentrations, experiments were also conducted with larger enzyme quantities (10 and 15 g/l). With the increase in enzyme quantity, while improvement in the yield was observed at low substrate concentrations (<0.5 M), the steep downward trend of the curves at higher substrate concentrations persisted, as shown in Figure 1, indicating that insufficient enzyme was not the main cause. Enzyme inhibition/inactivation by excess substrate may be a reason and this aspect was further investigated and reported below. The effect of substrate concentrations on the synthesis of flavor esters with *R. miehei* [5] and *Candida cylindracea* [11] lipases was reported earlier. The nature of alcohol has been shown to affect ester synthesis [37]. While higher ethanol concentrations led to lower ethyl butyrate yields, butanol had no inhibitory effect on butyl butyrate synthesis [37]. The presence of substitutions to either alcohol or acid also reduces enzyme activity [27,28].

Table 1 Effect of organic solvents on the synthesis of isoamyl butyrate

Solvent	log *P*	Percent esterification
Petroleum ether (40–60°C)	–	79.5
Petroleum ether (60–80°C)	–	77.6
Isooctane	4.51	69.9
Heptane	4.0	77.9
Hexane	3.5	90.9
Cyclohexane	3.2	66.2
Chloroform	2.0	67.6
Dichloromethane	1.3	2.8
Dioxan	1.1	2.4
Dimethyl sulfoxide (DMSO)	−1.3	0.0
Tetrahydrofuran (THF)	0.49	0.0

Reactions were carried out at 0.25 M substrate concentration and with 5 g/l immobilized enzyme at 40°C and at 150 rpm. Conversions shown were obtained in 24 h.

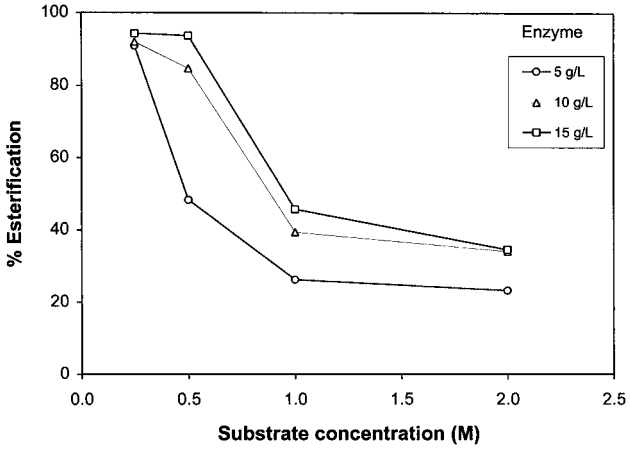


Figure 1 Effect of substrate concentration on isoamyl butyrate synthesis at different enzyme concentrations. Reaction conditions: equimolar alcohol and acid; 24 h; 40°C and 150 rpm.

Effect of substrate molar ratio

In esterification reactions, the equilibrium can be pushed forward by the use of excess molar quantities of nucleophile (alcohol) or by removing products from the reaction mixture [9]. However, excessive alcohol concentrations may slow down the reaction rates. Therefore, an optimum value exists for the actual excess of alcohol to be used in the esterification reaction under specified conditions. To check the actual beneficial effect of excess alcohol molar ratio, experiments were performed at four butyric acid concentrations (0.25, 0.5, 1.0 and 2.0 M) with varying alcohol concentrations under enzyme-limiting conditions (5 g/l). The results clearly show a steep increase in conversions with alcohol concentration (Figure 2). This increase was more marked at lower acid concentrations in that the span of alcohol concentration over which this increase in esterification occurred increased with acid concentration. At 1.0 M acid concentration, esterification continued to increase to a value of 65% even at 4.0 M alcohol and, more than a twofold increase in yield was observed compared to that at equimolar concentration. However, at 2.0 M butyric acid, this trend was different and the yields, after a small increase, actually reduced slightly beyond equimolar concentration, indicating probable inhibitory effect of butyric acid.

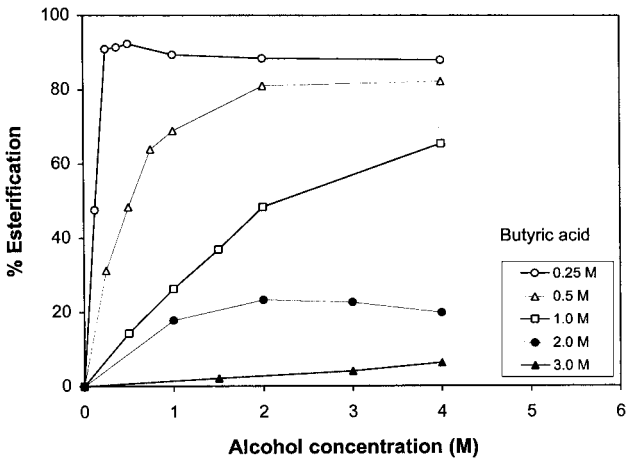
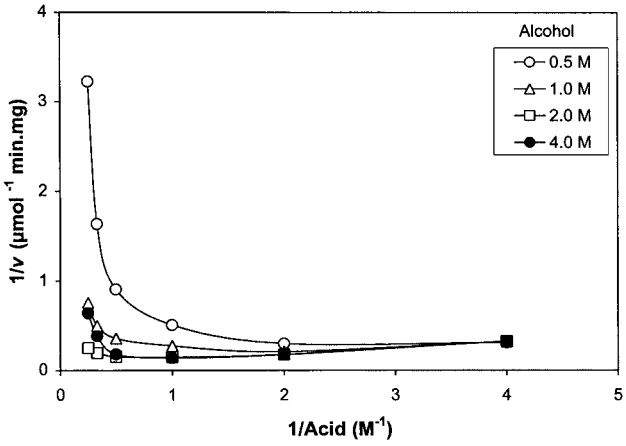


Figure 2 Effect of alcohol concentration on isoamyl butyrate yields at different butyric acid concentrations. Reaction conditions: enzyme, 5 g/l; 24 h; 40°C and 150 rpm.

The kinetics of the esterification reaction has been studied at different alcohol and acid concentrations. The results have been analyzed using double reciprocal plots of initial reaction velocities *versus* substrate concentrations (Figure 3a,b). The shape of the curves at higher concentrations of butyric acid (Figure 3a) indicates substrate inhibition which was stronger at low concentrations of isoamyl alcohol. Figure 3b shows no common intersection of the lines and therefore, a sequential mechanism can be ruled out [30]. As the alcohol concentration increased, the slope increased and the $1/\nu$ axis intercept decreased to a limit of $1/V_{max}$. These results support a Ping Pong Bi–Bi mechanism with dead-end inhibition by both the substrates. While it is reported that alcohols act as dead-end inhibitors of lipase (alcohol reacts with lipase to yield a dead-end lipase–alcohol complex), acid inhibition has not been observed. Acid, in fact, may also act as a competitive inhibitor by reacting with acyl–enzyme complex to yield a dead-end complex that can no longer react with alcohol to give an ester [31]. The present results support this view. The complete reaction rate equation for this



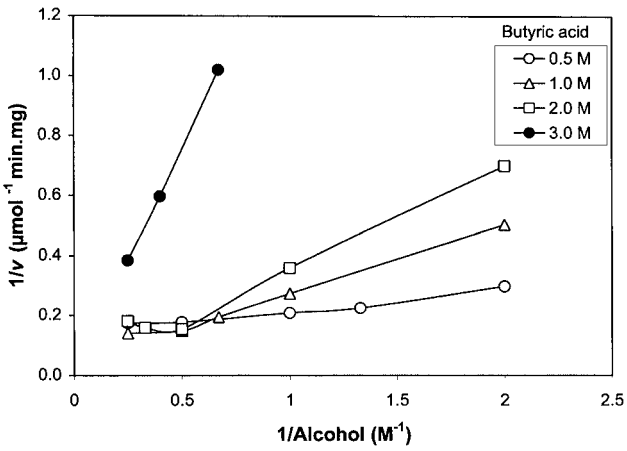


Figure 3 (a) Lineweaver–Burke plot of reciprocal butyric acid concentrations *versus* reciprocal initial reaction rates at fixed isoamyl alcohol concentrations. Reaction conditions: enzyme, 5 g/l; 40°C and 150 rpm. (b) Lineweaver–Burke plot of reciprocal isoamyl alcohol concentrations *versus* reciprocal initial reaction rates at fixed butyric acid concentrations. Reaction conditions: enzyme, 5 g/l; 40°C and 150 rpm.

mechanism with inhibition by both the substrates is given as [31]:

$$\nu = \frac{V^*_{max}\,[IAA][BA]}{[IAA][BA] + K^*_{M,BA}\,[IAA]\left(1+\frac{[IAA]}{K_{i,IAA}}\right) + K^*_{M,IAA}\,[BA]\left(1+\frac{[BA]}{K_{i,BA}}\right)} \cdots \quad (1)$$

where ν is the initial reaction rate (μmol/min mg), V^*_{max} is the maximum rate of reaction, $K^*_{M,BA}$ and $K^*_{M,IAA}$ are the Michaelis constants of acid and alcohol (M), $K_{i,BA}$ and $K_{i,IAA}$ are the inhibitory constants of acid and alcohol (M). The kinetic parameters in Equation 1 were evaluated through a regression analysis as: $K^*_{M,IAA}$=0.00306 M, V^*_{max}=11.718 μmol/min mg, $K_{i,BA}$=1.05 M; $K^*_{M,BA}$=0.00303 M. These kinetic parameter values indicate that while acid inhibition was apparent ($K_{i,BA}$=1.05 M), alcohol inhibition was less significant as the K_i value was high ($K_{i,IAA}$=6.55 M).

In contrast to our results, Marty *et al.* [21] reported Lipozyme inhibition by ethanol during the synthesis of ethyl oleate, while oleic acid showed no inhibition. This difference in behavior may be attributed to the more polar nature and shorter carbon chain length of ethanol (log P= $-$0.24) than that of isoamyl alcohol (log P=1.3). The shorter carbon chain length and more polar nature of butyric acid (log P=0.81) than that of oleic acid could also be a contributory factor. Moreover, they have employed a narrower range of substrate concentrations (0–0.05 M) than in our studies observing no acid inhibition but substantial inhibition by ethanol.

Several observations on alcohol inhibition effects have been reported in lipase-catalyzed esterification reactions. The presence of excess ethanol has been shown to drastically affect the yields of ethyl butyrate and other ethyl esters [7,11]. Recently, Duan *et al.* [6] reported inhibition of *C. antarctica* lipase by propanol. Butanol was also exerted inhibitory effects on the synthesis of butyl laurate [10]. In contrast to this observation, butanol had no inhibitory effects on the synthesis of butyl butyrate [11]. The inhibitory effects of isoamyl alcohol were also studied [30] in a transesterification reaction between isoamyl alcohol and ethyl acetate. However, ethyl acetate has been suggested to be relatively more inhibitory. Isoamyl alcohol, due to its lower polarity (log P=1.3) compared to other polar alcohols like ethanol (log P= $-$0.24) and butanol (log P=0.80), would probably retain the minimum water necessary for enzyme activity. We have experimentally verified this and have observed that the enzyme in contact with anhydrous isoamyl alcohol also performed equally well in the reactions, indicating that stripping of essential bound water from the enzyme did not occur. This was not the case with other polar alcohols.

On the other hand, we have observed a typical competitive inhibition by butyric acid in the present study. Butyric acid, a short-chain polar acid, concentrates in the microaqueous layer and causes a pH drop in the enzyme microenvironment leading to enzyme inactivation. Butyric acid binds to the acyl–enzyme complex unproductively to yield a dead-end intermediate that can no longer give rise to an ester. In the present study, we observed that high concentrations of butyric acid caused inactivation of the catalyst in addition to dead-end inhibition. The enzyme inactivation was confirmed by investigations on the activity of the enzyme recovered after completion of a reaction, which showed that lipase incubated with 2.0 M substrate did not show any appreciable esterification activity after recovery.

The influence of an acyl acceptor/nucleophile (i.e., alcohol) is also an important factor in understanding the mechanism of the reaction [20]. Alcohols like *n*-propanol, isopropanol, *n*-butanol, isobutanol, *n*-amyl alcohol, isoamyl alcohol and *S*(–)2-methyl-1-butanol were tested as acyl acceptors with butyric acid as the acyl donor. The reaction rates were not significantly affected by the nature of the alcohols except for isopropanol and isobutanol, in which significant decreases in reaction rate were observed (data not shown). Since all the isomers of pentanol: *n*-amyl (straight chain), isoamyl (γ-branching) alcohols and 2-methyl-1-butanol (β-branching) and most of the primary (1°) alcohols reacted with butyric acid resulting in similar reaction rates, the rate-limiting step can be said to be the acyl–enzyme formation rather than the deacylation step. The reaction rates were significantly higher with isoamyl alcohol, although it is a branched alcohol. This result supports the theoretical assumption that the inhibitory effect of substrates was eliminated or reduced when the side group was located far from the α-carbon atom. Isopropanol (2-propanol, 2° alcohol) was the most unreactive alcohol, most probably because of strong steric hindrance of the secondary alcohol function.

Effect of enzyme concentration and enzyme-to-substrate ratio

The effect of varying enzyme concentrations on the yield of isoamyl butyrate with substrate concentration as a parameter was investigated. A steep increase in the esterification with enzyme concentration was observed at most of the substrate concentrations tested. A tendency to steeply reach a saturation esterification value was noticed at lower enzyme concentrations when low substrate concentrations (0.25 and 0.5 M) were used. The increase in esterification was more gradual at higher substrate concentrations. The effect of substrate concentration on enzyme requirement to obtain a given conversion range (50–90%) was also studied. A near linear behavior in enzyme requirement has been observed (data not shown) with the increase in substrate concentration, the highest enzyme concentration of 60 g/l being required at 2.0 M substrate to achieve 88% conversion.

While enzyme concentration is an influential parameter, the enzyme-to-substrate ratio (E/S ratio, g/mol) is probably a more relevant parameter. Although enzyme percent (w/w) (of alcohol and acid) has been generally used in the literature [32], we have adopted E/S ratio mainly to eliminate the alcohol term and represent the actual quantity of the enzyme present *versus* acyl donor (acid). Accordingly, 24 h conversions of different substrate

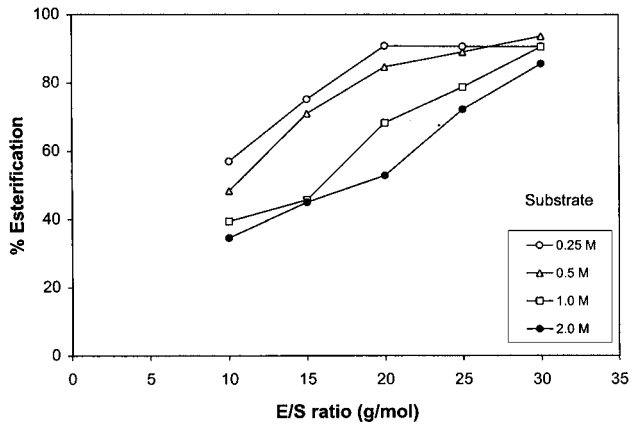


Figure 4 Effect of enzyme-to-substrate (E/S) ratio on isoamyl butyrate synthesis. Reaction conditions: equimolar alcohol and acid; 24 h; 40°C and 150 rpm.

concentrations (0.25, 0.5, 1.0 and 2.0 M) at various E/S ratios are plotted in Figure 4. Esterification generally increased with E/S ratio and an E/S ratio of 20 and above gave maximum yields. Also, E/S ratios required for a given conversion increased with substrate concentrations.

In general, it should be noted that for enzymatic esterification reactions, the lipase concentrations reported in the literature for achieving higher yields are often too high and substrate (and hence product) concentrations relatively too low (<0.5 M) for industrial exploitation. While the feasibility of lipase-catalyzed synthesis of isoamyl butyrate has been demonstrated earlier [17,18,37], the amount of lipase (mainly free lipases) employed was rather high (E/S ratios of 50, 100 and 200 g/mol employing 0.25 M substrate). In comparison, our results demonstrate the use of low E/S ratios (<20 g/mol) at all substrate concentrations.

Time course of esterification reaction

Figure 5 illustrates the time course of the isoamyl butyrate synthesis catalyzed by Lipozyme IM-20 at different substrate concentrations (E/S=20 g/mol). Esterification increased steeply with time during early stages of the reaction to attain a major part of the final conversion in 24 h and gradually reached the equilibrium maximum at 48 h, remaining constant thereafter. Allowing the reactions to proceed for 10–14 days resulted in no further change in conversion. The equilibrium could, however, be pushed towards synthesis by removing the products (water or ester in the present case) or by employing excess nucleophile. The favorable effect of excess nucleophile was already discussed in the preceding section and the effect of water removal is discussed in the subsequent section. While maximum conversions exceeding 85% were obtained with 0.25, 0.5 and 1.0 M substrate, conversion decreased to 52% at 2.0 M substrate (Figure 5). At low substrate concentrations (<0.5 M), conversions higher than 95% were not observed, probably due to the accumulation of water of reaction. The results represent a significant improvement in the process mainly in terms of reaction time and use of low enzyme quantities in comparison with the previous reports on isoamyl butyrate [17,18,37], which required large quantities of enzyme.

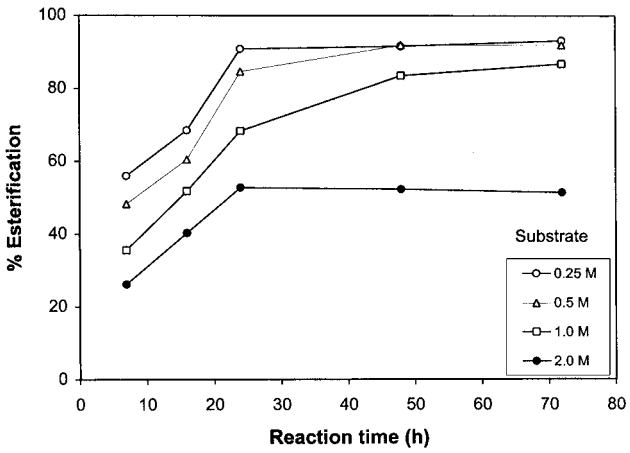


Figure 5 Time course of isoamyl butyrate synthesis at different substrate concentrations. Reaction conditions: equimolar alcohol and acid; E/S ratio, 20 g/mol; 40°C and 150 rpm.

Effect of water of reaction

The water content of the enzyme (Lipozyme IM-20) used in the present study was approximately 10% w/w and has been reported to be active in nearly anhydrous reaction mixtures [33]. The water content of the original enzyme was considered sufficient to provide the needed aqueous microenvironment and therefore, no additional water was added to the reaction mixtures before starting the reactions, while addition of water/buffer (≥0.02%) led to aggregation of enzyme particles and thus poor conversions. Addition of 0.01% water, although it did not cause any aggregation of enzyme particles, had no favorable effects (results not shown).

Esterification is an equilibrium reaction and water is formed in equimolar proportions to ester. The accumulation of this water invariably drives the equilibrium in the reverse direction (hydrolysis of the ester formed). The amount of water of reaction, corresponding to the extent of esterification calculated and given in Table 2, is seen to be considerable and increased with enzyme content. At a given substrate concentration, water of reaction increased with enzyme quantity indicating that more enzyme can tolerate relatively higher amounts of water. The tolerance of the enzyme to water may depend on substrate as well as enzyme concentration (Table 2). Increase in enzyme concentration resulted in enhanced product (ester/water) formation. However, ester formed per unit enzyme decreased while ester concentration increased (at all substrate concentrations tested) to reach nearly complete esterification (90–95%) or saturation esterification (incomplete) value.

When a large amount of water is formed, the immobilization support adsorbs this water and the enzyme becomes fully hydrated by many layers of water. These aqueous layers act as a barrier between the enzyme surface and the bulk reaction medium, thereby reducing enzymatic activity [7]. This water also drives the equilibrium in the reverse direction to hydrolysis of the ester formed, a likely reason for the reduced ester yield even at relatively higher enzyme levels (52% at 2.0 M using 40 g/l enzyme). A high enzyme level of 60 g/l was required to reach 88% esterification. These results provide experimental evidence of shifting reaction equilibrium because of unfavorable effects of the product (water), which has been reported in the literature on theoretical grounds [19].

At 2.0 M substrate concentration and an E/S ratio of 20 g/mol, the conversions reached equilibrium just after 24 h and remained constant thereafter (Figure 5). There are several possible explanations for the observed equilibrium: (i) a hydrolysis reaction, which may be triggered by the large quantity of accumulated water (>15 ml/l); (ii) slower diffusion of the substrates through the multilayered microaqueous interface that surrounds the enzyme; and (iii) the effect of excess water on the equilibrium of acyl–enzyme complex (E–S) formation and a consequent increase in the rate of the hydrolysis of the E–S complex releasing free acid again and hence reducing the net rate of esterification [34].

Protein molecules in their fully active native state are surrounded by a hydration shell which is composed of water molecules attached to the protein surface mainly by hydrogen bonds. This structure is indispensable for supporting the native protein conformation. If an organic solvent of highly polar nature is present in the system, its molecules tend to displace water from the hydration shell, thus distorting finely balanced interactions responsible for maintaining the native conformation of the protein molecule. Hydrophobic solvents like hexane can

Table 2 Effect of water of reaction on the synthesis of isoamyl butyrate

Substrate (M)	Enzyme (g/l)	Percent esterification	Water (ml/l)
0.25	1	40.6	1.827
0.25	2	47.9	2.156
0.25	2.5	78.9	3.551
0.25	3	80.5	3.623
0.25	4	90.7	4.082
0.25	5	93.2	4.194
0.25	10	94	4.23
0.5	5	58.4	5.256
0.5	7.5	93.5	8.415
0.5	10	96.7	8.703
0.5	12.5	95.4	8.586
0.5	15	96.3	8.667
1.0	5	23	4.14
1.0	10	39.4	7.092
1.0	15	71.6	12.888
1.0	20	86.8	15.624
1.0	25	87.1	15.678
1.0	30	91.1	16.398
1.0	40	93.4	16.812
2.0	5	21.6	7.776
2.0	10	35.2	12.672
2.0	15	41.9	15.084
2.0	20	43.9	15.804
2.0	30	47.7	17.172
2.0	40	51.4	18.504
2.0	50	71.6	25.776
2.0	60	88.2	31.752

Reactions were carried out at 40°C and 150 rpm for 72 h.

dissolve only a small amount of water and thus do not disturb the microaqueous layer. However, complications arise in reactions (such as esterification) where water is a product. As the more polar reactants (alcohol and acid) are converted into a less polar product (ester), the water of reaction is partitioned towards the biocatalyst and accumulation of water eventually results in lower reaction rates, decreased equilibrium conversions and decreased enzyme stability. In fact, the enzyme after reaction with 2.0 M substrate has shown no esterification activity after recovering it from the reaction mixture. This finding is in agreement with the results of Carta *et al.* [3], who reported that water accumulation drastically affects long-term stability of the enzyme. Therefore, it should be useful to remove the water of reaction from the system.

Removal of accumulated water

Since water is a product of the esterification reaction favoring the reverse hydrolysis reaction, it is important to remove it to achieve higher conversions. Several methods of water removal during reaction have been reported in the literature, such as vaporization, vacuum evaporation, air drying and addition of a molecular sieve [4]. In the present study, molecular sieve as a dehydrating agent has been studied. Four sets of reactions were performed at 0.25, 0.5, 1.0 and 2.0 M substrate concentrations using 5 g/l enzyme. The quantity of molecular sieve was varied in the range of 5–180 g/l and added after about 30–50% conversion since it adsorbs water strongly and may remove essential bound water from the enzyme if added initially, as reported by Monot *et al.* [23]. We also noticed lower equilibrium conversions when molecular sieve was added at 0 h (data not shown).

Molecular sieve addition had an increasing beneficial effect on esterification up to a certain level, after which it declined. The beneficial effect was more marked at higher substrate levels giving substantially improved conversions (>1.5-fold). This results from the stronger role played by water of reaction in stimulation of the reverse reaction (hydrolysis) at higher substrate concentrations, which is nullified by the removal of water by molecular sieve. The optimum amount of molecular sieve for maximum esterification increased from 60 g/l at substrate levels of 0.25 and 0.5 M to over 180 g/l at 2.0 M substrate.

Effect of temperature

The esterification reaction kinetics was studied with 0.5 M substrate and different temperatures under enzyme-limiting conditions (5 g/l). Upon increasing the reaction temperature from 25°C to 60°C, the initial reaction rate increased from 1.96 to 14.71 μmol/min mg enzyme (Figure 6). On the other hand, esterification yields passed through an optimum in the temperature range 30–35°C. Although Lipozyme IM-20 has been reported to be active in organic solvents and solvent-free conditions at temperatures as high as 90°C [15], reactions at higher temperatures were not attempted due to the low boiling point (69°C) of the solvent used (*n*-hexane) in this study. Hydrolytic enzymes, in general, have been shown to be thermostable even at 100°C in non-aqueous systems mainly for longer chain length substrates [38]. For the esters of butyric acid, there is diverse range of optimum temperatures reported in the literature; e.g., 50°C for butyl butyrate [35] and 30°C for ethyl butyrate [11].

Enzyme stability behavior at elevated temperatures is related to the maintenance of an intact hydration layer surrounding the enzyme while minimizing free water in the bulk reaction medium to prevent thermally induced deterioration of the catalyst [35]. Few reports [2,29] have indicated that an optimum amount of water in excess of that found in essentially dry solvent may be required to ensure enzyme activity, especially with strongly hydrophilic reactants. In these mixtures, hydrophilic substrates tend to strip the (stabilizing) bound water from the enzyme. Lower esterification yields at higher temperatures may also be explained by the fact that severe water removal from the system occurs at high temperatures resulting in a drastic decrease of the enzyme

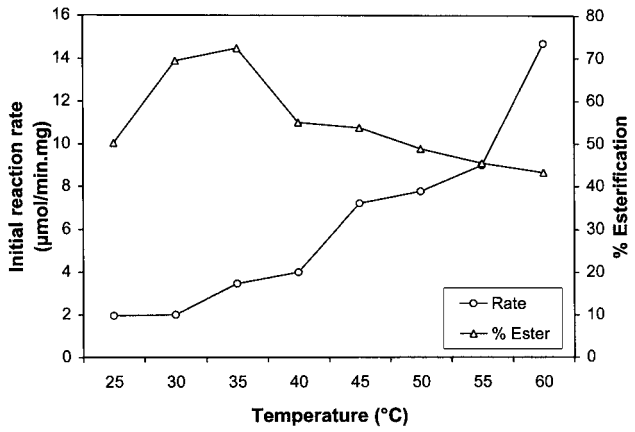


Figure 6 Effect of temperature on initial rates and yields of isoamyl butyrate synthesis. Reaction conditions: substrate, 0.5 M; enzyme, 5 g/l and 150 rpm.

activity. Similar observations were also reported by Pecnik and Knez [24].

The effect of temperature on the synthesis of isoamyl butyrate was observed to follow Arrhenius law with an activation energy (E_a) of 22.1 kcal/mol. No significant mass transfer (pore diffusion) limitations were observed. The enthalpy of the reaction (ΔH) was 21.5 kcal/mol (at 40°C) and the free energy of activation (ΔG) was −3.9 kcal/mol. The equilibrium constant (K_0) (at 40°C with 0.5 M substrate reaching 94% conversion) was 522.2 M^{-1}.

Conclusions

Lipozyme IM-20 effectively catalyzed the synthesis of isoamyl butyrate, a commercially important flavor ester that possesses banana flavor note, in *n*-hexane with high yields. The selection of various reaction parameters that maximize conversions at relatively low enzyme concentrations was investigated in detail. Substrate concentration showed a significant effect on esterification. Higher substrate concentration necessitated the use of more enzyme. Increase in the molar ratio of alcohol to acid favored enhanced esterification even under enzyme-limiting conditions. With an increase in alcohol concentration at fixed butyric acid concentration, yields improved (about threefold), indicating beneficial effects of employing excess nucleophile (alcohol). In contrast to several reports on alcohol inhibition [5–7,10,21], we observed acid inhibition and, to a small extent, alcohol inhibition. Isoamyl alcohol, being relatively non-polar compared to ethanol, propanol and butanol, retained the minimum bound water necessary for enzyme activity. It was possible to convert up to 2.0 M substrate employing a relatively low E/S ratio (<30 g/mol) and achieve more than 80% yields, which is significant compared to literature reports, which have employed high E/S ratios (50–200 g/mol) to convert substrate concentrations below 0.5 M. Water of reaction formed during the course of esterification accumulated in the system and resulted in a considerable decrease in equilibrium conversion. At all substrate concentrations, equilibrium was reached by 24 h. Water of reaction increased with both the enzyme and substrate concentrations. The equilibrium can be pushed to near completion in the case of low substrate concentration and the yields can be increased (>1.5-fold) in the case of high substrate concentrations by the removal of water using a molecular sieve.

Acknowledgements

The authors thank the Director, CFTRI, for providing facilities and Dr. S. Divakar for useful interactions and suggestions. S.H.K. is thankful to the Council of Scientific and Industrial Research, India, for the award of a Senior Research Fellowship.

References

1 Armstrong DW, B Gillies and H Yamazaki. 1989. Natural flavors produced by biotechnological processing. In: Flavor Chemistry Trends and Development. American Chemical Society, Washington, DC, pp. 105–120.
2 Ayala G, MT Gomez-Puyou, A Gomez-Puyou and A Darszon. 1986. Thermostability of membrane enzymes in organic solvents. *FEBS Lett* 203: 41–43.
3 Carta G, JL Gainer and AH Benton. 1991. Synthesis of esters using a nylon-immobilized lipase in batch and continuous flow reactors. *Biotechnol Bioeng* 37: 1004–1009.
4 Chen JP and JB Wang. 1997. Wax ester synthesis by lipase-catalyzed esterification with fungal cells immobilized on cellulose biomass support particles. *Enzyme Microb Technol* 20: 615–622.
5 Chulalaksananukul W, JS Condoret and D Combes. 1993. Geranyl acetate synthesis by lipase-catalyzed transesterification in supercritical carbon dioxide. *Enzyme Microb Technol* 15: 691–698.
6 Duan G, CB Ching, E Lim and CH Ang. 1997. Kinetic study of enantioselective esterification of ketoprofen with *n*-propanol catalyzed by a lipase in an organic medium. *Biotechnol Lett* 19: 1051–1055.
7 Dumont T, D Barth, C Corbier, G Branlant and M Perrut. 1992. Enzymatic reaction kinetic: comparison in an organic solvent and in supercritical carbon dioxide. *Biotechnol Bioeng* 39: 329–333.
8 Ergan F, M Trani and G Andre. 1990. Production of glycerides from glycerol and fatty acids by immobilized lipases in non-aqueous media. *Biotechnol Bioeng* 35: 195–200.
9 Ergan F, M Trani and G Andre. 1991. Use of lipases in multiphasic systems solely composed of substrates. *J Am Oil Chem Soc* 68: 412–417.
10 Gandhi NN, SB Sawant and JB Joshi. 1995. Studies on the Lipozyme-catalyzed synthesis of butyl laureate. *Biotechnol Bioeng* 46: 1–12.
11 Gillies B, H Yamazaki and DW Armstrong. 1987. Production of flavor esters by immobilized lipase. *Biotechnol Lett* 9: 709–719.
12 Hari Krishna S, B Manohar, S Divakar and NG Karanth. 1999. Lipase-catalyzed synthesis of isoamyl butyrate: optimization by response surface methodology. *J Am Oil Chem Soc* 76: 1483–1488.
13 Hari Krishna S, B Manohar, S Divakar, SG Prapulla and NG Karanth. 2000. Optimization of isoamyl acetate production by using immobilized lipase from *Mucor miehei* by response surface methodology. *Enzyme Microb Technol* 26: 131–136.
14 Hoq MM, H Tagami, T Yamane and S Shimizu. 1985. Some characteristics of continuous glyceride synthesis by lipase in a macroporous hydrophobic membrane bioreactor. *Agric Biol Chem* 49: 335–342.
15 Knez Z, M Leitgeb, D Zavrsni and B Lavric. 1990. Synthesis of oleic acid esters with an immobilized lipase. *Fat Sci Technol* 92: 169–172.
16 Laane C, S Boeren, K Vos and C Veeger. 1987. Rules for optimization of biocatalysis in organic solvents. *Biotechnol Bioeng* 30: 81–87.
17 Langrand G, C Triantaphylides and J Baratti. 1988. Lipase catalyzed formation of flavor esters. *Biotechnol Lett* 10: 549–554.
18 Langrand G, N Rondot, C Triantaphylides and J Baratti. 1990. Short-chain flavor esters synthesis by microbial lipases. *Biotechnol Lett* 12: 581–586.
19 Martinek K, AN Semenov and IV Berezin. 1981. Enzymatic synthesis in biphasic aqueous–organic systems: I. Chemical equilibrium shift. *Biochim Biophys Acta* 658: 76–89.
20 Martinelle M and K Hult. 1995. Kinetics of acyl transfer reactions in organic media catalyzed by *Candida antarctica* lipase B. *Biochim Biophys Acta* 1251: 191–197.
21 Marty A, W Chulalaksananukul, RM Willemot and JS Condoret. 1992. Kinetics of esterification in supercritical carbon dioxide. *Biotechnol Bioeng* 39: 273–280.
22 Mittelbach M. 1990. Lipase-catalyzed alcoholysis of sunflower oil. *J Am Oil Chem Soc* 67: 168–170.
23 Monot F, F Borzeix, M Bardin and JP Vandecasteele. 1991. Enzymatic esterification in organic media: role of water and organic solvent in kinetics and yield of butyl butyrate synthesis. *Appl Microbiol Biotechnol* 35: 759–765.
24 Pecnik S and Z Knez. 1992. Enzymatic fatty ester synthesis. *J Am Oil Chem Soc* 69: 261–265.
25 Prapulla SG. 1995. Studies on microbial flavor production. Ph.D. Thesis, University of Mysore, Manasagangothri, Mysore.
26 Prapulla SG, NG Karanth, KH Engel and R Tressl. 1992. Production of 6-pentyl-α-pyrone by *Trichoderma viride*. *Flavor Fragrance J* 7: 231–234.
27 Rangheard MS, G Langrand, C Triantaphylides and J Baratti. 1989. Multi-competitive enzymatic reactions in organic media: a simple test for the determination of lipase fatty acid specificity. *Biochim Biophys Acta* 1004: 20–28.
28 Rangheard MS, G Langrand, C Triantaphylides and J Baratti. 1992. Multi-competitive enzymatic reactions in organic media: application to the determination of lipase alcohol specificity. *Enzyme Microb Technol* 14: 966–974.

29 Reslow M, P Adlercreutz and B Mattiasson. 1988. The influence of water on protease-catalyzed peptide synthesis in acetonitrile/water mixtures. *Eur J Biochem* 177: 313–318.
30 Rizzi M, P Stylos, A Riek and M Reuss. 1992. A kinetic study of immobilized lipase catalyzing the synthesis of isoamyl acetate by transesterification in *n*-hexane. *Enzyme Microb Technol* 14: 709–714.
31 Segel IH. 1975. Enzyme kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. John Wiley and Sons, New York, pp. 827–830.
32 Shieh CJ, CC Akoh and LN Yee. 1996. Optimized enzymatic synthesis of geranyl butyrate with lipase AY from *Candida rugosa*. *Biotechnol Bioeng* 51: 371–374.
33 Valivety RH, PJ Halling and AR Macrae. 1992. *Rhizomucor miehei* lipase remains highly active at water activity below 0.0001. *FEBS Lett* 301: 258–260.
34 Valivety RH, PJ Halling and AR Macrae. 1992. Reaction rate with suspended lipase catalyst shows similar dependence on water activity in different organic solvents. *Biochim Biophys Acta* 1118: 218–222.
35 Welsh FW and RE Williams. 1990. Lipase-mediated production of ethyl butyrate and butyl butyrate in non-aqueous systems. *Enzyme Microb Technol* 12: 743–748.
36 Welsh FW, WD Murray and RE Williams. 1989. Microbiological and enzymatic production of flavor and fragrance chemicals. *CRC Crit Rev Biotechnol* 9: 105–169.
37 Welsh FW, RE Williams and KH Dawson. 1990. Lipase-mediated synthesis of low molecular weight flavor esters. *J Food Sci* 55: 1679–1682.
38 Zaks A and AM Klibanov. 1984. Enzymatic catalysis in organic media at 100°C. *Science* 224: 1249–1251.