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BIOCATALYSIS

Enzymatic Synthesis of Butyrates of Fusel Oil

V. S. Gamayurova*, K. L. Shnaider, and M. J. Jamai*****

Kazan State Technological University, Kazan, 420015 Russia

**e-mail: gamaur@kstu.ru*

***e-mail: 0202-84@mail.ru*

****e-mail: mjj82@mail.ru*

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Abstract—Many esters derived from lower aliphatic acids and alcohols have pleasant fruity aromas. These compounds are common in the plant kingdom. Some are chemically synthesized and used as fragrances. In this work, fruity flavored butyric esters are produced using lipase agents. The substrate is an alcohol-containing fraction obtained by distilling fusel oil, a waste product of the alcohol industry (T_b = 120–140°C). The yield of the target products in the presence of pancreatic lipase and the lipase from the *Candida rugosa* yeast is ~94.0% under the determined optimum conditions.

Keywords: butyrates, synthesis of aromatic substances, pancreatic lipase, lipase from the *Candida rugosa* yeast

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INTRODUCTION

Aromatic substances are widely applied in perfumery and in the development of fragrances for detergents, cosmetics, household chemicals, and medicines for external use [1, 2].

Esters are one of the most important and widely used groups of aromatic compounds. They frequently occur in nature but can also be synthesized chemically. Esters are mainly produced by esterification, i.e., reversible interaction between carbonic acids and alcohols during acidic catalysis (the catalysts are HCl, H_2SO_4, arylsulfonic acids, boron trifluoride, and sulfonic acid cation-exchange resins). Another way of synthesizing esters that are difficult to obtain via direct esterification is transesterification, catalyzed by bases. Other approaches are also used: the effect of carboxylic anhydrides and acyl chlorides on alcohols (in the presence of bases); interactions between carbonic acids and olefins (acidic catalysis, elevated pressure); and the carbonylation of alcohols in the presence of olefins (catalyzed by rhenium and cobalt carbonyls) [3]. However, economic and environmental concerns determine the need and feasibility of replacing chemical synthesis with enzymatic process. Lipase is one of the best enzymes for the wide practical implementation of processes in organic synthesis.

Lipase has very broad substrate specificity: it recognizes a broad spectrum of structurally complex fats, alcohols, and carboxylic acids as substrates. Many lipolytic enzymes are active and stable in organic solvents; they require no cofactors and produce hardly any byproducts [4, 5].

In this work, we present experimental data on synthesizing butyrates of fusel oil using lipase agents.

The alcohol-containing fraction obtained by distilling fusel oil at a boiling temperature of 120–140°C was chosen as the substrate. This fraction consists entirely of alcohols. The main components are isoamyl alcohol (87.42%) and butyl alcohol (10.56%); their total content is 98.00%. Other alcohols are present in trace amounts.

The second substrate was butyric acid. This acid is commercially produced and widely available. Many of the esters formed by this acid and aliphatic alcohols are aromatic solutions, which are applied mainly as fragrances for detergents, food industry, or perfume compositions.

EXPERIMENTAL

The following lipase agents were used in this work:

- a freeze-dried commercial preparation of *lipase from porcine pancreas, Type II* (United States), with activity of 25.0 U/mg protein (determined using olive oil as substrate); and
- a freeze-dried commercial preparation of *lipase from Candida rugosa*, *Type VII* (Japan) with activity of 346.5 U/mg protein (determined using olive oil as substrate).

The lipase preparations were chosen for the following reasons. The synthetic activity of the enzyme preparation from *Candida rugosa*, which is mostly used for the hydrolysis of lipids [6–8], is still insufficiently explored, although this preparation is one of

Table 1. Composition of the fraction isolated from fusel oil (T_b = 120–140 °C)

Component	Content, %
Ethanol	0.180
Propanol-1	0.680
2-Methylpropanol-1 (isobutyl alcohol)	9.410
Butanol-1	1.150
Isoamyl alcohols	87.420
Secondary amyl alcohols	0.001
Tertiary amyl alcohols	0.001
Pentanol-1	0.050
Isomers of C_6 alcohol	0.110
Hexanol-1	0.280
C_7 alcohols	0.210
C_8 alcohols	0.480
2-Propenol-1 (allyl alcohol)	0.007
Unidentified components	0.020
Total	99.999

the most common among lipases. Pancreatic lipase is inexpensive and easily available.

Our source of alcohols was the fraction with T_b = 120–140°C, obtained via the distillation of fusel oil. The composition of this fraction was determined by means of gas–liquid chromatography. The components of the fraction were analyzed on a Crystall-2000 chromatograph. The composition of the fraction is given in Table 1.

The butyric acid of high-purity grade used in this work met the requirements of TU (Technical Specifications) 6-09-530-75.

The activities of the lipase preparations was determined using a modified Ota–Yamada technique [9].

A 40% emulsion of olive oil was used as the substrate. The emulsion was prepared by adding 37.5 mL of 3% gelatin solution to 25 mL of olive oil. Emulsification was achieved by a Cameron CBL-1210 dispersant in 5 min.

To prepare the enzyme solution, 10 mg of lipase preparation were added to 2.5 mL of phosphate-citrate buffer (pH 7.0–7.4) and 7.5 mL of water.

The substrate (2.5 mL) was supplied with 2.0 mL of phosphate–citrate buffer (pH 7.0–7.4). The resulting mixture was incubated at 37°C for 10 min. Then 1 mL of the enzyme solution was added to the mixture and thoroughly stirred. The mixture was kept at 37°C for 60 min. The reaction was then stopped immediately by adding 15 mL of 96% ethanol.

Titration was performed with an aqueous solution of NaOH (0.05 N) in the presence of 1% phenolphthalein until a persistent pink color was obtained.

The control contained no enzyme. The enzyme solution was added to the control sample immediately before titration.

The reaction mixture was a macroheterogeneous two-phase system of the liquid–solid phase type. The lipase preparations were suspended in an organic solvent without additional immobilization. In these experiments, the enzyme concentration was varied by suspending 5 to 30 mg/mL of the reaction mixture. The reagent mixture contained a 0.1 M solution of butyric acid in an organic solvent (hexane, heptane, decane, or benzene). The consumption of the fusel oil was determined from the content of its main component, isoamyl alcohol. The ratio of butyric acid to isoamyl alcohol was varied from 1 : 1 to 1 : 4. Enzymatic synthesis was conducted for 4–48 hours at temperatures ranging from 15 to 40°C.

The amount of produced ester was determined titrimetrically from the change in the amount of acid in the system. Titration was conducted with a 0.1 N alcohol solution of NaOH (in 80% ethanol) in the presence of 1% phenolphthalein at 20°C until a persistent pink color was obtained. The control sample contained no enzyme. The enzyme solution was added to it immediately before titration.

The yield of esters (Y, %) was calculated using the formula

$$Y = \frac{(C - S)}{C} \times 100,$$

where S is the amount of 0.1 N alcohol solution of NaOH spent on the titration of the sample, and C is the amount of 0.1 N alcohol solution of NaOH spent on the titration of the control, mL.

To obtain the product, the suspended enzyme was filtered through a Type 1 Whatman filter paper after the reaction. The organic solvent was separated from the reaction mixture via distillation under vacuum. The formation of butyrates was confirmed by IR spectroscopy on a Lumex instrument (InfraLUM).

All experiments were performed in 12 replicates. The results were processed using the statistical tools of the Statistica package. The significance of the difference between the control and experimental results was estimated using Student's t-test.

RESULTS AND DISCUSSION

The enzymatic synthesis of butyrates, performed without using an organic solvent as the reaction medium, showed that the process results in rather low yields: 14.0 ± 0.5% for the lipase from the *Candida rugosa* yeast and 5.8 ± 0.4% for the pancreatic lipase (the acid/alcohol ratio was 1 : 2; the reaction temperature was 30°C; and the reaction time was 24 h).

Table 2. Effect of the solvent on the enzymatic synthesis of butyrate

Solvent	Yield of butyrates, %	
	pancreatic lipase	lipase from the *Candida rugosa* yeast
Hexane	**93.8 ± 0.2**	**93.6 ± 0.0**
Heptane	93.9 ± 0.2	93.6 ± 0.2
Decane	94.5 ± 0.0	93.7 ± 0.7
Benzol	73.7 ± 0.7	87.6 ± 0.4

Reaction time, 24 h; alcohol : acid ratio, 1 : 2; the temperature, 30°C; amount of enzyme, 10 mg/mL.

Table 3. Effect of the butyric acid/alcohol ratio on the yield of butyrates in enzymatic synthesis

Acid/alcohol ratio (M/M)	Yield of butyrates, %	
	pancreatic lipase	lipase from the *Candida rugosa* yeast
1.0 : 1.0	82.9 ± 1.7	75.3 ± 1.8
1.0 : 1.5	92.5 ± 0.1	91.6 ± 0.4
1.0 : 2.0	**93.5** ± 0.4	**94.5** ± 0.2
1.0 : 2.5	**94.4** ± 0.2	**94.3** ± 0.1
1.0 : 3.0	91.3 ± 1.6	90.5 ± 1.1
1.0 : 4.0	87.9 ± 0.3	72.7 ± 0.0

Reaction time, 24 h; temperature, 30°C; amount of enzyme, 10 mg/mL; reaction medium, hexane.

In subsequent experiments, the process was conducted in an organic solvent chosen on the basis of [10, 11] and newly obtained data on the effect of the solvent on the yield of butyrates of fusel oil alcohols. We chose a solvent that had no inhibiting effect on the enzyme.

Data on the effect of organic solvents on the yield of butyrates produced by lipase preparations are shown in Table 2.

The obtained data show that solvents of the alkane class (C_6–C_8) produced yields of more than 90.0%. Hexane was chosen for subsequent studies as the most available of these solvents. Benzene produced lower yields, due probably to inhibition of the enzymes.

The process was then optimized using the acid/alcohol ratio, the content of enzymes in the system, the reaction temperature, and the reaction time. The obtained data are given in Table 3. They show that raising the alcohol content in the medium increases the yield, which is maximal at an acid/alcohol ratio of 1 : (2.0–2.5). When using pancreatic lipase and lipase from the *Candida rugosa* yeast, the maximum yield of butyrates was 94.5%. Raising the content of alcohol in the mixture again reduced the yield of the desired product.

It is known that enzymes are catalytically active in a certain temperature range. However, the temperature optimum can shift in an organic solvent. Data on the temperature dependence of the yield of butyrates are given in Fig. 1. The lipase from the *Candida rugosa* yeast has a pronounced temperature optimum at 30°C. Raising the temperature again reduced the yield of the enzymatic reaction by 20%. The pancreatic lipase synthesizes butyrates with a sufficiently high yield over the range of investigated temperatures.

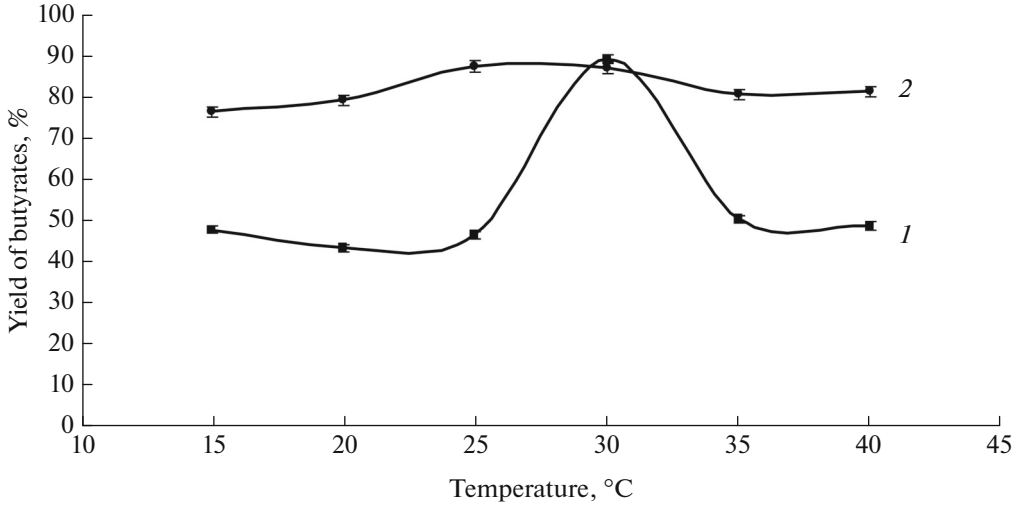

Fig. 1. The effect of the temperature on the yield of butyrates in reactions of synthesis catalyzed by (*1*) the lipase from the *Candida rugosa* yeast and (*2*) pancreatic lipase. Reaction time, 24 h; acid/alcohol ratio, 1 : 2; amount of enzyme, 20 mg; reaction medium, hexane.

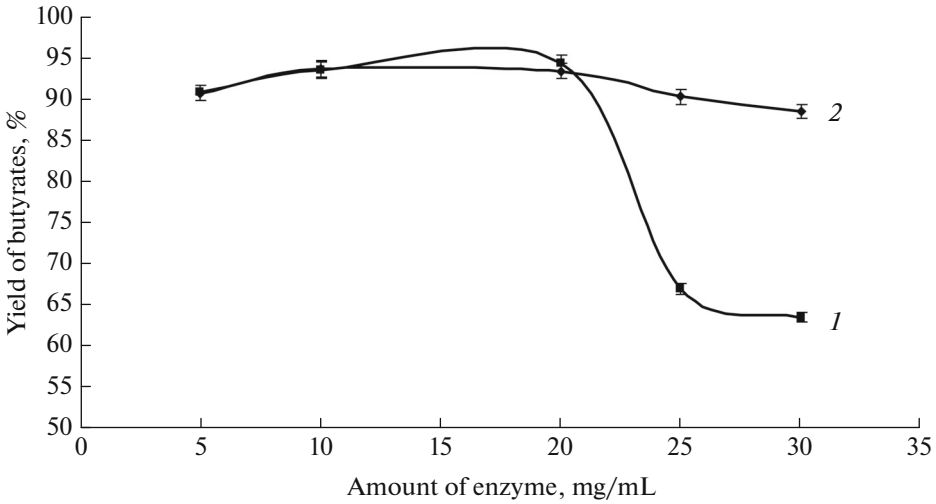

Fig. 2. The effect of the amount of enzyme on the yield of butyrates in reactions of synthesis catalyzed by (*1*) lipase from the *Candida rugosa* yeast and (*2*) pancreatic lipase. Reaction time, 24 h; acid/alcohol ratio, 1 : 2; temperature, 30°C; reaction medium, hexane.

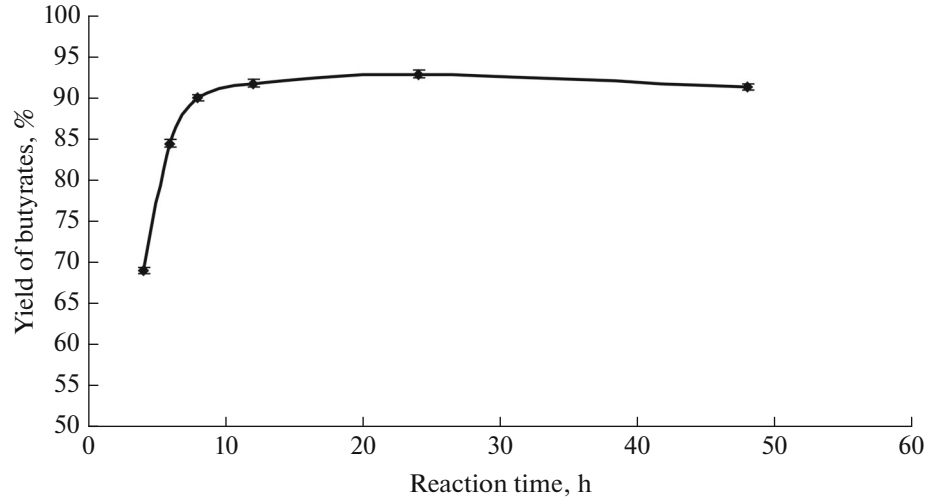

Fig. 3. The effect of the reaction time on the yield of butyrates in the synthetic reaction catalyzed by pancreatic lipase. Amount of enzyme, 10 mg/mL; acid/alcohol ratio, 1 : 2; temperature, 30°C; reaction medium, hexane.

The change in the enzyme content in the system can accelerate the rate of enzymatic catalysis. In macroheterogeneous two-phase systems of the liquid–solid phase type, however, increasing the concentration of enzymes does not always provide the desired result. We therefore studied the impact of the content of enzyme on the yield of the target products (Fig. 2).

Figure 2 shows that the yield of butyrates in the synthesis catalyzed by the enzyme preparations of the pancreatic lipase and the lipase from the *Candida rugos* yeast is maximal at an enzyme content of 10 mg/mL of the reaction mixture. At this content of enzyme, the yield of the target product was 93.8% for the pancreatic lipase and 93.6% for the lipase from the *Candida rugosa* yeast.

When the lipase from the *Candida rugosa* yeast is used, the increased concentration of the enzyme in the medium reduces the yield of the ester to a greater extent. For instance, when the enzyme concentration in the system is 25 mg/mL, the yield of butyrate falls by a factor of 1.4 relative to the maximum value. The reduction in the product yield upon increasing the enzyme concentration could indicate that the number of molecules located far from the interface in large grains of the enzyme preparation do not participate in the enzymatic reaction.

Our study of the dependence of the yield of butyrates on the reaction time showed that the yield of butyrates reaches 90.0% in as little as 8 h of enzymatic synthesis (Fig. 3).

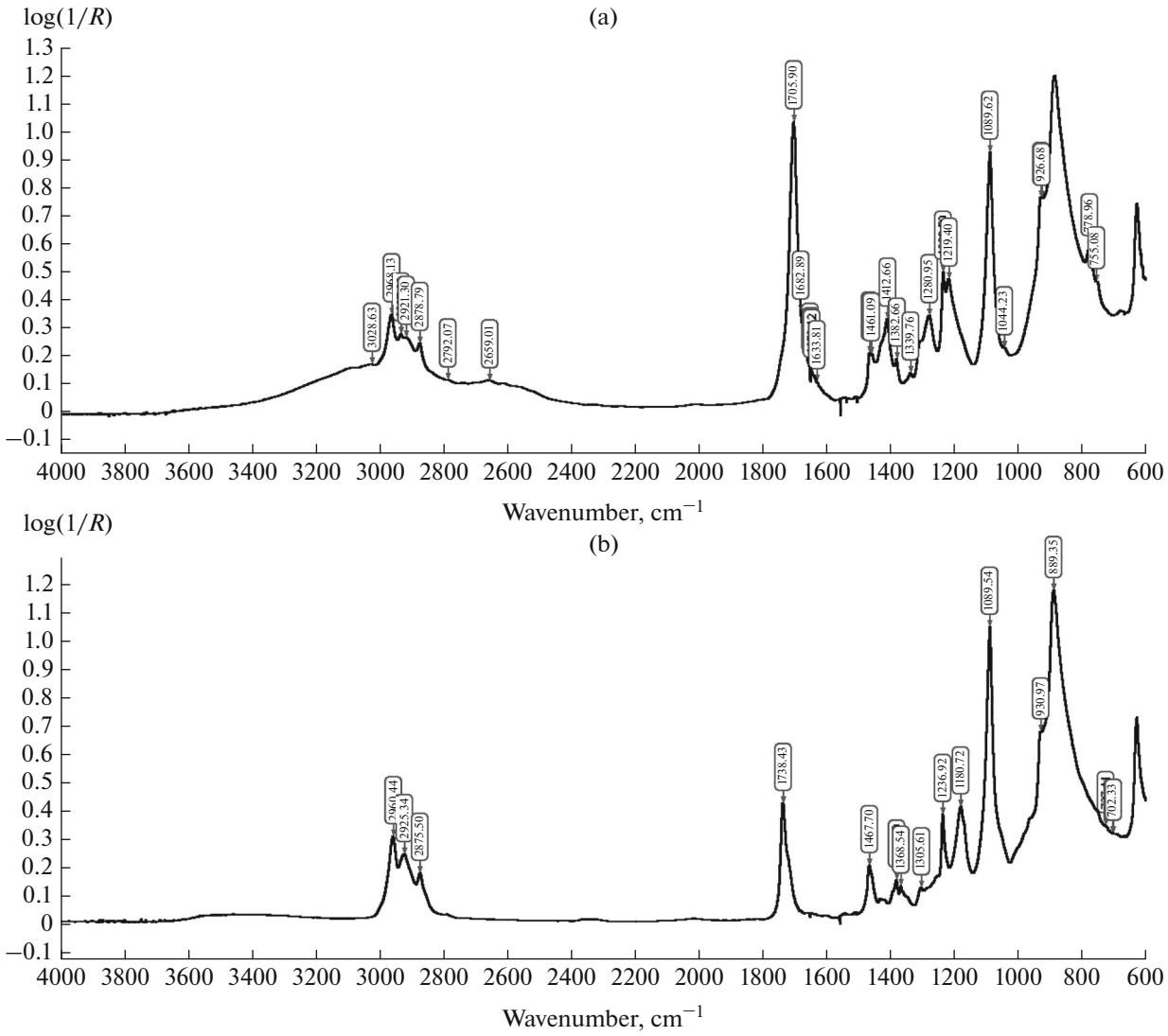

Fig. 4. IR spectra of (a) butyric acid and (b) butyrates.

After distilling off the excesses of alcohol and hexane under vacuum, the esters were analyzed via IR spectroscopy (Fig. 4). Analysis of the spectra showed that the stretching vibrations $\nu_{C=O}$ of the carboxyl group in the acid produce an intense band at 1705.9 cm^{-1}, while $\nu_{C=O}$ in the ester is shifted by almost 30 cm^{-1} and is observed at 1735.6 cm^{-1}. An intense signal of the ester bond ν_{COC} is found at 1180.7 cm^{-1} in the spectrum of the ester; this signal is missing from the spectrum of the acid. The stretching vibrations ν_{OH} are observed in the spectrum of the acid at 2659 cm^{-1}; these vibrations are missing of the spectra of the ester. These data clearly confirm the formation of esters during the enzymatic esterification of butyric acid.

CONCLUSIONS

The esterification of fusel oil alcohols by butyric acid in the presence of pancreatic lipase and lipase from the *Candida rugosa* yeast proceeds with a high yield (~94.0%) at a temperature of 30°C, a reaction time of 24 h, an acid/alcohol ratio of 1.0 : (2.0–2.5), and an enzyme content of 10 mg/mL. It was shown that enzymatic synthesis can be used to produce esters of butyric acid and lower alcohols, and can be an alternative to chemical methods for their preparation.

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