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PRODUCTION OF FLAVOR ESTERS BY IMMOBILIZED LIPASE

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SUMMARY

Candida cylindracea lipase adsorbed to silica gel produced a variety of flavor esters when hydrated and shaken in n-heptane containing substrates. Scale-up production of ethyl butyrate was examined in a packed column with recycling of n-hexane containing substrates. Increased substrate concentrations were stimulatory up to a point after which inhibition and enzyme destabilization in repeated runs occurred.

INTRODUCTION

Natural flavor esters extracted from plant materials are often either too scarce or expensive for commercial use. Esters produced from natural substrates by biocatalysts (considered "natural" by some regulatory agencies) can satisfy increasing commercial demand. Lipases can catalyze ester formation in an organic solvent capable of extracting the ester (Gatfield and Sand, 1984, Marlot et al., 1985, Patterson et al., 1979). We have previously shown that Candida cylindracea lipase adsorbed to silica gel could be repeatedly used for ethyl butyrate production in non-polar solvents provided that the enzyme was hydrated at the end of each batch process (Gillies et al., 1987). The C. cylindracea lipase exhibits a wide pH range (pH 2 to 8.5) of esterase activity (Tomizuka et al., 1966) and can transesterify a wide range of fatty acids from butter triglycerides (C4 to C18) to ethanol (Kanisawa, 1983). The present communication describes the characterization and potential of this immobilized lipase for commercial production of a variety of flavor esters.

MATERIALS AND METHODS

Enzyme Immobilization

Candida cylindracea lipase (glycerol-ester hydrolase, EC 3.1.1.3) (Sigma Chemicals Co.) was suspended in water to a concentration of 1-5% (w/v). The supernatant (recovered by decantation) was mixed with silica gel (Davisil 646; 60 mesh, pore diameter 150 A, provided by Grace Industrial Chemicals) (in the ratio of 3 mL supernatant to 1 g silica gel) and left at 25°C for 3 h. The gel was then washed three times with water on a glass-sintered funnel and excess liquid was removed by suction

with a water aspirator for a few minutes. The hydrated immobilized lipase was then ready for application.

Ester production in a shaken flask

Hydrated immobilized lipase was reacted with substrates dissolved in n-heptane in the ratio of 1 g to 4 mL. Reaction mixtures were incubated at 30°C with shaking at 200 rpm. At various times, samples (0.5 mL) were taken from the heptane phase and were added to heptane (1 mL) containing 1-hexanol (0.05% (w/v)) as internal standard. Ester, alcohol and acid were assayed using a FID gas chromatograph equipped with a glass wide bore capillary column (Supelcowax 10).

RESULTS AND DISCUSSION

Effect of substrate concentration

Since ethyl butyrate is in high demand as a component of pineapple-banana flavors in food industries, its production was used as a model system to study the effect of the substrate concentration. Immobilized lipase was prepared by soaking the support in a 1% lipase solution. Adsorbed lipase was hydrated and shaken in heptane containing equimolar concentrations (0.25 M) of butyric acid and ethanol. After 24 h reaction, 44% of the butyric acid was converted to ethyl butyrate. The limited synthesis was due to the unavailability of ethanol because no ethanol was found in the heptane phase. Since silica gel absorbs ethanol, excess ethanol is necessary. Keeping the butyric acid concentration at 0.25 M, the effect of higher ethanol concentrations ranging up to 1.2 M was studied. Figure 1 shows that with the use of 0.4 M ethanol, greater conversion of butyric acid to ester occurred, but higher ethanol concentrations were inhibitory to ester production.

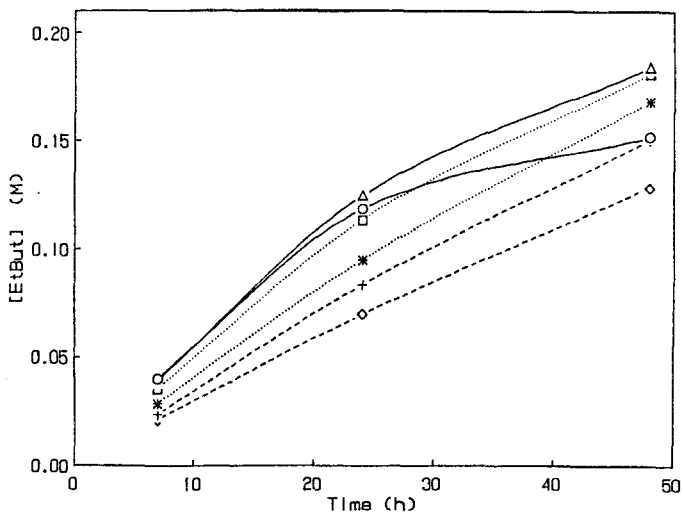

Fig. 1. Effect of ethanol concentration on ethyl butyrate production by C. cylindracea lipase. Immobilized lipase was reacted in heptane containing 0.25 M butyric acid and ethanol varying in concentration as follows: 0.25 M (○); 0.4 M (△); 0.6 M (□); 0.8 M (✳); 1.0 M (+); 1.2 M (◇).

In order to increase ester production rate, enzyme loading of the support was increased. Silica gel was soaked in different concentrations of lipase, was washed, then shaken in heptane containing 0.4 M ethanol and 0.25 M butyric acid. Production rate was increased by using up to a 5% lipase concentration, above which there was no significant increase.

Since initial ethanol concentration of 0.4 M and enzyme loading using a 5% lipase solution showed improved results, these conditions were used to study the effect of butyric acid concentration on ester production as well as stability of the immobilized lipase in repeated runs. Figure 2 shows that in the first run the rate of ester production during 22 h was higher when a higher concentration of butyric acid was used. However, the percent conversion of acid was lower for the 1 M and 0.5 M butyric acid concentrations (39% and 80% respectively) than the 0.25 M and 0.13 M butyric acid concentrations (total conversion). Another disadvantage of using high butyric acid concentrations (> 0.5 M) is instability of the immobilized enzyme, that is, decreasing ester production during repeated runs. Stable ester production was observed with use of lower butyric acid concentrations. For these reasons, the use of 0.25-0.5 M butyric acid is recommended. For optimal production of other esters, these effects of substrate concentration have to be taken into account.

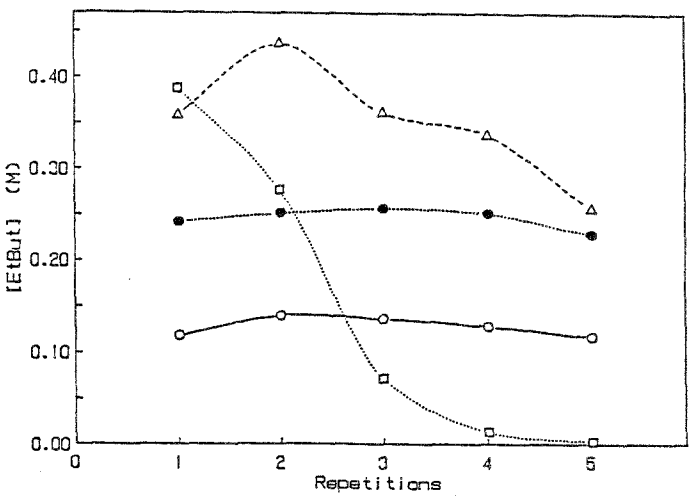

Fig. 2. Effect of butyric acid concentration on production of ethyl butyrate by immobilized C. cylindracea lipase. Immobilized lipase was reacted for 22 h periods repeatedly in heptane containing 0.4 M ethanol and butyric acid as follows: 0.13 M (○); 0.25 M (●); 0.5 M (△); 1.0 M (□).

Effect of temperature

Commercially, use of ambient temperature (20-30°C) is economical. Figure 3 shows that ester production using the present immobilized lipase system was optimal between 20-30°C whereas it was inhibited at higher temperatures.

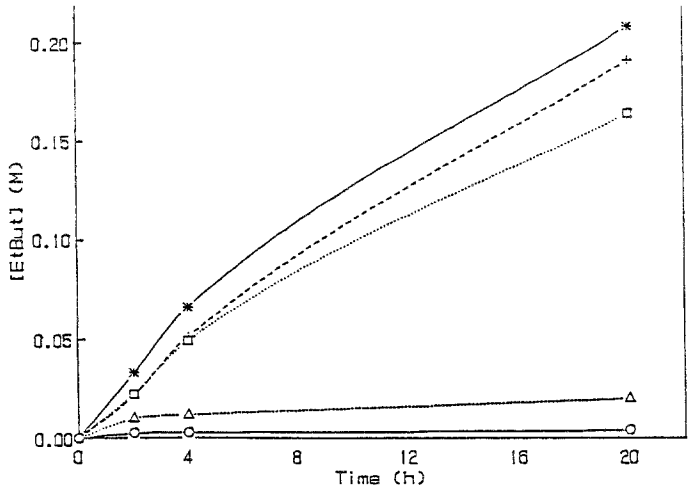

Fig. 3. Effect of temperature on production of ethyl butyrate by immobilized C. cylindracea lipase. Immobilized lipase was shaken with 0.4 M ethanol and 0.25 M butyric acid at: 60°C (○); 50°C (△); 40°C (□); 30°C (*); 20°C (+).

Demonstration of production of other flavor esters

The substrate specificity of this system will determine its commercial potential for producing a variety of flavor esters. Table 1 shows that this immobilized system produced a variety of esters from different alcohols and acids. Production at 24 h does not necessarily show the end point of the reaction. For example, ethyl hexanoate production continued to 79% conversion after 48 h. Isoamyl and isobutyl acetate production was slow with 46 % conversion after 72 h. The differences in the rate of ester production can be ascribed to specificity of the enzyme, and differences in substrate solubility (e.g. ethyl lactate and ethyl cinnamate were not produced probably due to the low solubility of the acids in heptane).

Table 1. Ester production by immobilized C. cylindracea lipase using a range of substrates. The molar concentration is that of ester in the heptane phase after 24 h. Percent molar conversion is that of initial acid to ester after 24 h.

ESTER	(M)	% CONVERSION
Ethyl propionate	0.19	76
Ethyl butyrate	0.25	100
Ethyl hexanoate	0.09	44
Ethyl heptanoate	0.21	84
Ethyl octanoate	0.26	100
Ethyl laurate	0.13	52
Ethyl isobutyrate	0.18	72
Ethyl isovalerate	0.01	3
Isobutyl acetate	0.06	25
Isoamyl acetate	0.06	24
Isoamyl butyrate	0.22	91

Packed bed column operation

Data so far obtained in shake flasks can be translated into stirred tank operation which will however cause abrasion of the silica gel, resulting in loss of enzyme. Therefore, a packed bed reactor was chosen as most appropriate for large scale use of the immobilized lipase.

The reactor consisted of a glass column (10 mm I.D.) packed to a height of 15 cm with 3.6 g of air-dried immobilized enzyme. Water was passed through the column and then removed by suction. Three bed volumes of substrate (0.4 M ethanol, 0.25 M butyric acid) in hexane were circulated through the column by means of a peristaltic pump. Hexane was used as the solvent because it has been approved for commercial extraction of flavor compounds. Figure 4 shows the kinetics of ethyl butyrate production on the 15 cm column with nearly quantitative conversion of the butyric acid after 24 h.

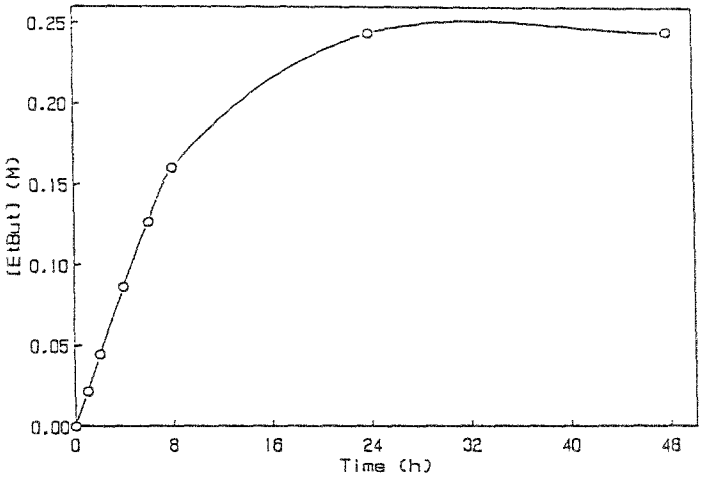

Fig. 4. Kinetics of ethyl butyrate production with immobilized C. cylindracea lipase in a packed column. Substrate in hexane was recycled at a space velocity of 400 h^{-1}.

The same kinetics of ester production were observed when the space velocity was lowered from 400 h^{-1} to 20 h^{-1}. However, space velocities lower than 20 h^{-1} showed decreased ester production rates. Thus, a space velocity of 20 h^{-1} is sufficient for the present system.

This packed bed system can be readily scaled up to commercially available large-radius columns of the same 15 cm bed height. This system can also be easily automated for continuous batch processing. The hexane/substrate solution can be circulated through the column until conversion of acid is complete, then passed to another unit operation for separation of ester and recovery of solvent for recycling. After rinsing the column with a water stream, a stream of fresh substrate can be directed into the circulating system.

In summary, immobilized lipase prepared by simple adsorption to

silica gel shows potential for commercial production of a wide variety of flavor esters when used in a packed column biphasic system as described.

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