0273-2289/99/77–79/0817/$12.50

Fusel Oil as Precursor for Aroma Generation by Biotransformation Using Lipase

Scientific Note

Heizir F. De Castro,* Regina Y. Moriya, Pedro C. Oliveira, and Cleide M. F. Soares

Department of Chemical Engineering, Faculty of Chemical Engineering of Lorena, PO Box 116, 12600-000, Lorena, S. Paulo, Brazil, E-mail: decastro@eabygold.com.br

Abstract

The feasibility of using mixtures of C4 and C5 chain-length aliphatic alcohols from fusel oil as the bulk organic media for lipase-mediated synthesis of laurate esters was assessed. Reaction mixtures consisted of lauric acid, lipase, solvent (if added), and appropriate amount of fusel oil (previously dehydrated with inorganic salts and molecular sieves). The influence of the reaction conditions such as substrate concentrations and temperature were investigated. Increased molar ratio of acyl donor to acyl acceptor allowed the esterification to proceed with no need for solvent addition.

Index Entries: Fusel oil; lipase; esterification; laurate esters.

Introduction

In countries where fuel ethanol is produced in large scale (over 13 billion L/alcohol/yr), such as Brazil, by-product utilization has become an important issue to make the ethanol production less polluting and more profitable *(1,2)*. Among these, fusel oil obtained at the rectification stage is currently used as raw material for the production of amyl and butyl alcohols *(2)*. However, only a small fraction (less than 25%) of the total fusel oil generated (around 30 million L/yr) is processed for this purpose *(3)*. The main components in fusel oil are ethanol, butanol and isoamyl alcohol, the remaining constituents being small proportions of other secondary alcohols and water *(3)*. Owing to its rich alcohol composition and availability in large quantities, fusel oil can be considered an inexpensive source of

*Author to whom all correspondence and reprint requests should be addressed.

starting material for the production of important natural flavor compounds by chemical synthesis *(2)*.

Possible alternatives for the production of flavoring compounds are biotechnological methods and the use of so-called biocatalyst for their synthesis: plant cell and tissue cultures, microorganisms and enzymes *(4,5)*. At the moment, microbial processes seem to be the most promising for the production of pure flavor compounds. Thus, natural isobutanol, which is one of the components of fusel oil, can be converted by certain *Acetobacter* strains into natural isobutyric acid. The other fusel alcohols such as 3-methyl butanol and 2-methyl butanol can be converted by the yeast *Hansenula mraki* into the corresponding acetates in a fairly high yield (more than 90% for 3-methylbutanol acetate). The esters produced volatilize during the aerobic process and are adsorbed on activated carbon. The resulting concentrate obtained by desorption of the activated carbon, can be used as a natural banana aroma *(6)*.

Another way of making natural esters is by exploiting the synthetic capabilities of a particular class of enzymes, mainly lipases. This property of some lipases has been known for a few years and is well established *(7–12)*. The lipase-catalyzed synthesis of more than 50 flavoring esters have been described to date *(5)*, and in principle, the reaction can be canted out in a mixture of alcohol and carboxylic acid with or without solvents, resulting in very high productivities and yields *(8–10)*. Even so, only a few researchers have previously developed a lipase-mediated process in nonaqueous reaction fluid for the conversion of fusel-oil alcohols to their respective carboxylic esters *(3,13,14)*. This may be associated to the technical problems involved when natural mixtures of alcohols are used in the esterification reactions, such as the competition between two or more acyl acceptors for the active site of the enzyme and high water levels. In the case of fusel oil, which has in its composition a mixture of both primary and secondary alcohols, a further complication may arise because secondary alcohols esterifications are strongly dependent on the size of the fatty-acid chain. The yields tended to decrease with shorter fatty acids; the lowest yields being obtained with acetic acid *(15,16)*. Therefore, the selection of the acyl donor is a crucial step to turn out the substrate into a suitable conditions required for lipase-mediated synthesis.

Although ester production by enzymatic process has been reported by several researchers, most of the published works are concerning the use of synthetic media *(7–9)* and little information is available for substrate derived from industrial residues. In this work, fusel oil from sugar-cane fermentation was used as a precursor for the preparation of other flavorants. For reasons associated with technical and cost feasibility, lauric acid was chosen as acyl donor. This kind of fatty-acid compromise suitable surface active material for shifting the reaction equilibrium towards the lipase-catalyzed esterification of secondary alcohols and can be readily obtained from babassu coconut (regional Brazilian fruit containing about 45% of lauric acid). Provided the starting materials could be available at low cost,

the process may gain industrial importance, particularly for laurate esters such as butyl and isoamyl, which are commercially produced as flavor-active materials.

Material and Methods

Materials

The enzyme, Lipozyme IM[20] (24 BIU/g) was kindly donated by Novo Nordisk (Denmark) and used as supplied (10% moisture content). Fusel oil was supplied by local alcohol distillery (São Paulo, Brazil) having the following composition (in w/v): ethanol (6%), n-propanol (1.7%), isobutanol (1.5%), n-butanol (10%), and isoamyl (52%). Reactants (n-butanol, isoamyl alcohol, approx 95%, and lauric acid, approx 99%, were purchased from Merck). Dry n-heptane, dried over molecular sieves, was used as the solvent for all experiments performed, except where noted otherwise. Inorganic salt (Na_2SO_4) and 0.32-cm molecular sieves (aluminum sodium silicate, type 13 X- BHD Chemicals, Canada) were used as dehydrated agents. Other reactants were purchased from Aldrich Chemical Company (Milwaukee, WI).

Methods

The high water levels originally present in the fusel oil were decreased by performing dehydration techniques with inorganic salts and molecular sieves as described by Valivety et al. *(17,18)*. The substrate used for esterification reactions consisted of appropriate amounts of dehydrated fusel oil, lauric acid, and heptane (if added). The level of Lipozyme used was 10% of the total substrate weight following the manufacturer's recommendation *(18)*. Esterifications substrates were preconditioned by incubating the substrate for 30 min with agitation at the reaction temperature prior to the addition of Lipozyme.

The enzymatic reactions were performed in 50-mL round-bottom flasks with magnetic stirrer (100 rpm). Except where noted, the reactions were carried out at 40°C. The reactions were monitored by measuring reactants concentrations by gas chromatography using a 6-ft 5% DEGS on Chromosorb WHP, 80/10 mesh column (Hewlett Packard, Palo Alto, CA), and hexanol as an internal standard. Water concentrations in the liquid and solid phases were measured by Karl Fischer method using the Karl Fischer Tritrator (Mettler DL 18). The results were evaluated by calculating the alcohols conversion rates, as previous described *(12)*.

Results

Ester formation by lipases has been shown to follow classical Michaelis-Menten kinetics with the intermediate formation of an acyl-enzyme complex *(19)*. When two or more acyl acceptors with the same acyl group are present in the reaction mixture, the competitive factor is used to describe

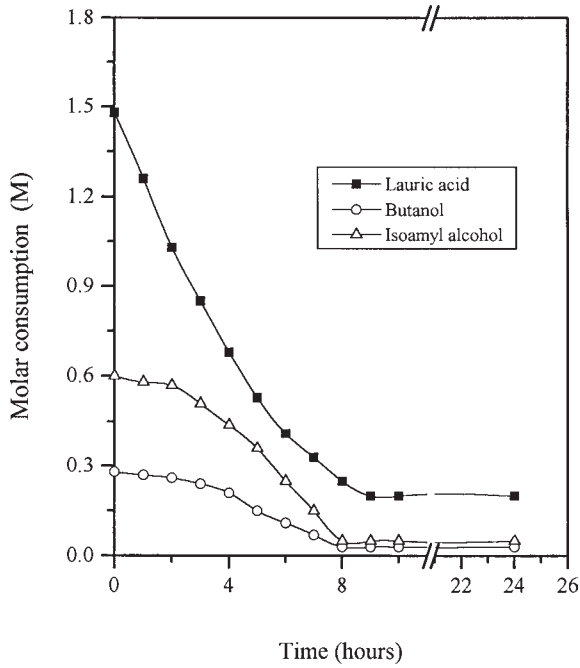

Fig. 1. Progress of the esterification reaction of n-butanol (0.3 *M*) and isoamyl (0.6 *M*) in competition for a single acyl donor (1.5 *M*). Substrates were made with standard reagents using heptane as solvent for simulating the composition of fusel oil. Reaction was carried out at 40°C.

the kinetics of the reaction *(19,20)*. Therefore, a necessary first step in assessing the feasibility of the lipase-mediated synthesis of fusel-oil esters was to verify the competition towards the enzyme active site when a mixture of acceptor alcohols are present at the same time in the reaction medium. In this approach, a standard substrate prepared with analytical reagents was used to simulate the original composition of fusel oil in terms of alcohol structures. Primary alcohol (n-butanol) and secondary alcohol (isoamyl) at concentrations of 0.3 *M* and 0.6 *M*, respectively, were mixed in n-heptane in the presence of 1.5 *M* lauric acid and incubated at 40°C. The other alcohols (propanol, isobutanol, and amyl alcohols) were omitted in this test because their low concentrations are supposed to give only a neglected interference in the reaction rates. The progress of the esterification reactions using this simulating substrate is shown in Fig. 1. High conversion rates of both butanol and isoamyl was attained after an incubation period of about 8 h. Although a simultaneous utilization of both alcohols was observed, isoamyl was the faster-reacting compound of the mixture. Similar profile was found when the substrate was prepared by using fusel oil instead of standard reagents (Fig. 2). These results suggest that lauric acid is a powerful acyl group in terms of selectivity and reaction rates. This allowed the first step of lipase kinetics to be enhanced, following the mechanism described by Rangheard et al. *(19)*. According to these authors, for multiple substrate

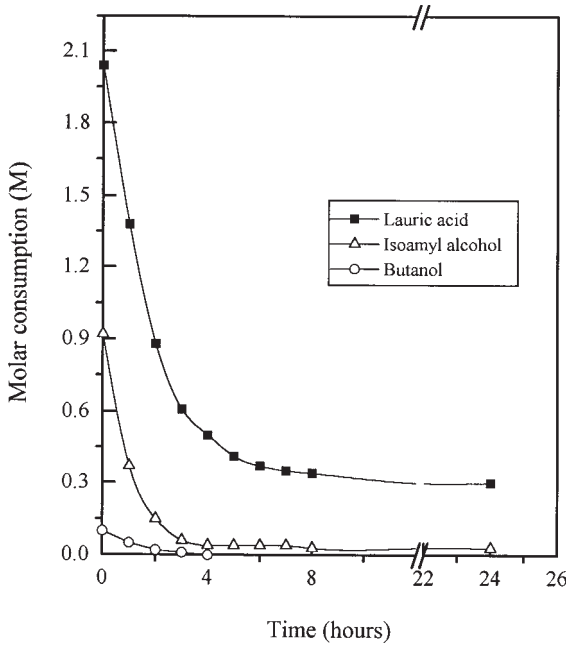

Fig. 2. Progress of the esterification reaction of butanol (0.1 *M*) and isoamyl (0.6 *M*) in competition for a single acyl donor (2.0 *M*). Substrates were prepared by mixing 4.0 mL of fusel oil with 16 mL of heptane. Reaction was carried out at 40°C.

competitive reactions, the rate limiting step is the acyl-enzyme formation. Consequently, the competition reaction is only a function of the nature of the different acyl donors and not of the acceptor used. In fact, by using appropriate amounts of the right acyl donor, the selectivity of enzyme for secondary alcohols was clearly demonstrated. This is in agreement with reported papers that claim that lauric and phenyl valeric acids showed better performance when compared to shorter fatty acids *(16,20)*. It is also probable that under the conditions of high acyl donor/acyl acceptor molar ratios the esterification was not affected by the presence of water, which is expected to play a role in this process. This was further confirmed by running a set of experiments in which the effect of molar ratio between acyl donor and acyl acceptors (Fig. 3) was evaluated. The results indicated that increased molar ratio of acyl donor to acyl acceptor allowed to attain high reaction rates and minimized the reverse reaction. Therefore, acyl donor in excess was used in most of the other experiments.

Having established these preliminary parameters, further experiments were carried out to determine the maximum amount of fusel oil that can be used to perform the lipase reverse action with high yield. This was done by gradually decreasing the amount of heptane used for diluting the fusel oil, up to a level where the esterification can be proceed with no need of solvent addition. In this set of experiment runs, a reaction temperature of 40°C was used for comparison of the effects of increasing fusel-oil pro-

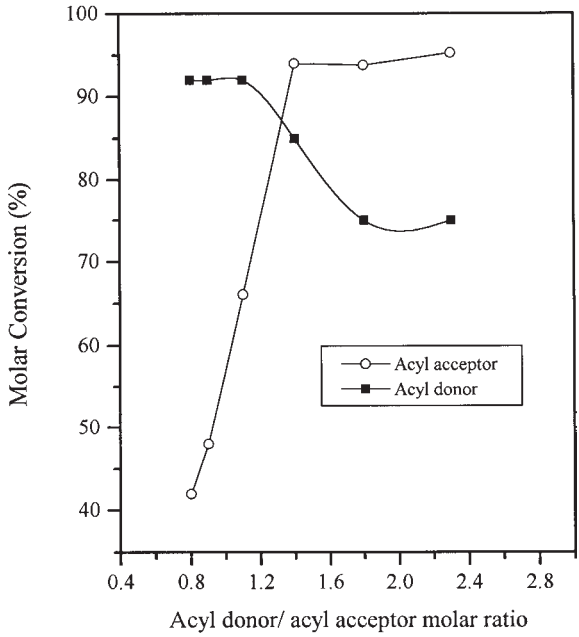

Fig. 3. Effects of the ratio of acyl donor to acyl acceptors on the molar conversion (after 24 h reaction). Substrates were prepared by mixing appropriate amounts of fusel oil, lauric acid, and dry heptane. Reactions were carried out with 10% Lipozyme content at 40°C.

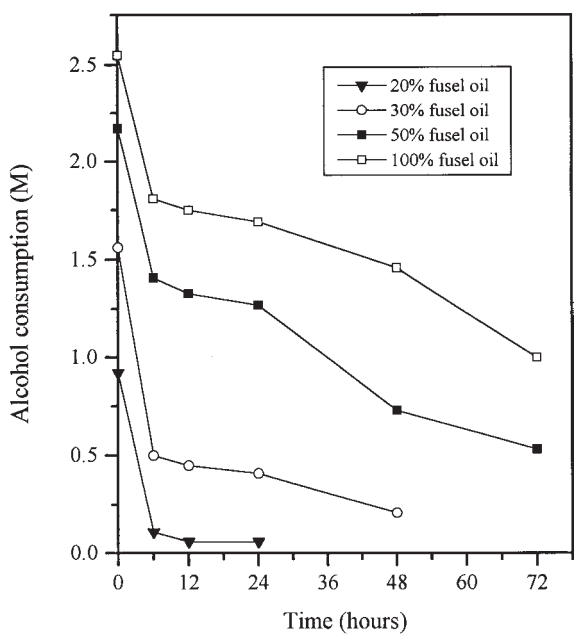

Fig. 4. Effect of the ratio of fusel oil to heptane on the isoamyl alcohol consumption as a function of time. Temperature was 40°C and 10% Lipozyme content was used.

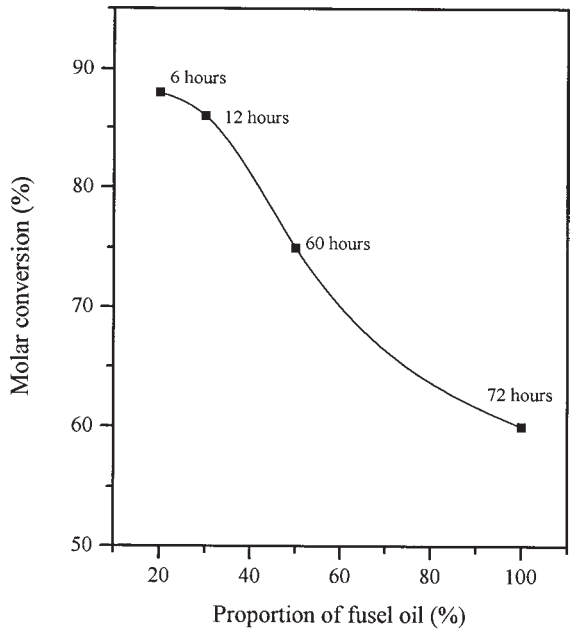

Fig. 5. Correlation between molar conversion and proportion of fusel oil in the reaction medium. (Data used with permission from Fig. 4.)

portions (20–100%) on the esterification rate with lauric acid by Lipozyme. The completion of the reaction was very dependent on the amount of fusel oil (Figs. 4 and 5). Conversion rates over 85% were achieved in less than 12 h when low fusel-oil concentrations were used (20 and 30%). Reactions carried out with higher fusel oil concentrations (50 and 100%), even at appropriate conditions, required longer reaction times in order to attain conversion rates lower than 75% (Fig. 5).

Taking advantage the special properties of Lipozyme such as, high activity and stability at temperatures up to 60°C, it is reasonable to expected an improvement on the esterification performance by simply adjusting the reaction temperature. Five reaction temperatures were investigated (Fig. 6), temperatures higher than 60°C were not used since this compromises the activity of the Lipozyme for another subsequent run. Although the activity of Lipozyme depends on the reaction temperature being maximum at 70°C, for long-term operations, it is recommended to use it at 60°C, as the slightly lower activity at 60°C is counterbalanced by an equally slower deactivation of the enzyme *(9,18)*.

As expected, the esterification performance was improved by increasing the incubation temperature up to 60°C. As shown in Fig. 6, the time required to attain high conversion rates with the solvent-free system gradually decreased when the temperature of incubation increased from 40–60°C. Under appropriate conditions, which are fully described in Table 1, efficient esterification of fusel oil was achieved. A typical solvent-free esteri-

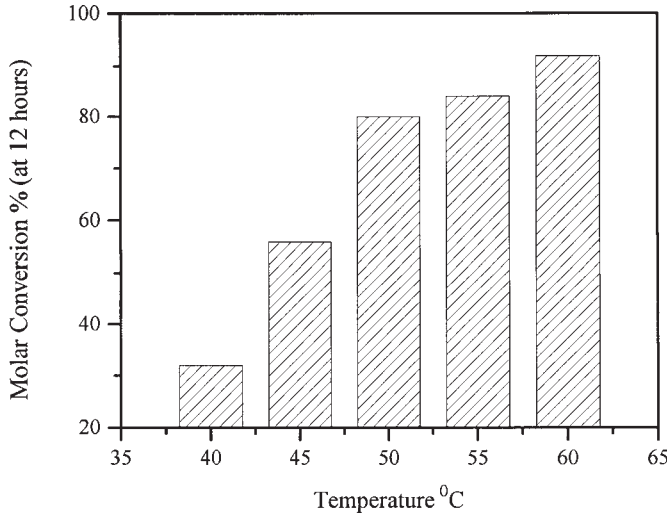

Fig. 6. Effect of temperature on the isoamyl molar conversion attained after 8 h of reaction. Fusel oil (5 g) was mixed with 8.8 g of lauric acid and allowed to heat at the required temperature for 30 min before the addition of 1.25 g of Lipozyme. Temperature reactions were: 40, 45, 50, 55, and 60°C.

Table 1
Summary of the Achieved Results Using Fusel Oil as a Precursor for Ester Formation

Tested parameters	Established conditions
Dehydration technique	Molecular sieves and inorganic salts
Molar ratio (acyl donor: acyl acceptor)	>1.5
Temperature	60°C
Enzyme concentration	10% total weight reactants
Water removal during batch runs	Procedure unnecessary

fication of fusel oil with lauric acid is given in Fig. 7. These reaction characteristics were representative of the results seen under the optimized conditions used in this work, and were observed in several reactions. In most reactions, very low levels of other laurate esters were formed, because high proportions of lauric acid (average of 3.5 *M*) decrease the original fusel alcohol concentrations to a levels of about 2.2 *M* for isoamyl alcohol and 0.2 *M* for butanol.

Discussion

Esters are important components of natural aromas, contributing to the flavor in most fruits and many other foods *(4,5)*. With the increasing demand for natural products, the food industry is interested in the use of biotechnological route to produce ester flavors *(6)*. In this context, it is not

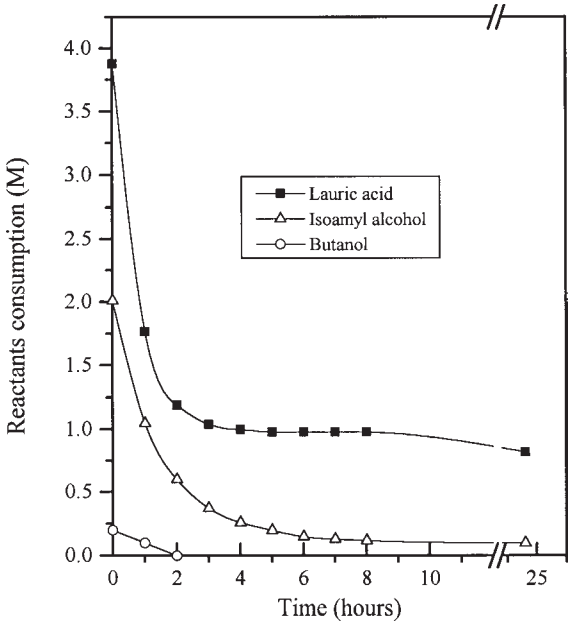

Fig. 7. Progress of typical solvent-free esterification reaction of fusel oil with lauric acid. Fusel oil (5 g) was mixed with 8.8 g of lauric acid and allowed to heat at 60°C for 30 min before the addition of 1.25 g of Lipozyme. Reaction was carried out at 60°C.

surprising that a great deal of research has been focused on the application of enzymes to catalyze various reactions *(7)*.

Lipase-catalyzed esterification between free acids and alcohols is a common strategy for the acylation of primary alcohols while still remaining a difficult task for secondary alcohols *(15)*. For the esterification of secondary alcohols, the choice of an appropriate acyl donor influences significantly the enzyme reactivity. Longer-chain fatty acids (C > 10) were found to be good substrates for the esterification reactions. The use of middle-chain fatty acids (10 < C > 5) resulted in low product yield, and short-chain fatty acids such as acetic acid showed very low reactivity *(16,20)*.

Previous attempts by other groups to enzymatically esterify fusel oil with carboxylic acids have been restricted to short-chain acids (mainly acetic and butyric acid), which usually gave low yields *(13,14)*.

To enhance the enzyme selectivity towards for esterification of isoamyl alcohol, the main constituent of fusel oil, lauric acid was used as acyl donor. The determination of several parameters, demonstrated the feasibility of the production of isoamyl laurate from fusel oil and lauric acid, using Novo's Lipozyme as catalyst. Under the experimental conditions, esterification conversion yields over 92% was obtained in less than 8 h reaction, at 60°C. The molar ratio between lauric acid and isoamyl alcohol should be greater than 1.5 in order to minimize the reverse reaction. Although other parameters in this biotransformation must still be studied—for instance,

other model system involving fusel oil/acyl donor combinations—the present communication should contribute to increase the relevant information concerning the ester production from industrial residues, by biotechnology processes.

Acknowledgments

The authors gratefully acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação de Amparo à Ciência do Estado de São Paulo (FAPESP) for their financial support.

References

1. Bichara, J. and Pizysienzning Filho, J. (1991), *Saneamento Ambiental* **11,** 14–23.
2. Laluce, C. (1990), *Crit. Rev. Biotechnol.* **11,** 149–161.
3. Macedo, G. A. and Pastore, G. M. (1997), *Ciência e Tecnologia de Alimentos* **17(2),** 115–119.
4. Gatfield, I. L. (1995), *Food Sci. Technol. Today* **9,** 237–240.
5. Jensen, B. F. and Eigtved, P. (1990), *Food Biotechnol.* **4,** 699–725.
6. Jansen, L., De Pooter, H. L., Schamp, N. M., and Vandamne, E. J. (1992), *Process Biochem.* **27,** 195–215.
7. Faber, K. (1997), in *Biotransformations in Organic Chemistry*, Faber, K., ed., Springer-Verlag, Berlin, pp. 255–271.
8. Welsh, F. H., Murray, W. D., and Williams, R. E. (1989), *Crit. Rev. Biotechnol.* **9,** 105–169.
9. de Castro, H. F. and Anderson, W. A. (1995), *Quimica Nova* **18,** 544–554.
10. Novo Nordisk Bioindustrial Group (1994), *Biotimes* **3,** 6.
11. Vulfson, E. N. (1993), *Trends Food Sci. Technol.* **4,** 209–215.
12. de Castro, H. F., Pereira, E. B., and Anderson, W. A. (1996), *J. Braz. Chem. Soc.* **7,** 219–224.
13. Welsh, F. W., Williams, R. E., Chang, S. C., and Dicaire, C. J. (1991), *J. Chem. Technol. Biotechol.* **52,** 201–209.
14. Welsh, F. W. and Williams, R. E. (1989), *J. Food Sci.* **54,** 1565–1568.
15. Molinari, F., Gandolfi, R., and Aragozzini, F. (1996), *Biotechnol. Tech.* **10,** 103–108.
16. Fukui, S. and Tanaka, A. (1987), *Methods Enzymol.* **136,** 293–302.
17. Valivety, R. H., Halling, P. J., and Macral, A. R. (1992), *Biochimica et Biophysica Acta* **1118,** 218–222.
18. Novo Nordisk Bioindustrials Inc. (1992), Product Information, B347C-GB, December.
19. Rangheard, M. S., Langrand, G., Triantaphylides, C., and Barati, J. (1989), *Biochimica et Biophysica Acta* **1004,** 20–28.
20. Triantaphylides, C., Langrand, G., Millet, H., Rangheard, M. S., and Buono, G. (1988), Bioflavour'87, Walter de Gruyter, Berlin, pp. 531–542.