Lipase-Catalyzed Esterification of Lactic Acid with Straight-Chain Alcohols

Torben H. Roenne[a], Xuebing Xu[b], and Tianwei Tan[a,*]

[a]Department of Biochemical Engineering, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, People's Republic of China, and [b]Food Biotechnology and Engineering, BioCentrum-DTU, Technical University of Denmark, 2800 Lyngby, Denmark

ABSTRACT: Enzymatic synthesis of esters of lactic acid and straight-chain alcohols with different chain lengths (C_6–C_{18}) were investigated in batch reactions with hexadecanol (C_{16}) as the model alcohol. Cyclohexane was the best solvent for higher ester yields, and the best biocatalyst was the immobilized *Candida antarctica* lipase B (Novozym 435) as well as the textile-immobilized *Candida* sp. lipase. A method was established to obtain ester yields in the range of 71 to 82% for the different alcohols, and the most favorable conditions for the esterification reaction using Novozym 435 were an equimolar ratio of lactic acid to alcohol, each at a concentration of 120 mM each; a 50°C reaction temperature; 190 rpm shaking speed; and the addition of 100 mg molecular sieves (4 Å) for drying. The ester yield increased with increasing lipase load, and a yield of 79.2% could be obtained after 24 h of reaction at 20 wt% of Novozym 435. The immobilized *Candida* sp. lipase prepared in the laboratory also could be used to produce esters of lactic acid and straight-chain alcohols, but it had a much lower activity than Novozym 435 with a temperature optimum of 40°C.

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KEY WORDS: *Candida antarctica* lipase, *Candida* sp. lipase, esterification, lactic acid esters, Novozym 435.

Because of its humectant properties, lactic acid has been used in the formulation of both cosmetic and pharmaceutical products. However, being an α-hydroxy acid, lactic acid has limited applications because it penetrates too quickly into the deep epiderm and causes skin irritation if administered at very high concentration (>10%) (1,2). One way to overcome this problem is to neutralize the acid group in lactic acid by reacting it with an alcohol. Esters thus formed can be used as humectants in cosmetic and pharmaceutical formulations (3). Esters of lactic acid also have been used in the food industry as surfactants in cereals and dairy products. In addition, lactic acid esters can act as bactericidal and fungicidal agents (4).

A major concern with reactions involving lactic acid is that it contains both a hydroxyl and a carboxylic acid functional group and may therefore act as both acyl donor and nucleophile. It can thus undergo self-polymerization, which is favored at high temperatures and in poorly hydrated media, to form linear polyesters and lactones (5). Furthermore, since lactic acid is a very polar compound it is not very soluble in nonpolar solvents. This is unfortunate since more highly nonpolar solvents are more favorable for enzymatic esterification (6).

Only a few reports have been published concerning the enzymatically catalyzed esterification reaction of lactic acid and alcohols (7,8). From *et al.* (7) have described the lipase-catalyzed esterification reactions of lactic acid in *n*-hexane using Novozym 435. They obtained high yields of ester products with the short-chain fatty alcohol butanol (89%), but the yield was considerably lower for longer-chain alcohols (28 and 11% for octanol and decanol, respectively). Torres and Otero (8) obtained good esterification yields (94–96% in 48 h) between lactic acid and longer-chain fatty alcohols (C_8–C_{16}) in acetonitrile, showing a possibility of using polar solvents.

Although the synthesis of lactic acid esters has been demonstrated, the procedures to be used have not yet been evaluated systematically. The purpose of this work was to further investigate the enzymatic production of esters of lactic acid and straight-chain alcohols in order to find a robust reaction system that can be used with both short- and long-chain alcohols (C_6–C_{18}). Furthermore, laboratory-immobilized *Candida* sp. lipase was evaluated for the synthesis. The studies were conducted with hexadecyl lactate as the model ester for parameter evaluation.

MATERIALS AND METHODS

Materials. Novozym 435, lipase B from *Candida antarctica*, immobilized on a macroporous polymer based on methyl and butyl methacrylic esters; Lipozyme TL IM, a lipase from *Thermomyces lanuginosa*, immobilized on silica granules; and Lipozyme RM IM, a lipase from *Rhizomucor miehei,* immobilized on a macroporous resin, were all a generous gift from Novozymes A/S (Bagsværd, Denmark). *Candida* sp. 99-125 was produced in the laboratory and immobilized by adsorption on a cotton-textile membrane (see below). Soybean powder was obtained from Shandong, China. Soybean oil was purchased from the local supermarket. KH_2PO_4, $(NH_4)_2SO_4$, and polyethylene glycol-6000 were all obtained from Beijing Yili Chemical Co. Ltd. (Beijing, China). Gelatin and lecithin were both obtained from Shanghai Chemical Co. Ltd. (Shanghai, China). Lactic acid (purity 80%) was obtained from Gaofeng (Beijing, China). All alcohols used had purities between 98 and 99% except decanol, which had a purity of 96%. Silica gel and

*To whom correspondence should be addressed.
E-mail: twtan@mail.buct.edu.cn

molecular sieves (4 Å) were obtained from Qingdao Huiyang Chemical Co. Ltd. (Qingdao, China). The internal standard, *n*-octadecane (purity >99%) was purchased from Merck (Hohenbrunn, Germany). All solvents used were of analytical grade and were dried over molecular sieves before use.

Candida sp. lipase fermentation and immobilization. Candida sp. lipase was obtained as described by Tan *et al*. (9). The fermentation of *Candida* sp. was carried out in a 30-L reactor at 28°C for 120–140 h under stirring at 350 to 500 rpm and with an air flow of 1 volume per minute per volume of batch (VVM). The culture medium was composed of 4% (wt/vol) soybean powder, 2.5% (wt/vol) soybean oil, 0.1% (wt/vol) KH_2PO_4, and 0.1% (wt/vol) $(NH_4)_2SO_4$. The fermentation broth (lipase activity: 8000 U/mL) was centrifuged at 2000 rpm for 20 min, and the lipase in the supernatant was precipitated by the addition of 3 vol of acetone. The precipitate was then washed with acetone and dried at room temperature. The resulting powder was ready for immobilization.

Cotton-textile (10 g) was presoaked for 1 h in 20 mL of an aqueous solution consisting of 5% (wt/vol) gelatin, 2% (wt/vol) lecithin, 2% (wt/vol) polyethylene glycol-6000, and 1% (wt/vol) magnesium chloride and then left to air-dry at room temperature. Supernatant (2 mL) from the fermentation broth was added over 1 g of the above-dried support, which was then dried at room temperature to a constant weight. The resulting textile was cut into small pieces (~0.25 cm^2) and stored at 0°C. The activity of the lipase thus immobilized was determined to be 3000 U/g by using the olive oil emulsion method (10). One unit of activity is equivalent to the amount of enzyme required to liberate 1 µmol FFA per minute from olive oil at 37°C.

Esterification. Ester synthesis was carried out in 50-mL stoppered flasks with 10 mL of solvent. Unless otherwise stated, the reaction was performed with 30 mM of lactic acid, 30 mM of hexadecanol, 100 mg molecular sieves, 20 mg internal standard, and 20 mg Novozym 435. The mixture was incubated for 24 h on an orbital shaker at 50°C and 190 rpm.

GC. Aliquots of the reaction mixture were withdrawn and analyzed by GC. A GC-2010 gas chromatograph (Shimadzu, Tokyo, Japan) equipped with a capillary column (25 m × 0.5 mm, 0.5 µm film thickness; CBP-20, Shimadzu) and an FID was used. Injection was done in a split mode (1:2), and the injector and detector temperatures were 280 and 285°C, respectively. The oven temperature was initially at 120°C and subsequently was increased at 15°C/min to 230°C, where it was held for 13 min. Nitrogen was used as the carrier gas at a flow rate of 6.21 mL/min. *n*-Octadecane, the internal standard, was added to the reaction mixture prior to reaction. Ester formation was calculated as being equivalent to alcohol consumed. A typical chromatogram is seen in Figure 1.

Ester yield calculation. Ester yields were calculated as the molar percent reduction of alcohols after reaction. Experimental repeatability was conducted under default conditions as already described. The absolute SD of ester yields was less than 0.57% for ester yields below 20% and 1.43% for ester yields above 20%.

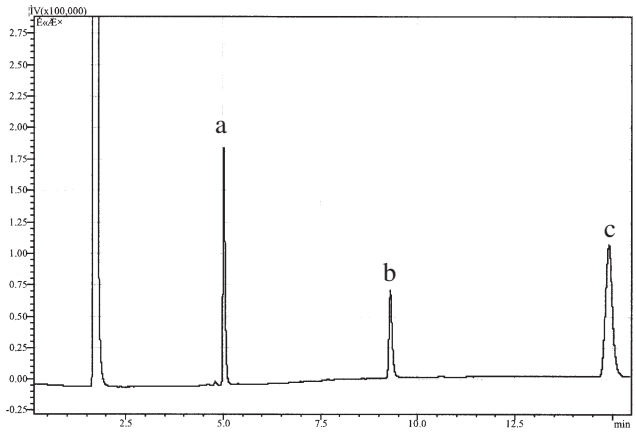

FIG. 1. Typical chromatogram used for monitoring the reaction progress. The different peaks represent (a) internal standard (*n*-octadecanane), (b) hexadecanol, and (c) hexadecyl lactate. See Materials and Methods section for details.

RESULTS AND DISCUSSION

A basic profile of reaction time course provides much information of reaction progress and basic kinetics. Following default conditions in the Materials and Methods section, a time course of the esterification between lactic acid and hexadecanol is seen in Figure 2. A high conversion rate is seen up to 4–6 h of reaction, and equilibrium is reached after 24 h.

Effect of solvent. Organic solvents having a range of log *P* values were screened for their suitability in the synthesis of lactic acid esters of straight-chain alcohols. Organic solvents with a log *P* below 2 are generally considered unsuitable for biocatalysis because they can strip off essential water present around the enzyme as a microaqueous layer, thereby affecting the active conformation of the enzyme. Nonpolar solvents, such as hexane (log *P* = 3.5), are unable to strip off any water from the enzyme, and they usually preserve catalytic activity (6,11–13).

Table 1 is a list of the solvents used together with their resulting ester yields. The highest ester yield (79%) was observed in cyclohexane, followed by yields of 72 and 62% in toluene and benzene, respectively. In polar solvents such as acetonitrile and acetone, the yields were more moderate, i.e., 27 and 12%, respectively, unlike those previously reported (8). Cyclohexane was the only nonpolar solvent that gave good yields; all the other nonpolar solvents gave very low yields (1–6%). Cyclohexane, like the other nonpolar solvents, was unable to solubilize lactic acid completely. It is speculated that this property allows the excess lactic acid to be deposited on the bottom of the reaction flask instead of on the enzyme support, where it would block access of the lipase to the active site. In addition to this effect, cyclohexane must possess some other favorable properties, such as a suitable aqueous solubility (log *S*), which makes it superior to the other nonpolar solvents for lactic acid esterification.

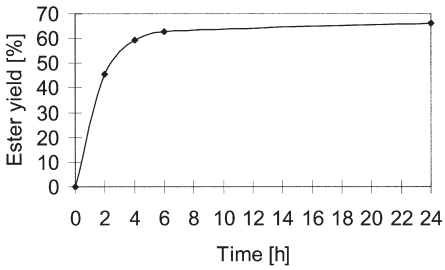

FIG. 2. Time course for the esterification between lactic acid and hexadecanol in cyclohexane catalyzed by Novozym 435. Reaction conditions: 120 mM of both lactic acid and hexadecanol; 20 wt% (based on reactants) Novozym 435; 100 mg internal standard (*n*-octadecane); 200 mg molecular sieves; 10 mL cyclohexane; 24 h; 50ºC; and 190 rpm.

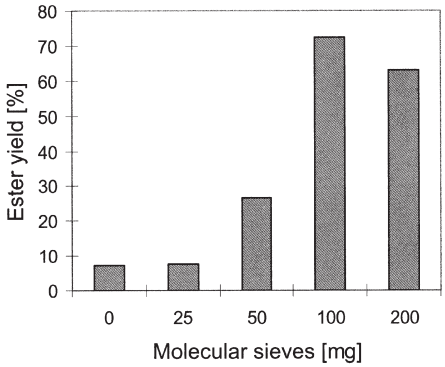

FIG. 3. Effect of adding molecular sieves to the reaction mixture. Reaction conditions: 27 mg (30 mM) lactic acid; 36 mg (15 mM) hexadecanol; 20 wt% (based on reactants) Novozym 435; 20 mg internal standard (*n*-octadecane); 10 mL cyclohexane; 24 h; 50ºC; and 190 rpm.

Lipase screening. Four immobilized lipases were screened for their catalytic efficiency in the esterification of lactic acid and hexadecanol. Novozyme 435 was the best lipase, with a 79.2% yield. Both Lipozyme TL IM and Lipozyme RM IM showed little activity (0.03% yield). When 200% enzyme load was used for *Candida* sp. lipase, a 33.0% yield was obtained. The high lipase load is justified because only a crude purification of the lipase was performed prior to immobilization on the textile, resulting in a lower activity compared with that of the commercial lipases. The results are not surprising since Novozym 435 previously was found to be superior in esterification reactions (5,7,15).

Effect of water absorbents. In general, a low water activity favors esterification in the presence of a lipase in an esterification system (14). Water formed during the esterification reaction between lactic acid and hexadecanol needs to be removed in order to drive the reaction toward esterification. Addition of molecular sieves to the reaction system could overcome this problem (8). We therefore investigated the effect of adding varying amounts of molecular sieves to the system (Fig. 3). The presence of molecular sieves can have a dual effect on the ester yield. Not only can it drive the reaction toward ester formation, but also it can strip water from the enzyme. A balance between the positive and negative effect was found at 100 mg where the highest ester yield (72.4%) was observed for this reaction system.

Effect of enzyme load. In terms of production cost, the impact of lipase is crucial. We therefore investigated the effect of enzyme load on the ester yield for Novozym 435 and the *Candida* sp. lipase. The results are seen in Table 2. Novozym 435 clearly has the higher activity of the two lipases. For 50% enzyme load, the ester yield is 30.8% for Novozym 435 compared with an ester yield of 2.9% for the *Candida* sp. lipase. However, when we increased the amount of *Candida* sp. lipase to 300%, an ester yield of 49.5% was obtained. For both lipases an increase in ester yield was seen when the enzyme load was increased. This is in agreement with results obtained by Wei *et al.* (2), who used acetone or acetonitrile as the solvent but contradicts the results of Kiran *et al.* (16), who found that in chloroform the highest yield of lauroyl lactate was obtained at a low enzyme-to-substrate ratio. The latter authors speculate that more lactic acid will bind to the enzyme at higher enzyme concentrations thereby depleting the free lactic acid available for the esterification (16). Solvent differences may be the reason for this phenomenon.

Effect of temperature. The effect of temperature on the catalytic activity of the two selected lipases was investigated at four levels each (30 to 60°C). As seen from Figure. 4, the highest yield (79.2%) was obtained at 50°C by using Novozym 435, whereas the *Candida* sp. lipase showed a temperature optimum

TABLE 1
List of Solvents Screened and Their Ester Yields[a]

Solvent	Log *P*	Log *S*[b] (mol/L)	b.p. (ºC)	Ester yield (%)
Acetonitrile	–0.33		82	27
Acetone	–0.24		56	12
tert-Butanol	0.35	0.63	78.0–81.0	6
Benzene	2.0	–1.64	80	62
Toluene	2.5	–2.21	110	72
Petroleum ether	≈3		60–90	2
Cyclohexane	3.2	–3.10	81	79
n-Hexane	3.5	–3.84	68	1
n-Heptane	4.0		98	1

[a]Reaction conditions: 27 mg (30 mM) lactic acid, 72 mg (30 mM) hexadecanol, 20 wt% (based on reactants) Novozym 435, 20 mg internal standard (*n*-octadecane), 100 mg molecular sieves, 10 mL solvent, 24 h, 50°C, and 190 rpm.
[b]Calculated according to Reference 17.

TABLE 2
Effect of Enzyme-to-Substrate Load on the Yield[a] of Hexadecyl Lactate

Enzyme load (%)	Ester yield (%) Novozym 435	Enzyme load (%)	Ester yield (%) *Candida* sp.
5	3.8	50	2.9
10	5.1	100	4.4
20	17.2	200	20.5
50	30.8	300	49.5

[a]Reaction conditions: 27 mg (30 mM) lactic acid, 36 mg (15,mM) hexadecanol, 20 mg internal standard (*n*-octadecane), 10 mL cyclohexane, 24 h, 50ºC, and 190 rpm.

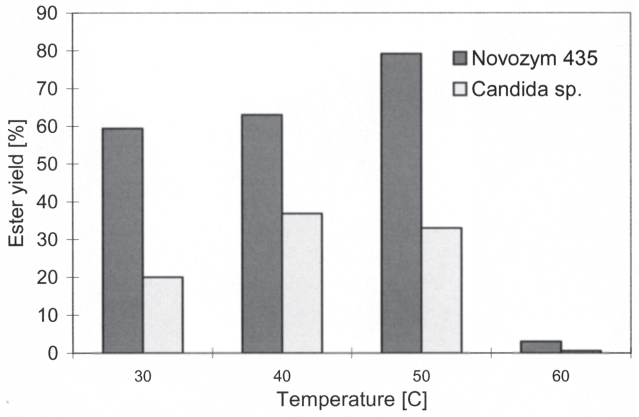

FIG. 4. Effect of temperature on ester yields obtained from Novozym 435 and *Candida* sp. lipase. Reaction conditions: 27 mg (30 mM) lactic acid; 72 mg (30 mM) hexadecanol; 20 or 200 wt% (based on reactants) of Novozym 435 or *Candida* sp. lipase, respectively; 20 mg internal standard (*n*-octadecane); 100 mg molecular sieves, 10 mL cyclohexane; 24 h; and 190 rpm.

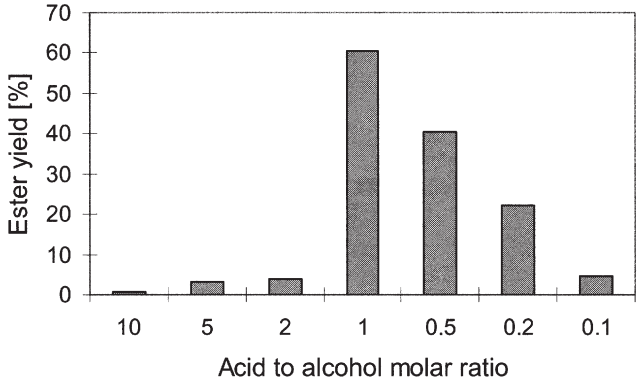

FIG. 5. Effect of the molar ratio of reactants in the enzymatic synthesis of hexadecyl lactate. Reaction conditions: 27 mg (30 mM) lactic acid and 72 mg (30 mM) hexadecanol; (molar ratio 1:1); 20 wt% (based on reactants) Novozym 435; 30 mg internal standard (*n*-octadecane); 100 mg molecular sieves; 10 mL cyclohexane; 24 h; 50ºC; and 190 rpm.

at 40°C with a yield of 36.8%. Both lipases showed a very low yield at a reaction temperature of 60°C, possibly due to inactivation.

Effect of molar ratio of lactic acid to alcohol. In this series of experiments, the ratio between the two reactants, lactic acid and hexadecanol, was varied while the other parameters were held constant. A substrate concentration of 30 mM for both substrates was used for a ratio of 1:1. The alcohol concentration was then held constant while increasing the lactic acid concentration to obtain a ratio of 10:1, and vice versa, to obtain a ratio of 1:10. An enzyme load of 20 wt% was used for all experiments. The results, shown in Figure 5, indicate that the most favorable acid to alcohol ratio was 1, yielding 60.4% of ester. In the presence of excess lactic acid, the ester yields were very low, decreasing steeply from 4.0 to 0.3% for ratios of 2 and 10, respectively. This effect can best be explained by the change in enzyme-to-substrate ratio. The yield also decreased in the presence of excess alcohol, but not quite so drastically, the lowest yield being 4.6% at a ratio of 1:10.

Influence of the substrate concentration. The goal of this series of experiments was to find the substrate concentration that gave the highest ester yield. Using a lactic acid-to-alcohol ratio of 2, we successively increased the lactic acid concentration from 30 to 360 mM. Torres and Otero (5) identified the importance of water content for the reaction yield and the use of an increasing amount of molecular sieves to maintain a high yield. The content of molecular sieves was thus increased from 100 to 350 mg from the first to the last experiment. As seen from Figure 6, the highest yield (78.9%) of hexadecyl lactate was obtained by using a lactic acid concentration of 120 mM. A further increase to 360 mM gave rise to only a relatively small reduction in the yield (65.6%), but at a concentration of 480 mM lactic acid, the yield became considerably lower (20%). In similar reactions, acetic acid has been shown to have an inhibitory effect on the lipase (15). Lactic acid may give rise to the same effect, since lactic acid is easily dissolved in water, and will make the micro-aqueous environment around the enzyme more acidic as the acid concentration is increased. This modification could lead to partial denaturation of the enzyme. On the other hand, the yields were significantly reduced as the substrate concentration was decreased from 120 to 60 mM (21.3%) and further to 30 mM (12.5%). The reason for this is unknown, but two points are worth mentioning. First, it may depend on the acid-to-alcohol ratio used in the experiments. As previously demonstrated in Figure 5, the ester yield was drastically lower for a ratio of 2 compared with that for a ratio of 1. This effect seems to be removed when the substrate concentration is increased. Second, perhaps it was due to a very dilute solution, where the enzyme does not come into contact with sufficient substrate.

Effect of alcohol chain length. Since esters other than hexadecyl lactate are of interest to the cosmetic, pharmaceutical, and food industries, we produced esters with other straight-chain alcohols. In this series of experiments, 30 mM each of

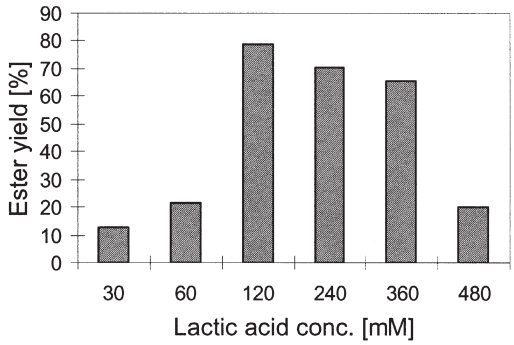

FIG. 6. Influence of the substrate concentration on the hexadecyl lactate yield. Reaction conditions: lactic acid to hexadecanol ratio of 2:1; 20 wt% (based on reactants) Novozym 435; 20, 40, 60, 80, 120, 150 mg internal standard (*n*-octadecane), respectively; 100 to 350 mg molecular sieves; 10 mL cyclohexane; 24 h; 50ºC; and 190 rpm.

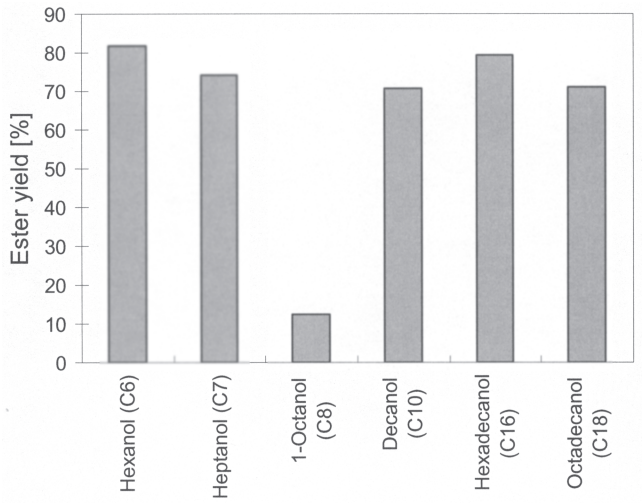

FIG. 7. Effect of alcohol chain length on ester yields. Reaction conditions: 30 mM lactic acid; 30 mM alcohol (either C_6, C_7, C_8, C_{10}, C_{16}, or C_{18}); 20 mg internal standard (*n*-octadecane); 20 wt% Novozym 435 (based on reactants); 100 mg molecular sieves; 10 mL cyclohexane; 24 h; 50°C; and 190 rpm.

alcohol and lactic acid were used with 20 wt% Novozym 435. All tested alcohols except for 1-octanol gave rise to ester yields in the range of 70.8 to 81.7% (Fig. 7). The ester yield using 1-octanol was only 12.3%, and further investigation of this is needed. Apart from the low ester yield for 1-octanol, Novozym 435 showed no apparent alcohol specificity and catalyzed the esterification with lactic acid with both short- and long-chain alcohols equally well. This is in accordance with the general view that Novozym 435 is a nonspecific lipase as well as the results obtained by Torres and Otero (8). However, it contradicts previous reports by From *et al*. (7), where high yields of butyl lactate were achieved, but low yields of octyl and decyl lactates. In general, our method has proven its overall applicability for preparing lactic acid ester having both short and long chain lengths.

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