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Full Length Research Paper

Comparison of lipase-catalyzed synthesis of cyclopentadecanolide under organic and biphasic systems

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Methyl 15-hydroxy-pentadecanate, which is made from *Malana oleifera* chum oil, is an ideal material to synthesize cyclopentadecanolide, an important macrocycle musk, with wide applications in the fields of perfume, cosmetic, food and medicine, etc. One kind of screened lipase from *Candida* sp.GXU08 strain was used to catalyze the synthesis of cyclopentadecanolide from methyl 15-hydroxy-pentadecanate. Traditionally, the catalytic reaction went on under organic solvent system, while an ultrasonic technology was innovatively used in water/organic solvent biphasic system to systhesize cyclopentadecanolide and received good catalytic effect. Both organic and biphasic systems had the same optimal conditions as: substrate concentration= 8 mmol/l, T= 40°C, ω= 180 r/min, pH= 6.0 to 6.5, ultrasonic time of 30 min, output power of 200 W and using lipase solution/lipase power and reaction system twice greatly increased the production of cyclopentadecanolide. It showed that, the maximum production of cyclopentadecanolide was 47.77×10^{-3}mg/U in biphasic system under the optimal conditions, which was 3.285 times of that in the organic system. It was also found that the production of cyclopentadecanolide in biphasic system was always higher than that in the organic system, which saved the process of freeze drying and improved the production of cyclopentadecanolide.

It was verified that methyl 15-hydroxy-pentadecanate was directly cyclized into cyclopentadecanolide in the organic system while in the biphasic system, methyl 15-hydroxy-pentadecanate was hydrolysed into 15-hydroxypentadecanoic acid firstly and then cyclized into cyclopentadecanolide.

Key words: Lipase, cyclopentadecanolide, *Malana oleifera* chum, biphasic system, ultrasonic.

INTRODUCTION

Malana oleifera chum is a special plant growing throughout the south-east of Yunnan province and west of Guangxi province, China. It has been used as a kind of edible oil for decade. Local people who eat it much easily have symptom of chronic diarrhea, which have restricted the plant's utilization. It was shown that the *M. oleifera*, chum oil produced in Guangxi province contains about 50% tetracosenoic acid (Huang et al., 1998). After ozonization, reduction and esterification reaction tetracosenoic acid is turned into methyl 15-hydroxy-pentadecanate that is an ideal material to synthesize cyclopentadecanolide, which is an important macrocycle musk with wide applications in the perfume, cosmetic, food, medical fields, etc. If *M. oleifera* chum is used in producing cyclopentadecanolide, its value would be greatly increased, which would give a great boost to local economics. Some methods for synthesis of cyclopeantadecanolide from methyl 15-hydroxy-pentadecanate have been reported (Zhang and Huo, 1995). Chemical methods exhaust much energy and need harsh conditions. As a biocatalysis, enzyme has high efficiency, high selectivity and high specificity in

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Abbreviations: PPL, Porcine pancreatic lipase; **CCL,** cylindracea candida lipase; **PEL,** penicillium expansum lipase; **CSL GXU08,** *Candida* sp.lipase GXU08.

synthesizing macrolide under moderate conditions, which has been widely used in the fields of organic synthesis, fine chemical engineering and food processing (Liu et al., 2001, 2006; Zhao et al., 2006). Catalytic synthesis of macrolide in organic media by lipase has also been reported (Dong et al., 1998; Wang et al., 2004; Effenberger et al., 2000; Dietmar and Anthony, 2001; Ueji et al., 1999, 2003). Gatfield firstly used hydroxy-acid to synthesize lactone by mycetes lipase in organic media (Gatfield and Ann, 1997). Muscolactone and γ-butyrolactone can be respectively made by 15-hydroxypentadecanoic and tetrahydroxy butyrate. The condensation reaction between diol and diaacid can be catalyzed by lipase in isooctane, tetracol and cyclohexane. Pan et al. (1998) used methyl ω-hydroxytridecoic acid to synthesize lactone by PPL, CCL and PEL. Lactone with six molecules could be prepared with the conversion ratio of 9%. To the best of our knowledge, no other research about synthesis of cyclo-pentadecanolide from *M. oleifera* chum has been reported overseas except several Chinese articles published by our research team (Pan et al., 2004; Shen et al., 2007; Huang et al., 2006).

Some high-catalyzed strains which can catalyze the synthesis of cyclopentadecanolide from methyl 15-hydroxy-pentadecanate have been screened successfully in recent years. The reaction was achieved in organic media by lipase powder prepared through a low temperature freeze-drying process. Recently, the team has found that, much more cyclopentadecanolide could be detected in the product if the organic media was replaced by aqueous/organic biphasic system when other reaction conditions remained unchanged. The same results were also proved by a series of repeated experiment. No research on lipase-catalyzed synthesis of cyclopentadecanolide under biphasic system has ever been reported.

It was reported that, though enzyme showed special catalytic activity in non-aqueous media (Ye, 1992; Shen and Tian, 2003), it would exhibit inactivity in completely dried solvent. Sufficient water was important for lipase to retain its activity during the catalytical reaction. This article mainly studied the synthesis of cyclo-pentade-canolide by lipase in both organic system and aqueous/organic biphasic system.

Ultrasound waves are sound waves with frequencies above 16 kHz up to 10 Mhz (Patist and Bates, 2008; Brncic et al., 2010). The main effect that is causing physical and chemical changes within the treated ma-terial is a phenomenon called cavitation. In their work, Povey and Mason (1998) explained mechanism of ultra-sonic activity on some enzyme reactions. Since the change of the millnnium, high-power ultrasound has become an alternative industrial technology applicable to large-scale commercial applications such as emulsification, homo-genization, extraction, crystallization, defoaming, activation and inactivation of enzymes, particle size reduction, extrusion, and viscosity alteration (Feng et al., 2011; Patist and Bates, 2011; Zhang et al., 2005). This new focus can be attributed to significant improvements in equipment design and efficiency during the late 1990s. The objective of this article is to present examples of ultrasonic applications that have been successful at synthesizing cyclopentadecanolide.

As is known to us, lipase is easily dissolved in water rather than organic solvent in whereas methyl 15-hydroxy-pentadecanate is not. It was reported that, ultra-sonic technology had been widely used in bioreaction system and had achieved ideal results (Du et al., 2008; Hao et al.,2011). So in our study of biphasic system, an ultrasonic technology was firstly used to disperse lipase solution into the mixture of substrate and cyclohexane so that an aqueous/organic solvent biphasic system can be formed. The effects of reaction time, temperature, pH, concen-tration of substrate, repeated use of lipase solution and reaction system on the production of cyclopenta-decanolide were investigated and compared in both systems, from which a better method of synthesizing cyclopentadecanolide would be obtained. The research would offer some theoretical basis for future application and development of the technology to produce cyclopentadrcanolide and would be helpful for application of *M. oleifera* chum in macrocycle musk industry.

MATERIALS AND METHODS

Candida sp.GXU08 (CSL GXU08) was screened by us. An activity unit is the amount of the lipase which releases 1 mmol free fatty acid per minute from olive oil at pH 7.0 and at 40℃. The lipase solution was made from the strain through process of fermentation, rotary evaporatation and 0 to 70% $(NH_4)_2SO_4$ fractionation precipitation. The lipase powder was made from lipase solution through freeze drying. The substrate was 15-hydroxy-pentadecanate, which was made from *M. oleifera* chum oil through a series process of ozonization, reduction and esterification reaction. The standard sample of cyclopentadecanolide was greater than 98% in content and was bought from Askrich chemical company, Japan. Other chemicals and solvents used in this work were of analytical grade.

Lipase-catalyzed synthesis methods

The substrate (0.3 mmol) was dissolved in cyclohexane (30 ml) to form a solution of 10 mmol/L in dry Erlenmeyer flasks (500 ml). The lipase solution (5 ml, 1000 U) was dispersed into the above Erlenmeyer flask with the help of ultrasonic rapper to form an aqueous/organic solvent biphasic system while the lipase powder (1 g, 1000 U) was directly put into a Erlenmeyer flask. The tootle lipase activity was equal in the two systems. Then, the Erlenmeyer flasks were put on sorting table at 40℃, at 180 r/min for 72 h. The lipase powder was filtered out and the liapse solution was separated from the solvent by separatory funnel when the reaction was completed. The residual solvent was concentrated to less than 5 ml with rotatory evaporator. Cyclopentadecanolide from the concentrated solution was detected by gas chromatography (GC) and mass spectra (MS) on-line characterization. Its production was calculated through external standard method. The statistical

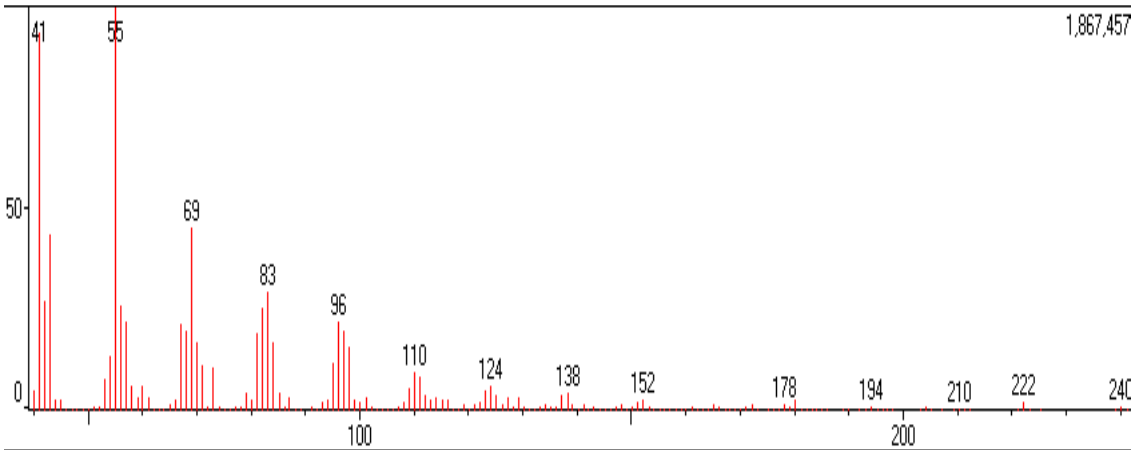

Figure 1. The mass spectrum of cyclopentadecanolide sample at retain time t= 11.703 min in gas chromatography.

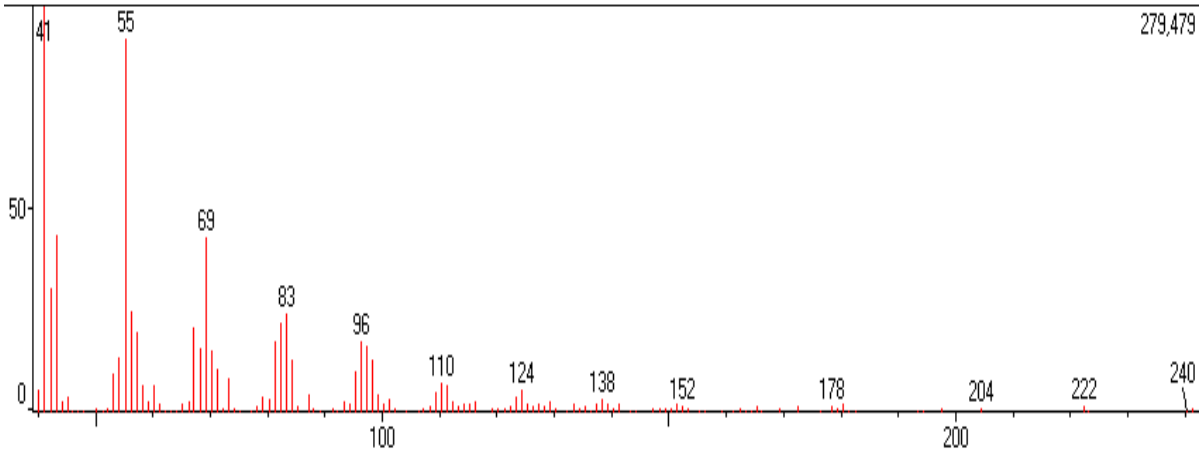

Figure 2. The mass spectrum of product at retain time t= 11.703 min in gas chromatography.

method of the experiment was single factor and every experiment data was the mean value with deviation of less than 5%.

RESULTS AND DISCUSSION

Structural characterization of product

sample and product reached 92% (Figures 1 and 2), which proved that the product was cyclopentadecanolide.

Effect of reaction time

In this catalytic reaction of synthesizing cyclopentadecanolide, a series of parallel experiment was conducted by respectively using CSL GXU08 lipase solution (5 ml, 1000 U) and lipase powder (1 g, 1000 U).

It observed that the production of cyclopentadecanolide per lipase activity unit increased with reaction time until it reached 72 h in the two curves (Figure 3). After 72 h, the production did not increase any more and the reaction nearly reached to an equilibrium state. So the proper reaction time was 72 h. It was observed that the production in the biphasic system was obviously higher than that of the organic system under same conditions.

Effect of reaction temperature

As a biocatalyst, enzyme also exhibits a temperature effect as normal catalyst. The reaction velocity usually increases with the temperature at the beginning and finally reaches a maximum value at certain temperature. Once the temperature exceeds some extend, the lipase will gradually inactivate and the reaction velocity will decrease until the reaction terminates completely.

As lipase is easy to be inactivated if the temperature is over 50°C, different temperatures such as 30, 35, 40 and 45°C was studied in this work. The production of

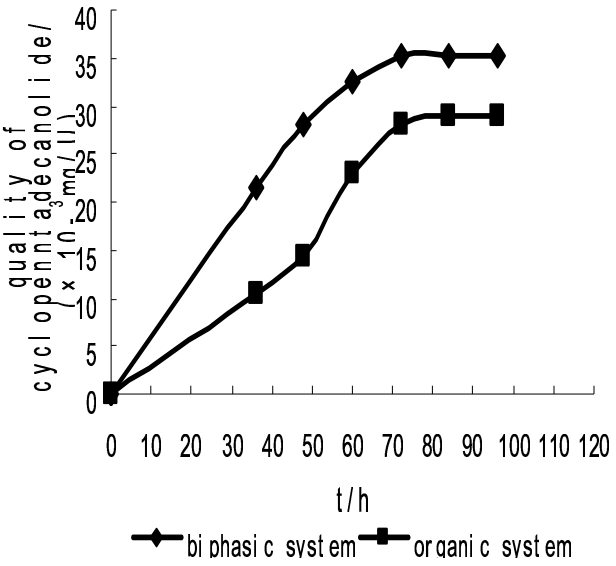

Figure 3. Effect of reaction time on the production of cyclopentadecanolide under the same conditions of T= 40℃, pH= 7.0, ω=180 r/min. Data presented are mean values with deviation of less than 5%.

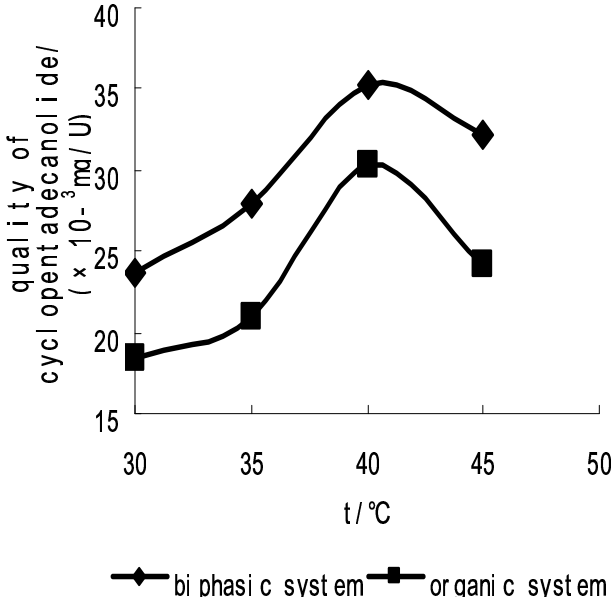

Figure 4. Effect of temperature on the production of cyclopentadecanolide under the same conditions of t= 72 h, pH= 7.0, and ω= 180 r/min. Data presented are mean values with deviation of less than 5%.

cyclopentadecanolide per lipase activity unit gradually increased with reaction temperature until it reached 40℃ (Figure 4). Once it exceeded 40℃, the production decreased instead in that part of the lipase gradually loosed activity. So the proper reaction time was both 72 h. It was obviously seen that the production in the biphasic system was higher than that of the organic system under same conditions.

Effect of substrate concentration

According to Michaelis-Menten equation: v = ks [E][S]/{ Km + [S] }, the concentration of substrate has great influence on the reaction velocity. Though increasing concentration of substrate can contribute to improve reaction velocity, it can also add the chance of producing inefficient compounds because of excessive substrate molecules centering on the lipase molecules, which would make the active site of liapse combine with two or more substrate molecules. Moreover, the higher concentration of the substrate is, the more impurity would exist. And the impurity might act as an anti-competitive suppressor to inhibit the reaction. Therefore, it is important to select proper substrate concentration to improve the reaction velocity. It should be noted that, the effect of lipase solution on the substrate concentrate was ignored in the biphasic system because 15-hydroxy-pentadecanate was almost insoluble in aqueous solution.

It showed that, the optimal concentration was 8 mmol/l when the maximum production of cyclopentadecanolide was obtained (Figure 5). The production in the biphasic system was higher than that of the organic system under same conditions.

Effect of pH

pH influenced both hydrolysis activity and catalytic activity of the lipase in the catalytic reaction. Table 1 showed the effect of pH on the hydrolysis activity of CSL GXU08 in the aqueous solution.

It obviously shown that the largest hydrolysis activity of CSL GXU08 would appear at pH= 6.0 to 7.0. Whether the lipase exhibited an optimal catalytic activity at this range as well as hydrolysis activity is shown in Figure 6.

It showed that the optimal pH in biaphasic system was 6.0 to 7.0, while it was 6.0 to 6.5 in the organic system. So the common optimal pH was 6.0 to 6.5, which also conformed to the range of optimal hydrolysis activity. The catalytic activity in the biaphasic system is apparently higher than that in the organic system under the same conditions.

Effect of reusing lipase

The lipase solution was separated through separatory funne.l The lipase powder was filtered with funnel after the reaction was completed and were put into new reaction system with other conditions unchanged. The operation was repeated for several times and the production of the cyclopentadecanolide from every

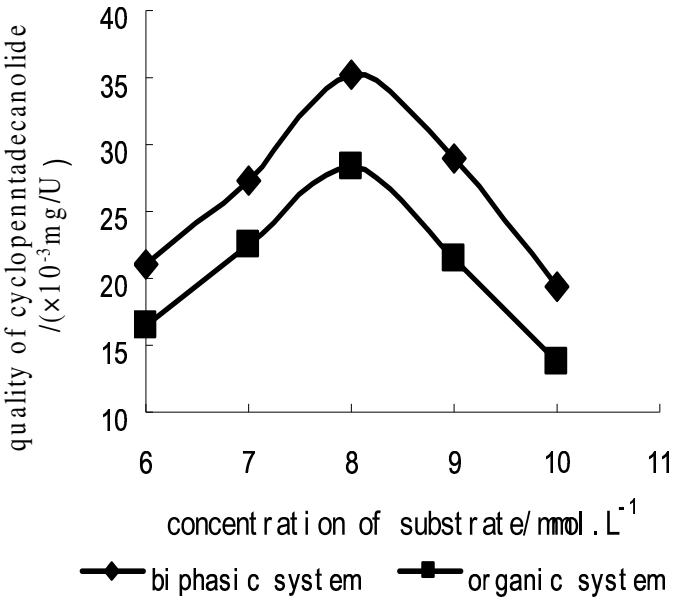

Figure 5. Effect of concentration of substrsate on the production of cyclopentadecanolide under the same conditions of T= 40℃, pH= 7.0, ω= 180 r/min and t= 72 h. Data presented are mean values with deviation of less than 5%.

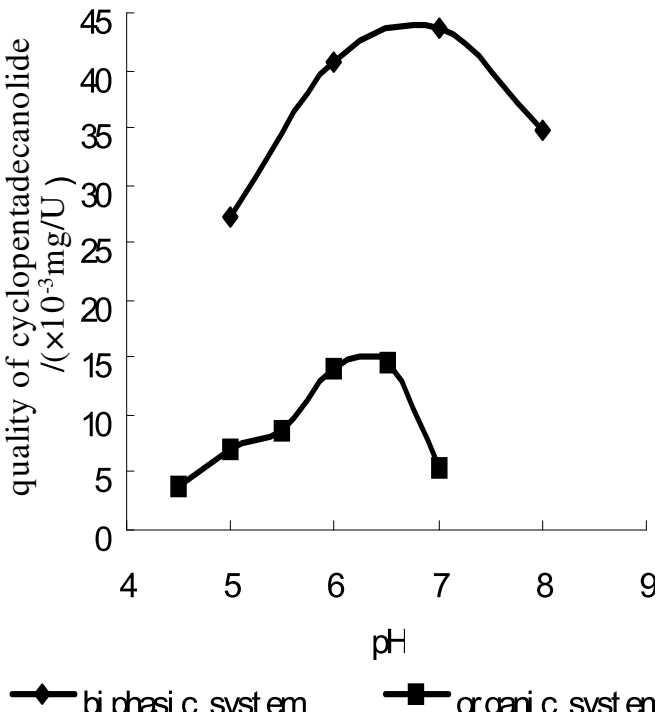

Figure 6. Effect of pH on the production of cyclopentadecanolide under the same conditions of T= 40℃, C_{SUB}= 8 mmol/l, ω=180 r/min and t= 72 h. Data presented are mean values with deviation of less than 5%.

Table 1. Effect of pH on the activity of CSL GXU08.

pH	Hydrolysis activity of GXU08/(u/ml)
4.0	0.5965
5.0	5.045
6.0	8.795
7.0	8.844
8.0	7.256
9.0	7.863
10.0	3.270
11.0	0.1557

reaction was separately detected. It was observed that the production was the largest when the lipase solution was used twice, which was nearly two times of that used once (Figure 7). This could be explained by the induced-fit hypothesis, which is when the lipase molecule combines with the substrate, some part of its active section will change in configuration, so that the catalytic-group and bonding-group needed in the reaction can be arranged and oriented correctly to fit with the substrate. At the same time, as the original electron distribution of lipase molecules changed, some chemical bonds of the substrate atoms would be easily induced to generate polarization phenomenon and tend towards an activated state, all of which would accelerate the reaction velocity. The first reaction was induced-fit process between lipase and substrate, while the second one was the accelerating process after induction. The reaction velocity will gradually decrease until the equation tended to equilibrium, which was why the production of cyclopentadecanolide would decrease after the third reaction. It could be concluded that, using the lipase solution and lipase power twice would improve the production. The catalytic activity in biaphasic system was also higher than that in the organic system under the same conditions.

Effect of reusing reaction system

The lipase solution after the reaction finished was separated and new lipase solution was put into the old reaction system and also for the lipase powder. It showed that, the total production of cyclopentadecanolide gradually increased, while the production made by the new lipase decreased when the reaction system was reused (Table 2). The production made by the second new lipase was much less than the first one and a little more than the third one in the two systems. The reason was that almost all the enzymatic reactions were reversible. The increasing production also accelerated the velocity of the reverse reaction until the reaction got to equilibrium, which caused the decrease of the production made by new lipase. So it was appropriate to use the reaction system twice in this work from economical consideration.

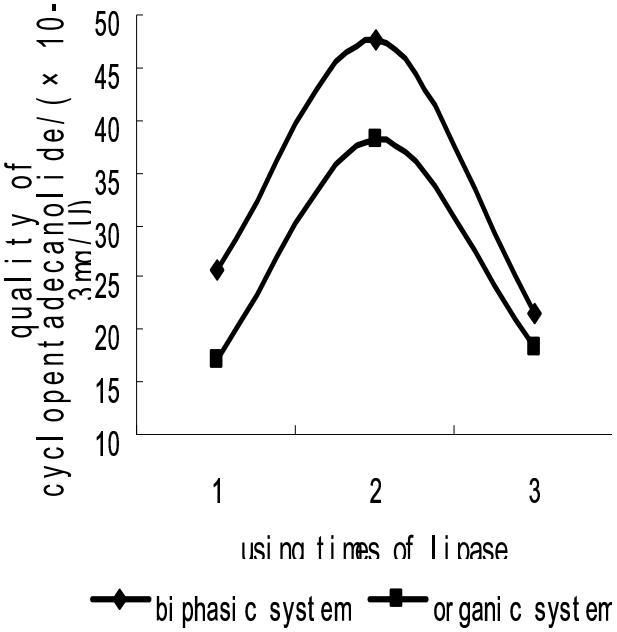

Figure 7. Effect of CSL GXU08 reusability on the production of cyclopentadecanolide under the same conditions of T=40°C, $C_{substrate}$ = 8 mmol/l, ω=180 r/min, pH= 6.5 and t= 72 h. Data presented are mean values with deviation of less than 5%.

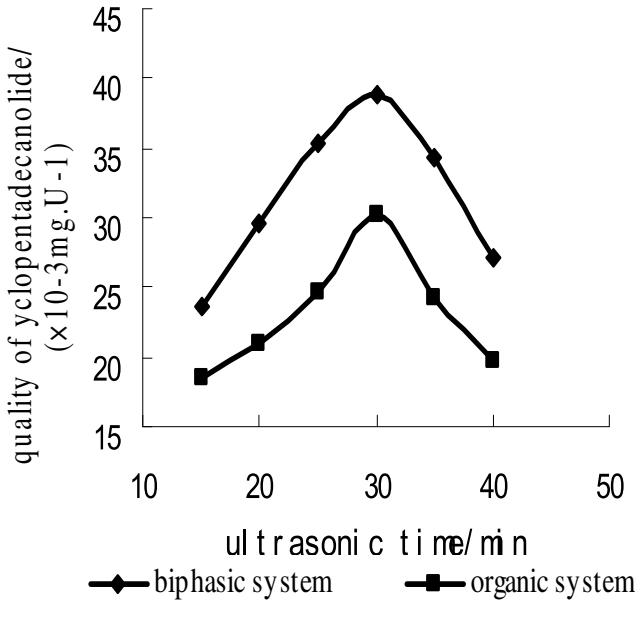

Figure 8. Effect of ultrasonic time on reaction.

Effect of ultrasonic parameter

As a kind of pre-treatment, ultrasound has great influence on quality of cyclopentadecanolide. So, different parameters of ultrasonic such as ultrasonic time and output power were used and are shown in Figures 8 and 9 under the same condition as earlier mentioned. The device was ultrasonic cleaner being used as an ultrasonic bath with maximum nominal output power of 300 W, work frequency of 40 HZ (module JD-300R, Ningbo Haishu Jinda Ultrasonic Equipment Co., Ltd, PRC).

It was shown that the optimal ultrasonic time in the biaphasic system was 30 min and the optimal ultrasonic output power was 200 W, so were the organic system. The catalytic activity in the biaphasic system was apparently higher than that in the organic system under the same conditions.

Reaction mechanism in two systems

In the organic solvent system, methyl 15-hydroxy-pentadecanate was proved to be directly cyclized to cyclopentadecanolide as the result of no hydroxypentadecanoic acid and other dimer or trimer found in the product (Shen et al., 2007). The reaction is shown in Figure 10.

There was large quantity of water in the biaphasic system. Methyl 15-hydroxy-pentadecanate was easily hydrolyzed to hydroxypentadecanoic acid. What was the possible reaction mechanism in the biaphasic system? To make out the question, two hypothesises were proposed as followings: (1) methyl 15-hydroxy-pentadecanate is directly cyclized into cyclopentadecanolide; (2) the reaction experienced two steps: Step A: firstly, methyl 15-hydroxy-pentadecanate was hydrolyzed into 15-hydroxypentadecanoic acid in the water; step B: 15-hydroxypentadecanoic acid was directly cyclized into cyclopentadecanolide.

In hypothesis (2), step A had been proved to be feasible. To verify step B, an experiment was designed. 15-hydroxypentadecanoic acid was used as the substrate instead of methyl 15-hydroxy-pentadecanate in the biaphasic system with other conditions unchanged. Cyclopentadecanolide was found in the product through GC-MS, which indicated the possibility of step B. The result is in agreement to the report of Pan et al. (2004) which showed that lipase can catalyze the synthesis of cyclopentadecanolide from 15-hydroxypentadecanoic acid. 15-hydroxypentadecanoic acid was found existing in the production during the reaction process in biphasic system. So, hypothesis 2 was verified to be feasible. It could be concluded from the result that, methyl 15-hydroxy-pentadecanate was hydrolysed into 15-hydroxypentadecanoic acid firstly and then cyclized into cyclopentadecanolide. The reaction in biphasic system is shown in Figure 11.

Conclusion

Such effects as reaction time, temperature, substrate

Table 2. Effect of the reusability of reaction system on quality of cyclopentadecanolide.

Use times of the system	Biphasic system		Organic system	
	Total quality of cyclopentadecanolide/×10^{-3}mg	Quality of cyclopentadecanolide made by new lipase /×10^{-3}mg	Total quality of cyclopentadecanolide/×10^{-3}mg	Quality of cyclopentadecanolide made by new lipase /×10^{-3}mg
1	31.92	31.92	24.52	24.52
2	37.05	5.13	28.04	3.52
3	37.97	0.92	28.82	0.78

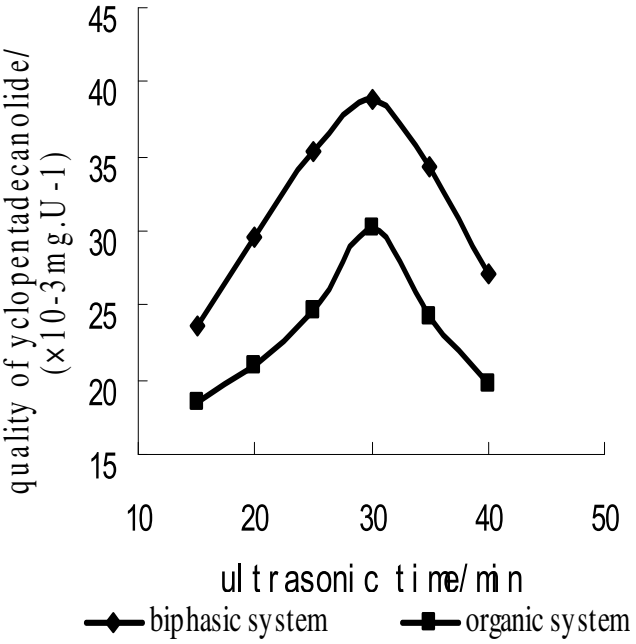

Figure 8. Effect of ultrasonic time on reaction.

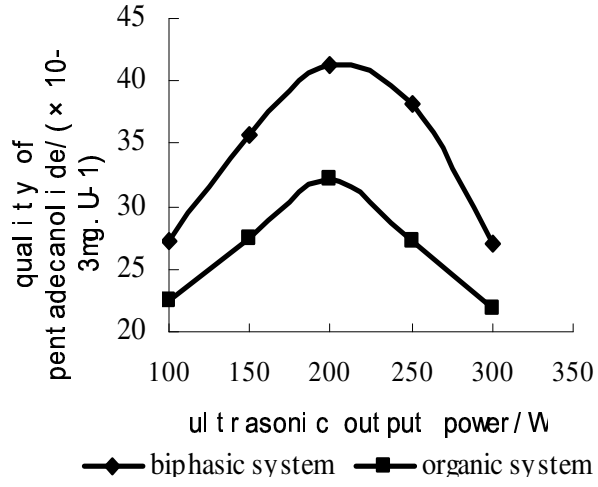

Figure 9. Effect of ultrasonic output power on reaction.

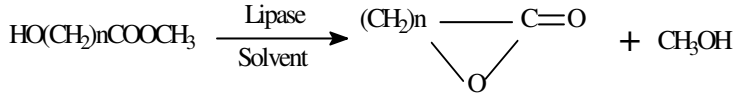

Figure 10. Reaction in the organic system.

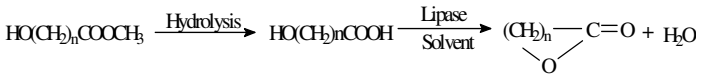

Figure 11. Reaction in the biaphase system.

supplied a useful reference for synthesis of valuable musk and has great significance to the development of local economics.

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