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# Production and regulation of different lipase activities from *Rhizopus chinensis* in submerged fermentation by lipids

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ABSTRACT

The fungal *Rhizopus chinensis* could produce several types of lipase, which were mainly intracellular. During the whole-cell lipase production by this strain in submerged fermentation, it was observed that two catalytic characteristics (hydrolytic and synthetic activity) of lipases were different with addition of lipids. The hydrolytic activity of the lipase was not induced by lipids efficaciously and could be detected regardless of whether substrate-related compounds were present. However, it was found that the induction of lipids for the synthetic activity lipase was significant, and that nearly no synthetic activity was detected while the medium contained no lipids. When only a little lipid (1 g/L) was added to medium, the synthetic activity increased sharply in the initial process of fermentation. Analysis of crude membrane-bound lipase by SDS-PAGE confirmed this induction. De novo biosynthesis of lipases, especially the lipase with synthetic activity occurred only when lipids existed. Cell growth and maltose repress the lipase production with synthetic activity, but have little influence on the lipase production with hydrolytic activity. Since the production process of mycelium-bound lipase with hydrolytic activity was different, it was reasonable to consider hydrolytic activity and synthetic activity for different application purposes. Whole-cell lipase obtained from fermentation process with high synthetic activity showed excellent catalytic ability in solvent free system on synthesis of ethylcaprylate and ethyloleate, the conversion could reach more than 90% in 5 h.



## 1. Introduction

In the past few years, lipases (triacylglycerohydrolases, EC 3.1.1.3) served as versatile biocatalysts capable of catalyzing numerous reactions. Among these reactions, organic synthesis reactions in non-conventional media catch more and more researchers' eyes. The main interest stems is their broad substrate specificity and exceptional high stability in organic solvents. Thus, not only hydrolysis but also synthesis reactions are possible in different media. Numerous important products, such as flavour esters [1], monoacylglycerols [2], optically pure building blocks [3] and also biodiesel [4] have been produced by these reactions. Regarded as naturally immobilized catalysts, cell-bound or intracellular microbial lipases have advantages over normal extracellular counterparts, and have attracted more attentions recently [5–12]. Despite most of studies were performed based on hydrolytic activity of these enzyme, nearly all of the mycelium-bound lipases were used in organic solvents to catalyze the synthetic reactions, due to their stability to organic solvents and thermal stability [12,13].

Hydrolytic activity and synthetic activity are often used to characterize a lipase catalytic ability, and the hydrolytic activity was mostly preferred. Generally, one lipase can catalyze its reaction in both directions, but some of enzyme can exhibit only one catalytic ability, while others show both under certain conditions. A problem, therefore occurs that synthetic activities of the enzymes in organic solvents do not correspond with the hydrolytic activities in aqueous solutions [14–17]. In order to investigate or improve the synthetic activity for lipase, studies for lipase production need to be performed based on synthetic activity. However, to our certain knowledge, scarcely has researchers investigated the lipase production in light of the synthetic activity. However, there exists also some reports about the whole-cell lipase with both hydrolytic and synthetic activity. Most of the strains were selected with high hydrolytic activity towards olive oil or tributyrin, although the ester

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synthesis ability was also studied [18,19]. However, whether the two catalytic abilities during a microorganism fermentation process are coincident is still remained unclear.

The fungal strain *Rhizopus chinensis* CCTCC M201021 was isolated from *Da Qu* (a kind of traditional leaven for production of Chinese liquor) by our laboratory, and the whole-cell lipase produced was proved to be an efficient biocatalyst in biosynthesis of short-chain fatty esters in *n*-heptane [9]. In our previous studies, we found that whole-cell lipases with hydrolytic activity and synthetic activity have different fermentation characteristics [20], and enzyme with synthetic activity in the whole-cell was located as a membrane-bound lipase by the treatments of Yatalase [21]. We also have purified two intracellular isoenzymes from this strain, the molecular weight of one enzyme is 39 kDa and another is 59 kDa. Both of them possess high hydrolytic activity, but nearly no synthetic activity was detected [22]. One of lipase gene from this strain has also been cloned and expressed in *Pichia pastoris*, the expression protein is 37 kDa, hydrolytic activity is 367 U/mL, and also no synthetic activity was detected (in press article in Chinese High Technology Letters, Chinese). From these results, it can be concluded that several types of lipases might be produced by this microorganism, the same as *Candida rugosa*, which can produce at least five types of lipase [23]. Therefore, in order to explain the difference between the whole-cell lipase with hydrolytic and synthetic activities produced by *R. chinensis* during the fermentation, and to regulate the target lipase production, the different effects of lipids on the whole-cell lipase production with two catalytic abilities (hydrolytic and synthetic) were performed in the present work. Meanwhile, the possible regulation mechanisms for the enzyme with synthetic activity were discussed.

## 2. Material and methods

### 2.1. Microorganism and culture conditions

*R. chinensis* CCTCC (China Center for Type Culture Collection) M201021 was studied to produce whole-cell lipase in submerged culture. The fermentation cultures were carried out in Erlenmeyer flasks (250 mL) containing 20 mL fermentation medium. The composition of basal medium contained only $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $K_2HPO_4$ (3 g/L), maltose (0.5 g/L), peptone (40 g/L), pH was adjusted to 5.5. Different lipids were added to the basal medium with different concentration and at certain time.

After sterilization, the medium was inoculated with spores from a fresh potato dextrose agar slant (3-day-old) with spore concentration of $4.25 \times 10^8$ spores/L. Fermentations in shake flask were carried out in a rotary shaker at 30 °C under shaking speed of 200 rpm for certain time. Three flasks were taken per time for triplicate analysis. The cultivated mycelium was separated from the culture by filtration, washed twice with tap water and once with 25 mM phosphate buffer (pH 7.50), The mycelia were then lyophilized for 24 h by a freeze drying system (Labconco, USA).

### 2.2. Biomass determination

The biomass was determined by weighing the dry cell after freezing-dry.

### 2.3. Oleic acid concentration assay

Ten millilitre fermentation broth was extracted by 2 mL hexane. Free fatty acids in the organic layer were determined by colorimetric method based on cupric soaps formation [24]. The latter are soluble in hexane. Intensity of blue color was measured at 710 nm. Standard curves prepared for linoleic and oleic acid (Sigma) were used to calculated the FFA content in lipid samples.

### 2.4. Lipase activity assay

Synthetic activity was measured by the ester-synthesis method in heptane according to the procedure described previously [21]. Octanoic acid (1.2 M) and ethanol (1.2 M) in heptane each of 0.5 mL were mixed. The reaction was started by adding 20 mg dry cell and incubated for 30 min at 40 °C with a shaking speed of 200 rpm. The reaction mixture was then filtered using 0.15 μm membrane to remove the cells. Samples (400 μL) were then drawn and mixed with 2-hexanol of 100 μL, as internal-standard, then analyzed by subsequent gas chromatograph (Agilent 6820, flame-ionization detector, 30 m × 0.22 mm PEG 20M (AC20) capillary column, Nitrogen was used as a carrier gas, and the injector and detector temperatures were set at 250 °C. Oven temperature by programmed temperature was started at 90 °C for 1 min before being elevated to 200 °C for 5 min at 10 °C/min). One unit of lipase synthetic activity was defined as the amount of enzyme which catalyzed to produce 1 μmol of ester per minute.

Hydrolytic activity was measured by hydrolyzing olive oil. Olive oil method was modified from olive oil emulsion method described by Salleh et al. [25]. The reaction mixture comprised 2.5 mL phosphate buffer (50 mM, pH 7.5) and 2 mL olive oil emulsion (olive oil/3% polyvinyl alcohol (w/v%), 1:3 (v/v)), 10 mg mycelium-bound lipase was added to start the reaction. The reaction was carried out for 15 min at 37 °C, with shaking at 150 rpm. The reaction was terminated by adding 7.5 mL ethanol, and the amount of free fatty acid released was measured by titration to pH 10.0. A unit of activity is equivalent to one micromole of free acid released per minute.

### 2.5. Preparation and analysis of crude membrane-bound lipase

One gram of grounded lyophilized mycelium from different culture conditions was treated by 15 mL acetone for 15 min under room temperature. The mixture was filtrated by vacuum filtration to remove the solvents, and dried for several hours. The dry cell was washed twice with 20 mL of 2.5 mM phosphate buffer (pH 5.5) and once with 20 mL of 2.5 mM phosphate buffer contained 0.1% Trition X-100 (pH 5.5). After centrifugation at 10,000 rpm for 10 min, the precipitate was shaken in 2.5 mM phosphate buffer contained 1.5% Trition X-100 (pH 5.5) for 4 h, the volume used was 10 times of the weight of acetone-treated dry cell. Supernatant was collected as membrane-bound proteins after centrifugation at 15,000 rpm for 20 min. Sample was analyzed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 13.5% separating and 5% stacking) according to Laemmli's method [26]. Protein concentration was determined using BCA (bicinchoninic acid) [27] Protein Assay Kit (Novagen, USA).

### 2.6. Induction of lipases formation by washed mycelium

This experiment is modified from the method by Rapp et al. [28], mycelium for 36 h old culture of *R. chinensis* on 1% (w/v) triolein was collected, and washed with deionized water and centrifugated to remove the triolein. Then, 5 g/L washed mycelium was incubated in 50 mM potassium phosphate buffer (pH 5.5) and culture medium, 1% of triolein and oleic acid were added accordingly.

### 2.7. Ester synthesis in solvent free system

Ester synthesis was carried out in 10 mL screw-capped test flasks by suspending lyophilized cells (80 g/L) in solvent free system. Each flask contains equivalent molar of acid and ethanol (2.88 g caprylic

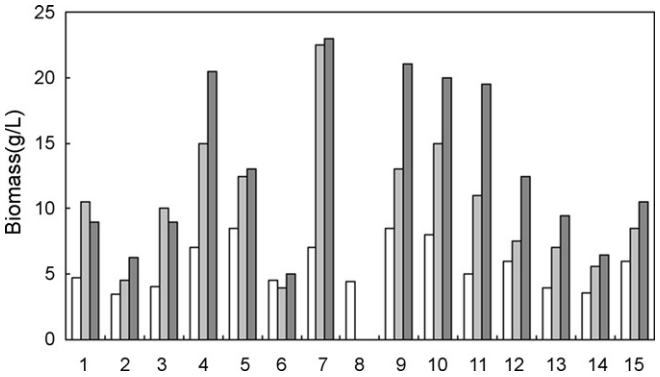


**Fig. 1.** Effect of different lipids and fatty acid on biomass production by *R. chinensis*: the fatty acid and lipids are: (1) lauric acid; (2) palmitic acid; (3) stearic acid; (4) oleic acid; (5) linoleic acid; (6) oleic alcohol; (7) olive oil; (8) tributyrin; (9) triolein; (10) soybean oil; (11) span 80; (12) Tween 80; (13) methyl stearate; (14) glycerol monostearate; (15) glycerol. Control is the medium without lipids and fatty acid adding, and the biomass is 4 g/L. (□) The medium contains 1 g/L lipids or fatty acid; ( ) the medium contains 10 g/L lipids or fatty acid; (■) the medium contains 20 g/L lipids or fatty.

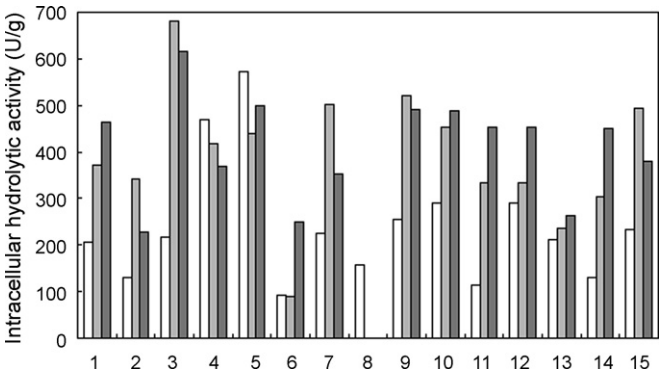


**Fig. 2.** Effect of different lipids and fatty acid on mycelium-bound lipase production by *R. chinensis*: hydrolytic activity. The fatty acid and lipids are: (1) lauric acid; (2) palmitic acid; (3) stearic acid; (4) oleic acid; (5) linoleic acid; (6) oleic alcohol; (7) olive oil; (8) tributyrin; (9) triolein; (10) soybean oil; (11) span 80; (12) Tween 80; (13) methyl stearate; (14) glycerol monostearate; (15) glycerol. Control is the medium without lipids and fatty acid adding, and the hydrolytic activity is 200 U/g. (□) The medium contains 1 g/L lipids or fatty acid; ( ) the medium contains 10 g/L lipids or fatty acid; (■) the medium contains 20 g/L lipids or fatty acid.

acid/1.164 ml ethanol; 2.82 g oleic acid/0.582 ml ethanol). The flasks were incubated on a reciprocal shaker (150 rpm) at 40 °C. The time course of the transformations was followed by GC analysis.

The chemicals and solvents used in this study were purchased from Sigma or Shanghai Chemical Co. (Shanghai, China) and of the highest purity available.

## 3. Result and discussion

### 3.1. Effect of different lipids and fatty acids on mycelium-bound lipase production

In our previous works, we found that the production of whole-cell lipase by strain *R. chinensis* CCTCC M201021 was induced by olive oil both in the solid-state and submerged fermentation [29,30]. In order to elucidate the mechanism of lipase production, either induced by the oleic esters or by a metabolic production derived from the oleic ester catabolism, different fatty acids and esters were tested. They were butyric acid, valeric acid, hexanoic acid, octanoic acid, capric acid, lauric acid, palmitic acid, stearic acid, oleic acid, linoleic acid, oleic alcohol, olive oil, tributyrin, triolein, soybean oil, span 80 (sorbitan oleate), Tween 80 (polyoxyethylenesorbitan monooleate), methyl stearate, glycerol monostearate and glycerol. The strain was found unable grow in the medium which content short chain fatty acid ($C \leq 10$), so no data were gained from these conditions. The results of the effects for lipase and biomass production by different lipids and fatty acids were depicted in Figs. 1–3.

Different amount of lipids and fatty acids were added to the basal medium with the concentration of 1 g/L, 10 g/L and 20 g/L, for most of chosen substrate the corresponding C/N ratio were about 1.20, 3 and 4.9 except for glycerol (1.1/1.98/2.95). Control is the medium without lipids and fatty acid, and the biomass is 3 g/L. The effects of different lipids and fatty acid on biomass production are presented in Fig. 1. Most of the lipids ($C \geq 12$) were found able to improve the growth of *R. chinensis*, and the biomass was increased sharply with the concentration increase of lipids. Among the lipids, oleic acid, olive oil, triolein, soybean oil and span 80 were the best carbon substrate for growth, and the highest 23 g/L biomass was obtained when 20 g/L olive oil was added. Lauric acid, palmitic acid, stearic acid also can improve the biomass production, but their effect on biomass production is not so remarkable as oleic acid. One possible reason is that they are all solids, and cannot disperse completely in the broth. Shimada et al. found that during the lipase production by *Geotrichum-Candidum*, the dispersal problem for solid fatty acid can be solved by adding amount of ethanol [31]. In terms of these results, it can be concluded that the biomass was affected by the C/N ratio and the type of substrate used. Even though some substrate (glycerol, oleic alcohol) with high C/N ratio (2.95, 5.03), the biomass is much lower when compared with other fatty acid with the same C/N ratio. Therefore the type of substrate used in the medium must be considered firstly, and then the C/N ratio.

The hydrolytic activity of mycelium-bound lipase was enhanced by the most of lipids used in this study, and the results were shown in Fig. 2. Stearic acid is the best inducer in all of the lipids, and the hydrolytic activity can retain at 681 U/g, which about 3 times than the control medium. Except for palmitic acid, oleic alcohol, tributyrin and methyl stearate, other lipids nearly have the same induction effect, which about 2 times than the control medium. Glycerol was also found to be a good inducer for the hydrolytic lipase production. The extracellular lipase production was different from the intracellular ones, almost no hydrolytic acitivities were detected (data not shown), since most of fatty acid and esters have no induction on the extracellular lipase. Salleh et al. also found that

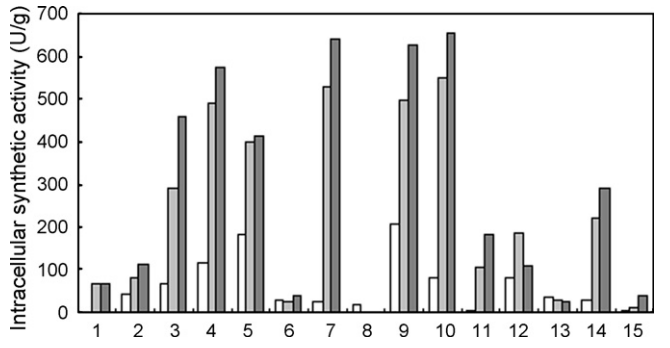


**Fig. 3.** Effect of different lipids and fatty acid on mycelium-bound lipase production by *R. chinensis*: synthetic activity. The fatty acid and lipids are: (1) lauric acid; (2) palmitic acid; (3) stearic acid; (4) oleic acid; (5) linoleic acid; (6) oleic alcohol; (7) olive oil; (8) tributyrin; (9) triolein; (10) soybean oil; (11) span 80; (12) Tween 80; (13) methyl stearate; (14) glycerol monostearate; (15) glycerol. Control is the medium without lipids and fatty acid adding, and the synthetic activity is 30 U/g. (□) The medium contains 1 g/L lipids or fatty acid; ( ) the medium contains 10 g/L lipids or fatty acid; (■) the medium contains 20 g/L lipids or fatty acid.

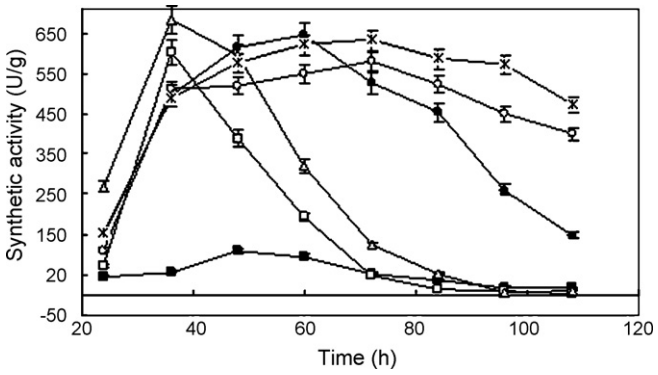


**Fig. 4.** Effect of triolein initial concentration in the lipase production process by *R. chinensis*: synthetic activity. (□) 0 g/L; (□) 1 g/L; (△) 5 g/L; (×) 10 g/L; (*) 20 g/L; (○) 30 g/L.

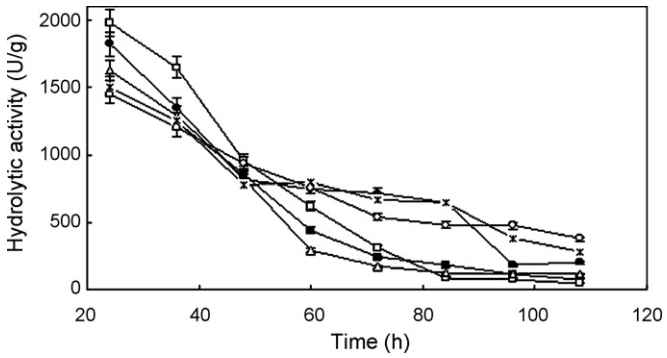


**Fig. 5.** Effect of triolein initial concentration in the lipase production process by *R. chinensis*: hydrolytic activity. (□) 0 g/L; (□) 1 g/L; (△) 5 g/L; (×) 10 g/L; (*) 20 g/L; (○) 30 g/L.

among lipid substrates glycerol was the only stimulator of extracellular lipase production for *Rhizopus oryzae* [25].

The effect of lipids on synthetic activity lipase production was depicted in Fig. 3, it can be observed that the induction of lipids for the synthetic activity lipase was different obviously from the induction for the hydrolytic lipase. Long chain ($C \geq 18$) fatty acids and their esters were excellent substrate for the lipase biosynthesis with high synthetic activities. They were stearic acid, oleic acid, linoleic acid, olive oil, triolein, soybean oil and glycerol monostearate. It is remarkable that lipids containing oleic acid had the most significant induction for this kind of lipase production. Other researchers also reported that, both extra- and intra-cellular lipase can be increased by the addition of triglyceride or fatty acid to the media, as well as olive oil and oleic acid [25,31,32]. Olive oil, the mostly used substrate in lipases' fermentation because of the high oleic acid content, serves not only as inducer for lipase production, but also as the carbon source for microorganism growth [33,34]. On the other hand, oleic acid is not the best carbon source for the lipase production. Possible reason is that oleic acid is an easily assimilable carbon source compared with olive oil. Azeredo et al. also found a completely different physiological behaviors were observed after the addition of easily (oleic acid and glucose) and complex (olive oil and starch) assimilable carbon sources to the liquid and solid media [35].

The lipase activity increased remarkably with the increase of lipids concentrations (from 0 g/L to 10 g/L), but when it reached 20 g/L, the lipase activity could maintain at a high level. The highest synthetic activity 694 U/g was obtained when 20 g/L soybean oil was added to the medium, and activity was 17 times higher than the control medium (40 U/g). Compared with 3 times increase in hydrolytic activity, the increase in synthetic activity is much higher. In order to study the two catalytic characteristics for the whole-cell lipase during the fermentation process, a substrate with high oleic acid content was chosen for the subsequent studies.

### 3.2. Batch studies on lipases production

Since the component of triolein is simpler than the olive oil and soybean oil, it was chosen to investigate the effect of lipids on the lipase production more in detail. Several batch fermentations were carried out in order to study the effect of the initial triolein concentration on lipase production by *R. chinensis*, Figs. 4 and 5 show the change of intracellular lipase production with hydrolytic and synthetic activities during the fermentations.

The initial concentration of triolein had significant effect on the lipase production with synthetic activity (Fig. 4). Maximum synthetic activity could only reach 109 U/g at 48 h when the medium without triolein, and decreased to 20 U/g after 108 h growth. When only 1 g/L triolein was added to the medium, the synthetic activity increased sharply to the maximal activity of 604 U/g at 36 h, and then decreased to 20 U/g at the end of fermentation. The more triolein contained in the initial medium, the longer the synthetic activity could maintain at a certain level. Decrease of activity was also found at 36 h, when the initial concentration of triolein was 5 g/L. And the same situation was 60 h for 10 g/L; 84 h for 20 g/L. Nearly no decrease of activity was found when the initial concentration of triolein was 30 g/L. Two reasons should be considered for this result. One is that protease would be synthesized which could decrease the lipase activity because of the different carbon source concentration. Gombert et al. have found that the type of carbon source supplemented to the basal medium determines the major enzymes produced in their research work [36]. But in our study, almost no protease activities were detected throughout all of the fermentations. Another reason is that the existence of triolein or oleic acid is very important for the lipase biosynthesis. The result of triolein consumption indicated that triolein was used up at early fermentation stage while 1 g/L and 5 g/L triolein in the medium, and could be maintained at a certain level when the initial concentration of triolein was higher.

Table 1 summarizes parameters in the batch cultures described above, the different initial triolein concentrations studied had some effects on microorganism growth. The maximum specific growth rate about 0.17 $h^{-1}$ was obtained when the initial triolein concentrations were 10 g/L and 20 g/L, which a little higher than 0.11 $h^{-1}$. In this table, the substrate consumption need to be considered. Not all of the lipids could be consumed by this strain when the initial triolein concentrations were more than 20 g/L. To our knowledge, the residual oleic acid is used to maintain the lipase activity. Thus the triolein concentration was not much lower or much higher and a suitable fed-batch fermentation should be performed to obtained high lipase production.

The same as the induction of lipids for hydrolytic and synthetic enzyme described above, the production process of mycelium-

**Table 1**
Effect of triolein initial concentration in the lipase production by *R. chinensis*: synthetic activity (*S* substrate consumed, *X* biomass, Lip maximum lipase yield, $\mu_{max}$ maximum specific growth rate).

| Parameter | Value, for an initial triolein concentration of | | | | | |
|---|---|---|---|---|---|---|
| | 0 g/L | 1 g/L | 5 g/L | 10 g/L | 20 g/L | 30 g/L |
| *S* (g/L) | 0 | 1 | 4.5 | 9.5 | 15 | 18.5 |
| *X* (g/L) | 3 | 5 | 11.5 | 16.5 | 24.5 | 26.8 |
| Lip (U/L) | 327 | 2114 | 4901 | 10,131 | 14,235 | 12,864 |
| $\mu_{max}$ ($h^{-1}$) | 0.157 | 0.11 | 0.12 | 0.167 | 0.173 | 0.1 |

bound lipase with hydrolytic activity was also different obviously from the lipase with synthetic activity. As shown in Fig. 4, the hydrolytic activity decreased at the beginning of fermentations, whether the medium contain triolein or not. High hydrolytic activity of 1246.5 U/g could be obtained as well, while no triolein was contained in medium. When triolein was added to the medium with certain concentrations, the hydrolytic activity was little higher than medium with no triolein, and the activity could be kept at relative high level at the end of fermentations. The hydrolytic activity decreased already at the beginning of fermentations, which different from most of other mycelium-bound lipase. We were also confused by this result, and the attempts to explain this phenomenon were also performed in our lab (unpublished data). The possible reason is that lipase with hydrolytic activity has allosteric modulation [37]. Lipase with hydrolytic activity has also been synthesized, the hydrolytic activity could not exhibit because of the allosteric modulation. Very low hydrolytic activity or no activity was detected in the fermentation broth, especially when the lipids were added to the medium. Similar phenomenon was also found in *R. oryzae* lipase production, the extracellular lipase secretion was strongly inhibited when olive oil was in the medium [38].

The same as the induction of lipids for hydrolytic and synthetic enzyme described above, the production process of mycelium-bound lipase with hydrolytic activity was also different obviously from the lipase with synthetic activity. As shown in Fig. 4, the hydrolytic activity decreased at the beginning of fermentations, whether the medium contain triolein or not. High hydrolytic activity of 1246.5 U/g could be obtained as well, while no triolein was contained in medium. When triolein was added to the medium with certain concentrations, the hydrolytic activity was little higher than medium with no triolein, and the activity could be kept at relative high level at the end of fermentations. The hydrolytic activity decreased already at the beginning of fermentations, which different from most of other mycelium-bound lipase. We were also confused by this result, and the attempts to explain this phenomenon were also performed in our lab (unpublished data). The possible reason is that lipase with hydrolytic activity has allosteric modulation [37]. Lipase with hydrolytic activity has also been synthesized, the hydrolytic activity could not exhibit because of the allosteric modulation. Very low hydrolytic activity or no activity was detected in the fermentation broth, especially when the lipids were added to the medium. Similar phenomenon was also found in *R. oryzae* lipase production, the extracellular lipase secretion was strongly inhibited when olive oil was in the medium [38].

The production of mycelium-bound lipase with synthetic activity relied completely on the sum of substrate-related compounds in the medium in this study. Oleic acid or the oleic acid esters were the best inducers for the lipase biosynthesis. Our results suggest that lipase with synthetic activity is completely an inducible enzyme: when the medium contains lipid, the enzyme is able to be synthesized, once the lipid is used up, the synthesis of this enzyme ceased immediately. To verify the induction of lipase production with synthetic activity in lyophilized mycelium, crude membrane-bound lipases from 72 h ferment cell with or without triolein were analyzed using SDS-PAGE. As shown in Fig. 6, lane 1 is crude membrane-bound protein purified from mycelium with triolein in the medium, a remarkable protein band of approximately 32 kDa was displayed in the lane 1, and there are also other band in this lane, but the concentration is lower relatively. The total protein concentration is 2.16 mg/mL, and the specific synthetic activity is 16 U/mg protein. Lane 2 is crude membrane-bound protein purified from mycelium without triolein in the medium, nearly no protein band appear at 33 kDa, and the other band is similar with the lane 1. The total protein concentration is 0.915 mg/mL, and the specific synthetic activity is 0.34 U/mg protein. During the purification process we also found that lipases with hydrolytic activity were mainly intracellular, and synthetic lipase was mainly bound to the cell membrane.

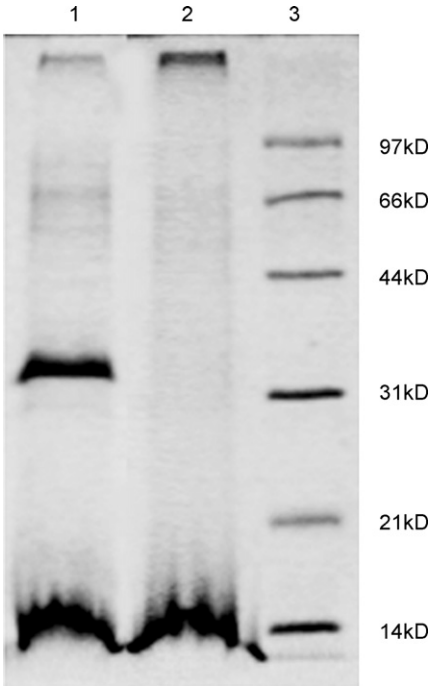


**Fig. 6.** Sodium dodecyl sulphate/polyacrylamide gel electrophoresis of crude membrane-bound lipase. Lanes: (1) medium contained 20 g/L triolein, (2) medium without triolein and (3) protein molecular weight marker.

Therefore, the protein obtained from membrane was remarkably different when the medium contained triolein or not. High concentration of membrane protein was biosynthesized when the triolein was added, and the synthetic activity is very high. Otherwise, the membrane protein was lower, and synthetic activity was also much lower while no triolein was added. These results confirmed the induction of lipase production with synthetic activity by lipids. Hama et al. reported that, the membrane-bound lipase from *R. oryzae*, was induced by olive oil, its the specific methanolysis activity of the cells showed a linear relationship with the concentration of the membrane-bound lipase [38]. Liew et al. [10] also find that, transesterification ability of membrane-bound lipase from *Rhizomucor miehei* was varied remarkably with different addition of oil. Although, the effect of oil and oleic acid on lipase production by *Rhizopus* sp. has been studied for several years [39–41], seldom did researchers concern the lipase production with synthetic activity in organic solvent. Therefore, it is necessary that the lipase produced from *R. chinensis* be studied in detail.

### 3.3. Induction of lipases formation by washed mycelium

To exclude the influence of fungal growth on lipases formation, washed mycelium was used to study the induction of lipases formation. The results were summarized in Table 2. After 24 h incubation the intracellular synthetic activity was increased 4.4- and 4-fold in the presence of triolein and oleic acid in the phosphate buffer system. But the corresponding intracellular and extracellular hydrolytic activity decreased sharply in this system. When the phosphate buffer was replaced by culture medium which can support cell growth, different result was obtained. The intracellular synthetic activity was much lower than it in the buffer system, however intracellular and extracellular hydrolytic activity were much higher than it in the buffer system. Even though differences existed

**Table 2**
Effect of triolein and oleic acid on the intracellular and extracellular lipases formation by washed mycelium. The washed mycelium was incubated in the buffer and medium with triolein and oleic acid for 24 h, and then the corresponding activities were detected. Comparison is the buffer without any substrate added, and untreated is the data detected at 36 h fermentation before washing.

| | Intracellular synthetic activity (U/g) | | Intracellular hydrolytic activity (U/g) | | Extracellular hydrolytic activity (U/ml) | |
|---|---|---|---|---|---|---|
| | Buffer | Medium | Buffer | Medium | Buffer | Medium |
| Triolein | 607 | 269 | 550 | 1267 | 3.33 | 8.33 |
| Oleic acid | 461 | 105 | 650 | 683 | 0.83 | 9.17 |
| Maltose | 60 | 71 | 557 | 1200 | 1.67 | 16.7 |
| Comparison | 60 | | 567 | | 6.6 | |
| Untreated | 137 | | 1267 | | 16.7 | |

between both systems, most of lipase activities were enhanced by triolein and oleic acid compare with the buffer system without any substrates. From these result, it is possible to conclude that oleic acid and its related substrate can induce or keep the lipase activities, but the induction is specific for the lipase with synthetic activity; Cell growth or maltose may repress the lipase production with synthetic activity, and have little influence on the lipase production with hydrolytic activity regardless of intracellular or extracellular. These results also confirm the conclusion described above. Similar results were also observed by Rapp [28] and Macris et al. [42]. Using the washed mycelium, Rapp and his coworkers found that catabolic repression of lipase formation in *F. oxysporum f.* sp. *vasinfectum* was mainly regulated by transcriptional control.

### 3.4. Fed-batch studied lipases production in shake flask

From the batch studies, it could be found that lipids are able to improve both the biomass and synthetic activity of whole-cell lipase, but the optimal concentration of lipids in the medium is not the same for the biomass and lipase production. Therefore, it could be concluded that there exists an optimal range of substrate concentration that should be taken into account when designing strategies of substrate feeding to improve lipase production.

Based on the result from Fig. 4, the initial medium contained 5 g/L triolein, and then another 5 g/L triolein was added to the medium at 30 h and 60 h, respectively. Fig. 7 shows the lipase production with synthetic activity by triolein feeding. When 5 g/L triolein was added to the medium at 30 h, the activity did not decrease at 36 h compared with the process only containing 5 g/L triolein in the medium, but maintained at a high level. After feeding the other 5 g/L triolein at 60 h, the synthetic activity also could be maintained at 650 U/g, even though there was a little decrease in activity. Biomass also increased by addition of triolein to the medium, the maximal 20 g/L biomass and 13000 U/L activity yield could be achieved at the end of fed-batch fermentation. The result

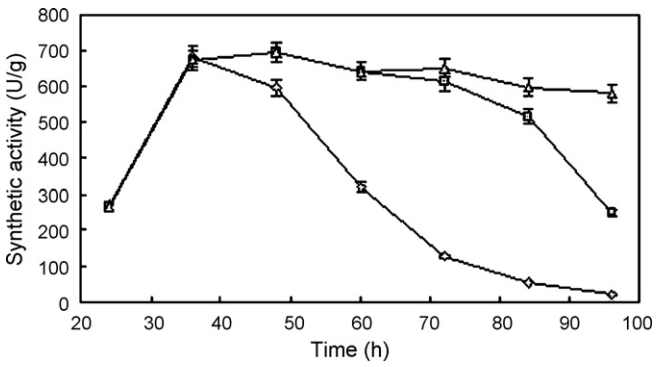


**Fig. 7.** Effect of whole-cell lipase production and cell growth by olive oil feeding. (○) initial medium with 5 g/L olive oil; (■) feeding 5 g/L olive oil at 30 h; (□), continued feeding 5 g/L olive oil at 60 h.

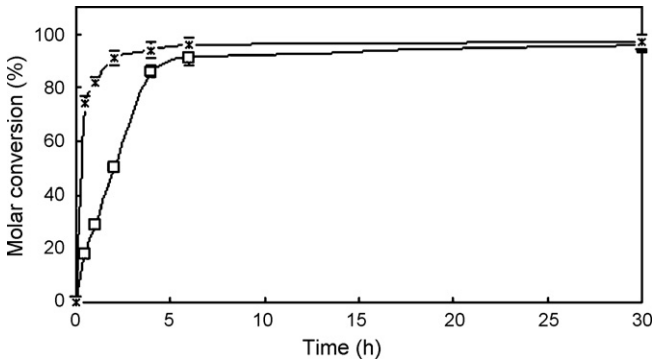


**Fig. 8.** Time course of esterification performed with lyophilized mycelium (80 g/L) in solvent-free system at 40 °C: ethylcaprylate (□); ethyloleate (*).

revealed that lipase with synthetic activity was improved by triolein feeding successfully, and the consumption of triolein was also saved by the fed-batch process. Reason for this result was that, both the decrease of activity by triolein consumption at low triolein concentration and the inhibition of activity at high triolein concentration could be avoided by fed-batch process.

Esterification by lyophilized whole-cell lipase in solvent free system

Numerous lipase catalysis reactions have been carried out in organic solvent, because of the significant advantages for the organic solvent system [43]. But the shortage of organic solvent is impossible to avoid: the solvent needs to be separated and regenerated; residual traces may remain in final product; the cost of the solvent itself, the increase in plant costs, and the cost of solvent recycling can lead to economic limits for the enzyme-catalyzed process [44]. Therefore, the solvent free system was developed as "more natural" process by many researchers [45–47]. In order to test the catalytic ability of mycelium-bound lipase with high synthetic activity from *R. chinensis* in the solvent free system, synthesis of ethylcaprylate and ethyloleate was performed by lyophilized mycelium. Reactions were carried out with 80 g/L mycelium-bound lipase, starting from an equimolar amounts ethanol and acid without organic solvent at 40 °C (Fig. 8). The result shows that, the mycelium-bound lipase is a strong catalyst for the esterification between the ethanol and long chain fatty acid. Only after 5 h, both of the conversion for ethylcaprylate and ethyloleate is more than 90%, even though the corresponding concentration of ethanol is 5 M and 2.6 M. Owing to the excellent catalytic ability of this enzyme, it can be applied to produce biodisel, flavour esters or other valuable esters in solvent free system.

## 4. Conclusion

The difference between hydrolytic lipase and synthetic lipase was obviously throughout the fermentation process for *R. chinensis*, and during the procedure of preparing the crude membrane-bound

lipase, we found that the intracellular protein from cell exhibited high hydrolytic activity, but nearly no synthetic activity was detected. It can be concluded that several lipases were produced by *R. chinensis*, in which, some of them only exhibited hydrolytic activity and some had synthetic activity in organic solvent. In order to improve the catalytic ability in organic solvent, the biosynthesis of lipase with synthetic activity need to be concerned, not the lipase with hydrolytic activity. Lipids can regulate the lipases production with synthetic activity, but have little influence on the hydrolytic lipase production. Base on the synthetic activity, lyophilized mycelium obtained from fermentation process exhibits high catalytic ability in solvent free system in esterification. Since the production process of mycelium-bound lipase with hydrolytic activity was different, it was reasonable to consider hydrolytic activity and synthetic activity for different application purposes.

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