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Biosynthesis of ethyl esters of short-chain fatty acids using whole-cell lipase from *Rhizopus chinesis* CCTCC M201021 in non-aqueous phase

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Abstract

A fungal strain capable of synthesizing ethyl esters of short-chain fatty acids was isolated from leaven (mouldy grains) samples through combined screening strategy of lipolysis and with esterification ability and was identified to be *Rhizopus chinesis* CCTCC M201021. When compared with other 10 commercial lipases, the whole-cell lipase of *R. chinesis* CCTCC M201021 (RCL) showed much higher ability in the synthesis of ethyl hexanoate during the screening of suitable lipases with a maximum yield of 96.5% after 72 h conversion using 0.5 M equal molar substrate concentration. Thereafter, the effect of important reaction parameters for enhancing ester formation by whole-cell RCL was investigated in this study. Higher esterification (>90%) was observed and maintained under chain length of carboxylic acids (C_2–C_8). Solvents with log *P* (*P*: partition coefficient) >2.0 enhanced RCL activity to give high conversion (>88.8%). Effect of temperature on reaction showed better esterification at 30–40 °C. Increase in concentration of substrates (ethanol and acid) from 0.2 to 1.4 M led to decrease in molar conversion from 96 to 85% in the synthesis of ethyl hexanoate. Changing the molar ratio of acid/ethanol from 1:1 to 1:3 at 0.6 M acid concentration resulted in a maximal conversion of 98.5% at the ratio of 1:1.3. It was observed that better esterification could be achieved with initial water activity (a_w) ranging from 0.66 to 0.97. A crucial enzyme concentration of 6 g/l was chosen for research after effect on esterification reaction was examined. The half-life period of esterification for different flavor ester production indicated that whole-cell RCL was more stable in batch esterification reaction within 840–975 h of half-life period for ethyl flavor esters.

Keywords: Whole-cell lipase; Esterification; Non-aqueous phase; *Rhizopus chinesis*; Flavor ester; Short-chain fatty acid

1. Introduction

With strong fruit flavor, ethyl esters of short-chain fatty acids are a large group of flavor and fragrance compounds widely used in food, beverage, cosmetic and pharmaceutical industries. Extracted from plant materials or produced by fermentation, natural flavor esters are often scarce and expensive for commercial application, which have great demands for them [1,2]. For example, ethyl haxanoate is a typical fragrance compound of Chinese liquor and Japanese sake with an annual demand of more than 2000 kl. Lipase-mediated synthesis of flavor esters has the potential of satisfying the increasing demand for natural flavor esters.

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Enzymatic esterification of flavor esters has the advantage of catalyzing reactions more specifically than chemical synthesis under mild condition and higher yield over microbiological production [3–5].

Lipases (triacylglycerohydrolases, EC 3.1.1.3) are the enzymes that had been used for the hydrolysis of acylglycerides in the oil chemical industries. In recent years, lipases have been found to catalyze reversible reactions such as esterifying reaction in aqueous, non-aqueous and solvent-free phases [6–8]. Lipase-mediated synthesis of aliphatic esters of longer chain substrates have shown their easy esterification abilities [9]. The synthesis of low molecular flavor ethyl esters from shorter chain substrate has been attempted. However, this attempt comparatively received less attention with no satisfaction as short fatty-acids easily strip the essential water around enzymes to cause their deactivation [10] or cause dead-end inhibition reacting with the serine residue at the active site of lipase [11]. In previous studies, almost all researchers did not succeed in synthesizing them with high yields (>80%) at more than 0.5 M substrate concentration [2,10,11], which keeps enzymatic pathway for synthesis of ethyl esters of short-chain fatty acids far from industries. It may be, therefore, relevant to note that: (1) lipases, whether free or immobilized, are much easy to denature since commercial lipases used for ester synthesis were produced from microorganisms selected mainly by their lipolysis abilities and (2) low molecular weight substrates are more water-soluble and have more difficulties to react with than do high molecular weight substrates (less water-soluble) in non-aqueous phase. Being active and stable to catalyze esterification of ethanol with short-chain fatty acids in a non-aqueous phase, lipase-producing and solvent-stable lipase strains should be specifically screened for synthesis of flavor esters. Fungal lipase is an important enzyme showing their ability not only in lipolysis application but also in esterification application because it has been used in the production of many fermented foods for many years.

The objectives of this study were to: (1) isolate lipase-producing strains with highly esterifying activity in non-aqueous system; (2) identify suitable lipase source for flavor ester synthesis; (3) investigate various reaction parameters affecting the esterification of ethanol with short-chain fatty acids by whole-cell lipase, including chain length of carboxylic acids (C_2–C_8), reaction temperature, solvent, substrate concentration, the ratio of acid to ethanol, and initial water activity; and (4) observe lipase stability in batch esterification reaction.

2. Materials and methods

2.1. Materials and medium

All chemicals and solvents used in this study were purchased from Sigma, Wako Pure Chemical Industries, Ltd. (Osaka) or Shanghai Chemical Co. (Shanghai) and of the highest purity available. Lipozyme®IM (RML, immobilized lipase from *R. miehei*) and Novozym®435 (CAL, immobilized lipase from *Candida antarctic*) were donated by Novo Nordisk A/S (Bagsvaerd, Denmark), CCL (lipase from *C. cylindracea*), RAL (lipase from *Rhizopus arrhizus*) and PPL (lipase from *Porcine Pancreas*), were purchased from Sigma; CLL (lipase from *C. lipolytica*) was purchased from Genencor Biotechnology Co. (USA); RCL (lipase from *R. chinesis*) was prepared in our laboratory as shown in Section 2.4. Other lipases were gifts from Biotechnology Research Center of Toyama Prefectural University (Japan).

Plate medium for isolation is potato dextrose agar containing 1% sodium deoxycholate and 150–200 u ampicillin. Medium for screening contains (g/l): soybean powder, 1.0, peptone 1.0; soluble starch, 1.0; K_2HPO_4, 0.2; and $MgSO_4{\cdot}7H_2O$, 0.1; pH 5.0–6.0. Fermentation medium (g/l): soybean powder, 1.0; peptone, 4.0; corn starch, 2.0; K_2HPO_4, 0.2; $MgSO_4{\cdot}7H_2O$, 0.1; pH 5.0–6.0.

2.2. Isolation of ester-producing strains with high esterifying activity in non-aqueous media from leavens

Leaven samples, obtained from distilleries, were collected, diluted with saline, and spread on plate medium to ensure the viability of colonies after incubation at 28–30 °C. Screening work was divided into two steps for lipolysis (hydrolytic) and esterifying (synthetic) activities. Firstly, isolated fungal strains from plates were inoculated into 10 ml screening

medium and incubated in shaking tubes for 3 days at 28–30 °C and 150 rpm. Supernatant of screening medium were collected and filled into holes of two kinds of different plate medium (plate medium (A) containing 1% glycerol tributyrin and plate medium (B) containing 1% olive oil with 0.001% fluorescent dye rhodamine). After 24 h incubation at 35 °C, plate A was subjected to observation and plate B was subjected to UV irradiation (350 nm). The microorganisms forming clear zone diameters of holes close around the colonies in plate A or fluorescent in zone diameters of holes close around the colonies in plate B indicated their lipase production ability used as a parameter to examine the size of zone diameter of holes [13,14]. Secondly, selected strains with higher lipase activity were inoculated into 25 ml screening medium and incubated in 250 ml shaking flasks at 35 °C and 150 rpm for 3 days. Collected by centrifugation, mycelium was washed with 100 mM phosphate buffer (pH 7) and then dried by a frozen drying system (Labconco, USA) to prepare whole-cell lipase. The strains having higher synthetic activities were selected for subsequent research by recognition of esterifying ability in solvent.

2.3. Assay of lipase hydrolytic and synthetic activity

Hydrolytic activity was measured by the olive oil emulsion method [12]. 4 ml 3% PVA/olive oil (3:1) was incubated with 5 ml 20 mM phosphate buffer (pH 7.5) in a 100 ml flask at 40 °C for 10 min. One milliliter of enzyme solution was added to give a final volume of 10 ml. After 15 min, the reaction was stopped by adding 15 ml ethanol. The amount of free fatty acid was titrated with 0.05N NaOH solution to pH 10. Control samples were given similar treatment but treated without enzyme. Synthetic activity was measured by ester-synthesis method in solvent. 50 mM hexanoic acid and ethanol were added into a 150 ml flask containing 10 ml heptane. The reaction was started by adding 0.3 g enzyme and incubated for 2 h at 35 °C with a shaking speed of 200 rpm. Filtered using 0.15 μm membrane to remove the cell, samples were drawn with a syringe for subsequent gas chromatograph. Blank samples were treated using similar method.

2.4. Whole-cell lipase and cell-free lipase preparation

The strain was inoculated in a test tube containing10 ml fermentation medium. Cultivated at 35 °C for 2 days at a shaking speed of 250 rpm, the strain was transferred to a 500 ml shaking flask containing 100 ml fermentation medium for further cultivation at 150 rpm under the same condition as above. The cultivated cells were collected by centrifugation at 8000 rpm for 5 min after 2 days, and washed twice with 0.85% NaCl solution, frozen-dried for 1 day and stored in a desiccator.

After collecting mycelia, cell-free lipase preparation was performed briefly by treating supernatant with ammonium sulfate. A fraction of 20–65% saturation was collected. After dialysis, the enzyme solution was subjected to a DEAE-Cellufine column (5 cm × 35.0 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 6.0). The column was washed with the same buffer, enzyme was eluted by a NaCl linear gradient (0.2–1.0 M). Concentrated with 65% saturation of ammonium sulfate, enzyme solution was dialyzed against 5 mM potassium phosphate buffer and frozen-dried.

2.5. Esterification reaction

Unless otherwise stated, a typical esterification reaction for flavor ester synthesis was conducted in 100 ml capped flasks with 15 ml heptane containing equal concentration of 0.60 M substrates and 6 g/l lipase. The mixture was incubated in batches, shaken at 150 rpm at a temperature of 30 °C for 72 h. The molar conversion was defined as (molar of ethyl ester/molar of initial fatty acid) × 100%. Repeated batch reaction was used for the measurement of half-life and catalyzed by using the collected whole-cell lipase, which was washed with fresh solvent and adjusted for water activity before using again. The half-life of lipase in batch reaction was defined as the time when batch molar conversion drops to half of the original conversion.

2.6. Adjustment of water activity of lipase and substrates

To obtain a defined initial a_w of substrates and lipase, short-chain fatty acids and ethanol and lipase were equilibrated separately in desiccators containing

saturated salt solutions for 3 days at 25 °C. Initial a_w of substrates and lipases were measured according to the Karl Fisher's method using a KF DL18 Mettle Toledo apparatus (Spain) after equilibrating separately in desiccators containing saturated salt solution at 25 °C for 4 days. The salts used were $Ca(NO_3)_2$ ($a_w = 0.09$), $MgCl_2$ ($a_w = 0.30$), $Zn(NO_3)_2$ ($a_w = 0.42$), Mg $(NO_3)_2$ ($a_w = 0.50$), $NaNO_3$ ($a_w = 0.66$), NaCl ($a_w = 0.75$), and K_2SO_4 ($a_w = 0.97$). A desiccator containing pure water was used for a_w of 1.0.

2.7. Product analysis method

Aliquots of 100 μl reaction mixture were withdrawn periodically and filtered by membrane. Each sample was diluted in 5 ml heptane containing 0.1 ml of 10% 2-ethyl-butyric acid as an internal standard. Analysis was done by injecting a sample of 1 μl into a gas chromatograph (HP6890) equipped with an SE-30 fused silica capillary column (30 m × 0.25 mm i.d.) and an hydrogen flame-ionization detector and an HP Vectra XA integrator (Hewlett-Packard, Avondale, PA). Oven temperature by programmed temperature was held at 50 °C for 3 min before being elevated to 150 °C for 2 min at 10 °C/min. Injector and detector temperatures were set at 250 °C and the carrier gas was nitrogen.

3. Results and discussion

3.1. Isolation and preliminary identification of strain

Leavens have been used in production of fermented foods for several thousand years in oriental countries. *Da Qu*, a kind of leaven for Chinese liquor making, was selected as microbial source in this study. Among 707 fungal strains isolated from leavens of different distilleries, 25 moulds had activity of both hydrolytic olive and tributyrate after the first screening. These were subjected to secondary observation with respect to their ability to synthesize ethyl hexanoate in solvent phase. Whereas, as few as four moulds showed higher esterifiying ability, the mould with highest conversion was the strain numbered as Y-92 from Yanghe distillery. It is worth being aware of finding useful strains producing lipases in *Da Qu* since Chinese liquor characterizes high content of fatty esters as flavor components and leaven with various microorganisms and more stable enzymes have been produced from uncooked wheat, and soybean.

The taxonomic studies of the mould indicated that the mycelial fungal belongs to *R. chinesis*. The morphological, physiological and cultural characteristics showed that the mould has stolonate mycelia with rhizoide, and straight brownish-white sporangiophores, subglobose columellae, a dense light brownish-gray turf, a litter acid and no acetion production with maximum growth temperature of 45 °C in Pfeffer solution. Identified by China Center for Type Culture Collection, the strain was designated as *R. chinesis* and preserved in an acquired number of CCTCC M201021. The lipase from *R. chinesis* CCTCC M201021 was chosen for further studies.

3.2. Comparative analysis of ability to synthesize esters by lipases from different microbial sources

For screening suitable lipases for the synthesis of short-chain fatty-acid esters, 10 lipases from different sources [Lipozyme®IM (immobilized lipase from *Rhizomucor miehei*), Novozym®435 (immobilized lipase from *C. antarctic*), CCL (lipase from *C. cylindracea*), PPL (lipase from *P. Pancreas*), RAL (lipase from *R. arrhizus*), CLL (lipase from *C. lipolytical*), PSL (lipase from *Pseudomonas* sp.), ANL (lipase from *Aspergillus niger*), PNL (*Phycomyces nitens*), and RCL (whole-cell and cell-free lipase from *R. chinesis*)] were examined for their ability to catalyze the esterification of ethanol with hexanoic acid in heptane at the normal temperature of 30 °C with an equal molar substrate concentration of 0.5 M and 6 g/l of enzyme added. Among these lipases, RCL, RML, PPL, RAL, and PSL presented higher synthetic activity for the incorporation of hexanoic acid into ethanol with a mean molar conversion yields of 80% (Table 1). The maximum yield was obtained by whole-cell lipase (RCL) with 96.5% conversion yield in concentration of 96.1 g/l ethyl hexanoate in reaction solvent. It was shown that substrate specificity of lipases varied considerably, and was dependent on the sources of lipases. Therefore, it was identified further that the strategies of screening lipase-producing mould, from leavens and secondary screening by their ability to synthesize flavor esters in solvent phase, succeeded significantly in isolation of microorganisms producing

Table 1
Effect of various sources of lipases on the synthesis of ethyl hexanoate in heptane

Lipase source[a]	Molar conversion (%)	Relative activity (%)
RCL (whole-cell)	96.5	100.0
RCL (cell-free)	84.9	88.0
RML	91.5	94.8
CRL	75.4	78.1
PPL	87.7	90.9
CLL	32.6	33.8
CAL	61.2	63.4
PNL	18.2	18.9
MJL	16.0	16.6
RAL	80.0	82.9
ANL	18.5	19.2
PSL	87.1	90.3

Reaction condition: equal molar ethanol and hexanoic acid concentration of 0.5 M and 6 g/l of enzyme at 150 rpm and 30 °C for 72 h.

[a] RCL, *R. chinesis* CCTCC M201021; RML, *R. miehei* (Lipozyme® IM); CRL, *C. rugosa*; PPL, *P. Pancreas*; CLL, *C. lipolytical*; CAL, *C. antarctic* (Novozym® 435); PNL, *P. nitens*; MJL, *Mucor javanicus*; RAL, *R. arrhizus*; ANL, *A. niger*; PSL, *Pseudomonas* sp.

solvent-tolerant lipase with higher activity. In the study on different lipase preparation from *R. chinesis* CCTCC M201021 (whole-cell and cell-free), interesting observation was made. Cell-free lipases from *R. chinesis* CCTCC M201021 did not express the same ability to ester synthesis as whole-cell lipase in spite of the fact that the same unit was added. On the other hand, there was a much lower activity of extracellular lipase than that of whole-cell lipase as this lipase was mainly membrane-bound enzyme (data not shown). Accordingly, the whole-cell RCL was selected for subsequent experiments.

3.3. Effects of chain length of carboxylic acids on the flavor ester synthesis by the whole-cell RCL

For investigation of the effect of the short-chain fatty acids on conversion of reaction, esterification reactions of C2–C8 carboxylic acids with ethanol were carried out in heptane. In general, lipases exhibited a distinct specificity as regards the chain lengths of substrate acids for synthesis in solvent phase [8,15]. For esterification of ethanol with short-chain aliphatic acid, it was shown in Table 2 that not only higher esterification were achieved by the whole-cell RCL lipase with longer chain length of acid components, but also better esterification by whole-cell RCL lipase for synthesis of ethyl esters of shorter chain fatty acid in solvent phase with molar conversion (>90%), with the exception of propionic acid. For example, there were 93% conversion for ethyl octanoate and 91.5% for ethyl acetate. In our previous research, the chain length of carboxylic acids had great influence on conversion by other commercial lipases [8]. The discovery that high yields of ethyl acetate was achieved in a solvent system demonstrated further that whole-cell lipase has specificity to short-chain fatty acids and an advantage over cell-free lipase in esterification reaction of short-chain fatty acids and ethanol. Whole-cell RCL might be regarded as an immobilized lipase on cell, which was helpful to form more stable conformation of lipase and to protect enzyme from stripping the essential water of lipase in non-aqueous phase to cause final denaturalization.

Table 2
Effect of chain length of carboxylic acid on the synthesis of various flavor esters

Fatty acid	Molar conversion (%)
Acetic acid	91.5
1-Propionic acid	82.6
Butyric acid	91.8
Valeric acid	93.5
Hexanoic acid	96.2
Heptanoic acid	91.3
Octanoic acid	93.0

Reaction condition: equal molar ethanol and hexanoic acid concentration of 0.5 M and 6 g/l of enzyme at 150 rpm and 30 °C for 72 h.

3.4. Effect of solvents

It is well-known that catalytic behavior of lipases in non-aqueous enzyme reaction depends mainly on non-aqueous environment surrounding the enzyme [14,16]. Effects of various solvents, ranging from polar to non-polar, on conversion yield were performed in the ethyl hexanoate synthesis by whole-cell form *R. chinesis*. The log *P*-value of the solvents, the value of a logarithm to the partition coefficient of a given solvent between water and 1-octanol in a two-phase system, is usually a widely used parameter to express solvent polarity and their possible effects on enzyme

Table 3
Effect of solvents on the synthesis of ethyl hexanoate by whole-cell lipase from *R. chinesis*

Solvent	Log *P*	Molar conversion (%)	Relative conversion (%)
Ethanol	−0.242	16.7	17.3
Acetone	−0.23	13.8	14.3
Tetrahydrofuran	0.49	18.9	19.6
Pentanol	1.30	17.5	18.2
Benzene	2.00	88.8	92.1
Cyclohexane	3.20	93.3	96.8
Hexane	3.50	95.8	99.4
Heptane	4.00	96.4	100.0
Octane	4.50	92.9	96.4
Nonane	5.10	93.2	96.7
Dodecane	6.60	95.2	98.8

Reaction condition: equal molar ethanol and hexanoic acid concentration of 0.5 M and 6 g/l of enzyme at 150 rpm and 30 °C for 72 h.

activity in non-aqueous phase [16]. The results in Table 3 showed that better esterification with more than 88% yield was obtained in the case of solvents with a log *P*-value >2.0, which gave excellent conversion. The poor esterification yield were obtained only when using solvents with log *P*-value <2. These results were different from normal observations because the use of a solvent with log *P*-value >4.0 usually gave rise to better esterification [16]. This phenomenon might be explained that cell membrane provided lipase more tolerant ability to polarity, that enhanced the protection of lipase by preventing solvents from easy stripping the essential water around enzyme, which however needs further investigation. It can be seen clearly in Table 3 that excellent conversion was given to the esterification in both hexane and heptane with maximum yield of up to about 96%.

3.5. Effect of temperature

We carried out the esterification of ethanol with hexanoic acid at equal molar 0.50 M in heptane containing 6 g/l of lipase at temperature ranging from 25 to 40 °C. As the reaction temperature increased, reaction rates also increased. It was shown in Fig. 1 that whole-cell lipase from *R. chinesis* CCTCC M201021 has an optimum temperature ranging from 30 to 40 °C with the maximal conversion yield of 96.4% in solvent after 72 h, which is identical to the optimal temperature of

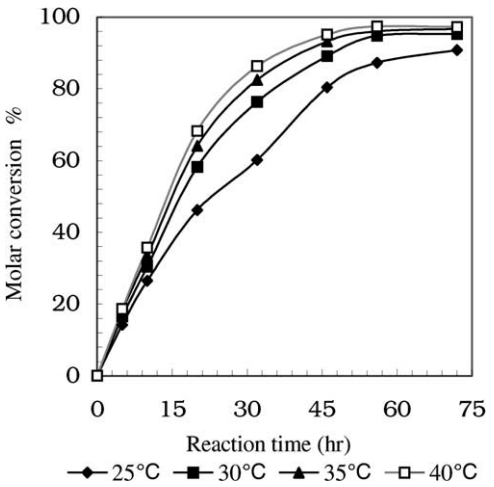

Fig. 1. Effect of reaction temperature on the synthesis of ethyl hexanoate by whole-cell lipase from *R. chinesis*; Reaction condition: equal molar ethanol and hexanoic acid concentration of 0.5 M and 6 g/l of enzyme at 150 rpm.

the enzyme in aqueous phase (data not shown). Therefore, as high as 96% conversion yield was obtained at the temperature of 30 °C, which was taken as optimal and operative parameter because of consideration of the enzyme's reuse.

3.6. Effect of substrate concentration and ethanol to acid molar ratio

The substrate concentration afforded by a biological catalyst is an important parameter affecting enzyme activity and potential application. The effect of varying concentrations of substrates (ethanol and hexanoic acid) in the rang of 0.2–1.4 M each has been carried out on synthesis of ethyl hexanoate at lipase concentration of 6 g/l using equal molar proportions of ethanol and hexanoic acid. Increase in substrate concentration was found to decrease the conversion yields of esterification reactions (Fig. 2). A higher conversion of over 96% observed at 0.6 M substrate slipped slightly to 85% with higher substrate level of 1.4 M, showing that productivity was as high as about 165 g/l esters although there are no research reports showing such a high yield. Similar trends were noticed with respect to the synthesis of other ethyl esters, from ethyl acetate to ethyl octanoate (results not shown). It indicated that being more tolerant to polar

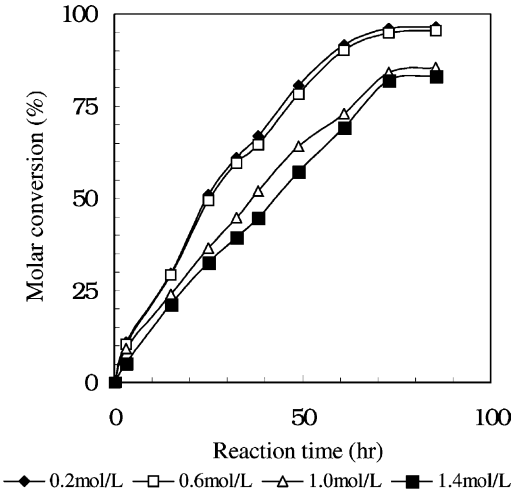

Fig. 2. Effect of acid (ethanol) concentration on the synthesis of ethyl hexanoate by whole-cell lipase from *R. chinesis* CCTCC M201021; Reaction condition: equal molar ethanol and hexanoic acid concentration and 6 g/l of enzyme at 150 rpm and 30 °C.

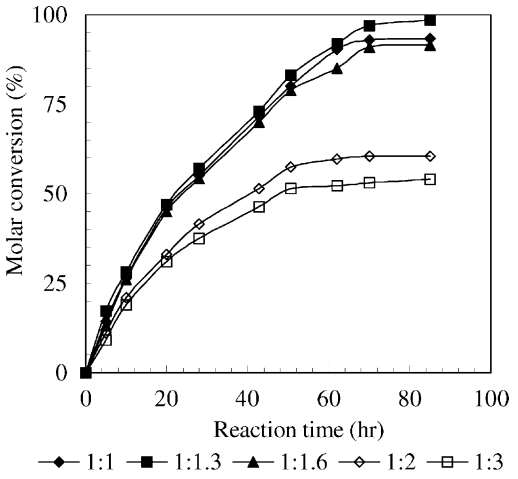

Fig. 3. Effect of substrate molar ratio (acid to ethanol) on the synthesis of ethyl hexanoate by whole-cell lipase from *R. chinesis*; Reaction condition: hexanoic acid concentration of 0.6 M and 6 g/l of enzyme at 150 rpm and 30 °C.

substrates was the characteristic of whole-cell lipase from *R. chinesis*. The fugal cell, that lipase was bound to, may be said to have the ability to provide suitable microenvironment when enzyme existed in solvent system and to inhibit the accumulation of polar substrate from damaging microaqueous layer [16]. The experimental observation identified and supported this assumption.

Considering cost of substrates and substrate inhabitation, the effect of ethanol to acid ratio on the esterification yield was investigated by fixing acid concentration at 0.6 M and varying ethanol from 0.60 to 1.8 M. It can be seen in Fig. 3 that a maximum yield of ethyl hexanoate was achieved with 98.5% conversion with excess ethanol and molar ratio of acid to ethanol was 1:1.3. Increase in ratio of acid to ethanol from 1:1.3 to 1:3, however, decreased in conversion from 98.5 to 50% since ethanol in excess formed the dead-end complex to inhibit conversion [17].

3.7. Effect of initial water activity

When the initial a_w was at different a_w level, esterification reaction for synthesis of ethyl hexanoate was carried out with 0.6 M equal molar substrate concentration and 6 g/l of lipase at 30 °C. The effect of initial a_w on reaction was showed in Table 4 that better esterification occurred to reaction with initial a_w ranging from 0.66 to 0.97, indicating that whole-cell RCL has wider extent of initial a_w than that previously reported [18,19]. The whole-cell lipase from *R. chinesis* gave rise to expanding range for a_w in non-aqueous enzyme reaction although it is generally suggested that reaction system in solvent at initial a_w (0.75) would produce better esterification [19].

Table 4
Effect of initial water activity on synthesis of ethyl hexanoate by whole-cell lipase from *R. chinesis*

Salt	Water activity (a_w)	Molar conversion (%)	Relative conversion yield (%)
$Ca(NO_3)_2$	0.09	32.5	33.8
$MgCl_2$	0.30	30.8	32.0
$Zn(NO_3)_2$	0.42	27.8	28.9
Mg $(NO_3)_2$	0.50	22.2	23.1
$NaNO_3$	0.66	92.1	95.7
NaCl	0.75	95.8	99.6
K_2SO_4	0.97	96.2	100.0

Reaction condition: equal molar ethanol and hexanoic acid concentration of 0.6 M and 6 g/l of enzyme at 150 rpm and 30 °C for 72 h.

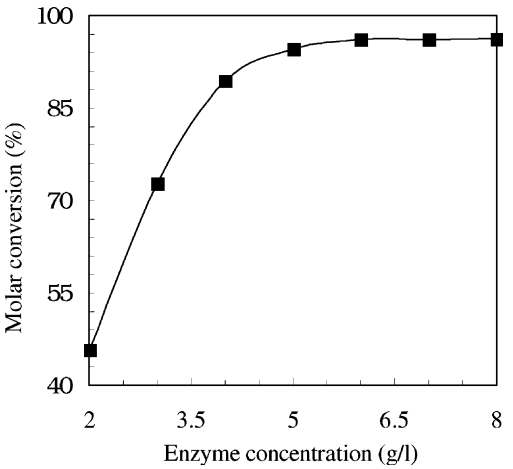

Fig. 4. Effect of enzyme concentration on the synthesis of ethyl hexanoate by whole-cell lipase from *R. chinesis*; Reaction condition: equal molar ethanol and hexanoic acid concentration of 0.6 M at 150 rpm and 30 °C for 72 h.

3.8. Effect of enzyme concentration

Whole-cell RCL was added into esterification reaction system with different concentration levels from 2 to 8 g/l at a substrate concentration level of 0.6 M. Results in Fig. 4 showed that molar conversion increased to about 95% with lipase concentration at a lipase concentration of over 6 g/l with substrate concentration fixed at 0.6 M, and thereafter remained constant. A decrease in enzyme concentration decreased molar conversion from 95 to 46% with declining of enzyme concentration from 6 to 2 g/l. This can be explained by considering the fact that the use of enzyme in excess wound not yield any significance result with regards to conversion yield as it remain inside the bulk cell particles.

3.9. Operational stability of whole-cell lipase

The stability of the whole-cell lipase was examined by reusing lipase for subsequent batch reaction. Filtered out in every batch, collected whole-cell lipase was washed with fresh solvent and equilibrated in desiccator for adjustment of water activity to be further reuse. Reusability of the lipase in batch reaction at 0.6 M substrate concentration was expressed as half-life period (Table 5), indicating that whole-cell lipase behaved more stable and tolerant to solvent with as long as 840–981 h half-life period for C2–C8 short fatty-acid esters. Although half-life period of esterification reaction for ethyl propionate is a little lower than others, the possibility of an efficient reuse of whole-cell lipase from *R. chinesis* CCTCC M201021 brought about an innovation that is bound to be appealing usefully in practical situation.

Table 5
Half-life of whole-cell lipase from *R. chinesis* CCTCC M201021 in the repeated batch synthesis of ethyl hexanoate

Esters	Half-life (h)
Ethyl acetate	840
Ethyl propionate	775
Ethyl butyrate	975
Ethyl valerate	923
Ethyl hexanoate	981
Ethyl heptanoate	925
Ethyl octanoate	951

Reaction condition: equal molar ethanol and hexanoic acid concentration of 0.6 M and 6 g/l of enzyme at 150 rpm and 30 °C for 72 h.

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References

[1] F. Bjorkling, S.E. Godtfredsen, O. Kirk, Trends Biotechnol. 9 (1991) 360.
[2] W. Chulalaksanaukul, S. Condoret, P. Delorme, R.M. Willemot, FEBS Lett. 276 (1990) 181.
[3] R.J. Kazlauskas, Trends Biotechnol. 11 (1993) 439.
[4] T. Ota, S. Takano, T. Hasegawa, Agric. Biol. Chem. 54 (1990) 1571.
[5] J.C. Peter, Trends Biotechnol. 11 (1993) 478.
[6] J. Stofberg, F. Grundschober, Perfumer Flavorist 24 (1987) 27.
[7] F.W. Welsh, R.E. Williams, K.H. Dawson, J. Food Sci. 55 (1990) 1679.
[8] Y. Xu, K.C. Zhang, in: R. Whitaker, N.F. Haard, C.F. Shoemaker, R.P. Singh (Eds.), Food for Health in Pacific Rim, Food and Nutrition Press, CT, USA, 1998, p. 587.
[9] S.H. Krishna, S. Divaker, S.G. Prapulla, N.G. Karanth, J. Biotechnol. 87 (2001) 193.
[10] S.Y. Huang, H.L. Chang, M. Goto, Enzyme Microb. Technol. 22 (1998) 552.

[11] Y. Xu, D. Wang, in: J.J. Zhong (Ed.), Advances in Applied Biotechnology, East China University of Science and Technology Press, Shanghai, 2000, p. 206.
[12] H.H. Yeoh, F.M. Wong, G. Lim, Mycologia 78 (1986) 298.
[13] G. Kouker, K.E. Jaeger, Appl. Environ. Microb. 53 (1987) 211.
[14] B. Cambou, A.M. Klibanov, Biotechnol. Bioeng. 26 (1984) 1449.
[15] G. Langrand, N. Rondot, C. Triantaphylides, J. Baratti, Biotechnol. Lett. 12 (1990) 581.
[16] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechnol. Bioeng. 30 (1987) 81.
[17] S. Ramamurthi, A.R. McCurdy, J. Am. Oil Chem. Soc. 71 (1994) 927.
[18] Z. Yang, D.A. Robb, Biotechnol. Bioeng. 43 (1994) 365.
[19] P.J. Halling, Emzyme Microb. Technol. 16 (1994) 178.