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Green synthesis of (*R*)-3-TBDMSO glutaric acid methyl monoester using *Novozym* 435 in non-aqueous media†

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An efficient biocatalytic synthesis of (*R*)-3-TBDMSO glutaric acid methyl monoester (*R*-J_6), an important intermediate in the synthesis of rosuvastatin, has been developed using a green catalytic route in the presence of lipase, conducted under mild conditions without additional chiral reagents. Enzyme screening indicated Novozym 435 to be the most efficient biocatalyst for *R*-J_6 synthesis. Methanol, which was the most effective alcohol for synthesis of *R*-monoester, was identified as the best acyl acceptor by molecular docking. The optimal conditions for synthesis of *R*-J_6 were as follows: 50 g L^{-1} catalyst, 3 : 1 molar ratio of methanol : substrate, 200 g L^{-1} substrate, iso-octane as solvent, orbital shaking at 200 rpm, and an incubation time of 24 h at 35 °C. The key factor affecting the yield of *R*-J_6 was the molar ratio of methanol to substrate found by an orthogonal array experimental design. Consequently, the desired product, *R*-J_6, was afforded with a titer of 117.2 g L^{-1}, a yield of 58.6%, and productivity of 4.88 g L^{-1} h^{-1}. This green method holds promise for the preparation of kilogram quantities of (*R*)-3-substituted glutaric acid monoesters.

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1. Introduction

Statins are a class of pharmaceuticals that inhibit the enzyme hydroxymethylglutaryl-CoA reductase (HMGR) and are widely used as hypolipidemic agents to lower the level of cholesterol in the blood.[1] Clinical trials have confirmed that statins can adjust blood lipid levels[2,3] and reduce the risk of fatal and nonfatal cardiovascular disease.[4] In particular, rosuvastatin, the so-called "super statin", has high efficacy, few side-effects, low toxicity, and outstanding selectivity. Dose-for-dose, rosuvastatin is by far the most efficacious statin for reducing plasma low-density lipoprotein (LDL) cholesterol, reducing total cholesterol significantly, and the duration of inhibition is longer than for other statins such as atorvastatin, simvastatin, and pravastatin.[5,6] The market for cholesterol-lowering drugs is the largest in the pharmaceutical sector,[7] and industrial production of rosuvastatin is significant. Sales of rosuvastatin remain in the world's top ten, with annual sales of $5.3 billion recorded in 2013.[8]

Current industrial production of rosuvastatin is mainly by chemical synthesis,[9,10] in which the pyrimidine nucleus and chiral side chain are condensed using the Wittig reaction.[11] The chiral side chain acts as the functional group, presenting the pharmacophore for HMGR recognition.[12] (*R*)-3-Substituted glutaric acid monoesters are important intermediates for the assembly of the chiral side chain. In recent years, several effective methods have been used for the preparation of (*R*)-3-substituted glutaric acid monoesters, including chemical synthesis,[12] enzymatic methods[13] and chiral resolution.[14] Industrial production of (*R*)-3-substituted glutaric acid monoesters is mostly by chemical synthesis.[12,15,16] Green synthetic routes involving renewable raw materials and the replacement of environmentally "unfriendly" syntheses are receiving increasing attention. However, chemical syntheses of 3-substituted glutarates require extreme conditions, such as low temperature (−78 °C)[12] and expensive reagents (benzyl (*R*)-(−)-mandelate and $Pd(OH)_2$–C)[15,17] containing heavy-metals, which affects the quality of the chiral end-product, and high energy consumption; and the poor extraction and expensive additional purification steps made the whole process costly, which made the method unsuitable for large-scale *R*-J_6 preparation.

With high catalytic efficiency, mild reaction conditions, fewer side reactions, and environmental friendliness, biological catalysts have been widely applied to industrial production.[18–21]

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Enzymatic methods provide an alternative to traditional complex chemical synthesis.[22] Kinetic resolution by biological catalysts can remove one enantiomer from the racemate selectively and mildly, and the unwanted enantiomer can be separated. However, since the maximum theoretical yield is only 50%,[23] application of kinetic resolution on a large scale has been hampered. Hydrolysis of 3-substituted glutaric acid diesters using hydrolases or esterases has been applied to the preparation of (*R*)-3-substituted glutaric acid monoesters,[24] but, because the substrates are poorly soluble in water, the reaction is slow. To achieve higher yields, the hydrolysis has been conducted in a two-phase aqueous–organic system,[25] but yields are still limited because the reaction only occurs at the solvent interface.

The use of organic solvents in biocatalytic reactions has addressed the problem of low aqueous solubility of the substrate. A possible alternative for green production of (*R*)-3-substituted glutaric acid monoesters is acylation of alcohols with 3-substituted glutaric anhydride using biocatalysts (Scheme 1), which has a theoretical yield of 100%. Since suitable organic solvents[26] can enhance the "rigid" conformation of lipases,[27–29] improve heat resistance[18,30] and maintain high catalytic activity,[27] mono-esterification using lipases is more promising for industrial application. Unfortunately, a lot of work on preparation of 3-substituted glutaric acid monoesters has revealed that natural lipases favor the product with *S*-configuration (ESI data Table S1†).[22,23] The yield of *R*-J_6 was low and could not meet industrial demand, therefore enzymatic synthesis of (*R*)-3-substituted glutaric acid monoesters (*R*-monoester) by lipases has rarely been reported.

In this study, we have extended the scope of the synthesis of (*R*)-3-TBDMSO glutaric acid methyl monoester (*R*-J_6) using organic solvent by alcoholysis of 3-substituted glutaric anhydride with an *S*-selective lipase. The lipase from *Candida antarctica* (CALB) has been screened and employed for *R*-J_6 production with high catalytic efficiency.[31] Following selection of the best performing enzyme, the co-substrate (alcohol) that would be favored for synthesis of the *R*-monoester was selected as the acyl acceptor by molecular docking. The final objective was to optimize the reaction conditions. An efficient process for large-scale production of (*R*)-3-substituted glutaric acid monoesters by reaction of 3-substituted glutaric anhydride with alcohols has been developed.

2. Materials and methods

2.1. Materials

Novozym 435 (CALB, lipase from *C. antarctica* immobilized on a macroporous anionic resin) was purchased from Novozymes (Beijing, China). 3-TBDMSO glutaric anhydride (TBDMSO: *t*-butyl-dimethyl-silyloxy) was purchased from Yuchen Fine Co., Ltd (Henan, China). α-Chymotrypsin was purchased from Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). Isopropanol and *n*-hexane (HPLC grade) were purchased from Sigma (St Louis, USA). Other chemicals and solvents (analytical grade) were from local suppliers (Wuxi, China). Standards of (*R*)-3-TBDMSO glutaric acid methyl monoester and racemic 3-TBDMSO glutaric acid methyl monoester were obtained as a gift from Chanyoo Pharmatech Co., Ltd (Nantong, China). The Chiralpak AD-H column (4.6 × 250 mm) was purchased from Daicel Chiral Technologies (Shanghai, China).

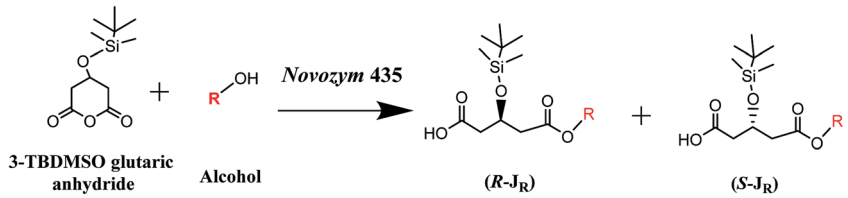
2.2. Analytical procedure

Methyl esters in the reaction mixture were analyzed by high performance liquid chromatography (HPLC) using a Daicel Chiralpak AD-H column (4.6 × 250 mm) and an ultraviolet detector supplied by Agilent. The mobile phase consisted of 96% hexane and 4% isopropanol with 0.02% (v/v) acetic acid, which was filtered through a 0.45 μm membrane. A 10 μL sample was injected into the column with a detection temperature of 25 °C and a flow rate of 1 mL min^{-1}. The run time was 15 min.

R-J_6 and racemic 3-TBDMSO glutaric acid methyl monoester (racemic J_6) were the internal standards. Under these conditions the retention times were as follows: *R*-J_6, 7.7 min; (*S*)-3-TBDMSO glutaric acid methyl monoester (*S*-J_6), 8.3 min. Aliquots (50 μL) of the reaction mixture were taken and solvent removed in an oven at 70 °C. Each sample was diluted with 1 mL of mobile phase then filtered through a 0.22 μm membrane.

2.3. Molecular docking of alcohols

The crystal structure of CALB[32] [PDB: 1TCA] was taken from the Protein Data Bank (http://www.rcsb.org/pdb/explore/explore.do?structureId=1TCA). In the molecular docking, a series of alcohols were used as acyl acceptors. Three-dimensional structures of the ester products were obtained from Chemoffice Ultra 11.0 then minimized using CHARMM.



Scheme 1 Enzymatic preparation of rosuvastatin side-chain intermediate (*R*-J_R) from 3-TBDMSO glutaric anhydride using *Novozym* 435. When R is CH_3–, *R*-J_R = *R*-J_6; alcohol can be methanol, ethanol, *n*-propanol, *n*-butanol, iso-butanol, *tert*-butanol, hexanol, benzyl alcohol, 1-phenylethanol or 2-phenylethanol.

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The compounds were docked into the CALB binding site to determine docking energies and hydrogen bonding. Only the ligand molecules were considered flexible during the docking simulation, and only the free energy of the best pose was taken for comparison.

2.4. Esterification reaction

A typical esterification reaction was conducted in 10 mL capped flasks using methyl *tert*-butyl ether (MTBE) as solvent, 3-TBDMSO glutaric anhydride as substrate, and Novozym 435 as catalyst (Scheme 1). Crushed 3 Å molecular sieves were activated by heating in an oven at 100 °C for at least 3 days. The organic solvent was dried over 3 Å molecular sieves for 72 h before use. The activated molecular sieves (2.5 g, Aldrich, 15–20 wt% based on substrate) were added to the reaction mixture to absorb water generated during the esterification. The mixture was incubated for 24 h on an orbital shaker (200 rpm) at 35 °C.

2.5. Statistical analysis

Three different factors (molar ratio of methanol to substrate, Novozym 435 concentration and 3-TBDMSO glutaric anhydride concentration) were explored using an L_9-orthogonal array design. The design was developed and analyzed using Design-Expert 8.0 software. All measurements were taken in triplicate and experiments were repeated three times to evaluate the standard deviation.

3. Results and discussion

3.1. Screening of the catalysts for *R*-J_6 production

According to the literature[33,34] there are four lipases (Lipozyme TLIM from *Thermomyces lanuginosus*, porcine trypsin, Novozym 435 from *C. antarctica*, and α-chymotrypsin) that can be used as biocatalysts for asymmetric alcoholysis of 3-substituted glutaric anhydride. The performance of the four lipases in the synthesis of *R*-J_6 was investigated and the results are shown in Table 1. Trypsin and α-chymotrypsin performed poorly, giving product titers below 10 g L^{-1}. Novozym 435 gave the best results, with the titer of *R*-J_6 reaching 12 g L^{-1} and productivity increased to 0.49 g L^{-1} h^{-1}. Based on these results, Novozym 435 was selected as the catalyst for *R*-J_6 production.

The effect of Novozym 435 concentration on *R*-J_6 production is shown in Fig. 1. As the concentration of Novozym 435 increased from 0 to 60 g L^{-1}, the titer of *R*-J_6 increased. The *R*-J_6 titer reached 15.7 g L^{-1} when the concentration of Novozym 435 was 60 g L^{-1}, with a yield of 26.13% and productivity of 0.65 g L^{-1} h^{-1}. The *R*-J_6 titer increased 11-fold compared with that obtained when 10 g L^{-1} Novozym 435 was used. CALB crystal structures suggest a catalytic mechanism somewhat similar to the serine proteases, with the Ser^{105}–His^{224}–Asp^{187} triad as the catalytic centre.[32,35,36] The mechanism of (*R*)-3-TBDMSO glutaric acid methyl monoester production by Novozym 435 has been studied by molecular dynamics simulation (more information showing in ESI Scheme S4†).

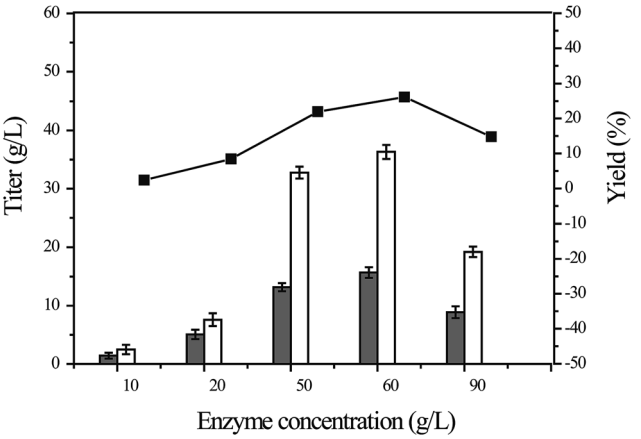

Fig. 1 Effect of Novozym 435 concentration on *R*-J_6 production. (▓) *R*-J_6 titer, (□) *S*-J_6 titer, (—■—) yield of *R*-J_6.

3.2. Effect of the acyl acceptor on *R*-monoester production

The ester bond between the alcohol and 3-hydroxy glutaric acid is hydrolyzed in the final step of rosuvastatin synthesis (Scheme S2†). However, alcohols can affect the interactions between product enantiomers and the enzyme. A series of alcohols were used as acyl acceptors in the molecular docking (Scheme 1), and the results are shown in Table 2. The larger the E_R/E_S ratio, the more stable the predicted transition state of the *R*-isomer. Of the alcohols studied, the best E_R/E_S ratios were for methanol (1.072) and *tert*-butanol (1.043). Therefore, methanol and *tert*-butanol were chosen as the acyl acceptors.

Table 1 Performance of different lipases in the synthesis of *R*-J_6[a]

Entry	Enzyme	Time (h)	*R*-J_6 titer (g L^{-1})	Productivity (g L^{-1} h^{-1})
1	Novozym 435	24	11.72	0.49
2	Lipozyme TLIM	24	10.56	0.44
3	α-Chymotrypsin	24	7.92	0.33
4	Trypsin	24	6.64	0.28

[a] Reaction conditions: 100 g L^{-1} 3-TBDMSO glutaric anhydride, 3 : 1 molar ratio of methanol to substrate, 30 g L^{-1} enzyme, MTBE as solvent at 30 °C with a shaking speed of 200 rpm.

Table 2 Results of molecular docking

Entry	R-OH	Energy of *R*-isomer (kcal mol^{-1})	Energy of *S*-isomer (kcal mol^{-1})	E_R/E_S
1	Methanol	−97.74	−91.187	1.072
2	Ethanol	−96.00	−109.65	0.876
3	*n*-Propanol	−100.70	−101.06	0.996
4	*n*-Butanol	−99.896	−107.47	0.930
5	Iso-butanol	−84.362	−87.93	0.959
6	*tert*-Butanol	−107.84	−103.37	1.043
7	Hexanol	−85.98	−113.09	0.760
8	Benzyl alcohol	−96.55	−102.17	0.945
9	1-Phenylethanol	−113.46	−115.89	0.979
10	2-Phenylethanol	−107.87	−115.27	0.936

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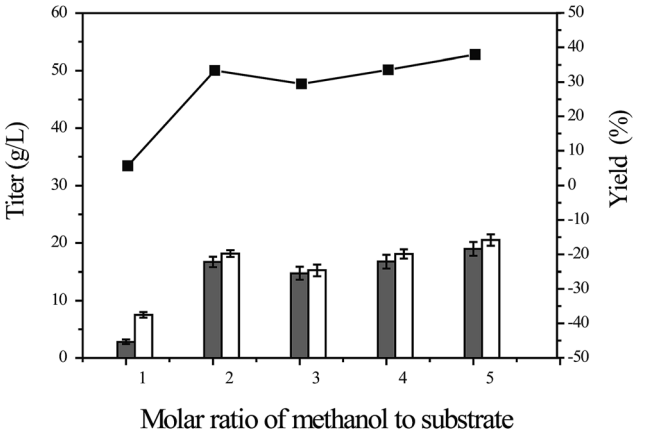

Fig. 2 Effect of methanol : substrate molar ratio on *R*-J_6 production. (▭) *R*-J_6 titer, (▭) *S*-J_6 titer, (—■—) the yield of *R*-J_6. Reaction conditions: 3-TBDMSO glutaric anhydride (50 g L^{-1}), the solvent was MTBE, 60 g L^{-1} Novozym 435. Methanol was added in one portion at the beginning of the reaction.

However, the shorter the carbon chain of the alcohol, the higher the rate of transesterification would be.[37] For the realization of large-scale production and reducing cost, methanol (the cheaper and the shortest carbon chain) was selected as the best acyl acceptor. To investigate the effect of the methanol concentration on *R*-J_6 production, the substrate was alcoholyzed at 30 °C for 24 h with various amounts of methanol by Novozym 435. The results (Fig. 2) show that when the molar ratio of methanol to substrate was 1 : 1, the *R*-J_6 titer was below 5.0 g L^{-1}. Of great interest, when the molar ratio of methanol to substrate reached 2 : 1, the titer and yield of *R*-J_6 rose to 16.7 g L^{-1} and 33.4%, respectively, while productivity reached 0.695 g L^{-1} h^{-1}.

3.3. Effect of reaction conditions on *R*-J_6 production

The effect of reaction temperature on *R*-J_6 production was investigated (Fig. 3A). The titer of *R*-J_6 increased continually as the temperature increased from 22 to 35 °C, and the productivity of *R*-J_6 reached 0.71 g L^{-1} h^{-1} at 35 °C.

The effect of solvents with different log *P* values on *R*-J_6 production are shown in Fig. 3B. No *R*-J_6 was detected in the solvent-free reaction (only methanol, substrate and enzyme in the reaction system). The *R*-J_6 titer was 21.5 g L^{-1} when iso-octane was used as the solvent; the productivity and the yield were 0.895 g L^{-1} h^{-1} and 35.7%, respectively.

The effect of substrate concentration is presented in Fig. 3C. The results show that the *R*-J_6 titer increased as the substrate concentration increased from 40 to 200 g L^{-1}. At a substrate concentration of 200 g L^{-1}, the *R*-J_6 titer was up to the maximum value, 67.1 g L^{-1}, 3.7-fold higher than that at 40 g L^{-1} substrate. However, the yield of *R*-J_6 decreased as the substrate concentration increased (40–200 g L^{-1}). Excess substrate had a negative effect on the yield of *R*-J_6. When the substrate concentration was above 200 g L^{-1}, the yield of *R*-J_6 dropped below 30%, therefore the optimal concentration was 200 g L^{-1}.

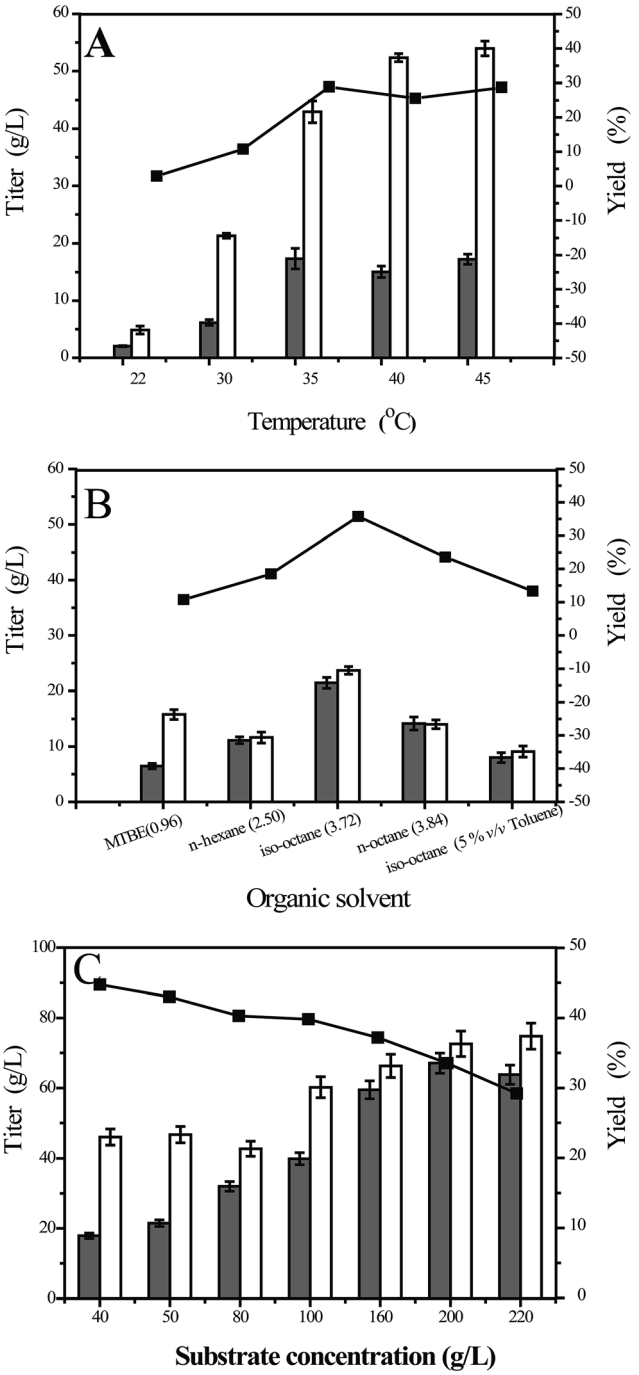

Fig. 3 Effect of reaction conditions on *R*-J_6 production. (▭) *R*-J_6 titer, (▭) *S*-J_6 titer, (—■—) the yield of *R*-J_6. (A) Effect of temperature. Reaction conditions: the solvent was MTBE, 3-TBDMSO glutaric anhydride (60 g L^{-1}), 60 g L^{-1} Novozym 435, 2 : 1 molar ratio of methanol to substrate. (B) Effect of different organic solvents: MTBE (log *P* = 0.96), *n*-hexane (log *P* = 2.50), cyclohexane (log *P* = 3.00), iso-octane (log *P* = 3.72), *n*-octane (log *P* = 3.84); reaction conditions: 3-TBDMSO glutaric anhydride (60 g L^{-1}), 60 g L^{-1} Novozym 435, 2 : 1 molar ratio of methanol to substrate, at 35 °C with a shaking speed of 200 rpm. (C) Effect of substrate concentration. Reaction conditions: the solvent was iso-octane, 60 g L^{-1} Novozym 435, 2 : 1 molar ratio of methanol to substrate, at 35 °C with a shaking speed of 200 rpm. Methanol was added in one portion at the beginning of the reaction.

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3.4. Statistical analysis

The optimum values of the molar ratio of methanol to substrate (molar ratio), Novozym 435 concentration (catalyst), and 3-TBDMSO glutaric anhydride concentration (substrate) were examined using an orthogonal array design (Table 3). The order of the effect of the factors on *R*-J_6 production was molar ratio > substrate > catalyst. The molar ratio of methanol to substrate was the main factor for *R*-J_6 production. In this study, the optimal conditions for synthesis of *R*-J_6 were as follows: 50 g L^{-1} catalyst, 3 : 1 molar ratio, and 200 g L^{-1} substrate. The *R*-J_6 titer was 117.2 g L^{-1}, and the yield was 58.6%. Under the optimal conditions, production of *R*-J_6 with time is shown in Fig. 4, indicating a maximum at 24 h. The biocatalytic process described in this study achieved a highest synthesis rate of 4.84 g L^{-1} h^{-1}, and thus has great potential for large-scale production of (*R*)-3-TBDMSO glutaric acid methyl ester, the purity of the desire product was up to 98%. The *R*-J_6 titer can be maintained at 90 g L^{-1}, therefore, the biocatalyst (Novozym 435) can be reused at least four times (Fig. 5).

R-J_6 has been prepared by using the lithium salt of benzyl (*R*)-(−)-mandelate,[12,17] three additional steps were required to generate *R*-J_6 (see ESI data Scheme S1†), the yield was only 42.3% from 3-TBDMSO glutaric anhydride, isolation and purification and waste treatment were difficult. α-Chymotrypsin with *R*-selective has been used to prepare *R*-isomers by hydrolysis of diethyl-3-hydroxyglutarate, however, the maximum *R*-isomer titer was 32.5 g L^{-1}, and the productivity was only 0.68 g L^{-1} h^{-1}.[38] The 3-substituents group of the substrate significantly affect the enzyme activity and selectivity, the α-chymotrypsin performed a low catalytic efficiency on the substrate whose 3-substituent was TBDMSO.[39] The *R*-isomer productivity was only 0.33 g L^{-1} h^{-1} by α-chymotrypsin while the 3-substituent was TBDMSO in our study.

Typical enzymatic process goals are a substrate loading > 100 g L^{-1}, reaction time < 24 h, conversion > 98%, and enantiomeric excess (ee) > 99%.[40] In our study, the synthesis of *R*-J_6 in non-aqueous media requires only a single step, and the titer of *R*-J_6 was up to gram scale. The desired products can be isolated, purified, and dried easily, and the organic solvents can be recycled by vacuum distillation with no wastewater discharge. Substrate concentration was up to 200 g L^{-1} and the reaction time was 24 h, but the yield of *R*-J_6 was only 58.6% and the ee value was low. The *S*-isomer (*S*-J_6) that was also produced can be used to assemble other statins and their derivative products[33] such as hapalosin,[41] iostatine[33] and dolastatin.[42] In order to obtain the statin skeleton of *R*-J_6 in high optical purity, further studies could explore isolation of *R*-J_6 from the enzymatic conversion solution containing racemic J_6 by dynamic kinetic resolution[43–46] using vinyl acetate as acyl donor[47] (Scheme S3†). Novozym 435 can be reused directly for dynamic kinetic resolution by filtering without any pretreatment, making the whole process green. To enhance the yield and further reduce costs of *R*-J_6 production and simplify the process, we are doing our best to change the enantioselectivity of CALB, and wanna to obtain a catalyst with high *R*-selectivity on *R*-J_6 preparation by directed evolution.

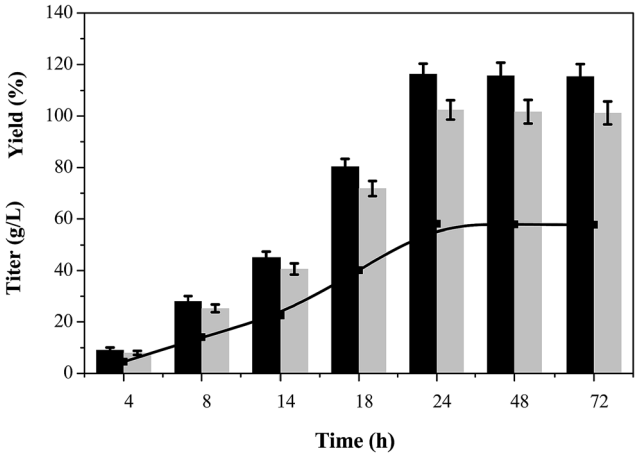

Fig. 4 Time course of *R*-J_6 production under optimized reaction conditions. (■) *R*-J_6 titer, (■) *S*-J_6 titer, (—■—) the yield of *R*-J_6. Reaction conditions: 3-TBDMSO glutaric anhydride (200 g L^{-1}), the solvent was iso-octane, 50 g L^{-1} Novozym 435, 3 : 1 molar ratio of methanol to substrate, at 35 °C with a shaking speed of 200 rpm. Methanol was added in one portion at the beginning of the reaction.

Table 3 Orthogonal array design to improve *R*-J_6 production

	Factor			*A*	*B*	*C*	
Run	*A*	*B*	*C*	Substrate (g L^{-1})	Molar ratio	Catalyst (g L^{-1})	*R*-J_6 titer (g L^{-1})
1	1	1	1	150	1	50	23.12 ± 1.2
2	1	2	2	150	2	60	50.83 ± 1.5
3	1	3	3	150	3	70	87.44 ± 2.4
4	2	1	2	200	1	60	37.44 ± 2.0
5	2	2	3	200	2	70	73.99 ± 2.4
6	2	3	1	200	3	50	117.19 ± 2.9
7	3	1	3	250	1	70	36.79 ± 1.8
8	3	2	1	250	2	50	73.11 ± 1.7
9	3	3	2	250	3	60	108.24 ± 2.7
Range				22.41	71.84	5.64	
Rank				2	1	3	
Optimization				200	3	50	

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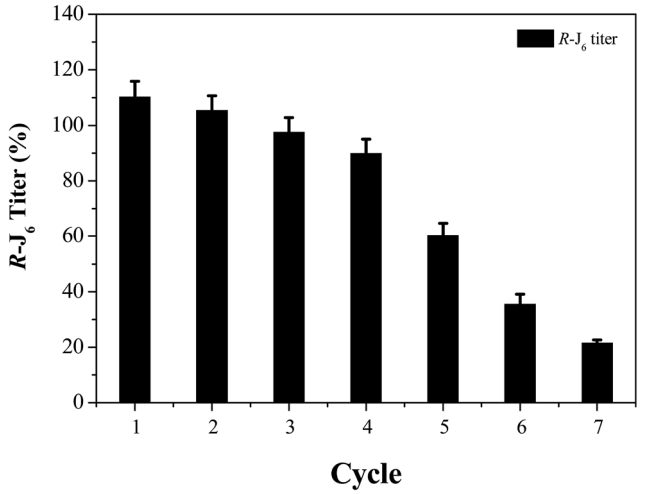

Fig. 5 Operation stability of *Novozym* 435 on *R*-J_6 production.

4. Conclusions

In summary, different acyl acceptors have been screened based on molecular docking,[48] and methanol was chosen as the best acyl acceptor. Our experiments suggest that several lipases, especially lipase from *Candida antarctica*, catalyze (*R*)-3-TBDMSO glutaric acid methyl ester (*R*-J_6) production *via* esterification in high yields. A method for enzymatic synthesis of *R*-J_6 in non-aqueous media has been described. The desired product, *R*-J_6, was afforded with a titer up to 117.2 g L^{-1} and a yield of 58.6%; the productivity of *R*-J_6 was improved tenfold from 0.49 g L^{-1} h^{-1} to 4.88 g L^{-1} h^{-1}. The biocatalyst (CALB) with high efficiency[49,50] and selectivity[51–53] can be reused at least four times (Fig. 5). Besides the advantages of this method, the highest enantiomeric excess (ee = 22%) is still moderate and ongoing studies are underway to circumvent this limitation. Compared with chemical syntheses and enzymatic hydrolysis, this method is a green chemical process with significant potential for industrial application.

5. Acknowledgements

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