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Biocatalytic synthesis of (−)-1-trimethylsilylethanol by asymmetric reduction of acetyltrimethylsilane with a new isolate *Rhodotorula* sp. AS2.2241

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Abstract

A new isolate, *Rhodotorula* sp. AS2.2241, capable of reducing acetophenone and α-bromoacetophenone with high stereoselectivity, was further studied to exploit its potential in the asymmetric reduction of silicon-containing ketones to silyl alcohols. After encapsulation, the cells were used in an aqueous or an aqueous/organic solvent biphasic system for the asymmetric reduction of acetyltrimethylsilane (ATMS) to prepare (−)-1-trimethylsilylethanol [(−)-TMSE]. It has been found that higher product yield and product enantiomeric excess could be achieved with immobilized cells in an aqueous/organic solvent biphasic system. For optimization of the reaction, various influential variables, such as the volume ratio of the aqueous phase to the organic phase, the hydrophobicity of the organic solvent, reaction temperature, buffer pH of the aqueous phase and the shaking speed, were examined with respect to the initial reaction rate, the product yield and the enantiomeric excess (e.e.) of TMSE formed. All the factors mentioned above had influences on the reaction to some extent. Isooctane was found to be the most suitable organic phase for the reaction. The optimum volume ratio of the aqueous phase to the organic phase, reaction temperature, buffer pH and shaking speed were 1/1, 40 °C, 6.5 and 150 rpm, respectively under which the product yield and the product enantiomeric excess were as high as 99 and 90%, which are much higher than those previously obtained by enantioselective reduction of acylsilanes.

Keywords: *Rhodotorula* sp.; Asymmetric reduction; Acetyltrimethylsilane; (−)-1-Trimethylsilylethanol; Aqueous/organic solvent biphase

1. Introduction

Recent investigations have clearly established that enantiomers of a certain racemic pharmaceutical can present different pharmacokinetics and bioavailability properties. The manufacture of the optically active form of drugs is consequently becoming a norm in the industry [1]. Optically pure compounds could be prepared mainly by either chemical or biological means. Usually, traditional chemical synthetic methods can ensure chiral purity only by using chiral feedstock chemicals owing to the difficulty and high cost of racemate resolution. Thus, the economic biocatalytic synthesis of either chiral feedstock chemicals or of the final chiral products can significantly advance current technologies. In recent years, for the preparation of chiral, non-racemic alcohols which are very important synthons for a large number of pharmaceuticals [2],

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many investigations have been carried out to exploit microbial reductases using either isolated enzymes or whole cell systems for asymmetric reduction of the corresponding prochiral carbonyl precursors. As these reactions often involve nicotinamide cofactors (usually NADPH), the use of whole cells rather than isolated enzymes is preferred to avoid the need for enzyme purification and cofactor regeneration [3]. Due to its wide availability and simplicity of use, yeast cells have been commonly used.

On the other hand, the interest in the use of unnatural or unconventional compounds as enzyme substrates is continuously growing in order to test the synthetic potential of biotransformations to produce useful organosilicon compounds, as well as to investigate in detail the mechanism of enzymatic catalysis [4–10]. In particular, the biotransformation of organosilanes with microbes has become the goal of extensive studies. Although highly enantioselective reduction of acylsilanes with aromatic substituents has been successfully performed, the product yield and the product enantiomeric excess (e.e.) for the reduction of other acylsilanes have proved to be disappointingly low [6].

Recently, we have succeeded in isolating a new yeast strain *Rhodotorula* sp. AS2.2241 which was capable of stereoselectively reducing acetophenone and α-bromoacetophenone into the corresponding alcohols [11]. Here, we report the study on its potential in reducing acetyltrimethylsilane (ATMS) to trimethylsilylethanol (TMSE).

2. Results and discussion

2.1. Comparison between free and immobilized cells

Encapsulation is a cost effective and facile method of immobilization [2] and was thus used in this study. As shown in Fig. 1, immobilized cells gave a lower initial reaction rate but a higher yield of TMSE than free cells after reaction for 2 h (89% versus 82%) and the product e.e. was a little higher when the reaction was performed with immobilized cells (63% versus 61%). Both alteration in substrate and product concentrations within the cells and variation in the enzyme activity caused by immobilization are responsible for this. In addition to the merit of easy separation, immobilization could enhance the tolerance of cells against unfavorable factors [12]. So immobilized cells were employed as the biocatalyst for further investigations. The product formed was mainly (−)-TMSE as indicated by the optical rotation and the average error of the product e.e. determination with GC is no more than 0.65%.

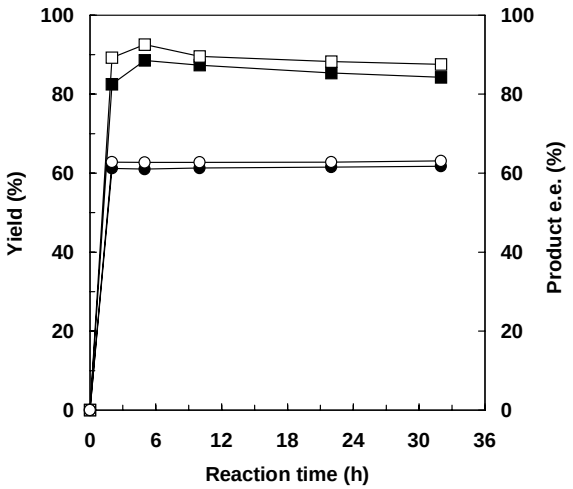

Fig. 1. Time course of the asymmetric reduction of acetyltrimethylsilane with free cells and immobilized cells in an aqueous phase. The reaction was performed at 30 °C and 150 rpm by adding 0.375 g free cells (■, ●) or 1.5 g immobilized cells (□, ○) into 10 ml Tris–HCl buffer (50 mM, pH 8.0) containing 14 mM acetyltrimethylsilane. *Symbols*: (■, □) the chemical yield; (●, ○) the product e.e.

2.2. Comparison of the reduction courses in an aqueous phase and an aqueous/organic solvent biphase

An obstacle in the bioreduction of acylsilanes in an aqueous phase is the pronounced substrate and product inhibition, which is often observed in such transformation. To reduce this limitation, an aqueous/organic solvent biphase was used for this biotransformation. As shown in Fig. 2, the reaction proceeded faster in the aqueous phase than in the aqueous/organic solvent biphase. This was probably due to the increased mass transfer limitation and lowered substrate concentration in the case of biphase. However, higher yield (91.4% versus 89%) and product e.e. (72.7% versus

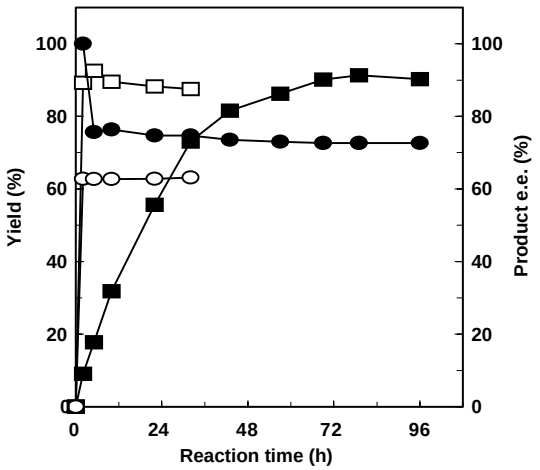

Fig. 2. Time course of the asymmetric reduction of acetyltrimethylsilane with immobilized cells in an aqueous/organic solvent biphase and in an aqueous phase. The reaction was performed at 30 °C and 150 rpm by adding 1.5 g immobilized cells into the biphasic system of 10 ml Tris–HCl buffer (50 mM, pH 8.0) and 10 ml *n*-hexane (■, ●) or the aqueous phase of 10 ml Tris–HCl buffer (50 mM, pH 8.0) (□, ○) containing 14 mM acetyltrimethylsilane. *Symbols*: (■, □) the chemical yield; (●, ○) the product e.e.

63.1%) could be achieved with the aqueous/organic solvent biphase. The in situ extraction of the product from the aqueous phase to the organic phase eliminated the product inhibition and shifted the equilibrium of the reaction towards the synthesis of TMSE, thus resulting in a higher yield, which otherwise could be lowered by the reverse reaction. At the same time, the product e.e. might be enhanced by the inactivation of the isoenzymes with different stereoselectivity from the reductases catalyzing the conversion of ATMS to (−)-TMSE. Furthermore, an advantage of employing an aqueous/organic solvent biphase is the ease with which the product could be isolated. Therefore, an aqueous/organic solvent biphase was chosen as the reaction system for the subsequent optimization of the reaction variables.

2.3. Volume ratio effect

The reaction rate, the yield and the product e.e. were examined in different biphasic systems with the volume of the organic phase fixed (10 ml) and that of the aqueous phase varied (2–30 ml). As can be seen in Table 1, the volume ratio of the aqueous phase to

Table 1
Effect of volume ratio of aqueous to organic phase on the reaction

V_a/V_o (ml/ml)	V_0 (mM/h)	Time (h)	Yield (%)	e.e. (%)
2/10	0.79	27/56	73.4/90.0	74.9/73.7
5/10	0.71	39/56	87.1/94.1	75.3/74.5
10/10	0.66	39/56	88.2/94.9	74.3/73.7
20/10	0.60	5/56	28.0/86.7	73.3/73.3
30/10	0.55	27/39	64.3/74.2	73.8/73.1

The reaction was carried out at 30 °C by adding 1.5 g immobilized cells into the biphasic system of 10 ml Tris–HCl buffer (50 mM, pH 8.0) and 10 ml *n*-hexane containing 14 mM acetyltrimethylsilane.

the organic phase (V_a/V_o (ml/ml)) influenced the reaction rate and the product yield more strikingly than the product e.e. Generally speaking, enzymes and viable cells may be denatured and inactivated once contacting with organic solvents due to the toxicity of organic solvents. So the reaction rate and the product yield were expected to increase with the increase of V_a/V_o because of the less chance for enzymes and viable cells to contact with organic solvents. The results obtained, however, were opposite to this expectation. The reason for this is most probably that immobilization increased the cells' stability and prevented the cells from direct contacting with organic solvents. On the other hand, the lower substrate concentration in the aqueous phase due to the increase of V_a/V_o resulted in a slower reaction rate. Moreover, the change of the substrate concentration in the reaction medium might influence the stereoselectivity of the reduction catalyzed by yeast cells, because of the difference in K_m and V_{max} of the several isoenzymes present in it [13]. The ratio of 10/10 was regarded as the optimum V_a/V_o, under which a high yield (94.9%) and a high product e.e. (73.7%) were achieved after a reaction time of 56 h.

2.4. Organic solvent effect

As the reaction medium, organic solvent affects the asymmetric synthesis of (−)-TMSE by several ways: selective denaturation of the enzymes involved in this reaction; variation of the partition of the substrate and the product; alteration of the equilibrium of the reaction; inactivation of the cells due to phasic and molecular toxicity. To achieve the full potential of the immobilized cells for asymmetric

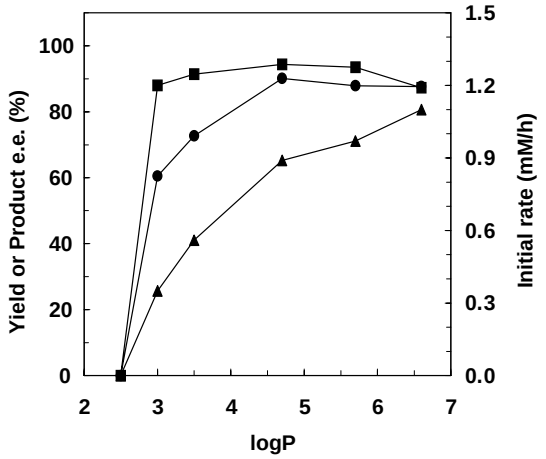

Fig. 3. Effect of organic solvent on the reaction. The reaction was performed at 30 °C and 150 rpm by adding 1.5 g immobilized cells into the biphasic system of 10 ml Tris–HCl buffer (50 mM, pH 8.0) and 10 ml organic solvent containing 14 mM acetyltrimethylsilane. *Symbols*: (■) the chemical yield; (●) the product e.e.; (▲) the initial reaction rate.

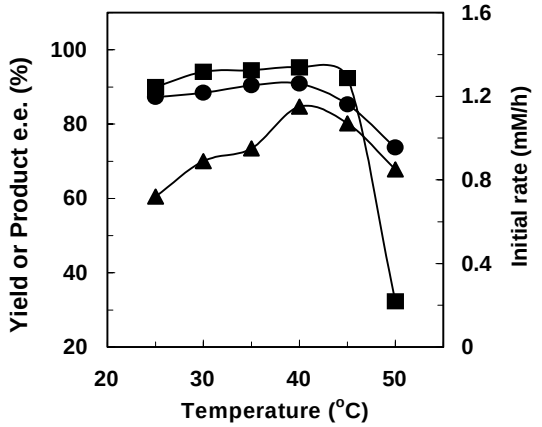

Fig. 4. Effect of reaction temperature on the reaction. The reaction was performed at various temperatures and 150 rpm by adding 1.5 g immobilized cells into the biphasic system of 10 ml Tris–HCl buffer (50 mM, pH 8.0) and 10 ml isooctane containing 14 mM acetyltrimethylsilane. *Symbols*: (■) the chemical yield; (●) the product e.e.; (▲) the initial reaction rate.

synthesis of (−)-TMSE, the effect of the hydrophobicity (expressed in log *P*) of different organic solvents on the reaction was investigated. As shown in Fig. 3, the reaction rate, the yield and the product e.e. could well be correlated with log *P* values of the organic solvents used as the organic phase. Over the range examined, the higher the log *P*, the higher the reaction rate. The product yield and the product e.e. increased with the increase of log *P* up to 4.7, above which, further increase in log *P* led to a slight fall in both the product yield and the product e.e. This could partly be explained by the higher toxicity of the solvents with lower hydrophobicity to the cells and the lower extraction ratio of the product by the solvents with higher hydrophobicity. Among the six organic solvents examined, isooctane (log *P* 4.7) was regarded as the most suitable one for the reaction in terms of the high product e.e. (90.1%) and the high product yield (94.4%).

2.5. Reaction temperature effect

It is well known that temperature has significant effect on the activity, selectivity and stability of a biocatalyst and the equilibrium of a reaction as well [14]. As can be seen in Fig. 4, an increase in temperature caused an obvious rise in the initial reaction rate and a minor increase in both the product yield and the product e.e. up to 40 °C. The initial reaction rate, the yield and the product e.e. dropped sharply when the temperature was above 40 °C, implying the inactivation of the desired reductases. Taking into account the initial reaction rate, the product yield and the product e.e., 40 °C was thought to be the optimum temperature for the reaction.

2.6. pH effect

Buffer pH influences not only enzymatic enantioselectivity and activity, but also the regeneration of the coenzymes, which in turn affects the reaction rates of isoenzymes [15]. Different isoenzymes have different optimum pH [16]. As a result, there exists an optimum pH under which the desired reductases are most active and the undesired isoenzymes show the lowest activity. Fig. 5 shows that the product e.e. was as high as 100% but both the initial reaction rate and the product yield were extremely low when the pH was 4.8, suggesting the thorough inactivation of the undesired isoenzymes and very low activity of the desired reductases. The optimal pH was 6.5 for the initial reaction rate, the product yield, the product e.e. and the cell stability.

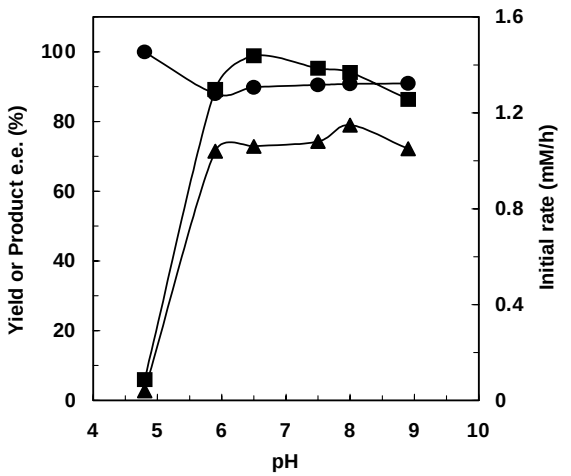

Fig. 5. Effect of pH on the reaction. The reaction was performed at 40 °C and 150 rpm by adding 1.5 g immobilized cells into the biphasic system of 10 ml 50 mM Tris–HCl buffer with various pHs and 10 ml isooctane containing 14 mM acetyltrimethylsilane. *Symbols*: (■) the chemical yield; (●) the product e.e.; (▲) the initial reaction rate.

2.7. Effect of shaking speed

Shaking speed affects the diffusion and partition of the substrate and the product in the reaction system and thus leads to changes in the initial reaction rate, the product yield and the product e.e. As shown in Table 2, the initial reaction rate increased rapidly with the increase of shaking speed when it was lower than 150 rpm, indicating that the mass transfer was the rate-limiting step. The optimal shaking speed was thought to be 150 rpm, above which little changes in the initial reaction rate, the product yield and the product e.e. were observed with further increase in shaking speed.

Table 2
Effect of shaking speed on the reaction

Shaking speed (rpm)	V_0 (mM/h)	Time (h)	Yield (%)	e.e. (%)
50	0.28	58	61.8	81.3
100	0.56	40	96.3	87.1
150	1.06	34	99.0	90.0
200	0.98	35	100.0	89.0

The reaction was carried out at 40 °C by adding 1.5 g immobilized cells into the biphasic system of 10 ml Tris–HCl buffer (50 mM, pH 6.5) and 10 ml isooctane containing 14 mM acetyltrimethylsilane.

3. Materials and methods

3.1. Biological and chemical materials

The yeast strain, *Rhodotorula* sp. AS2.2241, was from the State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology.

Medium for slant culture (SM): 1.0% glucose, 0.5% yeast extract, 0.5% peptone and 1.8% agar.

Fermentation medium (FM): 2% malt extract, 2% glucose, 1% peptone and 0.5% yeast extract.

ATMS, (±)-TMSE and *n*-nonane were purchased from Aldrich (Milwaukee, USA). All other chemicals were also from commercial sources and of analytical grade.

3.2. Immobilization

After cultivation for 20 h at 30 °C and 150 rpm, *Rhodotorula* sp. AS2.2241 cells were harvested by centrifugation (3500 rpm, 10 min), washed twice with deionized water and separated from the aqueous medium by centrifugation to give a cell wet mass (cwm) of 2–5 g per 200 ml. A 'homogenous' cell/sodium alginate suspension was prepared at 25 °C by mixing 10 g of a fresh cell suspension (5 g wet cells + 5 ml water) with 30 g of aqueous sodium alginate (2% (w/v)) solution (which was prepared by dissolving sodium alginate in deionized water, heating and stirring vigorously). The suspension was added dropwise through an injector to a gently stirred $CaCl_2$ solution (2%, aqueous) where the pearls of calcium alginate with a load of *Rhodotorula* sp. AS2.2241 cells precipitated. The pinhole size of the injector and the dropping rate were adjusted in such a way that the diameter of the pearls was around 1 mm. The pearls were kept in the $CaCl_2$ solution for another 1 h at 25 °C and collected by filtration, washed with water, re-suspended in an aqueous solution containing 20% glucose, 0.9% NaCl, and 0.05% $CaCl_2$ and stored at 4 °C for later use.

3.3. Asymmetric reduction with free cells or immobilized cells in an aqueous phase

To a 50 ml Erlenmeyer shaking-flask capped with a septum, was added 10 ml of a suspension of 0.375 g freshly harvested cells or 1.5 g immobilized pearls

containing 0.375 g free cells in Tris–HCl buffer (50 mM, pH 8.0) containing 20% glucose. After pre-incubated in a water-bath shaker at 30 °C and 150 rpm for 15 min, 0.14 mmol of ATMS was added and the incubation was continued. Samples were taken periodically and centrifuged (3500 rpm, 15 min) to remove the cells. The supernatant (500 μl) was mixed with 10 μl of *n*-nonane as the internal standard and extracted twice with 500 μl each of ethyl acetate which was subjected to GC analysis.

3.4. Asymmetric reduction in an aqueous/organic solvent biphase

The mixture of 10 ml organic solvent containing 10 μl *n*-nonane and a certain volume of 50 mM Tris–HCl buffer at a certain pH containing 1.5 g immobilized cells, 20% glucose and 0.05% $CaCl_2$ was pre-incubated in a 50 ml Erlenmeyer shaking-flask capped with a septum for 15 min at selected temperature and shaking speed. 0.14 mM ATMS was added and the incubation was continued. One hundred microliter of samples were periodically withdrawn from the organic phase with a syringe for GC analysis (above 99% ATMS and TMSE existed in the organic phase). Details about the organic solvent, the volume of Tris–HCl buffer, pH, temperature, shaking speed and substrate concentration were specified for each case. When the reaction was terminated, 5 ml of sample was taken from the organic phase and diluted to 20 ml with the same organic solvent to measure the optical rotation of the product TMSE on a Perkin-Elmer 241 polarimeter. For the study of organic solvent effect on the reaction, methylbenzene ($\log P$ 2.5), cyclohexane ($\log P$ 3.0), *n*-hexane ($\log P$ 3.5), isooctane ($\log P$ 4.7), *n*-decane ($\log P$ 5.7) and *n*-dodecane ($\log P$ 6.6) were employed.

3.5. GC analysis

GC analysis was performed with a HP4890D gas chromatography using a flame ionization detector and a chiral column (20% permethylated β-cyclodextrin 30 m × 0.32 mm, HP, USA). The column temperature was programmed as being kept at 64 °C for 5.7 min and then upgraded to 78 °C at a rate of 5 °C/min. Nitrogen was used as the carrier gas at 2 ml/min. Split ratio was 1:100 (v/v). The injector and the detector temperatures were both set at 250 °C. The retention times for ATMS, *n*-nonane, (+)-TMSE and (−)-TMSE were 3.37, 5.20, 5.42 and 5.75 min, respectively.

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