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Effect of phase composition on the bioconversion of methyltestosterone in a biphasic system☆

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Abstract

The bioconversion of methyltestosterone to methandienone by *Arthrobacter simplex* AS.1.94* was selected as model system for evaluating the effect of solvent nature and aqueous phase composition on the biocatalytic efficiency in aqueous–organic two-phase system. A series of organic solvents with varying $\log P_{oct}$ values, together with five different aqueous media, were selected as organic and aqueous phase. Tween-80 and menadione were added as surfactant and an external electron acceptor, respectively. The effect of volumetric phase ratio on biocatalytic activity was also investigated. The results showed that selection of organic phase must take a series of factors into account. The composition of the aqueous phase markedly influenced biocatalytic yields, particularly the addition of the menadione. In the system with carbon tetrachloride as the organic phase and phosphate buffer as aqueous phase, the volumetric phase ratio of 3_{org}:7_{aq} was feasible.

Keywords: *Arthrobacter simplex*; Methyltestosterone; Dehydrogenation; Biphasic system

1. Introduction

The dehydrogenation of methyltestosterone by *Arthrobacter simplex* AS.1.94* to produce methandienone, which is an important anabolic steroids [1], is of considerable commercial interest, as compared to the use of chemical synthesis dehydrogenation [2,3].

The methyltestosterone and other steroids involved in this bioconversion have a marked hydrophobic nature with low-water solubilities, which limits process productivity [4,5]. One of the technical solutions to this problem could be the application of two liquid-phase reaction system with substrate-solubilizing organic solvents [6]. The organic phase reservoir permits the storage of high concentration of substrates or products. Furthermore, this approach can realize in situ removal of the products, thus improving overall process productivity [7].

However, the disadvantage of organic–aqueous biphasic systems is the effect of solvent toxicity on the activity and stability of biocatalyst [8]. In whole-cell systems, the solvent might be incorporated within membrane lipids, which results in disruption of membrane functions, inactivation or denaturation of membrane bound enzymes, collapse of transport mechanisms, and even cell lysis [7,9]. Therefore, one of the key factors in the implementation of an effective aqueous–organic bioconversion is the selection of a proper solvent, which has to be biocompatible, to provide an adequate substrate pool and product sink, and to exhibit the required mass transfer characteristics. An empirical rule correlating the toxicity of the organic solvent with its $\log P_{oct}$ values is in current use, the $\log P_{oct}$ values being considered a measure of solvent hydrophobicity [10–13]. However, this correlation is not completely understood and does not hold true for all the organic solvents.

The present work used the dehydrogenation of methyltestosterone by *Arthroacter simplex* AS.1.94* (Fig. 1) as a model bioconversion to evaluate phase composition effects on dehydrogenation of steroid in biphasic systems with varying aqueous and organic phase components. During the experiment, some parameters being related to the phase composition effects were determined, such as solubility of steroid substrate, the saccharide metabolic activity retention (R, %) of *Arthrobacter simplex* AS.1.94* cells. The influence of surfactants, external electron acceptor and volumetric phase ratio in biphasic systems on the bioconversion rate were also examined.

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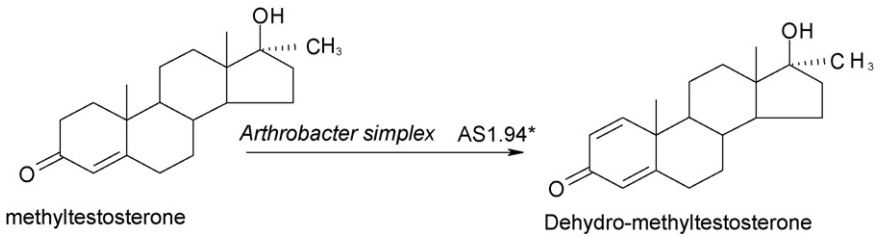

Fig. 1. C1,2-dehydrogenation of methyltestosterone by *Arthrobacter simplex* AS.1.94*.

2. Experimental

2.1. Chemicals

Methyltestosterone, methandienone and testosterone were supplied by Tianjin Pharmaceutical Company (China). Menadione was purchased from Sigma–Aldrich Co. Other salts and solvents were of analytic grade from Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (China).

2.2. Cell growth

Arthrobacter simplex AS.1.94* cells were cultivated in a 5 l fermentor (B. Braum Biotch., Germany) containing 3.5 l of growth medium (10 g/l glucose, 12 g/l corn steep liquor, 2.5 g/l yeast extract and 2.5 g/l KH_2PO_4). Temperature was maintained at 32 °C, pH at 7.0. The medium was agitated at 200 rpm and aerated at 1.5 l/min. The inoculum was grown in 1 l shake flasks containing 200 ml of the growth medium and cultivated for around 22 h under the same conditions. The steroid C1,2-dehydrogenase was induced with 0.3 g/l methyltestosterone about 8 h after inoculation. The culture was grown until an OD_{640} of 3.5 was reached after about 24 h incubation. Cells were collected by centrifugation at 9000 rpm and 10 °C, washed twice with 0.05 mol/l phosphate buffer (pH 7.0). The wet cells, approximately 150 mg dry cells/g were stored at −20 °C and thawed at 4 °C prior to use.

2.3. Solubility test

Known amount of methyltestosterone was shaken with 1.5 ml of the water-saturated solvent for at least 24 h at 33 °C. Undissolved steroid was sedimented by centrifugation and the clear supernatant was evaporated overnight at 80 °C and the residue redissolved in methanol. The samples were analyzed for steroid contents by high-pressure liquid chromatography (HPLC).

2.4. Saccharide metabolic activity retention (R, %) assay

The saccharide metabolic activity retention (*R*, %) assays were performed in the systems containing 7 ml of phosphate buffer, 3 ml of the organic solvent, and 5 g wet cell paste per litre. The liquid was incubated in a flask on a rotary shaker at 200 rpm and 33 °C for 4 h. Cells of the same concentration in 10 ml phosphate buffer were also incubated for comparison. After incubation for 4 h, *Arthrobacter simplex* AS.1.94* cells were collected by centrifugation, washed twice with phosphate buffer, while 10 ml of glucose solution (10 g/l) was added and incubated in the same conditions for 8 h. Samples (1 ml) removed from the flasks were performed as described by [14] to detect the concentration of glucose in samples. So, the consumption of glucose and the saccharide metabolic activity retention (*R*, %) were obtained by calculation. The saccharide metabolic activity retention (*R*, %) was considered to predict the tolerance of *Arthrobacter simplex* AS.1.94* cells to the solvent:

$$R\ (\%) = \frac{\text{consumption of glucose for the sample}}{\text{consumption of glucose for the comparison}} \times 100$$

2.5. Biotransformation

Two-phase bioconversion system containing 6 ml organic phase, 14 ml phosphate buffer, *Arthrobacter simplex* AS 1.94* cells of 5 g/l, methyltestosterone of 10 g/l and menadione of 0.6 mmol/l was incubated in orbitally agitated flasks at 200 rpm and 33 °C. Triplicate runs were carried out.

2.6. Steroid analysis

Samples (1 ml) removed from the shake flasks were centrifuged in Eppendorf centrifuge for separation of organic and aqueous phase. The carbon tetrachloride layer was drawn off using a glass syringe with a long needle and evaporated to dryness at about 80 °C. The residue was dissolved in methanol giving methyltestosterone and methandienone concentrations below 0.5 g/l. The sample solution was filtered through a 0.45 μm membrane filter. Samples (10 μl) were run through a column (Kromasil C18 5 μm, SSI) employing methanol (75%) and diluted water (25%) as mobile phase at a rate of 1 ml/min. Samples were detected at 241 nm and testosterone (0.4 g/l in methanol) was used as internal standard.

3. Result and discussion

3.1. Screening of solvents

The feasibility of biphasic bioconversion systems depends both on the organic phase toxicity and on its ability for acting as a reservoir of the substrate and product [8,15]. The series of solvents chosen for these experiments covered a wide range of $\log P_{oct}$ values (Table 1) [3,5,16], with special emphasis on the

Table 1
log P_{oct}, substrate solubilities, bioconversion rate, saccharide metabolic activity retention (R, %) screening for different organic solvents

Organic solvent	log P_{oct}[a]	Solubility of methyltestosterone (g/l)	R (%)	Bioconversion rate (%)
Ethyl acetate	0.68	61	6.7	6.5
Diethyl ether	0.85	69	8.0	5.2
Dichloromethane	1.5	64	7.0	20
Butyl acetate	1.7	68	5.0	22
Benzene	2.0	60	8.7	49
Chloroform	2.2	53	10	55
Toluene	2.9	46	50	74
Carbon tetrachloride	3.0	45	55	83
Hexane	3.5	0.48	60	31
Heptane	4.0	0.25	68	35
Octane	4.5	0.17	78	40
Dibutyl phthalate	5.4	0.35	80	46
Decane	5.6	0.086	76	46
Dodecane	6.6	0.064	78	50
Hexadecane	8.8	0.015	78	56

[a] log P_{oct} values came from Refs. [3,5,16].

more hydrophobic solvent which is often assumed to be biocompatible [10,17]. To test whether the log P_{oct} values could be used to predict organic phase feasibility for the bioconversion of methyltestosterone in this biphasic system, the dates of log P_{oct} values, substrate solubilities, saccharide metabolic activity retention (R, %) and bioconversion rate were described in Fig. 2.

It can be observed that the solubility of methyltestosterone tends to decrease with increasing log P_{oct} values of solvent, while the variation of the saccharide metabolic activity retention (R, %) with the solvent log P_{oct} values shows a "S" curve and the bioconversion rate exhibits a maximum at the solvent log P_{oct} values around 3. From this experiment dates, the more hydrophobic solvent with the higher log P_{oct} values is, the more tolerant the *Arthrobacter simplex* AS.1.94* cell could be. However, the bioconversion rate revealed no apparent correlation with the solvent hydrophobicity (log P_{oct} values). The reason could be supposed that the solubility of methyltestosterone was less in the more hydrophobic solvent with the higher log P_{oct} value, and the substrate reservoir effect of these solvents could not be realized. A similar result was obtained by Cruz et al. in whole-cell bioconversion of β-sitosterol in biphasic media [8]. Taking into account the bioconversion rate, the substrate solubility, the flammability and toxicity of the organic solvents, carbon tetrachloride was chosen as the optimum solvent for further studies.

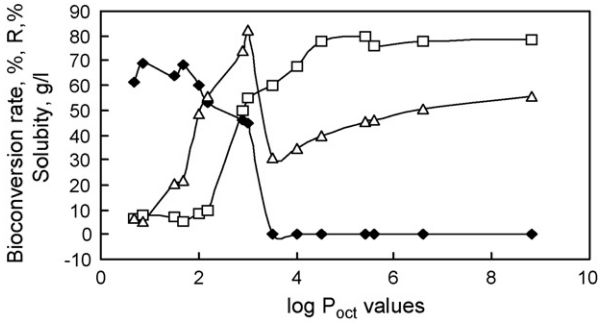

Fig. 2. Correlation between solubility of methyltestosterone (♦), saccharide metabolic activity retention (R, %) (□), bioconversion rate (△) and log P_{oct} values of different solvents.

3.2. Composition of the aqueous phase

The aqueous phase composition could be defined so as to provide the requirements for the prolonged maintenance of cell activity, while interacting suitably with the organic phase in terms of mass transfer and partition of the relevant solutes [7]. Considering this idea, parallel runs were carried out in aqueous-carbon tetrachloride systems, in which aqueous phase was sterile water (A1), phosphate buffer pH 7.0 (A2), culture (A3), Tris–HCl buffer pH 7.0 (A4), and growth medium (A5) (Fig. 3).

As can be observed, the bioconversion rates after 24 h using culture and growth medium as aqueous phase have an apparently less yield than the others, while using sterile water, phosphate buffer pH 7.0 and Tris–HCl buffer pH 7.0 as aqueous phase, the results showed there was no significant difference in the bioconversion rates. In the discussion of aqueous phase, however, Cruz et al. suggested that the complex aqueous media gave higher or

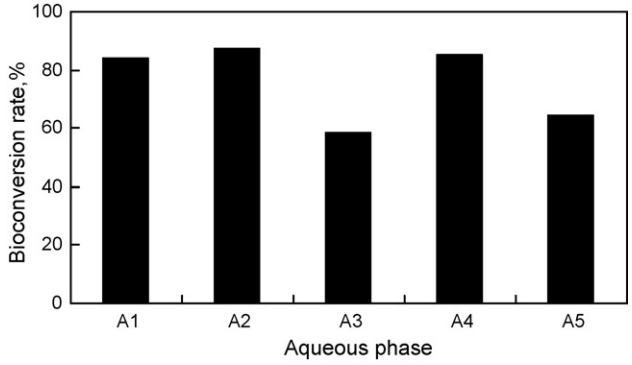

Fig. 3. Effect of different aqueous phase on the bioconversion of methyltestosterone by *Arthrobacter simplex* AS.1.94* in aqueous-carbon tetrachloride systems, in which aqueous phase was sterile water (A1), phosphate buffer pH 7.0 (A2), culture (A3), Tris–HCl buffer pH 7.0 (A4), and growth medium (A5).

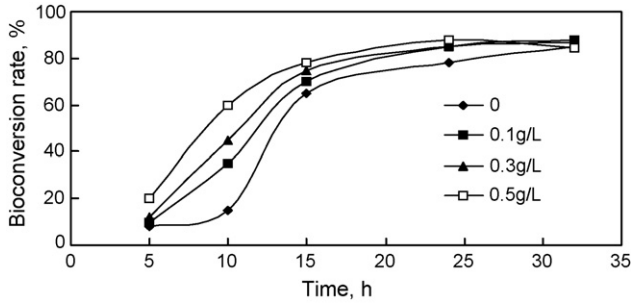

Fig. 4. Effect of surfactant concentration on the bioconversion of methyltestosterone. Tween-80 concentrations used were 0, 0.1, 0.3, 0.5 g/l. *Arthrobacter simplex* AS 1.94* cells of 5 g/l were shaken (180 rpm, 33 °C) in a biphasic system containing 6 ml carbon tetrachloride, 14 ml phosphate buffer, 10 g/l methyltestosterone and 0.6 mmol/l menadione.

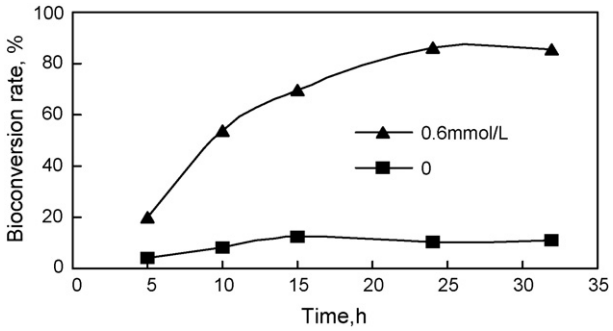

Fig. 5. Effect of menadione on the bioconversion of methyltestosterone. *Arthrobacter simplex* AS.1.94* cells of 5 g/l were shaken (180 rpm, 33 °C) in a biphasic system containing 6 ml carbon tetrachloride, 14 ml phosphate buffer and 10 g/l methyltestosterone.

comparable yield values [8]. Fig. 3 did not confirm the prediction of Cruz et al. [8]. This feature could presumably be ascribed to substrate mass transfer limitations or/and nutrients competing with the methyltestosterone, which decreased the biocatalysis activity of *Arthrobacter simplex* AS.1.94*.

3.3. Effects of surfactant and menadione in the biphasic system on bioconversion

Effect of surfactant concentration on the bioconversion of methyltestosterone in the biphasic system was further investigated. Results of the experiments were shown in Fig. 4. The conversion rates after 10 h varied between 15 and 58% for batches with 0 and 0.5 g/l of Tween-80 added, respectively. After 24 h, a conversion of around 85% was obtained for all four batches.

Bioconversion yield values after 24 h of reaction were minimally affected by the presence of surfactant in the aqueous phase, even though the initial activity was high. A similar result was obtained by Fernandes et al. for the C1,2-dehydrogenation of 6α-methyl-hydrocortisone-21-acetate in an organic–aqueous two-liquid-phase system [18]. On the other hand, the use of a biocompatible surfactant in the aqueous phase was apparently found to prevent *Arthrobacter simplex* AS.1.94* cells aggregation. In spite of that, the addition of surfactant to the aqueous phase was not necessary for the bioconversion of methyltestosterone in the biphasic system. As previous studies on the bioconversion of methyltestosterone by *Arthrobacter simplex* AS.1.94* cells in the biphasic system showed that the biocatalysis reaction occurred at the organic/aqueous interphase, the methyltestosterone could be available to *Arthrobacter simplex* AS.1.94* cells directly from the organic phase [2]. The transfer of substrate through miscellization was not only no beneficial, but also could decrease the substrate availability to the *Arthrobacter simplex* AS.1.94* cells.

In the biphasic system, the presence of organic solvent deactivate the cell's cofactor regenerating system [1], hence the use of external electron acceptor was necessary. Fig. 5 also showed that without addition of menadione, a conversion of around 20% after 24 h was reached and addition of more menadione leaded to higher initial rate and higher final conversions rate after 24 h, as compared to the yields in similar systems without menadione. Silbiger and Freeman [19] used menadione bisulfite in concentrations equimolar to hydrocortisone as an artificial electron acceptor. High conversion of around 90% was obtained at steroid concentrations of 1.2 g/l.

In the biphasic system presented here, menadione was used as an artificial electron acceptor. Indeed, an effective increase in the dehydro-methyltestosterone formation rate was obtained when the menadione concentration in the aqueous phase was increased from 0 to 0.6 mmol/l. At the concentration of 0.6 mmol/l, the dehydro-methyltestosterone formation rate exhibited a maximum (85%) (Fig. 6). With increasing the menadione concentration in excess of 0.6 mmol/l, a steep decrease in catalytic rate was observed, which could be attributed to excessive menadione concentration making the *Arthrobacter simplex* AS.1.94* cells' unstability.

3.4. Effect of volumetric phase ratio on bioconversion

A set of experiments was carried out to evaluate the effect of different volumetric phase ratio on the dehydrogenation of methyltestosterone using *Arthrobacter simplex* AS.1.94* cells in the biphasic system (Fig. 7). The bioconversion yields achieved were significantly higher in this systems with a volumetric phase ratio of 3_{org}:7_{aq} as compared to others. From the results obtained in Fig. 6, it was evident that the phase ratio had a marked effect

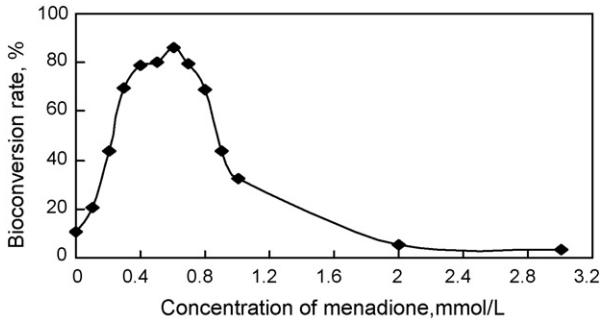

Fig. 6. Effect of menadione concentration on the bioconversion of methyltestosterone. *Arthrobacter simplex* AS 1.94* cells of 5 g/l were shaken (180 rpm, 33 °C) in a biphasic system containing 6 ml carbon tetrachloride, 14 ml phosphate buffer and 10 g/l methyltestosterone.

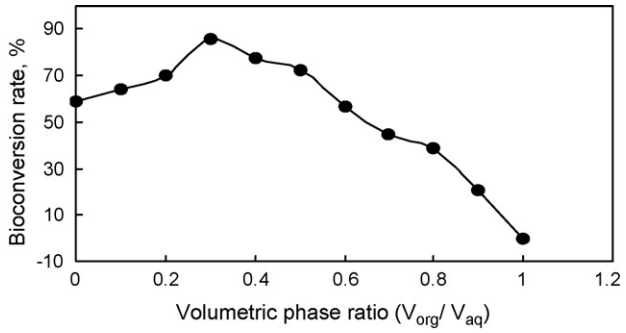

Fig. 7. Effect of volumetric phase ratio on the bioconversion of methyltestosterone. *Arthrobacter simplex* AS.1.94* cells of 5 and 10 g/l methyltestosterone and 0.6 mmol/l menadione were shaken (180 rpm, 33 °C) in the carbon tetrachloride-phosphate buffer two-phase system.

on the biocatalytic rate. A similar result was obtained by Cruz et al. [9] for the whole-cell bioconversion of β-sitosterol in aqueous–organic two-phase systems.

4. Conclusions

The C1,2-dehydrogenation of methyltestosterone to methandienone by *Arthrobacter simplex* AS.1.94* cells was achieved in high yield, with aqueous–organic systems using carbon tetrachloride (30%, v/v) as organic phase and phosphate buffer pH 7.0 (70%, v/v) as aqueous phase containing 0.6 mmol/l menadione as external electron acceptor. For screening organic phase, a series of factors must take into account, namely biocompatibility indicator parameters like $\log P_{oct}$ values, and solubility of methyltestosterone in the solvent, and flammability, volatility and toxicity to people. The composition of aqueous phase in the tested bioconversion systems markedly influenced biocatalytic yields. For the biphasic system, the use of external electron acceptor was necessary, however, the presence of a surfactant had no significant effect on the final conversion yield values. In this tested biphasic system, the volumetric phase ratio of 3_{org}:7_{aq} was feasible.

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