J. Microbiol. Biotechnol. (2016), 26(1), 72–79
http://dx.doi.org/10.4014/jmb.1506.06043

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Asymmetric Bioconversion of Acetophenone in Nano-Sized Emulsion Using *Rhizopus oryzae*

Qingzhi Li[1]*, Yang Shi[1], Le He[1], and Hui Zhao[2]

[1]*College of Life Science and Agronomy, Zhoukou Normal University, Zhoukou, Henan 466001, P.R. China*
[2]*College of Chemistry and Chemical Engineering, Zhoukou Normal University, Zhoukou, Henan 466001, P.R. China*

Received: June 17, 2015
Revised: September 17, 2015
Accepted: September 24, 2015

First published online
September 25, 2015

**Corresponding author*
Phone: +86-3948178261;
Fax: +86-3948319565;
E-mail: qingzhili0011@163.com

pISSN 1017-7825, eISSN 1738-8872

The fungal morphologies and pellet sizes were controlled in acetophenone reduction by *Rhizopus oryzae*. The acetophenone conversion and (*S*)-phenylethanol enantiomeric excesses (e.e.) reached the peak after 72 h of incubation when using pellets with 0.54 mm diameter, which showed an excellent performance compared with suspended mycelia, clumps, and pellets with 0.65 or 0.75 mm diameter. Furthermore, nano-sized acetophenone was used as a substrate to improve the performances of biotransformation work. The results showed that the conversion of nanometric acetophenone and (*S*)-phenylethanol e.e. reached the maximum (both >99%) after 32 h of incubation when using 0.54 mm diameter pellets, at least 24 h in advance of the control group. On the other hand, Tween 80 and 1, 2-propylene glycol showed low or no toxicity to cells. In conclusion, pellets and acetophenone nanoemulsions synergistically result in superior performances of acetophenone reduction.

Keywords: Acetophenone, bioreduction, conversion, enantiomeric excesses (e.e.), nanoemulsion, *Rhizopus oryzae*

Introduction

The chemical techniques used in the reduction of acetophenone are harmful to the environment, since acetophenone is a frequent organic pollutant in industry waste effluent [2]. Therefore, biotransformation, a key technology for the synthesis of fine chemicals and fuel materials in recent years, is becoming more and more significant from the viewpoint of green chemistry [7]. Biotransformation is a convenient technique for preparing chiral organic chemicals. The use of whole microbial cells is particularly beneficial to carry out the reduction, since the procedures do not require addition of cofactors for their regeneration [21]. However, the toxicity of aromatic ketones and aromatic alcohols influences the performance of the asymmetric reduction of the prochiral aromatic ketones [19].

Nanoemulsions, also referred to in the literature as mini-emulsions, emulsoids, unstable microemulsions, *etc*., are kinetically stable multiphase colloids with a droplet size in the nanometric scale, typically ranging from 50 to 500 nm [8]. Nanoemulsions can be prepared simply by blending oil, water, surfactant, and cosurfactant in the proper proportions, with mild agitation. Because nanoemulsification is a spontaneous process, the order of mixing the components is generally considered not to be crucial [12]. Nanoemulsions are widely used in the field of foods, beverages, and pharmaceuticals [1, 12, 13], since the nano-sized droplets lead to an enormous increase in interfacial areas [7], a more kinetically stable and optically transparent system [8], and higher solubility and bioavailability [7]. However, few studies have introduced nanoemulsions in the enantioselective bioreduction of acetophenone.

Herein, we studied the performance of acetophenone bioreduction by *Rhizopus oryzae* (Fig. 1A) using three fungal morphologies and three different sizes of pellets. Besides this, we observed the performances of the bioreduction work using nano-sized acetophenone as a substrate. The introduction of nanoemulsions provides a new method in acetophenone bioreduction.

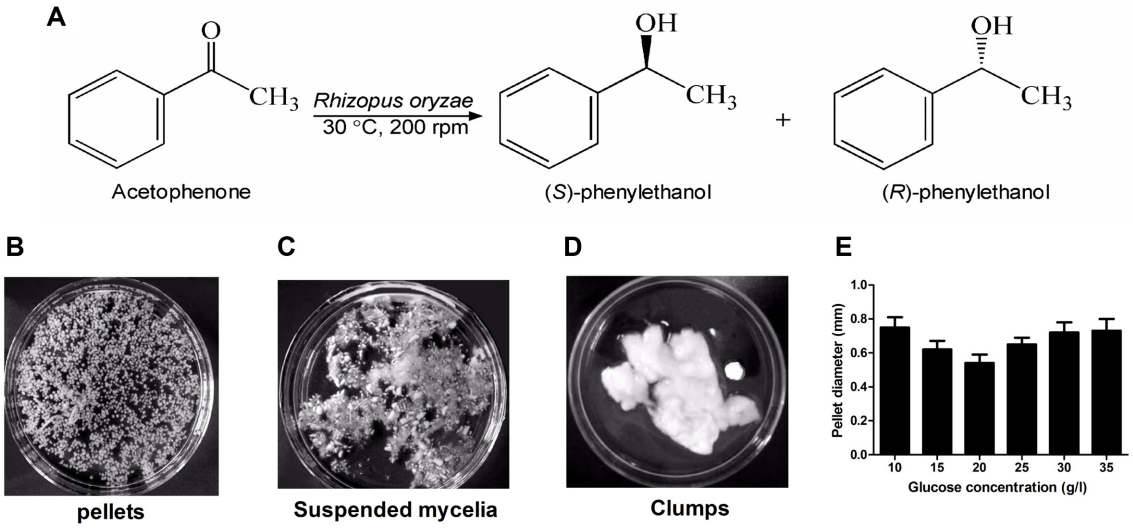

Fig. 1. *Rhizopus oryzae* was used for acetophenone reduction.
(**A**) Asymmetric reduction of acetophenone to (*S*)-phenylethanol and (*R*)-phenylethanol by *Rhizopus oryzae*. (**B**) Pellets were obtained when controlling the initial pH of the seed medium. (**C**) Suspended mycelia. (**D**) Clumps. (**E**) Glucose concentration affected the size of pellets when the pre-culture condition was set as follows: 1% (v/v) spore suspension, 250 ml shake flask containing 100 ml of seed medium, 30°C, 150 rpm agitation speed, initial pH 3.0, and 24 h of incubation. $n = 5$.

Materials and Methods

Reagents, Microorganism, and Culture Medium

The main reagents used in this study are as follows: acetophenone (analytical reagent, AR; Shanghai Chemical Reagents Corp., Shanghai, China), 1,2-propylene glycol (AR; Shanghai Chemical Reagents Corp.), Tween 80 (chemically pure reagent; Beijing Chemicals Company, Beijing, China), disodium hydrogen phosphate (AR; Beijing Chemicals Company), and sodium dihydrogen phosphate (AR; Beijing Chemicals Company). *Rhizopus oryzae* (ATCC 20344) was procured from the American Type Culture Collection (Rockville, MD, USA). The fungus was maintained on potato dextrose agar at 30°C for 7 days, and then stored at 4°C [5]. Seed medium included (g/l) urea 1.5, KH_2PO_4 0.6, $MgSO_4{\cdot}7H_2O$ 0.6, $ZnSO_4{\cdot}7H_2O$ 0.015, $FeSO_4{\cdot}7H_2O$ 0.0005, and five different concentrations of glucose (10, 15, 20, 25, and 30). Biotransformation medium contained (g/l) glucose 40, urea 0.05, $MgSO_4{\cdot}7H_2O$ 0.6, $ZnSO_4{\cdot}7H_2O$ 0.015, and $FeSO_4{\cdot}7H_2O$ 0.0005.

Preparation of Pellets with Different Sizes

The fungi were washed with deionized water and stirred with glass beads for 15 min to obtain the spore inoculum. A hemacytometer was used to count the number of spores under a microscope. The spore concentration was controlled to 1×10^6 spores/ml. Spores were inoculated at 1% (v/v) into a 250 ml shake flask containing 100 ml of seed medium (pH 3.0) and incubated at 30°C, 150 rpm for 24 h to obtain fungi with pellet morphology. Five different concentrations of glucose (10, 15, 20, 25, 30, and 35 g/l) in seed medium resulted in different pellet diameters.

Preparation of Acetophenone Nanoemulsions

Tween 80 and 1,2-propylene glycol were added to 100 ml of phosphate buffer (0.2 mol/l, pH 7.0). After dissolution, acetophenone was added dropwise. The mixed liquor was stirred with a magnetic stirring apparatus under 900 rpm for 4 h followed by detecting the size of acetophenone using a laser particle size analyzer (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The stabilization of 100 ml of acetophenone nanoemulsions in a 250 ml shake flask at 30°C, 200 rpm was evaluated by detecting the size of acetophenone in such a dynamic environment, and the stabilization in a static environment (without magnetic stirring) was also evaluated using the same method.

Biotransformation of Acetophenone

To assess the effects of pellets (0.75, 0.65, and 0.54 mm), suspended mycelia, and clumps on the performance of acetophenone bioreduction, the culture medium was prepared in phosphate buffer (0.2 mol/l, pH 7.0). Briefly, 50 ml of culture medium was filtered with a millipore filter and 1.5 ml of acetophenone was added dropwise. After the culture medium was added to a 250 ml shake flask under aseptic conditions, 10% (v/v) pellets or suspended mycelia, or 0.065 g of clumps was transferred into the shake flask and cultured at 30°C, 200 rpm for 120 h. Broth samples were collected every 24 h for analyzing the concentration of acetophenone, phenylethanol, and residual glucose. At the end of

incubation, the biomass was measured.

To assess the effects of pellets with 0.54 mm diameter on the performance of nano-sized acetophenone bioreduction, the culture medium was prepared in acetophenone nanoemulsions. After being filtered with a millipore filter, 50 ml of culture medium containing acetophenone nanoemulsions was added to a 250 ml shake flask under aseptic conditions. Next, 10% (v/v) pellets with 0.54 mm diameter were transferred into the shake flask, and cultured at 30°C, 200 rpm for 56 h. Meanwhile, 50 ml of culture medium containing acetophenone, Tween 80, and propylene glycol (without magnetic stirring) was used as a control. Broth samples were collected every 8 h for analyzing the concentration of acetophenone, phenylethanol, and residual glucose. At the end of incubation, the biomass was measured.

Analytical Methods

A gas chromatograph system (Shimadzu, Tokyo, Japan) with a DB-5 column (30 m × 0.25 mm nominal diameter × 0.25 μm film thickness) equipped with a flame ionization detector was used to determined the concentrations of acetophenone and phenylethanol. The conversion = $(C_0 - C)/C_0 \times 100\%$; (*S*)-phenylethanol enantiomeric excess (e.e.) = $(Cs - C_R)/(Cs + C_R) \times 100\%$. Here, C_0, C, C_S, and C_R represent the initial substrate concentration, the substrate concentration, the (*S*)-phenylethanol concentration, and the (*R*)-phenylethanol concentration, respectively.

The concentration of residual glucose was measured by using a biosensor (SBA-40C; Shandong Academy of Sciences, Jinan, China).

To calculate the biomass, the mycelia were washed twice with distilled water and the dried until constant weight at 95°C in order to achieve dry cell weight.

We quantified the pellet diameter (the average size of pellets) using a manual image analysis consisting of a camera, a microphotograph, and a PC with a frame grabber [22].

Droplet size distributions were analyzed using a laser particle size analyzer (Malvern Matersizer 2000; Malvern Instruments Ltd).

The values from the study were expressed as the mean ± SEM and analyzed by using SPSS ver. 16.0 (IBM, China). A P value < 0.05 was considered statistically significant.

Results

Effects of Pre-Culture Condition and Seed Medium on Fungal Morphology and Pellet Size

When incubated in a stirred shake flask, *Rhizopus oryzae* tends to grow in three fungal morphologies: clumps, suspended mycelia, and pellets [20]. To obtain these fungal morphologies, the pre-culture condition was assessed. Our preliminary study indicated that when the pre-culture condition was set as 1% (v/v) spore suspension, 250 ml shake flask containing 100 ml of seed medium, 30°C, 150 rpm agitation speed, and 24 h of incubation (data not shown), the fungal morphologies were dependant on the initial pH of the seed medium. Here, we used different initial pH of seed medium to control the sizes of pellets. As shown in Fig. 1B, initial pH 3.0 and 5.0 led to the formation of pellets and suspended mycelia, respectively, and initial pH 7.0 and 9.0 led to clump formation. It has been reported that pellet size plays a critical role in the production of organic acid by *Rhizopus delemar* [22]. Thus, we speculated that the pellet size could affect acetophenone reduction. As shown in Fig. 1C, different initial glucose concentrations had direct impacts on the pellet diameter. The pellet diameter altered in the range from 0.54 to 0.75 mm, when the glucose concentration was increased from 10 to 35 g/l.

Effects of Fungal Morphology and Pellet Size on the Performance of Acetophenone Reduction

We observed the effects of three diameters of pellets (0.75, 0.65, and 0.54 mm), suspended mycelia, and clumps on the performance of acetophenone bioreduction. The results showed that pellets had a better performance of reduction than suspended mycelia, but clumps were inferior to pellets and suspended mycelia. Moreover, the smaller pellets displayed excellent performance compared with the bigger ones. The pellets with 0.54 mm diameter reached the maximum conversion (>80%) after 72 h of incubation, but the pellets with 0.65 and 0.75 mm diameters needed more incubation time to reach the peak, along with poor conversion of less than 70%. Suspended mycelia and clumps did not achieve the maximum conversion in at least 120 h of incubation (Fig. 2A). The e.e. values of the (*S*)-phenylethanol were not statistically significant when pellets were used. However, suspended mycelia and clumps had lower e.e. values of the (*S*)-phenylethanol, showing a gradual descent with longer incubation time (Fig. 2B). The pellets had a faster rate of glucose consumption than suspended mycelia and clumps. The pellets with 54 mm diameter ran out of the glucose after 96 h of incubation, but the two bigger ones needed an extra 24 h (Fig. 2C). The initial biomass of all groups was 0.065 g. The pellets grew during the incubation period, and the biomass of suspended mycelia seemed unchanged. Nevertheless, the biomass of clumps decreased significantly (Fig. 2D).

Preparation and Assessment of Acetophenone Nanoemulsions

We optimized the fungal morphology and pellet size to get an excellent performance of acetophenone bioreduction. However, the bioreduction parameters were unsatisfactory. Although the asymmetric bioreduction of acetophenone by *Rhizopus oryzae* is one of the most promising methods, the acetophenone and phenylethanol are noxious to the cells.

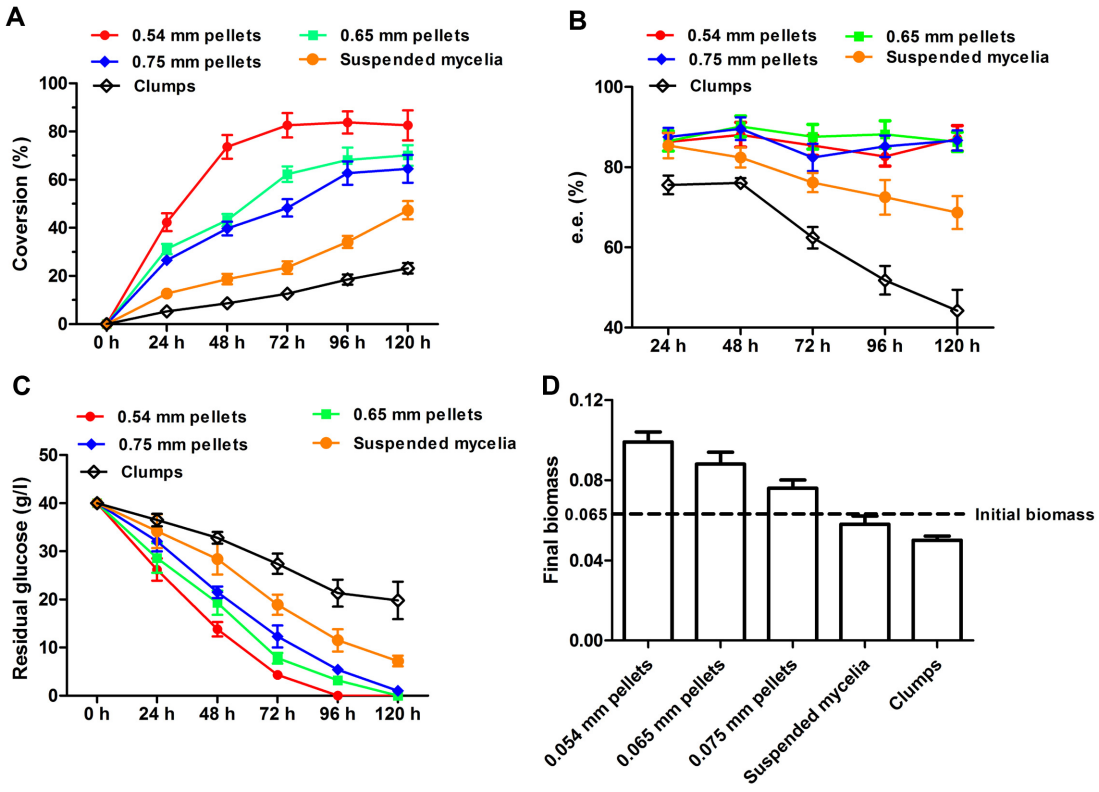

Fig. 2. The performances of acetophenone reduction when using pellets, suspended mycelia, and clumps.
(**A**) The maximum conversion (82%) was obtained at 72 h when using pellets with 0.54 mm diameter. Pellets with 0.65 and 0.75 mm diameter, suspended mycelia, and clumps needed more incubation time to reach their maximum conversion. $n = 5$. (**B**) (*S*)-Phenylethanol enantiomeric excesses (e.e.) reached about 85% when using pellets during the whole incubation periods. Suspended mycelia and clumps showed declined e.e. values. $n = 5$. (**C**) Pellets with 0.54 mm diameter consumed glucose faster than pellets with 0.65 and 0.75 diameters, suspended mycelia, and clumps, successively. $n = 5$. (**D**) More final biomass was obtained when using bigger pellets. However, the final biomass of suspended mycelia and clumps was decreased compared with the initial biomass. $n = 5$.

In consideration of the advantages of nanoemulsions, we prepared acetophenone nanoemulsions for the bioreduction work. Acetophenone nanoemulsions were prepared using the spontaneous emulsification method. As shown in Fig. 3A, 0.25 and 0.50 g of Tween 80 (in 100 ml of phosphate buffer) led to milky white emulsions; nearly transparent nanoemulsions were observed when using 1.0 g of Tween 80; stable transparent nanoemulsions were gained when using 1.5 and 2.0 g of Tween 80. Similarly, 1,2-propylene glycol and substrate acetophenone affected the formation of nanoemulsions in the same manner (Figs. 3B and 3C). The intensity and number distributions were measured using a laser particle analyzer. As shown in Figs. 3D and 3E, the intensity of nano-sized acetophenone distributed symmetrically, with the peak intensity at 70 nm. The number of nano-sized acetophenone distributed from 25 to 100 nm, with an average size of 53 nm. To estimate the stabilization of acetophenone nanoemulsions, we detected the size of acetophenone at every 2 days for 10 days after the preparation of acetophenone nanoemulsions. As shown in Fig. 3F, the size of acetophenone in the dynamic environment was larger than that in the static environment, but this was not statistically significant ($p > 0.05$). Usually, particle size distribution of the nanoemulsions is typically in the range of 20–200 nm [1, 13]. In this study, the average size of acetophenone droplets in the dynamic environment was 107 nm, while the average size in the static environment was 94 nm at day 10, which was completely satisfactory for the present experiment.

Nano-Sized Acetophenone Used as a Substrate for Bioreduction

We used nano-sized acetophenone as a substrate in the bioreduction work for the first time. As shown in Fig. 4A, nano-sized acetophenone reached the maximum conversion (near 100%) after 32 h of incubation (24 h in advance at

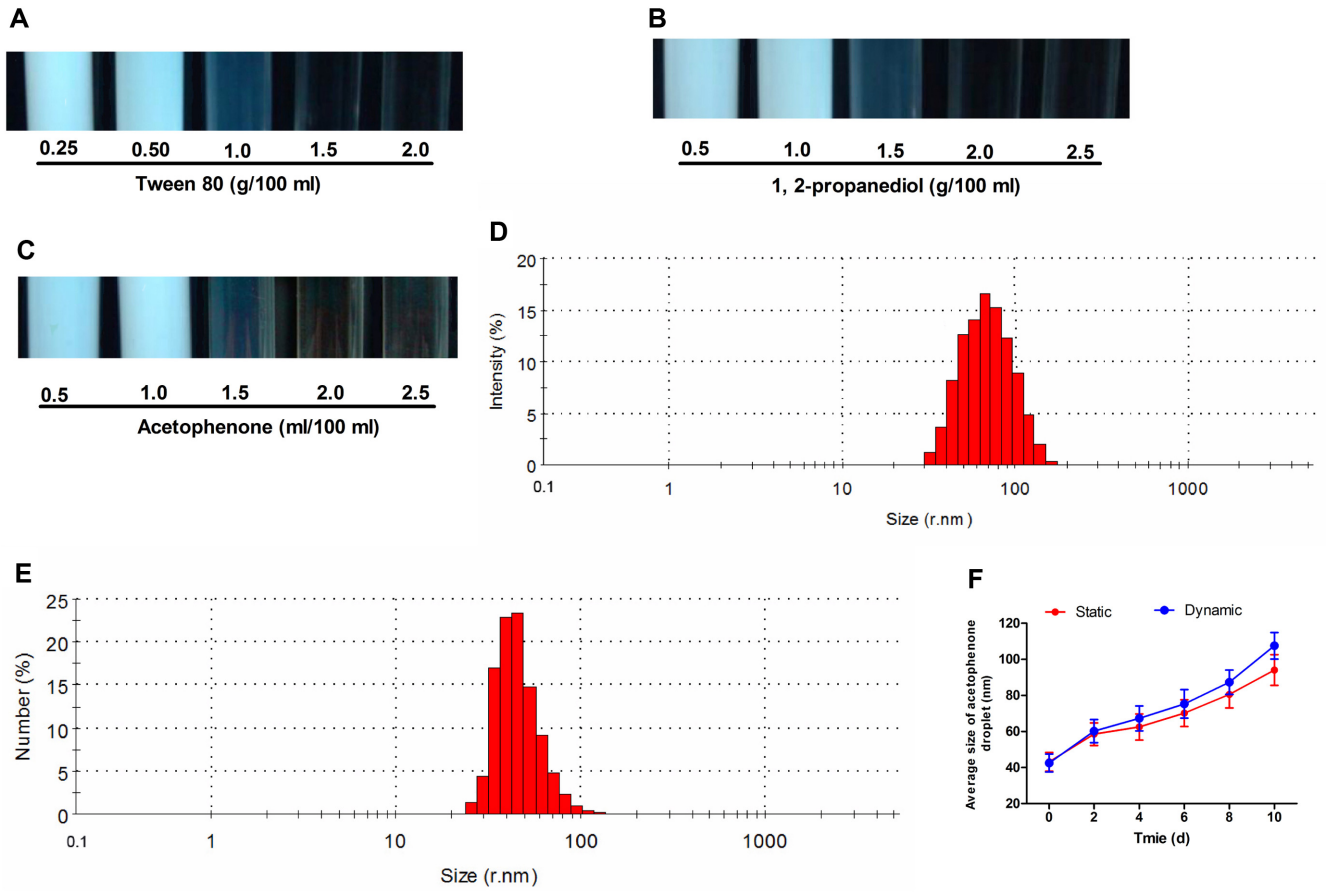

Fig. 3. Preparation of acetophenone nanoemulsions by the spontaneous emulsification method.
(**A**) Five different concentrations of Tween 80 were assessed when 1.5 g/100 ml of 1,2-propylene glycol and 1.5 ml/100 ml of acetophenone were used. Milky white emulsions were obtained when using 0.25 and 0.50 g/100 ml of Tween 80 and stable transparent nanoemulsions were gained when using 1.0, 1.5, and 2.0 g/100 ml of Tween 80. We chose 1.0 g/100 ml of Tween 80 finally. $n = 4$. (**B**) The effects of 1,2-propylene glycol on the formation of acetophenone nanoemulsions. $n = 4$. (**C**) The effects of substrate on the formation of acetophenone nanoemulsions. $n = 4$. (**D**) The intensity distribution of nano-sized acetophenone. The intensity distributed from 30 to 110 nm, with the peak intensity being at 70 nm. (**E**) The number of distribution nano-sized acetophenone. The number distributed from 25 to 100 nm, with an average size of 53 nm. (**F**) Acetophenone nanoemulsions were stable both in a static and a dynamic environment. $n = 5$.

least) when 0.54 nm pellets were used. The e.e. values of the (*S*)-phenylethanol were more than 99% when using nano-sized acetophenone as a substrate, but the e.e. values of the control group were near 85% (Fig. 4B). Besides this, the rate of glucose consumption was faster compared with the control group (Fig. 4C) and more final biomass was obtained in the process of nano-sized acetophenone bioreduction (Fig. 4D).

Tween 80 and 1,2-Propylene Glycol Had Low or No Toxicity to *Rhizopus oryzae*

To evaluate the toxicity of Tween 80 and 1,2-propylene glycol, we controlled their concentrations to observe their effects on the performance of acetophenone reduction. Transparent nanoemulsions were obtained when the concentrations of Tween 80 were 1.0, 1.5, and 2.0 g/100 ml, and the concentrations of 1,2-propylene glycol were 1.5, 2.0, and 2.5 g/100 ml. The conversion, e.e., residual glucose, and final biomass, when using different concentrations of Tween 80 and 1,2-propylene glycol in the transparent nanoemulsions, were all not statistically significant (Figs. 5A–5H), indicating that Tween 80 and 1,2-propylene glycol had low or no toxicity to *Rhizopus oryzae*. Interestingly, the milky white emulsions (0.5 g of Tween or 1.0 g of 1,2-propylene glycol) had poor performances of acetophenone bioreduction. These findings indicated that the toxicity of

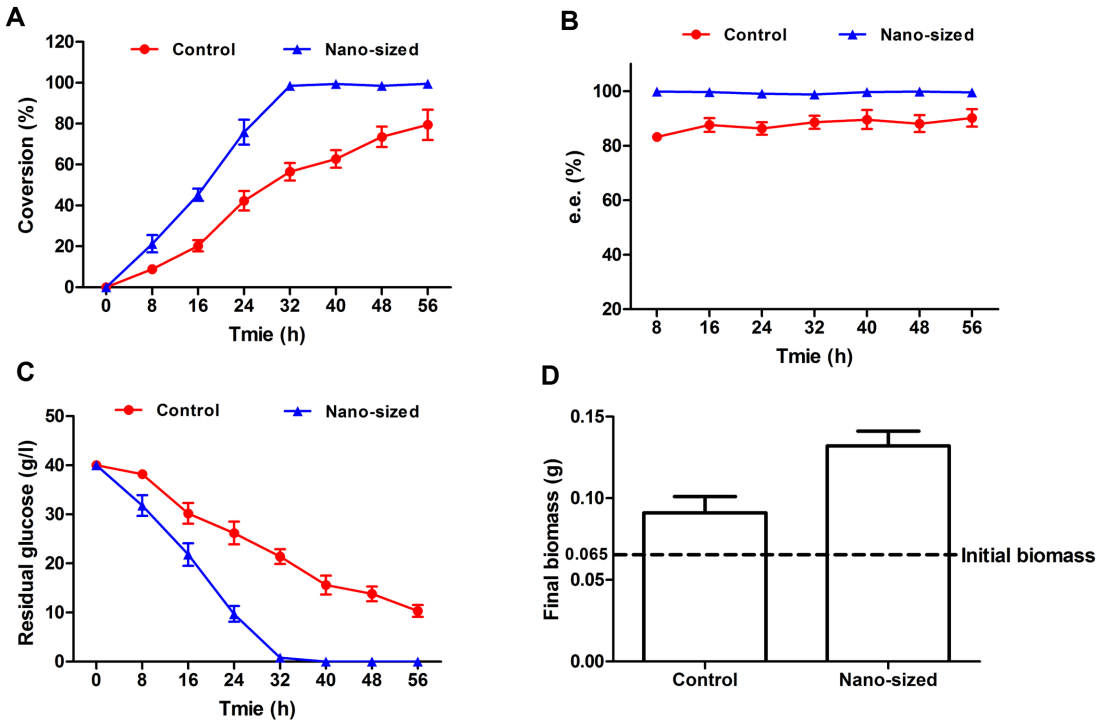

Fig. 4. Nano-sized acetophenone was used as a substrate for biotransformation work.
(**A**) The conversion reached near 100% when using nano-sized acetophenone as a substrate after 32 h of incubation. $n = 5$. (**B**) (*S*)-Phenylethanol enantiomeric excesses (e.e.) reached near 100% when using nano-sized acetophenone as a substrate. $n = 5$. (**C**) Pellets consumed glucose faster when using nano-sized acetophenone as a substrate. $n = 5$. (**D**) The final biomass was increased compared with the control group. $n = 5$.

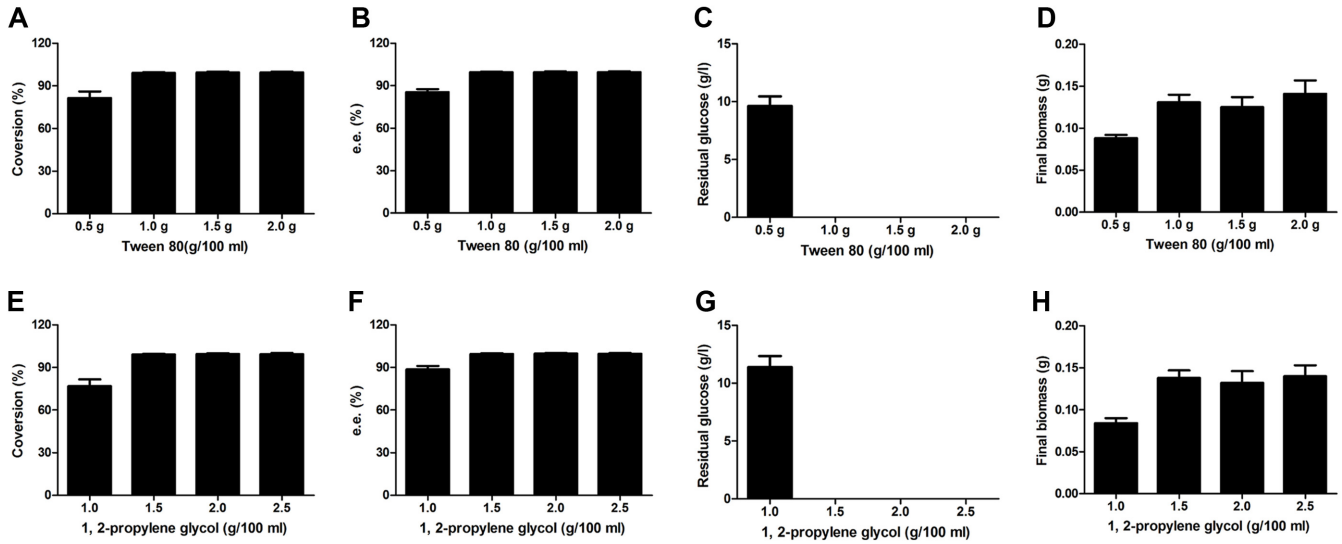

Fig. 5. Tween 80 and 1,2-propylene glycol had low or no toxicity to *Rhizopus oryzae*.
The toxicities of acetophenone, Tween 80 and 1,2-propylene glycol were evaluated after 56 h of incubation period. Milky white emusions (0.5 g/100 ml of Tween 80 or 1.0 g/100 ml of 1,2-propylene glycol) showed poor coversion (**A**, **E**) and e.e. (**B**, **F**), more residual glucose (**C**, **G**), and less final biomass (**D**, **H**) compared with transparent nanoemulsions, suggesting that acetophenone nanoemulsions had a lower toxicity to cells than milky white acetophenone emulsions. There was no difference in the performance of reduction when using 1.0, 1.5, and 2.0 g/100 ml of Tween 80 as well as 1.5, 2.0, and 2.5 g/100 ml of 1,2-propylene glycol, indicating that Tween 80 and 1,2-propylene glycol had low or no toxicity to *Rhizopus oryzae*. $n = 5$.

transparent acetophenone nanoemulsions to *Rhizopus oryzae* was lower than that of milky white acetophenone emulsions.

Discussion

The biotransformation by fungi provides an inexpensive, operationally simple strategy without pollution for the asymmetric reduction and hydrolysis of alkylaryl ketones as well as their corresponding acetates [9]. However, the analysis of products showed that only 78–88% e.e. could be obtained using *m*-methoxy acetophenone as a substrate [11]. In the process of industrial fermentation, pellets are often the preferred morphology [3]. Pellet formation is strongly dependent on the growth conditions, such as the initial pH values [15], the shaking frequency [21], the temperature [14], the volume of seed medium [10], the concentration of nitrogen source and carbon source [20], and so on. We compared the performance of acetophenone reduction using three fungal morphologies: clumps, suspended mycelia, and pellets. The results showed that pellets were the best option in consideration of the conversion, e.e., and incubation period. Furthermore, pellets with three different diameters were used to optimize acetophenone reduction. We found that the smallest pellets exhibited the best performance. The pellet size must be kept to a certain critical value to prevent oxygen limitation and keep the activity of *Rhizopus oryzae* [17]. Moreover, the removal of biomass after incubation will be easier when the pellet morphology is used [2, 10]. Previous studies showed that the fungal pellet diameter was connected with the fermentation performance, and the highest yield was obtained when using pellets of smaller diameter, which may be interpreted that the inner zone of larger pellets was relatively inactive [4], limiting internal mass transfer [17]. On the other hand, the smaller pellet had an increased surface area, which can contact with more substrate. Some studies found that a larger fungal pellet had higher specific glucoamylase activities, whereas a smaller fungal pellet had higher biodegradation rates [6, 22]. Even so, the bioreduction parameters using the smallest pellets were unsatisfactory.

Although the asymmetric bioreduction of the aromatic ketones with active whole cells is one of the most promising methods, the substrate and product are noxious to the cells [18, 19]. To resolve the bottleneck, Yang *et al*. [19] introduced an organic solvent to control the concentrations of the substrate in an aqueous phase and to remove the product from the aqueous phase in situ. Wang *et al*. [16] isolated a novel bacterial strain and optimized the conditions for bioreduction of 3,5-*bis*(trifluoromethyl) acetophenone. In addition, 2-propanol was used instead of glucose as the hydrogen donor, leading to an increase of the substrate concentration. In the present study, we used nano-sized acetophenone as a substrate to attenuate the toxicity of acetophenone to *Rhizopus oryzae*. Obviously, the conversion and e.e. values reached nearly 100%. The final biomass in the control group was decreased at the end of incubation compared with the initial biomass. On the contrary, the final biomass was increased when using nano-sized acetophenone as a substrate, suggesting that acetophenone nanoemulsions suppress the toxicity to cells. We also evaluated the toxicity of Tween 80 and 1,2-propylene glycol to cells. The results revealed that there was no difference in the conversion, e.e., residual glucose, and final biomass when using different concentrations of Tween 80 and 1,2-propylene glycol in the transparent nanoemulsions, suggesting that Tween 80 and 1,2-propylene glycol have no significant effect on acetophenone reduction.

Taken together, pellets and nanoemulsions both increase the interfacial areas and shorten the incubation time. In addition, nanoemulsions decrease the toxicity of acetophenone to fungal cells and elevate the solubility and bioavailability. These advantages synergistically result in superior performances of acetophenone reduction. The first attempt at bioreduction by *Rhizopus oryzae,* using nano-sized acetophenone as a substrate, seems to be practicable.

Acknowledgments

This study was funded by the Science and Technology Research Plan Funded Key Projects in Henan Province Department of Education (No. 13A150112).

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