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Efficient hydration of 2-amino-2, 3-dimethylbutyronitrile to 2-amino-2, 3-dimethylbutyramide in a biphasic system via an easily prepared whole-cell biocatalyst

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From an environmental perspective, utilizing nonconventional solvents (i.e. green solvents) with low ecological footprint is a highly beneficial alternative to the conventional organic solvents to form the reaction system. Nitrile hydratase (NHase, EC 4.2.1.84) catalyzed hydration of 2-amino-2, 3-dimethylbutyronitrile (ADBN) to 2-amino-2, 3-dimethylbutyramide (ADBA) in various green solvent-aqueous reaction systems were investigated in this study. After systematical optimization of reaction conditions, HFE-7100/H_2O (v/v, 10%) biphasic system was ultimately identified as a promising reaction system in the aspects of reducing product inhibition, avoiding substrate hydrolysis, facilitating product separation and solvent recovery. The average ADBA yield of entire batch reaction attained 97.3 %, which was obviously higher than those previously reported chemical or enzymatic methods. This is the first attempt to apply a fluorous solvent-aqueous biphasic system into a biocatalytic process, and the results suggests that proper fluorous solvent employed in the biphasic system is more accord with the requirement of green chemistry.

1. Introduction

Since they were commercialized in the 1980s by American Cyanamid Company (BASF), imidazolinone herbicides have become exceedingly effective herbicidal agents owing to their noteworthy advantages in strong selectivity, broad-spectrum characteristics, environmental safety and attractive price.[1,2] 2-amino-2, 3-dimethylbutyramide (ADBA) produced from the hydration of 2-amino-2, 3-dimethylbutyronitrile (ADBN), is a key intermediate in synthesis of those highly potent and broad-spectrum imidazolinone heribicides.[3-5]

Conventional chemical hydration is accomplished by using concentrated sulfuric acid with concentrated ammonia and dichloromethane in 100 °C, or by using hydrogen peroxide with concentrated ammonia and palladium-on-carbon (Table S1).[4-6] In addition to the harsh reaction conditions required, high concentration of sulfate and ammoniated effluent generated from the aforementioned two chemical ways are difficult to be removed in the downstream processing,[4-6] which would drive chemical ways to be eliminated eventually with the increasingly restrictive environmental regulations. Enzymes or whole-cell biocatalyst are not only specific for chemical reaction, more importantly, but also can catalyze at ambient temperatures and physiological pH, and be reused or biologically degraded.[7] From the perspective of green chemistry, these advantageous properties place enzymes or whole-cell catalyzed biotransformation be more eco-friendly along with the fact that they produce less or no toxic wastes, thus contribute a more sustainable productive process.[8, 9] A biocatalytic hydration method was exploited using whole-cell of *Rhodococcus boritolerans* CCTCCM 208108 in a 30/70 (v/v) *n*-hexane/H_2O biphasic system, and the ADBA concentration reached 50 g L^{-1} with a yield of 91 % after 19 h reaction at 10 °C.[10]

Using above-mentioned aqueous-organic solvent biphasic system or even single organic solvent system has been an extensively accepted approach to facilitate the recovery of products, overcome the toxicity and/or low solubility of substrates or products, and control the thermodynamic equilibrium toward synthesis rather than hydrolysis.[8,11,12] However, there are some inherent problems including biocatalyst deactivation, solvent toxicity and the fact that organic solvents are waste volatile organic compounds (VOCs).[12] With respect to green chemistry, substantial progresses have been made for identifying green solvents with a reduced ecological footprint and available physicochemical properties to substitute traditional organic solvents.[13] The use of reaction system composed by green solvents provides a greener choice, which can take into account both the solubility and hydrolysis of substrates/products and environment issues. Despite a considerable amount of green solvents such as ionic liquids,[14] fluorous solvents,[15] bio-based solvents[16] and super critical fluids[17] have been well established in the enzymes- or whole cells-catalyzed biphasic systems, there is still plenty of uncharted territory for exploring the usage of the cumulatively emerged green solvents in the nitrile hydratase (NHase) catalyzed process. To date, ionic liquid is the only one that successfully applied, in which conversion of 1, 3-dicyanobenzene to 3-cyanobenzamide and 3-cyanobenzoic acid using the NHase from *Rhodococcus* R312 in a [bmim][PF_6]/H_2O biphasic system.[18] In this work, we aim to develop a green

reaction system for efficiently enzymatic hydration of ADBA from ADBN using recombinant *Escherichia coli* (*E. coli*) cells harboring NHase.

2. Experimental

Chemicals

Green solvents were provided by Wuhu Huaren Science and Technology Co., Ltd (Wuhu, China), TCI (Shanghai, China), and Aladdin (Shanghai, China), respectively. ADBN and ADBA were purchased from Adamas (Shanghai, China) and Topbiochem Technology Co., Ltd (Shanghai, China), respectively. Fermentation medium compositions were industrial grade and purchased from local Chinese market.

Preparation of whole-cell biocatalyst and reaction systems

Recombinant *E. coli* cells harboring NHase from *Pseudonocardia thermophila* CGMCC 4.1303 was constructed as described in the "Electronic Supplementary Information". Cells were harvested by centrifugation, washed and resuspended with potassium phosphate buffer (100 mM, pH 7.0). 12 kinds of green solvents (Fig.1) were added to the corresponding volume of cells suspension to a concentration of 5-50 % (v/v). Each reaction experiment was performed as following conditions: ADBN (100 mM), temperature (30 °C), 100 mM phosphate buffer (pH 7.0), enzyme amount (20 U/mL), reaction volume (400 μL) and shaking speed (200 rpm). Samples were withdrawn at 0.5 h and 1 h, respectively, then centrifuged at 12, 000 rpm for 5 min, and analyzed by gas chromatography (GC).

GC analysis and NHase activity assay

ADBA was analyzed via a previously described method.[19] GC was carried out by Agilent 7820A (Agilent, USA) equipped with AT·FFAP column (30m×0.25mm×0.25mm; Lanzhou Institute of Chemical Physics, China). The temperatures of the injector and detector were set at 250 °C. The carrier gas nitrogen flow was 1.2 mL min^{-1}. After a 3 min solvent delay time at 150 °C, the oven temperature was increased at 10 °C min^{-1} to 200 °C, and then maintained for 5 min. The injection volume was 0.2 μL under a splitless mode.

Freshly harvested cells were disrupted using the ultrasonic cell disruptor (JY92-2D, Ningbo Scientz, China) on ice at 400 W for 60 cycles (working 5 s and intervals 5 s as one cycle). The disrupted cell debris was collected by centrifugation at 12, 000 rpm and 4 °C for 10 min, and the obtained supernatants were used for the NHase activity assay. 4 μL of ADBN was added into 400 μL of supernatants to an ADBN concentration of 80 mM, and the reaction was performed at 30 °C and 200 rpm. The reaction continued for 6 min, and then stopped by adding 10 uL of 6.0 M hydrochloric acid. The ADBA produced was determined by GC. One unit of NHase activity (U) was defined as the amount of wet whole cells (g) that required to produce 1μmol of ADBA per minute at 30 °C and pH 7.0. The enzyme activity of wet whole cells used was determined to be 109 U/g. The enzyme amount used in this work was expressed as U/mL according to the total enzyme activity (U) added into the reaction mixture (mL).

Effects of pH and temperature on the hydration of ADBN to ADBA

Effects of reaction temperatures with a range from 15 °C to 35 °C on the hydration of ADBN to ADBA were investigated in the HFE-7100/H_2O (10 %, v/v) biphasic system and glycerol carbonate/H_2O (5 %, v/v) homogeneous system as following conditions: ADBN (100 mM), 100 mM phosphate buffer (pH 7.0), enzyme amount (20 U/mL), reaction volume (400 μL) and shaking (200 rpm). Effects of reaction pH with a range from 5.0-8.0 on the hydration of ADBN to ADBA were also investigated in the above mentioned two reaction systems as following conditions: ADBN (100 mM), temperature (20 °C), enzyme amount (20 U/mL) reaction volume (400 μL) and shaking speed (200 rpm). Samples were withdrawn at different intervals, centrifuged and analyzed by GC.

Effects of ADBA and ADBN concentrations on the hydration of ADBN to ADBA

Effects of substrate concentrations (60-250 mM) on the hydration of ADBN to ADBA were investigated in the single aqueous media, HFE-7100/H_2O (10 %, v/v) biphasic system and glycerol carbonate/H_2O (5 %, v/v) homogeneous system, respectively, and each reaction experiment was performed using 15 U/mL, 20 U/mL and 30 U/mL of enzyme amounts, respectively, at 20 °C and 200 rpm in 100 mM phosphate buffer (pH 7.0). Detailed reaction process of each experiment was shown in the "Electronic Supplementary Information". Effects of product concentrations (0-180 mM) on the hydration of ADBN to ADBA were also investigated in aforementioned three reaction systems, and each reaction experiment was performed using 30 U/mL of enzyme amount and 80 mM of substrate concentration at 20 °C and 200 rpm in 100 mM phosphate buffer (pH 7.0) for 2 h. Samples were withdrawn at different intervals, centrifuged and analyzed by GC.

Batch reaction for the hydration of ADBN to ADBA

Batch hydration of ADBN to ADBA in the HFE-7100/H_2O (10 %, v/v) biphasic system was conducted as the following conditions: pH 7.0, ADBN (120 mM), enzyme amount (30 U/mL), temperature (20 °C), reaction volume (25 mL) and shaking speed (200 rpm). Samples were withdrawn at different intervals, centrifuged and analyzed by GC to determine whether the amount of product no longer increased. After reaction finished, the reaction mixture was centrifuged at 8, 000 rpm for 10 min, and the upper aqueous phase was separated from the HFE-7100/H_2O biphasic system, and collected for product separation and purification. 375 uL of ADBN to a final concentration of 120 mM plus 22.5 mL of distilled water (pH 7.0) were supplemented to the residual HFE-7100 phase and whole-cell for the next batch.

ADBA separation, purification and identification

After batch reaction finished, appropriate volume of hydrochloric acid (6.0 M) was added to the above collected aqueous phase (approximately 155 mL) to pH 4.0 for denaturing the proteins leaked from cells, and centrifuged at 12,000 rpm for 10 min. The obtained supernatant was treated with the activated carbon (2 %, w/v) at 50 °C and 180 rpm for 2 h to eliminate pigment and hybrid protein, and then evaporated under reduced pressure to remove the unconverted ADBN and water. The isolated white product was incubated with ethyl acetate at 50 °C for 0.5 h, and recrystallized at -20 °C. The crystals were collected by filtration, weighed and analyzed by GC. The structure of final product was elucidated by Time of Flight Mass Spectrometer (EI-TOF/MS). EI mass spectra were obtained from *m/z* 35-800 with

source temperature of 250 °C and an ionization voltage of 70eV.

3. Results and discussion

Screening of reaction systems

The hydration of ADBN to ADBA catalyzed by NHase is a well-known valuable reaction.[5,10] ADBN is very poorly water soluble while ADBA is the exact opposite, thus an aqueous-organic biphasic system where the organic phase acts as a substrate reservoir will greatly increase productivity. *n*-hexane was selected as the organic phase after screening a wide range of solvents in a previous work.[10] However, *n*-hexane is hazardous to operate with, particularly at large scale, on account of its toxicity and flammability. The potential replacement of *n*-hexane with green solvents would be considered highly necessarily from an industrial and environmental perspective. Various green solvents in a limited range of 5-50 % (v/v) were used to test their possibilities to construct a system for the hydration of ADBN to ADBA. The results shown in Fig.1 indicated that diverse green solvents formed reaction systems generated significantly different ADBA yield. Preliminary experiment in aqueous phase (pH 7.0, 100 mM sodium phosphate buffer) under the optimized conditions was examined to set a benchmark for comparison of subsequent reaction systems, and the yield of ADBA catalyzed by whole-cell harboring NHase reached 58.5±2.46 % and 62.4±3.24 % at 0.5 h and 1 h, respectively.

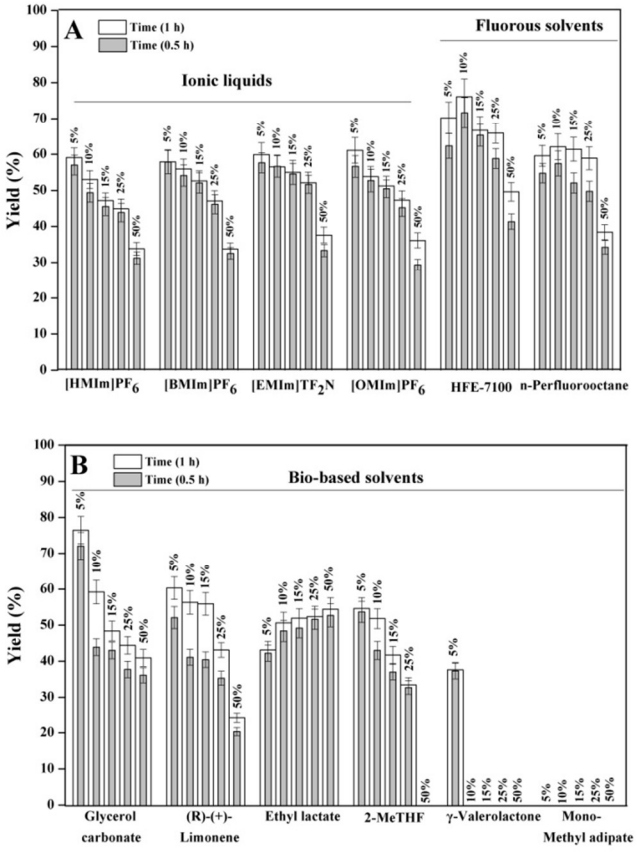

Fig.1. Screening of reaction systems. (A) Reaction in ionic liquids/H_2O (5-50 %, v/v) and fluorous solvents/H_2O (5-50 %, v/v); (B) Reaction in bio-based solvents/H_2O (5-50 %, v/v). Reaction conditions: ADBN (100 mM), temperature (30 ^oC), 100 mM sodium phosphate buffer (pH 7.0), enzyme amount (20 U/mL) reaction volume (400 μL) and shaking speed (200 rpm). Samples were withdrawn at 0.5h and 1.0 h, centrifuged and analyzed by GC.

Water plays crucial role in the NHase catalyzed hydration process, thus ADBA yield could be influenced directly by the alteration of water content of reaction systems. Except that in the ethyl lactate formed reaction system, the ADBA yield decreased gradually along with increasing the green solvents ratio of reaction systems (Fig. 1). High green solvents content of reaction system probably affected the NHase activity of whole-cell, which resulted in the decrease of catalytic efficiency. In the reaction systems formed by γ-valerolactone and mono-methyl adipate, it could be evidently observed that no ADBA was produced in the reaction course, suggesting the enzyme activities of whole-cell were completely deactivated when the ratios of these two solvents were over 10 % (v/v) in the reaction system. Among all reaction systems, HFE-7100 and glycerol carbonate formed reaction systems gave better catalytic performance than the single aqueous media on the hydration of ADBN to ADBA (Fig.1).

Quite a few studies have shown the potential to employ fluorous-organic solvents or single fluorous solvent as reaction media in the lipase-catalyzed reactions: (trans)esterification reactions in a perfluorocarbon-hydrocarbon homogeneous system using *Candida rugosa* lipase;[20] alcoholysis between vinyl cinnamate and benzyl alcohol catalyzed by a poly(ethylene glycol)-lipase PL complex in the fluorous (FC-77)/isooctane homogeneous system;[21] and kinetic resolution of racemic 1-phenylethanol catalyzed by Novozym 435 in three types of low-boiling hydrofluorocarbon compounds.[15] These reactions in the fluorous or fluorous-organic solvents exhibited markedly improved catalytic efficiency and generated better yields under the same conditions compared to the conventional organic or aqueous-organic solvents. However, either the problem of products separation or long reaction time largely hinder the practical application of these biocatalytic process in the perfluorinated compounds (PFCs) formed reaction systems.[8] Some of the negative aspects as described in a previous review also noted that PFCs could not be regarded as totally environmentally friendly solvent.[22,23] One possible solution for the replacement of PFCs is hydrofluoroethers (HFEs), which have been commercially introduced as a cost-effective and environmentally safe alternative to fluorous phase of a biphasic system with available miscibility properties.[24] In this work, ADBA yield of 71.4±4.27 % and 76.1±4.95 % achieved in HFE-7100/H_2O (10 %, v/v) biphasic system at 0.5 h and 1 h, respectively (Fig.1A). HFE-7100, one of fluorous solvents with low toxicity and global warming potentials, is not classified as a VOC solvent and is approved without restrictions under the Significant New Alternatives Program (SNAP) of US EPA,[25] suggesting that HFE-7100/H_2O biphasic system could be a promising green reaction system in industrial hydration of ADBN to ADBA.

Glycerol derivatives has been proved to be a suitable media for biocatalytic process, and many promising results were reported.[26] In this work, the yield of ADBA reached 72.3±3.76 % and 76.5±3.79 % at 0.5 h and 1 h, respectively (Fig.1B) in

homogeneous system formed by 5 % (v/v) of glycerol carbonate with 100 mM sodium phosphate buffer (pH 7.0). Glycerol carbonate is a potential low VOC bio-based solvent.[27] Ou et al. investigated the activities of *C. antarctica* lipase B (CALB) on transesterification of ethyl butyrate with *n*-butanol in glycerol carbonate compared to that of in aqueous phase, indicating that glycerol carbonate was a bio-based solvent of interest for biocatalysis in nonaqueous solvents.[28]

Since Erbeldinger *et al*.[29] in 2000 initially used thermolysin to

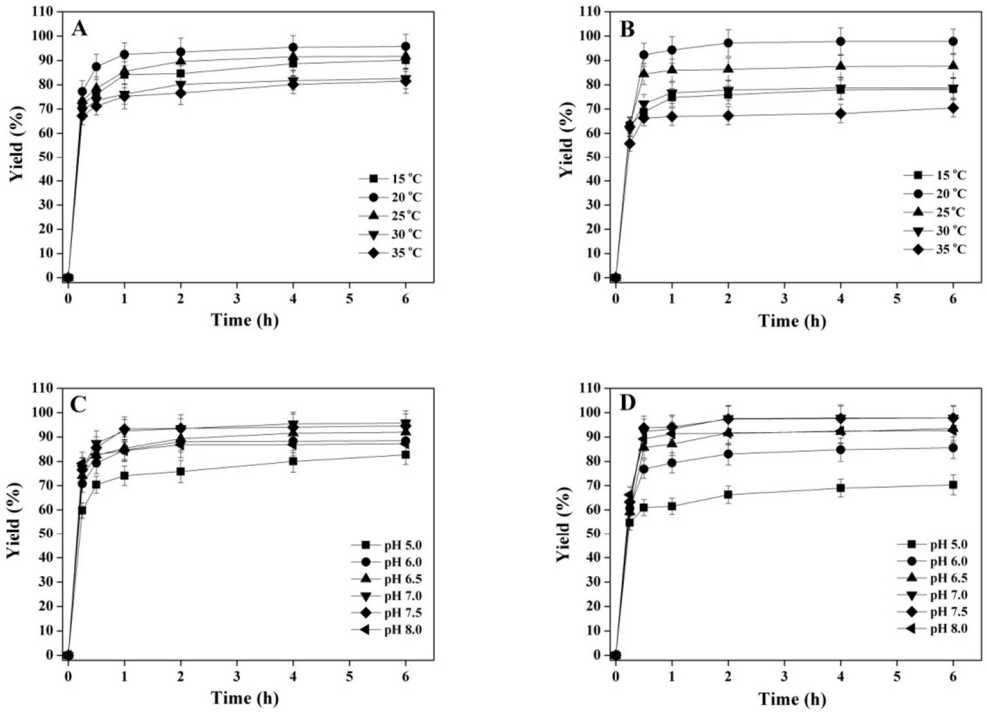

Fig 2. Influences of reaction temperature (A, B) and pH (C, D) on the hydration of ADBN to ADBA in HFE-7100/H_2O (10 %, v/v) biphasic systems (A, C) and Glycerol carbonate/H_2O (5 %, v/v) homogeneous systems (B, D). Reaction conditions used in A and B: ADBN (100 mM), pH 7.0, enzyme amount (20 U/mL) reaction volume (400 μL) and shaking speed (200 rpm). Reaction conditions used in C and D: ADBN (100 mM), pH 7.0, temperature (20 °C), enzyme amount (20 U/mL) reaction volume (400 μL) and shaking speed (200 rpm).

catalyze the formation of *Z* -aspartame in [bmim][PF_6], ionic liquids (ILs) have been used intensively over the last decade in many enzymatic transformations, typically centering on esterification, transesterification, alcoholysis, aminolysis, hydrolysis and polymerization.[30] However, as shown in the Fig. 1A, all enzymatic hydration of ADBN to ADBA occurred in ILs formed reaction systems did not exhibit a better catalytic performance compared to that of in the single aqueous media.

Effects of temperature and pH

In the process of NHase catalyzed hydration, the activity of enzyme is influenced directly by temperature and pH, thus affects the ADBA yield. According to above screening results, influence of temperature on the hydration of ADBN to ADBA was investigated in HFE-7100/H_2O (10 %, v/v) biphasic system and glycerol carbonate/H_2O (5 %, v/v) homogeneous system, respectively. As shown in Fig.2A and 2B, high or low temperature could result in the decrease of enzyme activity, and thus reduce the ADBA yield. Under high temperature (above 30 °C), naturally, the cyanogroup of ADBN would hydrolyze into hydrocyanic acid, then seriously inhibit the enzyme activity.[31, 32] Thus, a relatively low temperature without obviously decreasing enzyme activity was suitable. When temperature was 20 °C, the yield of ADBA achieved maximum in both reaction systems. The optimum temperature of the reaction was selected as 20 °C. Consequently, effect of pH on reaction course was investigated in above mentioned reaction systems. The results shown in Fig.2C and 2D indicated that an overly acid or alkali of reaction condition had a negative effect on the activity of the NHase, and thus led to the decrease of ADBA yield. The maximum ADBA yield was achieved at a range of pH 7.0-7.5 in above mentioned two reaction systems. Considering that the whole-cell was much stable in neutral pH condition (data not shown), therefore, pH 7.0 was chosen as the optimum reaction pH for further experiments. Under the optimal temperature and pH, the yields of ADBA in HFE-7100/H_2O (10 %, v/v) biphasic system and glycerol carbonate/H_2O (5 %, v/v) homogeneous system significantly increased compared to that before optimization (Fig.1) and that in single aqueous phase, and reached approximately 93.3 % and 94.1 % after 1h reaction, respectively (Fig.2C and 2D).

Effects of ADBN and ADBA concentration in different reaction systems

Having acquired the optimum temperature and pH, further experiments regarding the effects of the substrate, enzyme amounts and product concentrations on the hydration of ADBN to ADBA were carried out to determine the optimal reaction conditions. The detailed reaction courses were provided in the

"Electronic Supplementary Information", and the highest ADBA yield achieved in the shortest reaction time under various substrate concentrations were summarized in Fig.3A.

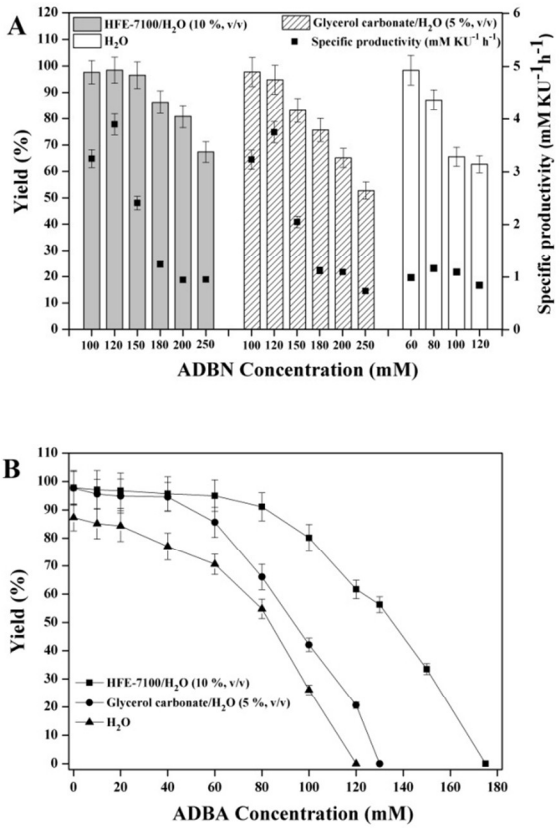

Fig 3. Influences of ADBN concentrations (A) and ADBA concentrations (B) on the hydration of ADBN to ADBA in different reaction systems. Reaction conditions used in (A): temperature (20 °C), pH 7.0, enzyme amount (30 U/mL), reaction volume (400 μL), shaking speed (200 rpm) and reaction time (h) is defined as the shortest time that required to obtain the highest yield of product. Specific productivity (mM KU^{-1} h^{-1}) was calculated according to the ADBA concentration (mM), enzyme amount (KU) and reaction time (h). Reaction conditions used in (B): ADBN (80 mM), temperature (20 °C), pH 7.0, enzyme amount (30 U/mL), reaction volume (400 μL), shaking speed (200 rpm) and reaction time (2 h).

Higher enzyme amounts could significantly reduce the reaction time (Fig.S2 and S3), and led to the increase of ADBA productivity (Fig.3A). Compared to that obtained in single aqueous media, ADBA productivity generated in HFE-7100/H_2O (10 %, v/v) biphasic system and glycerol carbonate/H_2O (5 %, v/v) homogeneous system increased approximately 4 and 3.3-fold, respectively (Fig.3A). Meanwhile, it could be observed that ADBA yield reduced gradually along with increase of substrate concentration, which was potentially attributed to the accumulation of product in aqueous phase to a certain concentration, and then triggered the inhibition of enzyme activity. On the premise of excluding the fact that the substrate was toxic to the NHase activity of whole-cell in 2h (Fig.S4), effects of product concentrations on the reaction course in aforementioned three reaction systems were investigated. In comparison with the single aqueous media, both of the two green solvents formed reaction systems alleviated the product inhibition in a varied degree (Fig.3B). According to the final ADBA yields achieved under different substrate concentrations, the threshold concentrations of the product that led to reaction be terminated in these three reaction systems were calculated to be approximately 70 mM, 130 mM and 170 mM, respectively (Fig.3A), which were consistent with the data of product inhibition as shown in Fig. 3B. However, there is an obvious discrepancy between these two values that ought to be same in aqueous system (approximately 70 mM in Fig.3A vs approximately 120 mM in Fig.3B), implying that in addition to product inhibition, there was other factor resulting in the inhibition of enzyme activity under high substrate concentration, which is likely to be caused by the hydrocyanic acid produced from the hydrolysis of the cyanogroup of the substrate as aforementioned.[30] Under the optimal reaction conditions, ADBA yields of 97.7% and 98.4 % were finally achieved in HFE-7100/H_2O (10 %, v/v) biphasic system and glycerol carbonate/H_2O (5 %, v/v) homogeneous system at higher substrate concentration (120 mM and 100 mM), respectively (Fig.3A). However, the ADBA yields in single aqueous media only attained to approximately 65 % at the substrate concentrations of 100 mM and 120 mM. Furthermore, the specific productivities obtained in HFE-7100/H_2O (10 %, v/v) biphasic system and glycerol carbonate/H_2O (5 %, v/v) homogeneous system were 3.98 mM KU^{-1} h^{-1} and 3.23 mM KU^{-1} h^{-1}, respectively, which were much higher than that (0.98 mM KU^{-1} h^{-1}) in single aqueous media. In the green solvents system constructed in this work, the substrate concentration used, the ADBA yield obtained and the specific productivity attained were all apparently higher than those reported previously (Table S1).[10]

Compared with glycerol carbonate/H_2O (5 %, v/v) homogeneous system and single aqueous phase, HFE-7100/H_2O (10 %, v/v) biphasic system exhibited several advantages, including catalyzing the reaction with a higher ADBA productivity; reducing product inhibition or substrate hydrolysis; highly beneficial for product separation and fluorous solvent recovery. As a water immiscible fluorous solvent, HFE-7100 served as a reservoir of the substrate in this fluorous-aqueous biphasic system. When the cells located in the interface of HFE-7100 and water, the substrate entered the cells and was converted into ADBA by NHase, and then the produced product diffused into the aqueous phase due to its high water solubility, thus facilitating the reaction to the desired direction. Given its apparent advantageous to this biocatalytic hydration process, it could be unequivocally concluded that HFE-7100/H_2O (10 %, v/v) biphasic system is an effective reaction system in the NHase catalyzed process, thus was selected for further batch reaction.

Batch reaction for the hydration of ADBN to ADBA

The ADBA yield remained unchanged under the optimized reaction conditions after substituting 100 mM sodium phosphate buffer (pH 7.0) with distilled water. Therefore, distilled water was chosen as the aqueous phase in batch reaction for benefiting the follow-up of product purification and process costs. Considering the production efficiency and product inhibition, 120 mM of substrate concentration was utilized to investigate the

reusability of the whole-cell biocatalyst in HFE-7100/H_2O (10 %, v/v) biphasic system. The ADBA yield decreased with a prolonged reaction time after seven batches reaction (Fig.4), which was mainly resulted from the leakage of NHase from the whole-cell into aqueous phase during the long operating course, and further confirmed by determination of the content of leaked protein in HFE-7100/H_2O (10 %, v/v) biphasic system (Fig.S4C). The total leaked protein content of aqueous phase reached approximately 7 % of total protein amount of whole cells after 8 batches, essentially consistent with the reduction trend of enzyme activity of whole cells (Fig.S4C and Fig.S5).

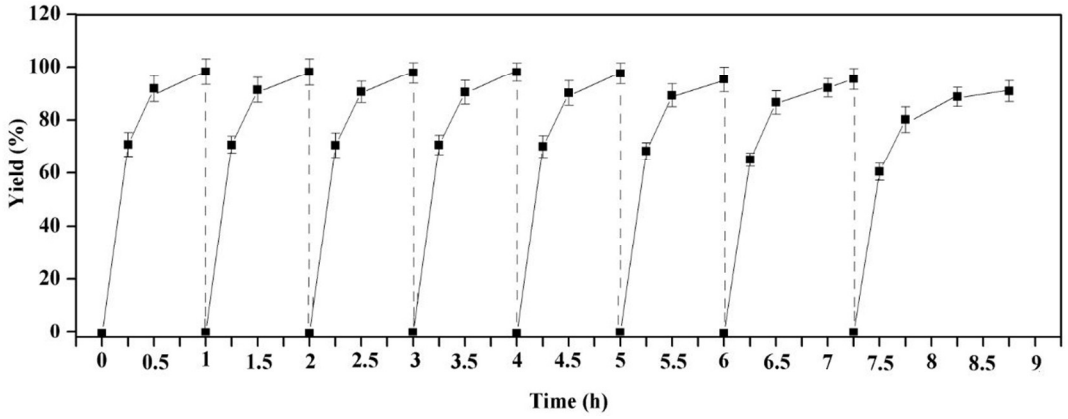

Fig 4. Batch hydration of ADBN to ADBA in HFE-7100/H_2O (10 %, v/v) biphasic system. Reaction conditions: pH 7.0, ADBN (120 mM), enzyme amount (30 U/mL), temperature (20 °C), shaking speed (200 rpm) and reaction volume (25 mL). After reaction finished, the reaction mixture was centrifuged at 8, 000 rpm for 10 min, and the upper aqueous phase was removed from the HFE-7100/H_2O biphasic system, and collected for product purification. 375 uL of ADBN to a final concentration of 120 mM plus 22.5 mL of distilled water (pH 7.0) were supplemented to the residual HFE-7100 phase and whole-cell for the next batch.

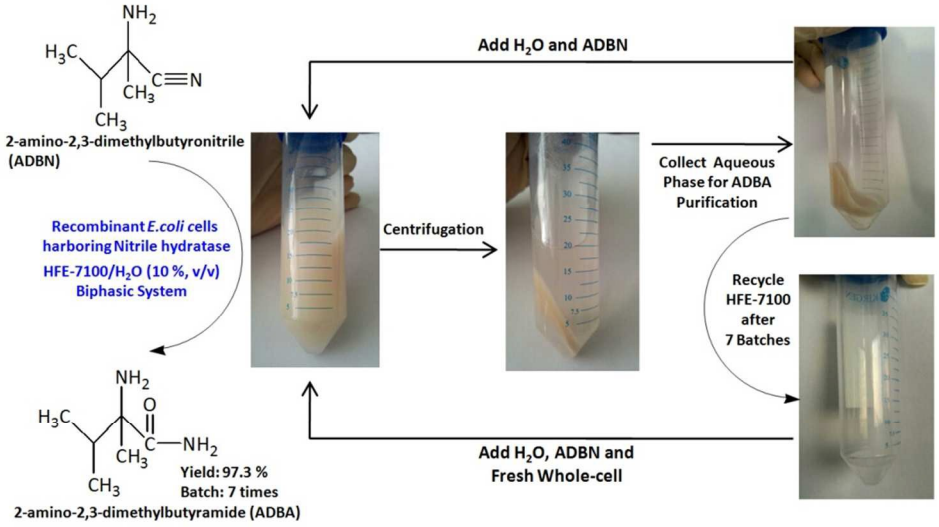

Fig 5. Scheme of batch hydration of ADBN to ADBA in HFE-7100/H_2O (10 %, v/v) biphasic system

The average ADBA yield of first seven batches still reached to 97.3 %, which is completely meet the industrial production demand. As depicted in Fig.5, the second round of reaction could be initiated by simply adding the substrate and distilled water after removing the aqueous phase which contained vast majority of product. The fluorous solvent could be easily collected and reused in another batch reaction (Fig.5), suggesting that HFE-7100 formed reaction system is a very favorable reaction media for industrial biocatalytic process for ADBA synthesis. To further reduce the product inhibition and the leakage of enzyme from whole cells, the strategy of in *situ* removal product using specific resins combined with the suitable method of whole-cell immobilization is now exploring in our lab.

ADBA separation, purification and identification

Aqueous phase contained product could be readily separated from centrifuged reaction mixture (Fig. 5). After removing the pigment, hybrid protein, and unconverted ADBN from the collected aqueous phase, the isolated white product was further purified using ethyl acetate, and recrystallized at -20 °C. The obtained crystals were collected, weighed and determined by GC, and a final isolated yield of 90.2 % with a purity of 98.5 % was achieved (Fig.S6). The structure of final product was confirmed by Time of Flight Mass Spectrometer (EI-TOF/MS) (Fig. S7).

Conclusions

We have demonstrated for the first time to apply a fluorous solvent/H_2O biphasic system into a biocatalytic process: hydration of ADBN to ADBA using an easily prepared, reasonably priced and reusable whole-cell biocatalyst. The results indicated that suitable fluorous solvent could be an excellent substitute to the conventional organic solvent to form biphasic system with water. HFE-7100/H_2O (10 %, v/v) biphasic system offers several process advantages such as the ability to conduct the reaction under high substrate concentration; reducing product inhibition and substrate hydrolysis; continuous bioprocess without wastes generated. Additionally, as a strictly green biphasic system, it complies with the requirements of green chemistry, and avoids the toxicity, flammability and volatility associated with conventional organic solvents, thus provides a sustainable approach for industrial biocatalytic synthesis of ADBA.

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Graphical abstract: