Ionic liquids/deep eutectic solvents enhance isoquercitrin production by the conversion of rutin extracted from grapefruit peel

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ABSTRACT: Whole-cell catalysis has been widely used in industry due to its diversity and ease of operation. It breaks through the limitations of complex extraction and high cost of enzymatic catalysis. But the catalytic efficiency of the whole-cell catalyst may be affected by the existence of the cell membrane to a certain extent. In this experiment, ionic liquid (ILs) and deep eutectic solvents (DESs) were used to treat cells expressing *α*-L-rhamnosidase (rhaB1), which increased the permeability of cell membrane to some extent, and effectively solved the problem of contacting enzymes with substrate. The results of single factor experiment showed that the optimum pH and temperature of the whole-cell catalyst were 6.5 and 40 °C, respectively. Under this condition, when the concentration of substrate rutin was 0.05g/L and the choline chloride/urea content was 6% (v/v), the rate of isoquercitrin reached a maximum, 93.05%. Therefore, it is effective to improve the catalytic performance of the whole cell catalyst by using a eutectic solvent to increase its permeability.
Keywords: biocatalysis, isoquercitrin, whole-cell, permeability, ionic liquids, deep eutectic solvents

Introduction

Pomelo, grapefruit, and other rutaceae plants are rich in flavonoids such as rutin, naringin, hesperidin. However, the scraps of fruit are often discarded in daily life, and the resources such as naringin have not been reused, which is a huge waste of resources. In order to make use of these flavonoids, using flavonoids in waste peels to produce substances which are rare in nature have been conducted. Hydrolysis of flavonoids by whole cell to synthesis rare products is a hot spot in biotransformation, because whole-cell catalysis eliminates the need for pure enzyme separation and purification processes and reduces the cost of production and cells are easily isolated from the product and easily recovered, increasing the recycling of the catalyst [1]. Additionally, enzymes protected by whole cells are more stable than when separated. However, the binding of the substrate to the active site of the enzyme is limited because of the presence of cell walls/membrane in the whole-cell catalyst. In addition, it is difficult for the product to flow out of the cell in time [2], which can easily lead to inhibition of the substrate and decrease the yield of the target product. Therefore, it is necessary to take measures to increase the contact between the enzyme and the substrate therefore increase the efficiency of whole cell catalysis.

At present, the most widely used methods are as follows: 1) Anchor the protein gene on the cell surface using cell surface display technology, enzymes and substrates can directly combine on the cell surface so catalytic process can be conducted; 2) Improve the cell permeability, which can increase the intracellular and extracellular mass transfer efficiency to enhance the enzyme's contact with the substrate. Although the former method can increase the exposure of the enzyme to the substrate to a certain extent, it needs to consider the size of the displayed protein molecule [3] as well as screening of target protein, and other complex molecular biological processes. Improvement of cell permeability is favored by more researchers because it allows small molecules and certain macromolecules to pass freely through the cell without destroying cellular organisms and can avoid complicated manipulations. Different strategies (Table 1) such as osmotic pressure has been reported in Ariane Peyret's research, after high-pressure progressing, the cell membrane permeability changes slightly (permeabilization from 0.48±0.01 to 0.48±0.04) [4], and small molecules easily enter the cell. Other methods including electric field [5], permeability reagent [6] and solvent medium have been applied to improve cell membrane permeability. What's more, mass transfer of substrates to products can be enhanced by modifying the microorganisms themselves. Overexpression of the transporter *ydeD* gene in *E. coli* facilitates cysteine efflux [7]. Cell permeability can also be controlled by regulating the expression of bioactive peptides. Chen [8] expressed bombesin II and maltose binding protein, and the *β*-glucuron aldolase activity was increased by 10 times when induced by 0.1 mmol/L IPTG. In addition, treatment of cells with ionic liquid has been reported that [PrMIm][BF_4] reduced the total cell lipid content by 8.3%, while the [PrMIm][PF6]-treated cells reduced by more than 17% [9], and the reduced lipids flowed out of the cell through the

enlarged cell membrane. Thus, the extracellular medium such as ionic liquids (ILs) and deep eutectic solvents (DESs) enhance cell permeability are easy to operate and efficient.

Table 1. Improvement strategies of cell permeability.

Methods	Process	Mechanism	Effect	Refs
Physical treatment	Osmotic pressure treatment	Osmotic pressure	Permeabilization changed from 0.48±0.01 to 0.48±0.04	[4]
	Electrical treatment	Electric field	Formation of micropores in phospholipids bilayer	[5]
Chemical regulation	Biological treatment reagents	Penetrant	Inhibition of membrane formation	[6]
	Solvent medium	Hydrophobic interaction	Total lipid content reduced by 8.3%	[9]
Molecular biology regulation	Change the membrane structure and composition	Gene mutation/knockout	Overexpression of ydeD	[7]
	Expression transporter	Channel protein	Catalytic activity improved 10 times	[8]

ILs and DESs are excellent promoters that have received widespread attention in recent years. ILs, also known as room temperature ILs, which are liquid composed entirely of organic cations and inorganic or organic anions at room temperature [10]. They are environmental friendly with low saturated vapor pressure, non-flammability, hydrophobicity, and good thermodynamic stability properties. ILs have been in biocatalysis, Zhang and his colleagues used ionic liquid [EMIM][BF_4] as solvent to synthesize Indolyl 4H-chromenes by lipase, and obtained a high yield (77-98%) [11], ILs show good advantages in promoting catalysis over catalyzed reactions without ionic liquids. Wang et al [12]. used free rhaB1 and ionic liquid [Toma] [Tf_2N] as cosolvent to hydrolyze rutin to generate isoquercitrin, and the yield of isoquercitrin was 98.3% indicating that ionic liquids are also catalytically effective accelerator. DESs, have been used for many years in separation processes, functions materials, chemical reactions, electricity chemical [13] since its discovery. and they have shown good application prospects because of its low cost, low toxicity and biodegradable peculiarities [14], which are very similar physic-chemical properties to ILs, making them a nearly 100% green solvent for atomic utilization. However, different ILs and DESs may have different degrees of toxicity to cells, and their own water may affect the results of cell catalysis. Therfore, it is necessary to screen ILs and DESs prior to the large-scale use of them.

Isoquercitrin has been used as an antithrombotic drug derived from flowers of malvaceae cotton, leaves of apocynaceae kenaf to treat hepatitis and cardiovascular and cerebrovascular diseases because of its antihypertensive, hypolipidemic, anti-oxidant, anti-inflammatory [15], anti-enzyme effect as well as other pharmacological efficacy. However, isoquercitrin is very rare in nature, and the yield of isoquercitrin by traditional chemical synthesis method is low, additionally, there are other disadvantages such as high cost and environmental pollution, which are contrary to the concept of developing green chemistry. Gong An et al [16] used immobilized naringinase to hydrolyze rutin in a microchannel reactor and the yield of isoquercitrin 92.24±3.26% was achieved, biotransformation can make up for the deficiency of traditional chemical production methods and can be promoted.

Imidazolium ILs stand out from numerous ion frontal fluids because of their simplicity of synthesis and low cost, and choline chloride based DESs are widely used due to their easy synthesis of raw materials. In this study, imidazolium ILs and choline chloride based DESs were applied to the directional hydrolysis of rutin extracted from pomelo peel for the synthesis of isoquercitrin hoping to provide new ideas for the reuse of flavonoids from pomelo peel and other pericarp.

Materials and methods

Reagents and chemicals

Five ILs (Table 2) and five DESs (Table 3) used in this study were purchased from Shanghai Cheng Jie Chemical Co., Ltd, and the whole-cell catalyst was prepared by this subject. Rutin was purchased from Shanghai Shun Bo Biological Engineering Technology Co., Ltd., and isoquercitrin standard was purchased from Sigma-Aldrich Corporation. The purity of the reagents used for HPLC detection is the chromatographic level and the other reagents are all analytically pure.

Preparation of whole cell

The strain BL21-pET21a-rhaB1 (preserved in the laboratory) was inoculated in LB liquid medium containing ampicillin resistance (50 ug/mL) (3~5 mL), then put in the incubator at 37 °C oscillating until the index period.

Hereafter, the bacterial (2%, v/v) was transferred to LB liquid medium (500 mL) containing ampicillin resistance (50 μg/mL), and cultured at 22°C until OD_{600} reached 0.6 to 0.8. Then IPTG 400 μM was added and the temperature was lowered to 17 °C for 16-20 h to induce expression of rhaB1. The liquid was centrifuged at 4 °C and 8000 rpm for 5 min to collect the cells. Then they were washed twice with PBS (pH 7.4) to obtain whole cells for catalysis.

Table 2. The ionic liquids used in the present study.

Name	Abbreviation	Molecular Formula	M_W/g·mol^{-1}
1-butyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]imide	[BMIM][Tf_2N]	$C_{10}H_{15}F_6N_3O_4S_2$	419.36
1-butyl-3-methylimidazolium hexafluorophosphate	[BMIM][PF_6]	$C_8H_{15}N_2F_6P$	284.20
1-Butyl-3-methylimidazolium tetrafluoroborate	[BMIM][BF_4]	$C_8H_{15}N_2BF_4$	226.02
1-Hexyl-3-methylimidazolium hexafluorophosphate	[HMIM][PF_6]	$C_{10}H_{19}F_6N_2P$	312.24
1-Ethyl-3-methylimidazolium hexafluorophosphate	[EMIM][PF_6]	$C_6H_{11}F_6N_2P$	256.13

Table 3. The deep eutectic solvents used in the present study.

Name	Abbreviation	T_f/°C	T_m/°C
Choline chloride/urea (1:2)	ChCl/U	12	25
Choline chloride/glycerin (1:2)	ChCl/GI	-35	--
Choline chloride/malonic acid (1:1)	ChCl/MA	10	--
Choline chloride/ethylene glycol (1:2)	ChCl/EG	-66	--
Choline chloride/acetamide (1:2)	ChCl/A	51	--

Treatment of whole-cell catalysts with ILs/ DES

Before adding the substrate, the mixture of whole-cell and ILs/DESs were put in a conventional reactor at 35 °C and 180 rpm for 30 min to change the permeability of cell membrane, then the mixture were centrifuged at 6000 rpm for 10 min, the supernatant was removed, in succession, the precipitate was washed by phosphate buffer (pH7.4) for 2-3 times to obtain treated catalyst.

Screening of ILs and DESs

E. coli strains were cultured as above, and ILs/DESs (1%, v/v) were added simultaneously with the addition of the inducer IPTG. Finally, the bacterial liquid was centrifuged at 6000 rpm for 10 min at 4 °C to collect the cells. Lately, the cells were resuspended after washing twice with PBS (pH 7.4) to prepare whole-cell catalyst. After that, the whole-cell catalyst was mixed with rutin (0.02g/L, v: v=1:1). Then, the mixtures were put into an batch reactor at 35 °C and 180 rpm for 3h, and the samples were taken every 30 min for HPLC detection.

Screening of the content of ChCl/U

The cultivated cells expressed rhaB1 were resuspended in citric acid-disodium hydrogen phosphate buffer and ChCl/U of 0%, 2%, 4% 6%, 8%, 10% (v/v) were added respectively. The mixture were then placed in a batch reactor at 35 °C, 180 rpm for 30min to obtain effective amelioration of cell permeability. Samples were separated immediately in a refrigerated high-speed centrifuge at 4 °C, 6000 rpm for 10 min and then washed with phosphate buffer (pH7.4) (repeat 2 times) to remove residual ChCl/U. Lately, equal volume substrate rutin was added. Thereafter, the mixtures were put into a batch reactor at 35 °C, 180 rpm for 3h. Then take the sample every 30 minutes for HPLC detection.

Analysis of rutin and isoquercitrin by HPLC

HPLC quantitative analyses were performed using a constant flow pump of H&E Pump P3000A (H&E Co., Ltd., Beijing, China) with a UV–VIS Detector (PLC-2, Biochem. Jinda, Ltd., Shanghai, China) and N-2000 workstation (Hangzhou Mingtong S&T Ltd., Hangzhou, China). The separation and determination of rutin and isoquercitrin with the method of an HPLC-UV were performed on an Alltima C_{18} column (250 mm × 4.6 mm, i. d.; 5 μm, W. R. Grace & Co., Deerfield, IL, USA), and it was maintained at 30 °C. The mobile phase used consisted of acetonitrile: (0.02%) phosphoric acid (volume ratio of 20: 80) at a flow rate of 1.0 ml·min^{-1} with detection of 360 nm. Before injection, all of the samples were filtered through a 0.45 μm filter. Isoquercitrin yield and rutin conversion were calculated as follows, respectively.

$$\text{Isoquercitrin yield}\left(\%\right)=\frac{\text{molar amount of isoquercitrin}\left(\text{mol}\right)}{\text{initial molar amount of rutin}\left(\text{mol}\right)}\times 100\% \quad (1)$$

$$\text{Rutin conversion (\%)} = \frac{\text{consumptive molar amount of rutin } (\text{mol})}{\text{initial molar amount of rutin } (\text{mol})} \times 100\% \quad (2)$$

Preparation of SEM samples

Equal volume of 2.5% glutaraldehyde was added to the samples, and they was placed in a refrigerator at 4 °C for 2-3 hours. Then, 0.1M phosphoric acid was added for low-speed centrifugation for 15 min and repeated 3-4 times. Then, ethanol of 30%, 50%, 70%, 80%, 90%, and 100% were used respectively for gradient dewatering, followed by drying, sticking and coating. Finally, the samples were observed on the scanning electron microscope (S-4800, Hitachi, Japan).

Reusability tests

Using the optimal conditions according to single factor tests, the whole cell catalyst was treated with ChCl/U. The substrate and the catalyst were mixed and placed in a conventional reactor for 2 hours. The reaction mixture was then taken out, and a portion of the supernatant was collected after centrifugation for HPLC detection. The precipitate was washed with phosphate buffer (pH 74) and then resuspended with this buffer. After adding a new substrate, the reaction was continued in the reactor and repeated 5 times.

Statistical analyses

Triplicate experiments were carried out for each parameter investigated. Standard deviation of the measures was calculated to check the reliability of the results. The differences in mean values were evaluated using the analysis of variance (ANOVA) method.

Results and Discussion

Screening of ILs and DESs

Fig.1A shows [BMIM][BF_4] had the highest inhibitory effect on cell growth and the isoquercitrin yield was only 23.4%. [BMIM][PF_6] showed enhanced effect with the highest isoquercitrin yield of 58.6%, which was increased by 8.9% compared to the control. The yield of products (except [BMIM][PF_6] group) was lower than the control (53.8%), indicating that the toxicity of ILs inhibits cell growth and the enzyme activity does not function properly. This is maybe ILs affected the three-dimensional structure of the enzyme, thus affecting their catalytic ability [17].

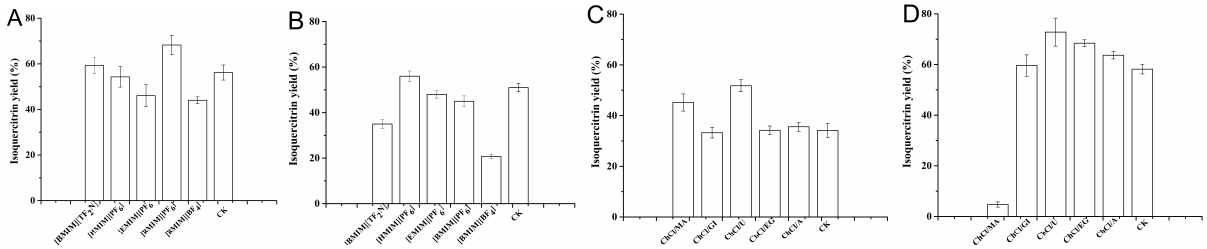

Fig. 1 Screening of ILs and DESs. Effect of ILs on growth of *E. coli* (A) and catalytic ability of cultured cells (B). Effect of DESs on growth of E. coli (C) and catalytic ability of cultured cells (D). Fermentation medium: 10 g/L of tryptone and NaCl, and 5 g/L yesat extract. Catalytic conditions: rutin concentration: 0.02g/L (pH 5.0); reaction temperature: 35 °C, rotation speed: 180rpm/min, time: 3h.

As shown in Fig. 1B, the synthesis of isoquercetin by whole cells expressing rhaB1 treated with [BMIM][BF_6] was 68.2%, which was higher than that of the blank control group (54.9%), and the yield increased by 23%. The yield of [BMIM] [BF_4] group was lowest (42.3%), which was significantly lower than that of the control, and decreased by 26.9%. The yield of isoquercitrin obtained by using the cells achieved from the ILs cultured strain as a catalyst were lower than that obtained by directly treating the cells with the ILs. This may be that ILs affect the expression of enzymes during cell growth, which in turn reduces catalytic efficiency. And the impact on cells that have already grown is relatively small.

As described in Fig. 1C, the catalytic ability of cells cultured with ChCl/GI, ChCl/EG and ChCl/A was slightly higher than that of the control (52.9%), while the cells treated with ChCl/MA and ChCl/U was significantly higher than that of the control, which the isoquercitrin yield was 61.5% and 66.7%, respectively. This may be attributed to that most of the raw materials for DESs are biocompatible, non-toxic, and greener. For example, choline chloride and urea are both biomass raw materials, so they may be less cytotoxic to cells [18]. At the same time, it could increase the permeability of the cell so that the enzyme and the substrate are in full contact [19].

Fig.1D shows that among the five DESs, there are four DESs could improve the catalytic properties of the cells. The ChCl/MA treated whole-cell catalyst had almost no catalytic ability, which the yield of isoquercitrin was only 4.7%, this probably because of its high acidity, which destroyed the surface charge of rhaB1 [20]. While the isoquercitrin yield of 72.8% with ChCl/U treatment was obviously higher than that of the control (32.8%). Therefore, ChCl/U was selected as the best solvent to increase cell membrane permeability in the selected ILs and DESs.

Effects of different contents of ChCl/U on the permeability of E. coli membrane structure

Fig.2A shows when the concentration of ChCl/U was less than 8%, the content of deoxyribonucleic acid and protein in the extracellular fluid increased with the increasing concentration of ChCl/U, indicating that nucleic acids and proteins could flow out of the cell. Deoxyribonucleic acid and protein have maximum UV absorption peak at OD_{260} and OD_{280}, respectively, and OD_{260} and OD_{280} of the extracellular fluid could reflect the state of cell membrane permeability [21]. In other words, ChCl/U had increased cell membrane permeability [22]. However, when the concentration of ChCl/U increased to 10%, the content of protein and nucleic acid in the extracellular fluid decreased, which may be a high concentration of DES, which is too toxic to the *E.coli* organelles, resulting in the inability of cell substances to flow out [23, 24]. Thus, it is necessary to understand the toxicity range of DESs to cells before applying them to improve cell permeability.

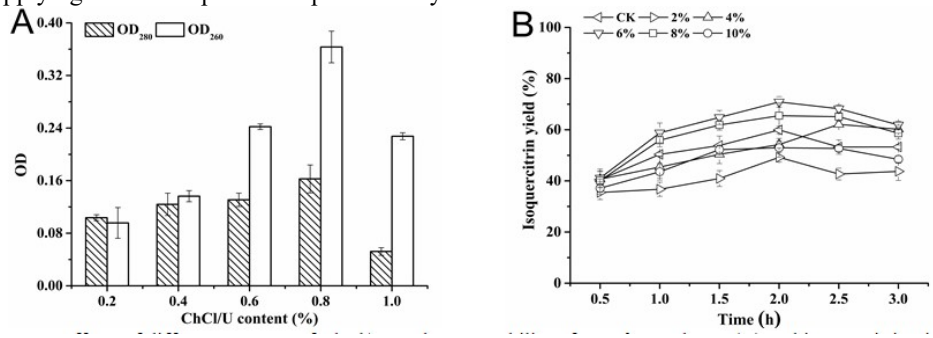

Fig. 2 Effects of different contents of ChCl/U on the permeability of *E. coli* membrane (A) and isoquercitrin yield (B). Treatment of cells with choline chloride/urea: The mixture of cells and ChCl/U were shaken in a batch reactor 180rpm/min for 30 min. Absorbance was measured at 260 and 280 nm after centrifugation of the supernatant. Catalytic conditions: rutin concentration: 0.02g/L (pH 5.0), reaction temperature: 35 °C, rotation speed: 180rpm/min, time: 3h.

Figs.2B shows the effect on the catalytic ability of of ChCl/U treated cells. The results showed that the isoquercitrin yields were lower than those of the blank control when the choline ChCl/U content was 2% and 4%. The yield reached a maximum of 70.87% at the ChCl/U content of 6% when reacted for two hours, and isoquercitrin yield showed a downward trend with the increase of ChCl/U content. More than this, when the content increased to 10%, the yield of isoquercitrin showed the lowest value of the selected conditions. These results indicate that excessive ChCl/U were detrimental to the reaction. Combined with Fig.2A, the conclusion that could be drawn is that when the content of ChCl/U was too high, its viscosity may affect the mass transfer rate. In addition, the high ionic strength in the reaction system may lead to a decrease in the conversion rate of the substrate [25]. So, content of 6% ChCl/U was selected for follow-up experiments.

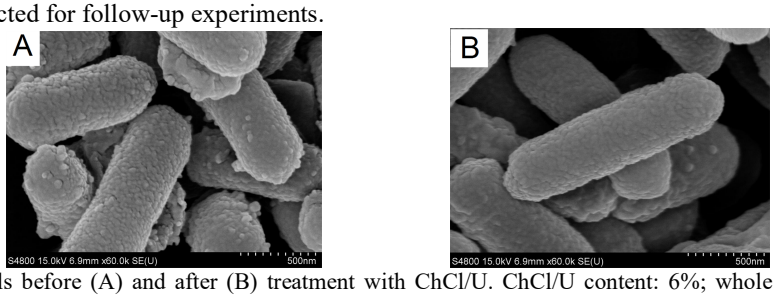

Fig. 3 SEM of *E.coli* cells before (A) and after (B) treatment with ChCl/U. ChCl/U content: 6%; whole cells concentration: 0.02g/ml; incubation temperature and time: 35 °C, 30min.

Fig.3 shows the surface of the treated cells is significantly smoother compared to the cells that have not been treated with ChCl/ U. This phenomenon could be explained that the addition of ChCl/U had disrupted the structure such as peptidoglycan on the surface of *E. coli* cells, so the surface of the cell became smooth. ChCl/U releases anions (i.e., Cl^-) and cations (cholinium) and H-bond donors (HBD) in aqueous solution [26]. Choline cations and Cl^- could interact with negatively charged carboxyl groups and positively charged amino groups rich in on the cell

surface, respectively, so the integrity of the cell membrane wass destroyed. combined with our experiment, treating the cells with a certain concentration of ChCl/U could cause damage to the cell membrane and thus increase its permeability [27], while also make the cells more active in promoting biotransformation.

Effect of temperature

Figs.4A and 4B show the effect of temperature (30-50 °C) on rutin conversion and isoquercitrin yield in the DES-containing buffer system during the whole-cell-catalyzed conversion of rutin. The concentration of rutin dissolved in phosphate buffer buffer (pH 5) was 0.02g/L. When the temperature reached 30 °C, the yield of isoquercitrin was only 13.6% at 0.5 h. The yield gradually increased as the temperature increased from 30 °C to 40°C, and the highest yield 71.56% was obtained at 40 °C within 2h. As the temperature continued to rise, the yield of isoquercitrin were significantly reduced. The yield of isoquercitrin was similar at 45 °C and 50 °C.

Temperature affects the catalytic efficiency by affecting the molecular transfer between the enzyme molecules and the hydrolyzed substrate in the solvent, and the proper temperature increase can accelerate the contact between the substrate and the catalyst [28]. When the temperature is higher than 45 °C, isoquercitrin yield was significantly reduced indicating that the whole-cell enzyme activity had been lost when the temperature exceeded 45 °C. Thence, the optimum temperature for whole-cell catalysis should be 40 °C, which is also relatively easy to achieve in industrial applications.

Effect of pH

As shown in Figs 4C and 4D, the effect of pH (5.0-7.0) on rutin conversion and isoquercitrin yield in the DES-containing buffer system during whole-cell catalytic conversion of rutin. Citric acid-disodium hydrogen phosphate buffer (pH5.0), 2-(N-Morpholino) ethanesulfonic acid (MES) buffer (pH5.5 and 6.0) and phosphate buffer (pH6.5 and 7.0) were prepared with 0.05 g/L of rutin, and the ChCl/U content were 6% (v/v). The results showed that when the pH of the buffer solution was 6.5, the yield of isoquercitrin reached 83.68%, which was the maximum. Since the pH value has a great influence on the enzyme and the substrate, which affects the conformation, active sites of the enzyme, thereby affecting its stability, indicating that the stability of rhaB1 in the cells was highest under this pH condition. which was consistent with the optimum pH of pure rhaB1 [29].

In addition, isoquercitrin yield were relatively high at pH 5.5-6.5 and the maximum yield at pH 5.5 and 6.0 was comparable to that at pH 6.5, elucidating that the whole cell catalyst had good activity under moderate acidic conditions (pH5.5-6.5). However, when the pH value gradually changes to peracid or alkaline environment, the enzyme activity was greatly lost, which may be due to the pH environment. This change would cause the hydration film or charge distribution of the protein surface layer to change, causing the structure of the protein to change, thereby deactivating the enzyme [30]. Therefore, the optimum pH environment of whole cell catalyst is 5.5-6.5, and the enzyme activity maintains good stability in this range.

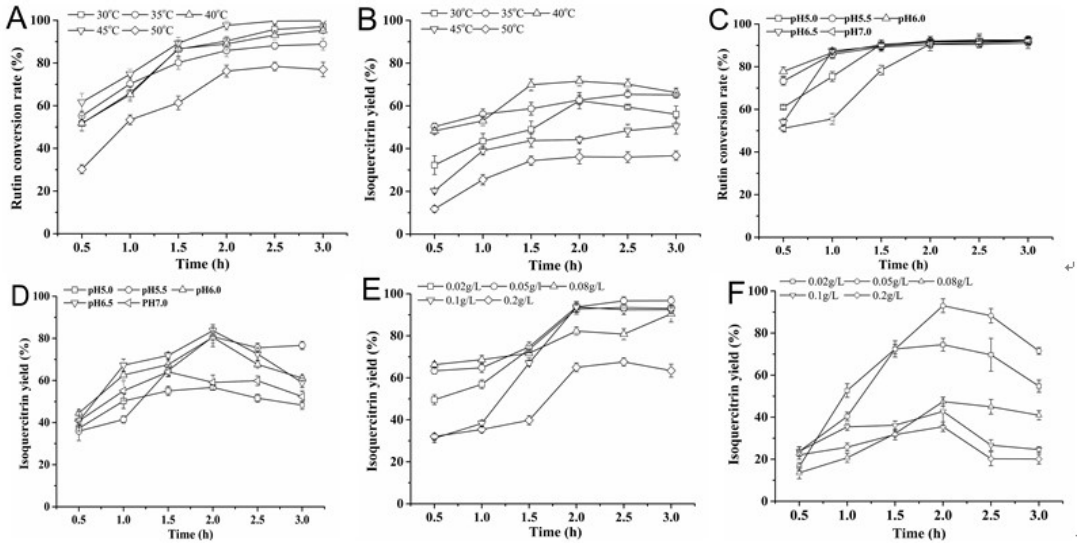

Fig.4 Effect of different factors on the enzymatic hydrolysis of rutin to synthesize isoquercitrin. Reaction conditions: pH (A) 5.0-7.0, rutin concentration: 0.02g/L, temperature: 35 °C. Rutin concentration (B): 0.02-0.2g/L, pH: 6.5, reaction temperature: 35 °C. Temperature (C): 30-50 °C, pH: 6.5, rutin concentration: 0.05g/L. All reactions were reacted in a batch reactor ,180 rpm/min for 3 hours.

Effect of substrate concentration

Figs 4E and 4F show the effect of rutin concentration (0.02-0.2g/L) on rutin conversion and isoquercitrin yield in the DES-containing buffer system during whole-cell catalytic conversion of rutin in a conventional reactor. Different concentrations of rutin was dissolving in citric acid phosphate buffer (pH6.5), and 6% ChCl/U was added. The results showed that the yield of isoquercitrin was negatively correlated with the substrate concentration after an hour of catalytic reaction. The yield of isoquercitrin reached its maximum at a substrate concentration of 0.05 g/L in 2h, which was 93.05%. When the substrate concentration was higher than 0.05 g/L, the yield of isoquercitrin decreased as the substrate concentration increased. The reason may be that a high concentration of the substrate blocked the originally permeated cell membrane, so that the substrate could not enter the cell or the enzyme could not pass through the cell membrane leading deviant contact between α-L-rhamnosidase and the substrate so that the reaction rate were reduced.

The inhibition of substrate and degradation of products may also be the cause of lower yield [31]. Compared with the substrate concentration of 0.05g/L, the yield of the product was relatively low when the substrate concentration was 0.02g/L, indicating that both the product and the substrate were unstable. Because rutin and isoquercitrin are flavonoids with high antioxidant activity, rutin could be easily hydrolyzed into hyperoside in natural conditions, and isoquercitrin could also be easily derivatized into other compounds [32, 33]. Therefore, starting from the angle of mass production of isoquercitrin and suitable substrate concentration, then the substrate concentration of 0.05g/L is the best choice.

Operational stability of whole-cell catalyst

Fig.5 shows that the whole cell catalyst was reused 5 times in a conventional reactor under optimal conditions. The yield of isoquercitrin was above 80% in the first two cycles, demonstrating the stability of the enzyme in the whole cell and the higher activity. Compared with the first two cycles, the isoquercitrin yield was reduced by about 30% in the subsequent reaction, which indicates that the stability of the whole cell catalyst has started to decline since the second cycle.

After treated with ChCl/U the permeability of whole-cell catalyst was increased, and the cell diameter became larger. On the one hand, it was favorable for the substrate to enter the cells binding to the enzyme [34], thereby improving the catalytic efficiency. On the other hand, the cell surface became thinner and the pore size increases. And during the progress of reuse, the cell wall could break down and the intracellular substances were lost, thus the catalytic performance of rhaB1 decreased with the loss of its microenvironment.

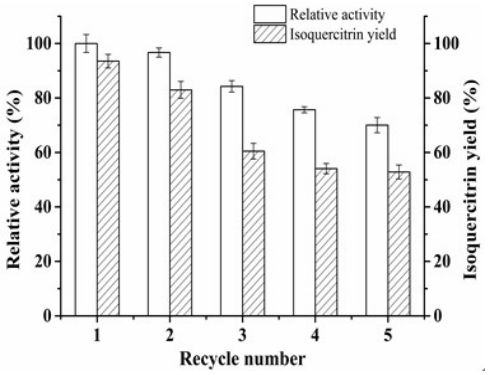

Fig.5 Effect of reusability of ChCl/U-treated whole-cell catalyst on the yield of isoquercitrin. Reaction conditions: catalyst concentration: 0.02g/ml; ChCl/U amount: 6%; substrate concentration: 0.05g/L; temperature: 40°C; rotational speed: 180rpm, each cycle reacted for 2 hours.

Comparison of enzyme rhaB1and whole-cell catalyst expressing rhaB1

Table 4 shows the comparison of hydrolysis of rutin to isoquercitrin in a conventional reactor between whole-cell catalyst prepared from recombinant *E.coli* and crude rhaB1. Isoquercitrin yield of the whole-cell catalyst treated with ChCl/U was slightly lower than that of crude rhaB1, but the reaction time was shortened by 4/5 and the substrate concentration was 5 times that of rhaB1. In addition, whole-cell catalyst have higher pH and temperature tolerance than crude rhaB1. Because rhaB1 existed in the intracellular are protected by microenvironment of cells, which are the key factors that ensure high catalytic activity, high selectivity, and high stability of entoenzyme [35]. The crude rhaB1 was very unstable after being detached from its physiological environment, and was easily affected by the surroundings [36]. Simultaneously, the conditions during the reaction will make the enzyme inactive and reduce the catalytic effect. In addition, the separation and purification of the enzyme were complicated, and it also

leaves contamination in the reaction system thus it is difficult to separate it from the product after the reaction and cannot be reused. The whole-cell catalyst was different from crude rhaB1 because of its form of existence. It can be used again after centrifugation and maintains a certain amount of enzyme activity. Therefore, the use of whole-cell catalysts has significant advantages in synthesis of isoquercitrin compared to crude rhaB1.

Table 4. Comparison of catalytic activity between crude rhaB1 and rhaB1whole-cell catalyst.

Catalyst type	pH_{opt}	T_{opt}/°C	t_{opt}/(h)	Substrate concentration $_{opt}$ (g/L)	Yield (%)
Whole-cell [a]	6.5	40	2	0.05	93.05±1.3%
Crude rhaB1 [b]	5.0	35	10	0.01	98.3±3.8%

[a] Reaction condition: rutin concentration 0.05 g/L, reaction temperature 40 °C, 180 rpm/min for 2 h, whole-cell catalyst (rhaB1 30 mg/mL) treated with 0.06 g/mL ChCl/U for 30min.
[b] Reaction condition: rutin concentration 0.01 g/L, reaction temperature 35 °C, 180 rpm/min for 10 h, crude rhaB1 30mg/mL, 0.02 g/mL. [Toma][Tf_2N]-buffer (pH 5.0) as the reaction medium.

Statistical analyses

The results of the one-way analysis of variance (ANOVA) are shown in Table 5. The effects of temperature and substrate concentration on the yield of isoquercitrin were extremely significant because the P value was less than 0.01. The effect of pH on yield was not significant because of the P value was 0.2, which was higher than 0.05.

Table 5. Single factor analysis of variance.

Factor	SS	*df*	MS	*F*	*P*
Temperature	4440.392	4	1110.098	11.575	0.000
pH	1083.846	4	270.962	1,.614	0.202
Substrate concentration	7105.594	4	1776.398	5.881	0.002

Conclusions

The best solvent ChCl/U was selected from five ILs and DESs to improve the catalytic ability of whole-cell catalysts expressing rhaB1. The appropriate ChCl/U content was 6%, and the optimum temperature and pH were 40 °C and 6.5 respectively which were higher than the crude rhaB1 (35 °C, pH5.0). The cellular microenvironment provided a corking protection for the rhaB1, hence, the tolerance to the external environment was improved, as a result, the substrate concentration also increased. The ChCl/U -treated whole-cell catalyst can effectively improve the permeability of the cell membrane so that improve the mass transfer of cells to exert its catalytic action in a greater extent. Thus, the application of DESs in the whole-cell biotransformation of rutin extracted from pomelo skin provided an effective way to recycle flavonoids in rutaceae plants.

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Electronic Supplementary Material

Table S1 Variance analysis of influence of temperature on isoquercitrin yield.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		4440.392	4	1110.098	11.575	.000
	Linear Term	Contrast	2118.239	1	2118.239	22.087	.000
		Deviation	2322.153	3	774.051	8.071	.001
Within Groups			2397.558	25	95.902		
Total			6837.951	29			

Table S2 Variance analysis of influence of pH on isoquercitrin yield.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		1083.846	4	270.962	1.614	.202
	Linear Term	Contrast	117.754	1	117.754	.701	.410
		Deviation	966.092	3	322.031	1.918	.152
Within Groups			4197.044	25	167.882		
Total			5280.890	29			

Table S3 Variance analysis of influence of rutin concentration on isoquercitrin yield.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		7105.594	4	1776.398	5.881	.002
	Linear Term	Contrast	4459.734	1	4459.734	14.783	.001
		Deviation	2645.859	3	881.953	2.920	.054
Within Groups			7552.035	25	302.081		
Total			14657.629	29			

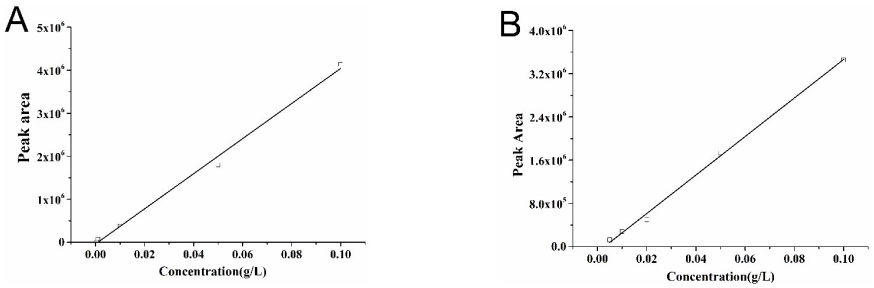

Fig. S1. The standard curves of rutin (A) and isoquercitrin (B).