Utilization of rutin extracted from pomelo peel using a PVDF biocatalytic membrane reactor coupled with reaction and separation

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Abstract: This study reported the catalytic conversion of rutin in a novel PVDF biocatalytic membrane reactor. Isoquercitrin, the natural rare flavonoid with more obvious pharmacological effects, was synthesized by using rutin extracted from abandoned pomelo peel. The PVDF hollow fiber membrane with a filtration precision of 0.1 μm was selected as the membrane module. Recombinant *Escherichia coli* whole cells were used as catalysts, and the product separation was effectively achieved in reaction-separation coupling system. The effects of extractant-substrate volume ratio, reaction temperature, pH value and substrate concentration on the whole-cell catalytic reaction were investigated. The optimum reaction conditions were as follows: at 40°C, extractant: substrate=3:4, pH=5.5, substrate concentration was 0.02 g/L. The maximum isoquercitrin yield obtained in the system was 87.92±2.93%. After five reaction run, the relative enzyme activity of the whole-cell catalyst remained above 50%. The membrane after 10 h of reaction was washed 7 times with 1% (v/v) aqueous HCl solution, and the membrane flux was restored to 75%. In order to evaluate the performance of the system, the isoquercitrin yield was further compared with that of the system without extractant under the same conditions. The results showed that the yield of the system with extractant showed an upward trend within 0-8 h, and reached the maximum at 8 h, which was 82.2±2.3%. On this basis, the catalytic reaction was no longer limited by factors such as product inhibition, and this method can be applied to other biocatalysis.
Keywords: rutin**,** whole-cell catalyst, biocatalytic membrane reactor, reaction-separation coupling

Introduction

Rutin is a kind of flavonoids extracted from plants, which has many biological activities and medicinal value. Rutin is widely distributed in the plant kingdom. Some traditional Chinese medicines all contain rutin, such as jujube, hawthorn, wolfberry, pomelo [1]. And, the rutin content in pomelo peel is rich. Every year, a large amount of pomelo peel waste is produced during the grapefruit processing [2]. Pomelo waste causes environmental pollution and great waste of resources. In order to make full use of the discarded resources, research on active ingredients in pomelo peel extracts has been widely carried out. Studies showed that it was feasible to hydrolyze rutin extracted from pomelo peel waste to isoquercetin catalyzed by rhamnosidase. As a natural rare flavonoid, isoquercitrin has many pharmacological effects such as anti-oxidation, blood sugar lowering and blood pressure lowering [3]. However, the pharmacological activity of isoquercetin with only one terminal rhamnose less than rutin was significantly higher than that of rutin [4]. Therefore, the use of extracts of pomelo peel waste produced by the citrus juice industry to effectively synthesize rare substances can alleviate environmental stress, and can more effectively utilize biological resources.

Traditional chemical synthesis of flavonoids often leads to the emergence of by-products. Compared with chemical transformation, enzymatic transformation has higher selectivity under mild conditions [5]. The enzyme has been completely inactivated before the end of the reaction in traditional batch reactor. In particular, the operation cost and product quality will be seriously affected by the filling of enzymes in the process of dealing with large-scale raw materials. During the continuous reaction process, the stability of the enzyme can be better guaranteed by immobilizing the enzyme on the insoluble carrier, and it is conducive to promoting the reuse of the enzyme and improving the production performance of the reaction system. The immobilized carriers have been reported as nanofibers, nanospheres / microbeads, gelatin and capsule [6]. Enzymes can be immobilized on these carriers by adsorption or covalent binding. However, due to the existence of steric hindrance or interface effect, there are some limitations in the application of immobilized enzymes [7]. Enzyme membrane reactor with synergistic catalysis and separation performance has shown great advantages in product synthesis and separation in practical applications. Among them, the membrane can selectively separate products and catalysts, and can also be used as a catalyst carrier [8]. According to the need of practical application, the enzyme molecule can be immobilized on the surface or pore of the membrane, and can even circulate freely in the interception side [9]. In the process of reaction, the pressure-driven cycle can increase the load of enzyme and ensure the activity of enzyme adequately.

The selection of membrane materials is very important for the reaction system. A good supporting material should have high porosity to reduce filtration resistance and avoid the degradation of overall mechanical properties caused by too large membrane holes [10]. At the same time, the tolerance of membrane materials to high temperature, acid and alkali, the degree of pollution resistance and the price are also the key factors to be considered. At present, polysulfone materials are widely used as membrane materials for sewage treatment, which has strong corrosion resistance to chemical reagents. However, its strong adsorptivity to organic substances, especially proteins, can easily lead to fouling and blockage of the membrane reactor [11]. Therefore, the application of polysulfone membrane materials in biocatalytic reactions is not advantageous. PVDF membrane is one of the preferred materials because of its good impact resistance, wear resistance and chemical stability [12].

Based on the above, the whole cell of *Escherichia coli* BL21-pET21a-*rhaB1* expressing rhaB1 was used as catalyst, and hollow fiber polyvinylidene fluoride (PVDF) microporous membrane was selected as module to prepare enzyme membrane reactor. Whole-cell catalysts are driven by peristaltic pumps to enter the membrane and form a stable enzyme membrane on the inner surface, thus eliminating the use of cross-linking agents and other stationary agents. In addition, in order to improve the product recovery, glyceride triacetate was used as extractant to enhance the enrichment of isoquercitrin. The performance of biomembrane reactor was investigated by enzymatic hydrolysis. The effects of different conditions on the yield of the product were investigated, and the inner surface of the membrane before and after the introduction was characterized. In order to evaluate the efficiency of the system, the reaction parameters of different systems were compared.

Materials and methods

Material

PVDF membrane was purchased from Guangzhou Hexin New Material Technology Co., Ltd., and PVC membrane was purchased from Shenzhen Jinglaiquan Water Purification Equipment Co., Ltd. Syringe (1 mL) was purchased from Zhenjiang Huadong Instrumental Glass Co., Ltd., and silicone tube was purchased from Cartral Fluid (Shanghai) Technology Co., Ltd., and epoxy resin glue was purchased from Shenzhen Mizhan Technology Co., Ltd.. Rutin was purchased from Shanghai Shun Bo Biological Engineering Technology Co., Ltd., and isoquercitrin standard was purchased from Sigma-Aldrich Corporation. The BL21-pET21a-*rhaB1* strain was previously saved in the laboratory.

Membrane selection

Comparing different membrane materials and taking the changes of enzyme concentration during the whole cell catalyst introduction as the indicator to determine the preparation time of the enzyme membrane.

Whole-cell catalysts preparation

The BL21-pET21a-*rhaB1* strain was inoculated in LB liquid medium containing ampicillin (50 μg/mL) and cultured in a 37°C constant temperature shaker for the indicated periods. Then, the bacterial culture was transferred to a new LB medium containing ampicillin (50 μg/mL) at 2% (v / v) transfer and cultured at 22°C for 8h until the OD_{600} reached 0.6 to 0.8. The inducer IPTG was added to a final concentration of 400 μM to induce expression of rhaB1 at 17℃ for 18-20 h. And the bacterial solution was centrifuged at 4°C and 5000 rpm/min for 15 min to collect the cells. Finally, the cells were washed 1-2 times with PBS (pH=7.4) and stored at -20 °C for later use.

The stored cells (4%, v/v) were uniformly mixed with choline chloride-urea (6%, v/v) and placed in a 35°C water bath shaker at 180 rpm/min for 30 min. Then, the mixture was centrifuged at 6000 rpm/min for 5 min to remove the supernatant, and the precipitate was washed 1-2 times with PBS (pH=7.4) to prepare a whole-cell catalyst.

Membrane reactor preparation

In this study, two ends of 4 strands of PVDF hollow fiber membranes were fixed by epoxy resin adhesive (Both ends were still unblocked), and the membranes were bonded with 1 mL syringe to prepare the membrane module. The membrane module was connected to the peristaltic pump, the substrate phase and the catalyst through a silicone tube. For the preparation of an enzyme membrane on the inner surface of the membrane, the whole-cell catalyst was introduced into the PVDF hollow fiber membrane at 30 rpm/min for 60 min under the impetus of peristaltic pump. The system is capable of maintaining a constant temperature and circulation of whole-cell catalysts. The configuration of the membrane reactor is given in Fig.1. Membrane modules after 1h and 10h of the whole-cell introduction was allowed to stand at room temperature for a while, and then broken by liquid nitrogen. The inner surface of the film was then characterized by field emission scanning electron microscopy.

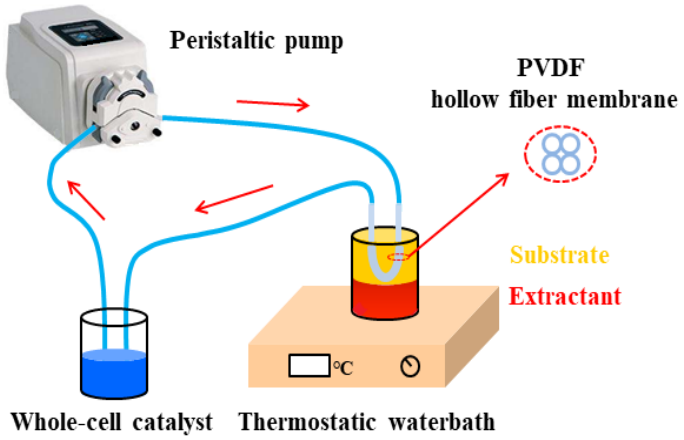

Fig. 1. PVDF Membrane Bioreactor set-up

Construction of reaction-separation coupling system

The PVDF membrane reactor is a combination of a membrane reactor and a recycle separation system. The rutin substrate solution was prepared with a citric acid-dibasic phosphate buffer solution (pH=5.0) for whole cell catalysis. Meanwhile, triacetin was used as an extractant, and extractants of equal volume with substrates were added to separate the product during the reaction. The samples were taken from the reactor every 2h for determination by HPLC, and the product yield was calculated. So as to understand the system efficiency, the reactions without an extractant was also carried out under the same operating conditions in the same reactor. And the product yield in the reaction system with or without the extractant was compared.

Optimization of whole cell catalytic reaction conditions

This study focused on increasing the conversion of rutin originated from pomelo peel extract through timely separation of products, and achieving multiple reuses in PVDF membrane reactor. The conversion of rutin to isoquercetin in enzymatic membrane reactor was analyzed by high performance liquid chromatography, and the effects of volume ratio of extractant to substrate, temperature, buffer pH and substrate concentration on isoquercetin yield were investigated to explore the optimal reaction conditions. Furthermore, flux and reuse times were determined to evaluate the selective characteristics and recovery properties of the membrane.

Before further study of the effects of different single factors, we conducted a large number of preliminary experiments. In order to verify that the whole-cell catalyst is immobilized on the inner surface of the membrane instead of impurities, the assay was performed using fluorescently labeled *rhaB1-EGFP* whole cells. After the whole cells were introduced into the hollow fiber membrane for 1 h, the membrane module was disassembled and soaked in ultrapure water, and then the soaking solution was observed under a 365 nm ultraviolet lamp [13]. Compared with the rhaB1 enzyme solution, the soaking solution emits green fluorescence under the irradiation of the ultraviolet lamp. The results proved that the whole-cell catalyst is fixed on the inner surface of the membrane.

Based on the above, rutin substrate solution of required concentration was prepared with MES buffer (pH=6). The volume ratio of catalyst to rutin was 1:1. The flow rate was 30 rpm/min, and the reaction temperature was 40°C. The volume ratio of extractant to rutin was 1:4, 1:2, 3:4 and 1:1. In order to study the effect of temperature on isoquercetin yield, the reactions were carried out at 30, 35, 40 and 45°C. Based on the results of the pre-test, the volume ratio of extractant to rutin was 3:4. Buffers used to prepare the substrate solution with different pH were prepared as citric acid-disodium hydrogen phosphate buffer (pH=5), MES buffer (pH=5.5, 6), disodium hydrogen phosphate-potassium dihydrogen phosphate buffer (pH=6.5). The different concentration of substrate affecting isoquercitrin yield was determined as 0.01 g/L, 0.02 g/L, 0.03 g/L and 0.04 g/L. The flow rate and the reaction temperature were the same as above.

All reactions were carried out for 10 h in membrane reactors. During the reaction, the samples were taken from the reactor every 2 h. Three parallel samples were set for each sample and analyzed by HPLC. Standard deviation of the measurements was calculated to check the reliability of the results. The differences in mean values were evaluated using the analysis of variance (ANOVA) method.

Membrane cleaning

Then, the membrane after 10 h was washed with 1% (v/v) HCl (pH=1.05), and the cleaning effect was judged by the membrane flux recovery rate. The membrane flux recovery rate was calculated from the measured weight of permeate sample, as shown in Eq.1

$$J = \frac{V}{T \times A} \tag{1}$$

$$n = \frac{J_n}{J_o} \times 100\% \quad (2)$$

Where V is the sampling volume (L), T is the total sampling time (h), A is the effective membrane area (m2), Jo and Jn are the initial membrane flux and membrane flux after each washing.

Reuse of enzyme membrane reactor

In addition, under the optimal reaction conditions obtained by the single factor test results, the whole-cell catalytic reaction was carried out in the enzyme membrane reactor, and the isoquercitrin yield and enzyme activity were measured after 6 h. Then, new identical substrate was replaced and the above reaction was repeated to count the recyclability of the whole-cell catalyst.

Results and discussion

Membrane material selection

Both microfiltration membranes and ultrafiltration membranes can be used in enzyme membrane reactors. Microfiltration membrane has a high permeability but is incapable of effectively trapping or immobilizing the enzyme catalyst [14]. But, ultrafiltration membranes can effectively retain enzyme molecules that are not adsorbed on the membrane surface. Common ultrafiltration membrane materials are shown in Table 1. And, PVDF hollow fiber membranes meet the needs of this study, which have high chemical stability, good anti-adsorption and anti-pollution properties. In addition, the diameter of E. coli whole-cell is 0.5×1~3 μm. The selected membrane material can't effectively trap the whole cell catalyst on the membrane inner surface unless its pore diameter is less than 0.5 μm. Since the organic solvent was used in this study, the PVDF membrane material with strong resistance to acid and alkali was selected as the membrane module for further research, which diameter was 0.1 μm.

Table 1 Comparison of common membrane materials

Material	Hydrophilic /hydrophobic	Density (g/cm^3)	Advantage	Aperture (μm)	Refs
PVDF	hydrophobic	1.77-1.80	High stability to temperature and chemicals	0.10	[15]
PVC	hydrophobic	1.38	High tensile and compressive strength	5.00	[16]
PES	hydrophobic	1.37	High mechanical and tensile strength	0.45	[17]
PAN	Hydrophilic	1.184	Strong anti-pollution ability	0.01	[18]
PP	hydrophobic	0.90	Heat resistance, non - toxic, good electrical insulation	1.00	[19]
PSF	hydrophobic	1.05	Chemical resistance	0.10	[20]

Construction of reaction-separation coupling system

As shown in Fig.2, the yield of isoquercitrin in the reaction system with or without added extractant is compared. The maximum yield obtained at 4 h was 44.5±6.1% in the system without extractant, and the product yield in the extractant group was 49.4±1.2%. There was no significant difference between the two group ($P>0.05$). As the reaction continues, the yield in the no-extractant system showed a downward trend. At 8 h, the yield dropped by 37%. However, the yield of the extractant system showed an upward trend within 0~8 h. The maximum yield obtained at 8 h was 82.2±2.3%. And, the difference between them was very significant ($P < 0.01$).

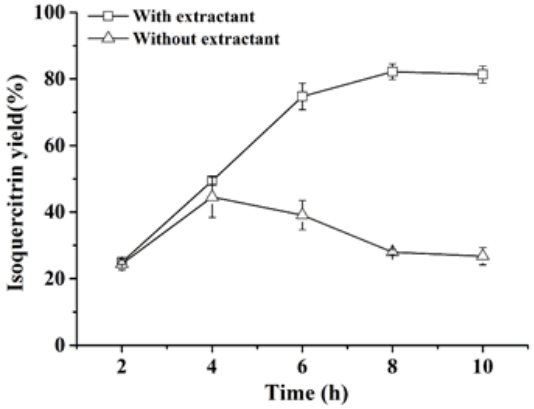

Fig. 2. Isoquercetin yield with and without extractant in the reaction system.

The results showed that in the system with extractant, the products were enriched in the extractant phase in time. The reactor maintained good material transport efficiency. Under the condition that the performance of the membrane module and the activity of whole-cell catalyst were not damaged, substrates were continuously hydrolyzed to products. However, in the system without extractant, the micropore on the inner surface of hollow fiber membrane was blocked prematurely because the product failed to be separated from the reaction system in time. As a result, the reaction was terminated due to the failure of the substrate to bind to the catalyst. Moreover, isoquercitrin was easily converted into hydroxypropyl derivative after long time retention [21], so the yield was not increased or even decreased. The addition of extractant increased the product yield by 1.8 times, which indicated that the reaction-separation coupling system was successfully constructed in the membrane reactor.

Effect of extractant to substrate volume ratio on isoquercitrin yield

Fig.3a shows the effect of the extractant to substrate volume ratio on isoquercitrin yield in the enzyme membrane reactor. When the volume ratio of extractant to rutin increased from 1:1 to 1:2, the yield of isoquercitrin increased from 41.2±3.1% to 60.2±2.1%. Moreover, the acquisition time of the maximum yield was shortened. The reason is that the increase of extractant accelerates the product enrichment and the product transfer to extraction phase accelerates the reaction to go forward [22]. Then, the isoquercitrin yield reached 61.9±4.5% when the volume ratio increased to 3:4. The maximum yield did not increase significantly and the acquisition time was delayed. Triacetin, as an extractant, is oil liquid with high viscosity. Its excessive addition may cause an increased probability of contact with the membrane, thus blocking the outer surface of the membrane and hindering the material transport inside and outside the membrane [23]. As the volume of the extractant was further increased, the yield of isoquercitrin dropped sharply and the maximum value was only 21.8±4.3%. This indicates that the membrane was further fouled with the increase of organic solvent, and the product was also contaminated to some extent. From the point of view of green chemistry, in order to avoid excessive use of organic solvents and shorten reaction time, the volume ratio of extractant to rutin was selected to be 1:2 for the further study.

Effect of temperature on isoquercitrin yield

Before the effect of temperature was determined in the PVDF membrane reactor, many preliminary experiments had been done. The suitable temperature of tubular PVDF membrane is 5°C~45°C, and the membrane will be deformed beyond this temperature range. The micropore in the membrane is blocked by extrusion, which hinders the mass transfer inside and outside the membrane [24]. Meanwhile, whole-cell death at temperatures above 50°C would also lead to a sharp decline in transformation efficiency. Therefore, the upper limit of temperature is determined to be 45°C. In addition, it was observed that the activity of α-L-rhamnosidase in the whole cell was very low at temperatures below 30°C. Therefore, the low temperature limit of this study was determined to be 30°C.

Fig.3b shows that the maximum yield of isoquercetin was obtained at 40°C. According to Arrhenius equation in an endothermic reaction, the reaction rate increased with temperature. When the temperature increased from 30°C to 40°C, the yield increased from 43.0±1.8% to 74.4±5.0%.The yield of the product reached a maximum at 40°C. When the temperature is low, the binding rate with substrate is relatively slow due to the low activity of enzymes in cells, which resulted in a low reaction rate. With the increase of reaction temperature to 40°C, the isoquercetin yield improved significantly and the maximum yield was 72% higher than the original yield. The temperature, rised to 45°C, is still within the optimum temperature range of whole-cell catalyst [25]. But the PVDF membrane gradually deformed. It was concluded that the effect of temperature on membrane materials was greater than that on enzymes. In order to ensure the performance of the membrane and the reaction as expected, the next step experiment was carried out at 40°C.

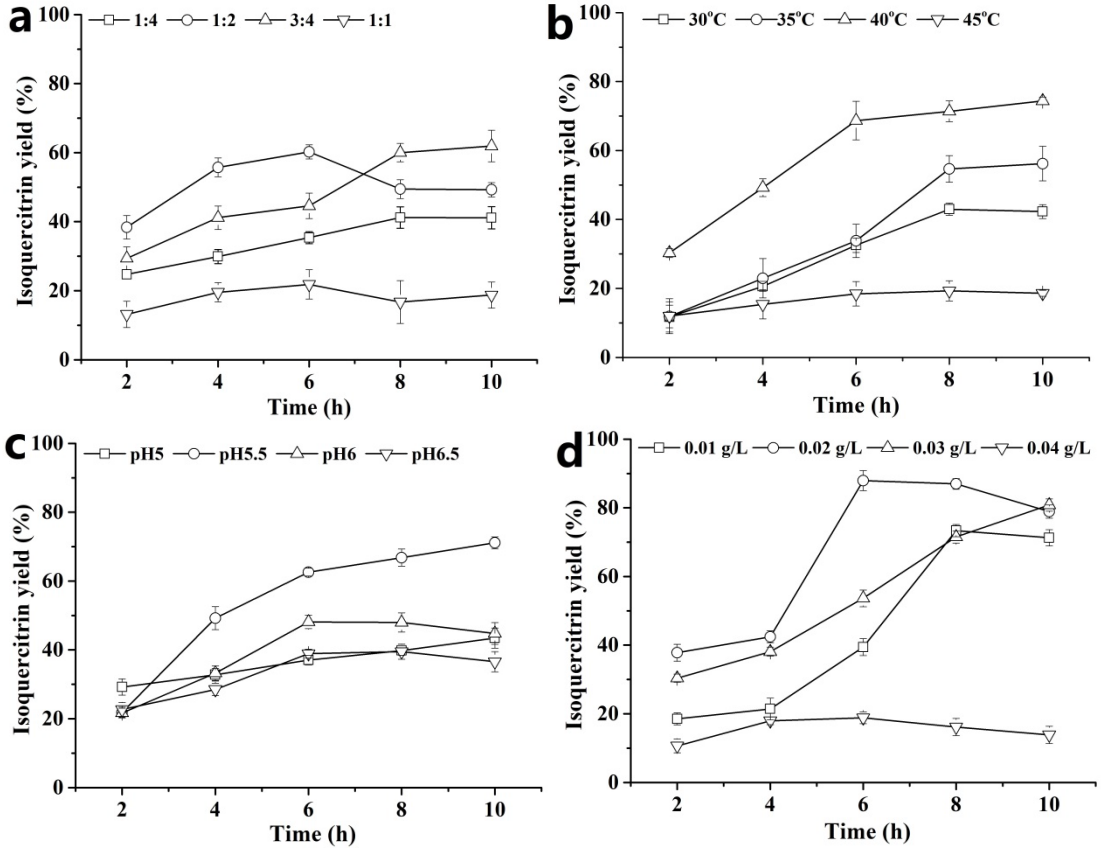

Fig. 3. Effect of different factors on the enzymatic hydrolysis of rutin to produce isoquercitrin in a membrane reactor. (a) Effect of extractant to substrate ratio on isoquercitrin yield at temperature 35°C, rutin concentration 0.02 g/L, pH=6.5; (b) effect of temperature on isoquercitrin yield at extractant: rutin=2:1, rutin concentration 0.02 g/L, pH=6.5; (c) effect of pH on isoquercitrin yield at extractant: rutin=2:1, temperature 35°C, rutin concentration 0.02 g/L; (d) effect of rutin concentration on isoquercitrin yield at extractant: rutin=2:1, temperature 35°C, pH=5.5. The flow speed was 30 rpm/min, reaction time was 10 h.

Effect of pH on isoquercitrin yield

Fig.3c shows the effect of pH on the yield of isoquercitrin in the enzyme membrane reactor. When the pH was 5, the maximum yield of isoquercetin was only 43.4+2.9%. With the increase of pH to 5.5, the yield of isoquercetin increased significantly and the maximum value was 71.1+1.7%.Buffer pH has important effects on the solubility of enzymes and substrates in many aspects, such as the conformation, catalytic activity and stability of enzymes. The solubility of rutin in buffer solution (pH=5) is low, so the reaction efficiency is low [26]. With the increase of pH, the yield of isoquercitrin increased significantly. The main reason is that the solubility of rutin increased and the activity of α-L-rhamnosidase at pH=5.5 is higher than that at pH=5.0 [27]. Both of which can increase the probability of binding of the enzyme to the substrate, thus promoting transformation and increasing product formation. However, the isoquercitrin yield in the enzyme membrane reactor gradually decreased while the pH value continued to rise. During the enzymatic reaction, the optimum pH of the enzyme is closely related to the PK value of the groups involved in catalysis and the substrates bound by the enzyme activity center [28]. Usually only one dissociation state is most favorable for the combination of enzyme and substrate, so the optimal pH value for the binding of enzymes to substrates was 5.5.

Effect of rutin concentration on isoquercitrin yield

The substrate concentration was another determinant factor for the performance of this reaction. The results of the membrane reactor with whole-cell catalyst are presented in Fig.3d. The figure shows information about the effect of substrate concentration on rutin conversion. Better results were obtained when the substrate concentration was 0.02 g/L, the maximum yield of isoquercitrin was 87.92±2.93%. When the substrate concentration increased from 0.01 g/L to 0.02 g/L, the yield of isoquercitrin increased gradually, and the acquisition time of the maximum yield was shortened. With the concentration of the substrate increased, the chance of enzyme molecule binding with substrate increased, which accelerated the reaction rate and increased the products released in the same time. When the substrate concentration continued to increase, the yield showed a downward trend, and the maximum product yield respectively decreased from the highest 87.92±2.93%

to 80.82±1.79% and 18.86±1.71%. Furthermore, substrate inhibition and product degradation are possible causes of product yield decline. Rutin can be hydrolyzed into hyperoside and paclitaxel, and the clogging of the membrane caused by high concentration of substrate can also lead to the degradation of the performance of the reaction system. Isoquercitrin can also be degraded to hydroxypropyl derivatives, resulting in a decline in yield [29]. Hence, 0.02 g/L is selected as the optimum substrate concentration to ensure a high yield of the product.

Comparison of parameters for synthesis of isoquercitrin in different reaction systems

Based on the reaction system of rutin hydrolysis to isoquercitrin, the catalytic effects of pure enzyme, enzyme crude and whole cell catalysts in different reactors were compared in Table 2. The whole cell of *Escherichia coli* BL21-pET21a-*rhaB1* expressing rhaB1 was used as catalyst in the PVDF membrane reactor. Compared with the reaction time of free enzymes in conventional reactor, the whole cell catalysis in membrane reactor was reduced from 10 h to 6 h. The products can be enriched and separated timely by adding extractive phase, which promoted the hydrolysis of rutin to produce isoquercitrin and shortened the reaction time. In addition, the influence of reaction conditions on free enzymes can easily lead to the decrease or even loss of enzymes activity in the process of reaction. It is difficult to achieve product separation and reuse because the separation and purification of enzymes are complex [30]. However, the whole-cell catalyst circulated in the hollow fiber membrane and formed a stable cell membrane on the surface. Meanwhile, the pH of the whole-cell catalyst (pH=6.5) and temperature resistance (40 ℃) were higher than rhab1 crude enzyme solution (pH=5.0, 35 ℃) under the protection of the cell microenvironment. This indicates that the whole-cell catalyst in the membrane reactor has better catalytic activity and stability. Even the whole-cell catalyst can be reused after centrifugation and maintains a certain enzyme activity. This further demonstrates a significant advantage in the catalytic synthesis of isoquercitrin on biofilms.

Table 2 Comparison of parameters for enzymatic synthesis of isoquercitrin in different reactor

Reactor and reaction system	Catalyst type	Substrate concentration (g/L)	Enzyme concentration (g/L)	T_{opt} (℃)	pH_{opt}	t_{opt} (h)	Yield (%)
Conventional reactor[a]	Hesperidinase	0.71	50	40	9.0	10	91.46±0.55
Conventional reactor[b]	Crude rhaB1	0.01	30	35	5.0	10	98.3±3.8
PVDF membrane reactor[c]	Whole-cell	0.02	30	40	6.0	6	87.92±2.93

[a] Reaction system: substrate, enzyme and ionic liquid.

[b] Reaction system: substrate, rhaB1 crude enzyme solution.

[c] Reaction system: substrates, whole-cell catalyst and extractant.

Morphology and inner surface characteristics of PVDF membrane

SEM images are shown in Figure 4, which represents the change in the internal surface morphology of the PVDF hollow fiber membrane before and after the whole-cell catalyst introduction. Fig.4a and Fig.4b represent the structures of untreated membranes. The micropores in the membrane are densely distributed and exhibit a rough irregular shape (Fig.4a). However, in a much smaller field of view, tooth shapes regularly arranged in the inner wall (Fig.4b). After 1 h introduction of the whole-cell catalyst, the whole cells polymerized with the membrane micropores and gelatinized on the inner surface of the membrane to form a smooth lay [31].

As can be observed from Fig. 4c and Fig. 4d, the number of membrane micropores is less than that of the untreated membrane. This indicated that some of the whole cells have been fixed to the inner membrane surface. Fig.4e and Fig.4f showed that the membrane porosity decreased significantly after 10 h. With continuous flow of catalyst, the number of cells and the viscosity of the solution membrane increased, which resulted in the slow movement of the molecules in the membrane [32]. Even the further solidification of the gelation polymer resulted in the formation of a tight network structure in the membrane. Ultimately, most of the substances in circulation were intercepted on the inner surface, which gradually restricted the separation of substances. And even the membrane blocked. Therefore, the membrane should not be used continuously for a long time, and it needs to be cleaned regularly for reuse.

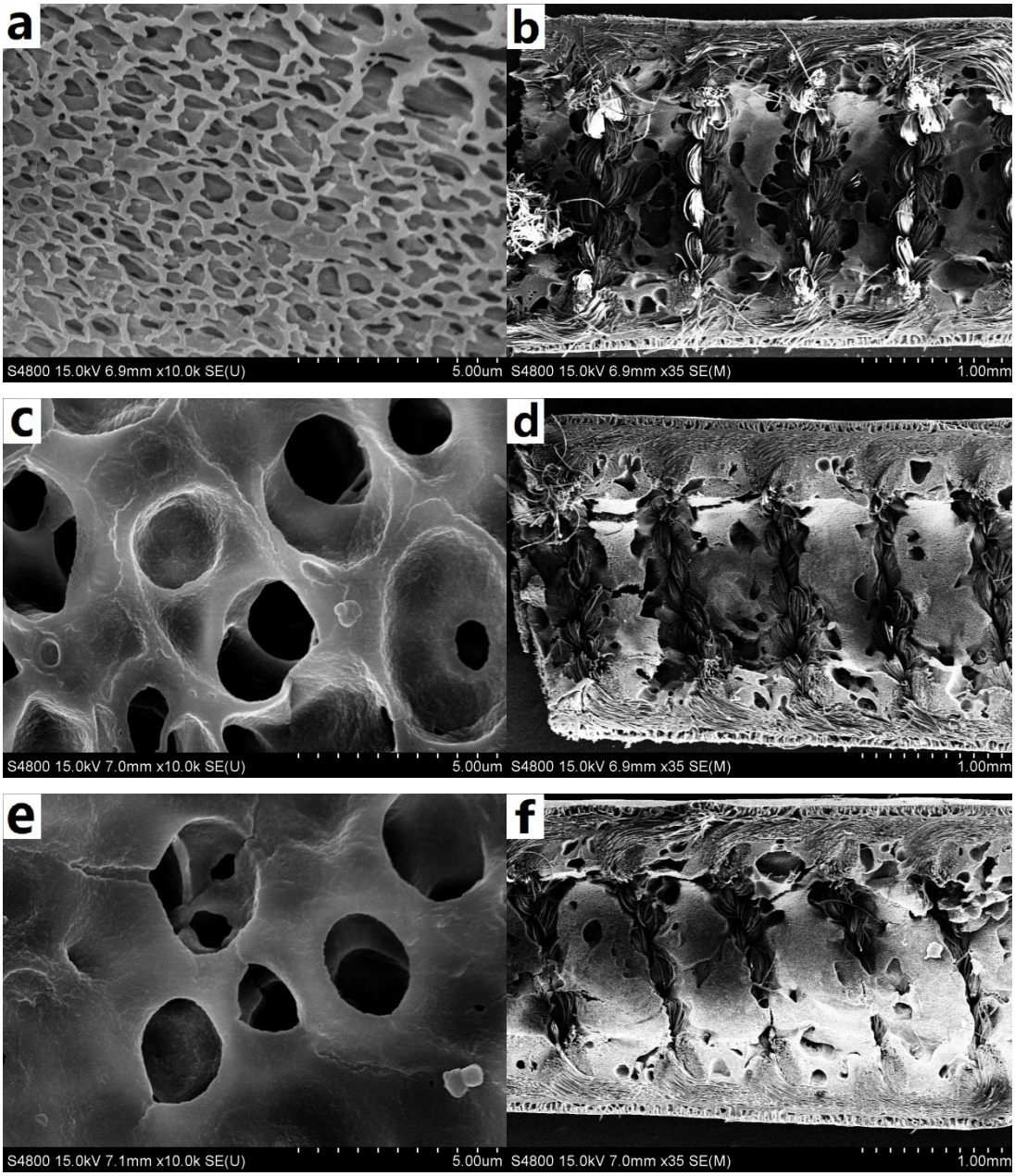

Fig. 4. SEM of the PVDF membrane before and after whole-cell introduction. The morphology of the membrane surface (a) and the channel (b) before the whole-cell introduction; The morphology of the membrane surface (c) and the channel (d) after 1 h introduction of the whole-cell; The morphology of membrane surface (e) and the channel (f) after 10 h introduction of the whole-cell. Whole-cell treatment conditions: ChCl/U 6% (v/v) mixed with 0.02 g/mL whole-cell and incubated in a water bath shaker at 35°C for 30 min.

Membrane fouling and cleaning

Fig.5 shows the flux recovery rate of PVDF hollow fiber membranes cleaned by HCl solution after 10 h of use. After 10 hours of enzymatic reaction, the flux recovery was not obvious after cleaning the membrane with 1% (v/v) HCl solution for three times. The recovery rate increased from only 25% to 33.3%. This indicates that the gel layer formed in the membrane is thick, even it is difficult to clean with hydrochloric acid solution. And the membrane flux of the PVDF membrane was restored to about 50% of the initial membrane flux after the fourth cleaning. However, after the 6th and 7th cleaning, the recovery rate of membrane flux did not increase continuously, and only 75% of the original membrane flux was restored after 7th cleaning. Fouling is caused by loosely adhered impurities, which can be removed by physical cleaning such as backwashing. Chemical cleaning methods are needed to remove the blocked membrane holes and strongly adhered impurities in the filtration process. Once irreversible fouling is formed, membrane cleaning becomes difficult or even unusable. Studies have shown that the more the amount of cleaning agent used, the better the recovery of membrane flux, and different types of cleaning agents (hydrochloric acid, sodium hydroxide, sodium hypochlorite) mixed use

can also achieve a better recovery rate [21]. However, the membrane flux can't be restored to 100%.

The PVDF membrane material is easily fouled due to the fact that the polyvinylidene fluoride is a hydrophobic material with low surface properties. Impurities are easily deposited on the surface of the membrane to form a gel, which limits the application of membranes in separation and concentration of natural products and water purification. It is necessary to improve its hydrophilicity to make up for this shortcoming. For example, a layer of hydrophilic thin film is formed on the inner surface of the membrane by filling SiO_2 particles [33]. So impurities are not easily accumulated to form a gel layer. Even if the layer is formed, it will gradually fall off under the long time or large fluidic hydraulic scour, thus enhancing the membrane's pollution resistance.

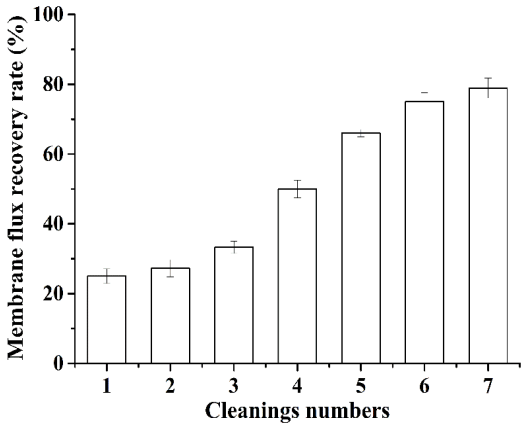

Fig. 5. Relationship between cleaning times and membrane flux recovery rate. Treatment conditions: extractant: rutin=2:1, temperature 40°C, rutin concentration 0.02 g/L, pH=5.5. The flow speed was 30 rpm/min for 10 h. The membrane washed with 1% HCl solution.

Reuse of enzyme membrane reactor

The whole-cell catalyst was reused in the enzyme membrane reactor and the relative enzyme activity is shown in Fig.6. The yields of isoquercitrin in the first two reactions were above 65% and the relative activity of the enzyme remained above 80%. After 5 times of repeated use, the relative viability of α-L-rhamnosidase in whole cells remained above 50%. The reason why the relative activity of enzymes keeps high may be that the enzymes are inside the cells. Because of the protection of the cell membrane, the fluid shear force in the reaction process was reduced, and the stability of the enzyme was guaranteed [34]. In addition, the permeability of cell membranes increased after choline-urea chloride treatment.

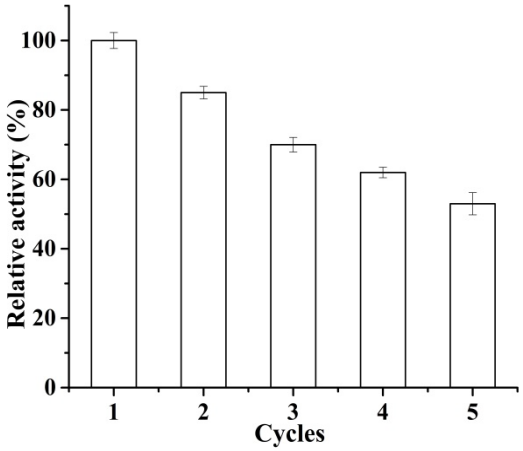

Fig. 6. The cycles and change of relative activity of whole-cell in enzyme membrane reactor. Reaction conditions: extractant: rutin=2:1, temperature 40°C, rutin concentration 0.02g/L, pH=5.5, the flow speed: 30 rpm/min, each reaction cycled for 6 h.

With the increase of reuse times, the cell membranes would rupture after repeated fluid impact, and the intracellular substances gradually lost and the enzyme activity decreased. Regular cleaning or ultrasonic treatment of the membrane can increase the reuse times of the membrane and increase the yield of the corresponding products. From the point of view of utilization and product benefit, the enzyme membrane reactor is suitable for reuse to some extent.

Conclusions

In this study, an enzymatic membrane reactor with reaction-separation coupling system was prepared using PVDF hollow fiber membrane as membrane module and whole cell as catalyst. This study focused on the reaction conditions of enzymatic hydrolysis of rutin to isoquercetin in the enzymatic membrane reactor. The membrane module was cleaned with 1% (v/v) hydrochloric acid solution after 10 h, and the repetitive advantage of whole cell catalyst in enzymatic membrane reactor was investigated. In the PVDF membrane reactor, the maximum yield of isoquercetin was obtained as 87.92±2.93% at 40 °C when the extractant-to-substrate ratio was 3:4, the buffer pH was 5.5, and the substrate concentration was 0.02g/L. The membrane module was cleaned after 10 h, and the membrane flux recovered to 75% of the initial value after 7 times of cleaning. Moreover, the enzyme activity remained at about 50% after 5 times of reuse in the enzyme membrane reactor. These results are very promising for a whole-cell catalytic reaction. On this basis, the catalytic reaction was no longer limited by factors such as product inhibition, and this method can be applied to other biocatalysis.

Abbreviations

RhaB1 – α-L-rhamnosidase gene
EGFP –Enhanced green fluorescent protein gene
E. coli –*Escherichia coli*
PVDF –Polyvinylidene fluoride
PVDF –Volatile organic compound
PVC –Polyvinyl chloride
PAN –Polyacrylonitrile
PES – Polyethersulfone resin
PP – Polypropylene
PSF – Polysulfone
*p*NPR – *p*-Nitrophenyl-α-L-rhamnopranoside
MES – 2-(N-Morpholino)ethanesulfonic acid
IPTG – Isopropyl-β-D-thiogalactopyranoside

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