Journal of the Serbian Chemical Society

JSCS-info@shd.org.rs • www.shd.org.rs/JSCS

J. Serb. Chem. Soc. *80* (7) 853–866 (2015)
JSCS–4764

UDC 547.9+544.473:577.15+547.918+
542.913+66.094.941
Original scientific paper

Ultrafast synthesis of isoquercitrin by enzymatic hydrolysis of rutin in a continuous-flow microreactor

JUN WANG[1,2], AN GONG[2], SHUANGSHUANG GU[2], HONGSHENG CUI[2] and XIANGYANG WU[1]*

[1]School of Environment and Safety Engineering, Jiangsu University, Zhenjiang 212013, P. R. China and [2]School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang 212018, P. R. China

(Received 15 September, revised 6 November, accepted 13 November 2014)

Abstract: Isoquercitrin is a rare flavonol glycoside with a wide range of biological activities and is a key synthetic intermediate for the production of enzymatically modified isoquercitrin. In order to establish an ultrafast bioprocess for obtaining isoquercitrin, a novel continuous flow biosynthesis of isoquercitrin using the hesperidinase-catalyzed hydrolysis of rutin in a glass–polydimethylsiloxane (PDMS) microreactor was first performed. Using the developed microchannel reactor (200 μm width, 50 μm depth and 2 m length) with one T-shaped inlet and one outlet, the maximum yield of isoquercitrin (98.6 %) was achieved in a short time (40 min) under the following optimum conditions: rutin concentration at 1 g L^{-1}, hesperidinase concentration at 0.1 g mL^{-1}, reaction temperature 40 °C, and a flow rate of 2 μL min^{-1}. The value of the activation energy, E_a, of the enzymatic reaction was 4.61 kJ mol^{-1}, and the reaction rate and volume productivity were approximately 16.1-fold and 30 % higher, respectively, than those in a batch reactor were. Thus, the use of a continuous-flow microreactor for the enzymatic hydrolysis of rutin is an efficient and simple approach to achieve a relatively high yield of isoquercitrin.

Keywords: biocatalysis; continuous flow; hesperidinase; isoquercitrin; microreactor.

INTRODUCTION

Isoquercitrin is a rare flavonol glycoside with a wide range of biological activities and is a key synthetic intermediate for the production of enzymatically modified isoquercitrin (EMIQ), a new multiple food additive. Due to its significant economic benefits and ecological acceptability when compared extraction from natural sources and chemical synthesis,[1] there is a respectable number of reports indicating that isoquercitrin can be efficiently synthesized from rutin

* Corresponding author. E-mail: wuxy@ujs.edu.cn or jimwang_js@hotmail.com
doi: 10.2298/JSC150914112W

(CC) 2015 SCS. All rights reserved.

using an enzymatic hydrolysis process under suitable reaction conditions.[2] Recently, several methods for the transformation of rutin to isoquercitrin have been investigated, including acid hydrolysis,[3] heating,[4] microbial transformation,[5] and enzymatic transformation techniques.[6] Among these methods, the biotransformation of rutin to isoquercitrin using selective hydrolysis would be a feasible procedure if the transformation could be performed at a reasonable cost.

The application of commercial hesperidinase as a biocatalyst in the biosynthesis of isoquercitrin by the selective biotransformation of rutin has many advantages when compared with crude and recombinant enzymes. Hesperidinase is more technically feasible for biological manufacturing approaches by controlling the pH instead of the temperature.[3,7] Consecutively, using an ionic liquid as a novel co-solvent in the [Bmim][BF_4]–glycine–sodium hydroxide buffer (pH 9; 10:90, *V*/*V*) to improve the isoquercitrin synthesis could allow for greater dissolution of the substrates and exercise a significant effect on the conversion, yield and selectivity of the enzymatic reaction system. These results indicated that ionic liquids could effectively enhance the selective synthesis of isoquercitrin and that the reaction process is simple and eco-friendly.[4,8] In addition, the reaction time necessary to achieve the highest isoquercitrin yield of 91.41 % was reduced from 30 to 10 h, while the conversion of rutin and the yield of isoquercitrin were increased by 1.67-fold and 2.33-fold, respectively.[9] However, the industrial production of isoquercitrin is hindered by a low reaction rate in a batch bioreactor. Therefore, a longer reaction time would significantly increase the overall production cost of preparing isoquercitrin. For this reason, a better method for a high-efficiency synthesis of isoquercitrin over a short time needs to be developed.

Due to the rapid heat transfer and mixing in microreactors, reactions can be performed significantly faster than those in batch reactors, typically with increases in both yield and selectivity.[10] Recently, the use of microreactor technologies within the scope of bioprocesses as process intensification and production platforms is gaining momentum.[11,12] Compared to traditional batch reactors, this trend can be ascribed to a particular set of characteristics of microreactors, namely the enhanced mass and heat transfer, combined with easier handling and smaller volumes.[13] Haswell and co-workers demonstrated that the aldol reaction between aldehyde and silyl enol ether in the presence of tetrabutyl ammonium fluoride (TBAF) reaches completion in only 20 min when using a microreactor, *versus* 24 h in a typical reactor.[14] Herein, miniaturized devices are gaining widespread use in biocatalysis because this approach contributes to the rationalization of process development with a significant reduction in the work force, in the quantity of reagents required and in waste production, concomitantly contributing to a significant cost reduction.[15] These microreactors have been shown to outperform conventional, large-scale vessels operating in the batch mode, given the

Available on line at www.shd.org.rs/JSCS/

(CC) 2015 SCS. All rights reserved.

favorable mass and heat transfer characteristics due to a large area to volume ratio and the possibility of operating in a continuous mode.[16] Under the correct conditions, microreactors can also offer better selectivity, improved yields over shorter periods, increased process control, greater safety, and flexible production.[17] Kanno and co-workers showed that an enzyme-catalyzed reaction performed homogeneously in a flow could yield higher conversions than that in the batch counterpart.[18] In this case, a solution of *α*-galactosidase in phosphate buffer at pH 8 was combined with a similarly buffered solution of *p*-nitrophenyl-*α*-D-galactopyranoside (PNPGal) in a 200 μm×200 μm microreactor. The authors were able to show that the hydrolysis in the microreactor was 5-fold faster than that in the analogous batch reaction performed in a micro-test tube.[19] However, no report has hitherto been published detailing the use of a continuous-flow microreactor to significantly enhance the hesperidinase-catalyzed synthesis of isoquercitrin.

The purpose of this study was to set up a continuous biocatalysis system for the selective and effective biotransformation of rutin to isoquercitrin in a glass–PDMS microreactor. The effects of the channel length of the microreactor, reaction temperature, rutin concentration and enzyme concentration on isoquercitrin yield were investigated. In addition, the activation energy value E_a of the enzymatic reaction was determined.

MATERIALS AND METHODS

Materials

Hesperidinase (contains both *α*-L-rhamnosidase and *β*-D-glucosidase activities, ≥1 units g^{-1} solid) produced by *Aspergillus niger* and standard isoquercitrin were purchased from Sigma (St. Louis, MO, USA). The ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]) was purchased from Shanghai Cheng-Jie Chemical Co. (Shanghai, China) and had a residual chloride content of less than 50 ppm. All the reagents used were of analytical grade except methanol and acetonitrile, both of which were of HPLC-grade (purchased from Tedia Co. (Fairfield, OH, USA)). All other solvents and reagents were of analytical grade. Water was purified using an Elga Purelab Option-Q purification system (Elga Labwater, High Wycombe, UK) and had a resistivity of not less than 18.0 MΩ cm. This water was used for cleaning procedures and in the preparation of all buffer solutions. All aqueous solutions were prepared with ultrapure water and filtered through a 0.45 μm membrane filter.

Hesperidinase-catalyzed synthesis of isoquercitrin in a continuous-flow microreactor

Hesperidinase-catalyzed synthesis of isoquercitrin was realized in a glass–PDMS microreactor with rectangular microchannels. The microreactor developed by the laser burn technology had a T-shaped inlet and an outlet channel. The main channel dimension was 200 μm wide, 50 μm deep and 2 m long (Fig. 1). The reaction substrate (rutin at pH 9 in glycine–sodium hydroxide buffer) and an enzyme solution containing 10 % [Bmim][BF_4] were both pumped into the microchannel by a two-channel syringe pump. The flow rates of the two phases were the same.

Available on line at www.shd.org.rs/JSCS/

(CC) 2015 SCS. All rights reserved.

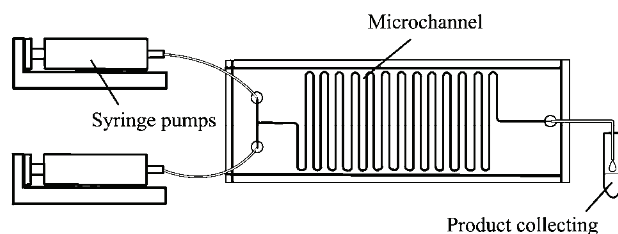

Fig. 1. Ultrafast synthesis of isoquercitrin through the enzymatic hydrolysis of rutin in a microreactor.

A scheme of isoquercitrin synthesis using hesperidinase-catalyzed hydrolysis of rutin is presented in Fig. 2, which shows the chemical structures of rutin, isoquercitrin and L-rhamnose.

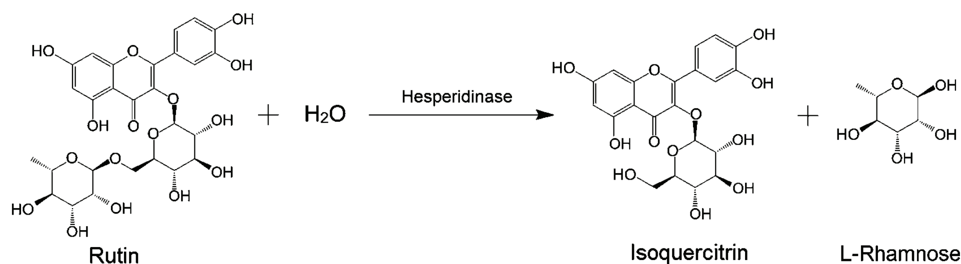

Fig. 2. Scheme of isoquercitrin synthesis using hesperidinase-catalyzed hydrolysis of rutin.

HPLC analysis and LC-MS analysis

HPLC quantitative analyses were performed using a constant flow pump (2PB0540, Beijing Satellite Factory, Beijing, China) with a UV–Vis detector (L-7420, Techcomp Co., Shanghai, China) and an N-2000 workstation (Hangzhou Mingtong S&T Ltd., Hangzhou, China). An Alltima C_{18} column (250 mm×4.6 mm, i.d.; 5 μm; from W. R. Grace & Co., Deerfield, IL, USA) was used, and the column was maintained at 30 °C. The separation and determination of rutin and isoquercitrin using the HPLC/UV method was performed on the Alltima C_{18} column with a mobile phase consisting of acetonitrile:0.02 % phosphoric acid solution (20:80, *V*/*V*) at a flow rate of 1.0 mL min^{-1}. Rutin and isoquercitrin were detected at 360 nm. All solutions were filtered through a 0.45 μm filter before injection. All of the experiments were performed in triplicate.

The isoquercitrin yield of the hesperidinase-catalyzed isoquercitrin synthesis was calculated using the following equation:

$$\text{Isoquercitrin yield (\%)} = \frac{\text{Moles of isoquercitrin}}{\text{Initial moles of rutin}} \times 100 \quad (1)$$

LC-MS was performed on a Thermo Fisher system. The LC equipment comprised a Finnigan MAT Spectra System P4000 pump, an autosampler with a 50 μL loop, a UV6000 LP diode array detector (DAD) and a Finnigan AQA mass spectrometer. LC separation was performed on the Alltima C_{18} column (250 mm×4.6 mm, i.d.; 5 μm). The mobile phases consisted of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). Separation was performed under the following conditions: 0–35 min, 6–100 % B; 35–40 min, back to 6

Available on line at www.shd.org.rs/JSCS/

(CC) 2015 SCS. All rights reserved.

% B. The column was equilibrated for 15 min prior to each analysis. The wavelength range of the DAD was 200 to 400 nm. The flow rate was 1.0 mL min^{-1} for LC, and the column remained at 40 °C during DA detection. Electrospray ionization (ESI) was performed using nitrogen to assist nebulization (1.0 mL min^{-1} flow rate). Selected ion monitoring (SIM) in the negative ion mode with 1.6 kV capillary voltage was used, and the temperature of the curved desolvation line (CDL) and heat block were both set at 200 °C. The data were processed using Xcalibur 1.2 software. The intense peaks at *m/z* 463.17 in the ESI-MS spectra under negative ion mode corresponded to the deprotonated $[M–H]^-$ of isoquercitrin.[9]

Kinetic analyses

To study the kinetics of the enzymatic synthesis of isoquercitrin, the reactions were performed at different temperatures. The temperature ranged from 25 to 40 °C because the hesperidinase could exhibit its optimal activity at 40 °C and a temperature higher than 40 °C might deactivate the enzyme. The reaction rates were calculated according to:

$$k = \ln\frac{a}{(a-x)}\frac{1}{t} \tag{2}$$

where k is the reaction rate constant (min^{-1}), a is the initial concentration of substrate (μmol L^{-1}) and x is the concentration of isoquercitrin at time t, where t is the reaction time.

Statistical analyses

Triplicate experiments were performed for each investigated parameter. The standard deviation of the values was calculated to check the reliability of the results. The differences in mean values were evaluated using the analysis of variance (ANOVA) method. Significance was determined at the 95 % level of probability.

RESULTS AND DISCUSSION

The effect of microchannel length

The continuous-flow microreactor in this study consisted of channel diameters of 50 μm deep and 200 μm wide. The effects of the microreactor channel length on isoquercitrin yield using a continuous-flow microreactor at different flow rates are shown in Fig. 3A. Increasing the channel length from 0.5 to 2 m had a beneficial effect on isoquercitrin yield. When the microfluid flow rate was 2 μL min^{-1}, the channel length was 2 m, and the residence time was 40 min, a maximum yield of 34.5 % was achieved. These results indicated that the shallow depth of the microreactor channels provided for very short diffusion lengths of reaction mixtures and induced the microfluid under laminar flow condition.[20] This is one of the great advantages of using microscale reactors for rapid reactions.

These results sufficiently confirmed that reducing the flow rate to induce a prolonged residence time would facilitate enzymatic reactions. However, when the liquid flow rate was too fast, the reaction droplets of hesperidinase increased friction with the inner wall of the channel. This phenomenon prompted the increased shear stress and local overheating and deactivated the enzyme.[21] Therefore, the yield of isoquercitrin was reduced.

Available on line at www.shd.org.rs/JSCS/

(CC) 2015 SCS. All rights reserved.

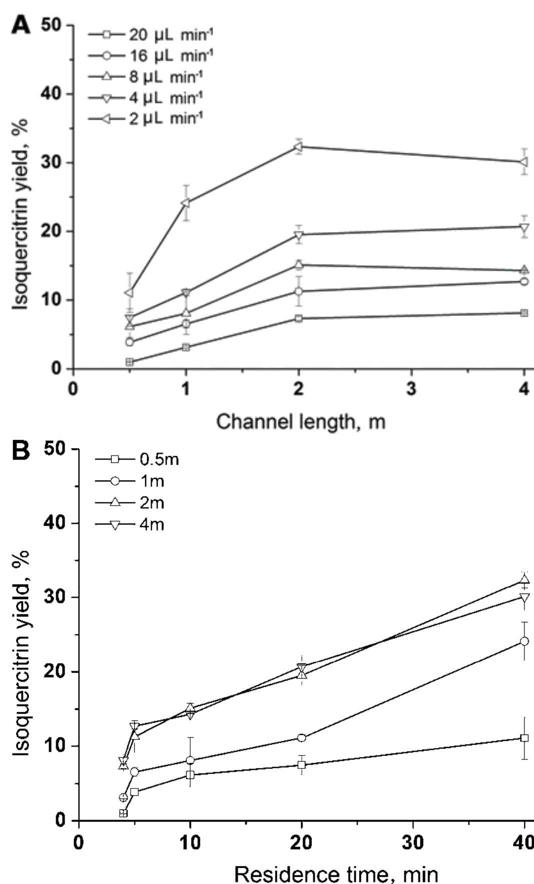

Fig. 3. Using the continuous-flow microreactor developed to synthesize isoquercitrin by the enzymatic hydrolysis of rutin. A) Effects of the channel length of the microreactor on the isoquercitrin yield at different flow rates; B) effects of residence time on the isoquercitrin yield. Reaction temperature: 40 °C; rutin concentration: 1 g L^{-1}; hesperidinase concentration: 0.01 g mL^{-1}.

The effects of residence time on the efficiency of isoquercitrin synthesis are shown in Fig. 3B. Using a 2 m long channel, the residence time (4–40 min) of the reaction mixture in the microreactor was tuned by varying the flow rates (20–2 μL min^{-1}). As expected, the reaction appeared to be favored by an increase in the residence time. When the enzymatic reaction was conducted at 20 μL min^{-1}, only a 29.0 % yield was obtained, while at 2 μL min^{-1}, a maximum yield of 34.5 % was achieved. Thus, a 2 m long channel was chosen for further experiments.

The effect of reaction temperature

The effects of temperature on isoquercitrin yield using a continuous-flow microreactor at different flow rates are shown in Fig. 4A. The isoquercitrin yield initially increased with temperatures ranging from 25 to 40 °C, whereas higher temperatures led to a decline in isoquercitrin yield and enzyme denaturation, which lowered the efficiency of enzymatic hydrolysis. The yield had a similar

(CC) 2015 SCS. All rights reserved.

tendency at different flow rates. An optimal temperature of 40 °C at 2 μL min^{-1} was chosen to promote the maximum yield of isoquercitrin.

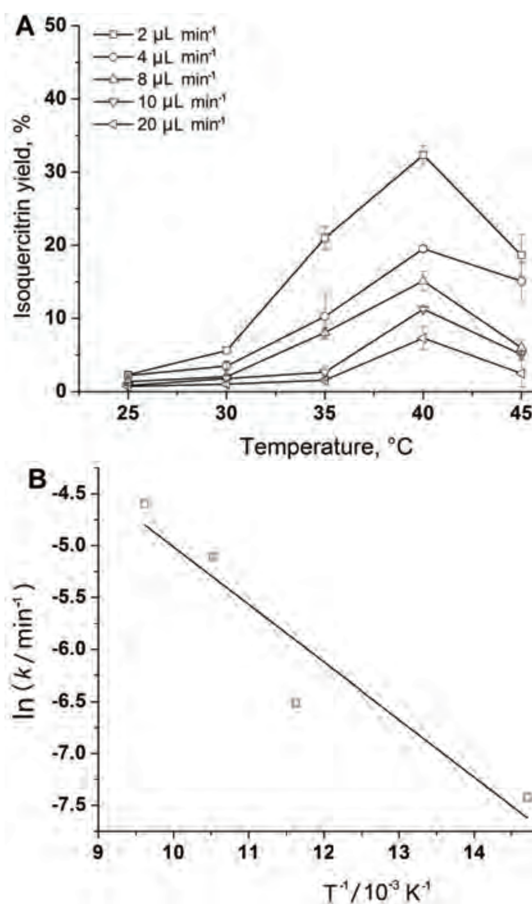

Fig. 4. A) Effects of temperature on the isoquercitrin yield and B) the Arrhenius plot of ln k *vs*. T^{-1} using the developed continuous-flow microreactor at different flow rates at 40 °C. Rutin concentration: 1 g L^{-1}; hesperidinase concentration: 0.01 g mL^{-1}.

An analysis was performed to study the effects of temperature on reaction rate and energy of activation in the enzymatic synthesis of isoquercitrin. Depending on the reaction rate constant values at various reaction temperatures, the value of energy of activation could be further estimated according to the Arrhenius equation:

$$\ln k = \frac{E_a}{RT} + \ln A \quad (3)$$

where k is the reaction rate constant, A is the frequency factor and E_a is the energy of activation.

An Arrhenius plot made based on ln k *vs*. the reciprocal of temperature is shown in Fig. 4B. The reaction rate increased with increasing temperature. In

Available on line at www.shd.org.rs/JSCS/

(CC) 2015 SCS. All rights reserved.

addition, the activation energy E_a could be obtained from the slope of the straight line. Thus, when the flow rate was 2 μL min^{-1}, the E_a of the reaction was calculated to be 4.61 kJ mol^{-1}. E_a represents the ability of the enzymatic reaction to overcome the original "energy barrier". In this continuous-flow microreactor, the E_a value is relatively small, and the enzymatic reaction occurs more easily. These results suggest that heat transfer was compromised in the developed microreactor with rectangular microchannels and that local temperature changes were significant. Heat transfer was enhanced as the flow rate increased; therefore, the heat dissipates faster from the active site of the enzyme,[22] which subsequently decreased the yield of isoquercitrin when the temperature exceeded 40 °C.

The effect of rutin concentration

The effects of the rutin concentration on isoquercitrin yield using a microreactor at different flow rates are shown in Fig. 5A. As expected, the yield of isoquercitrin increased with increasing inlet substrate concentration and lowering the flow rate. At inlet concentrations of rutin of 1 g L^{-1}, a yield of isoquercitrin of

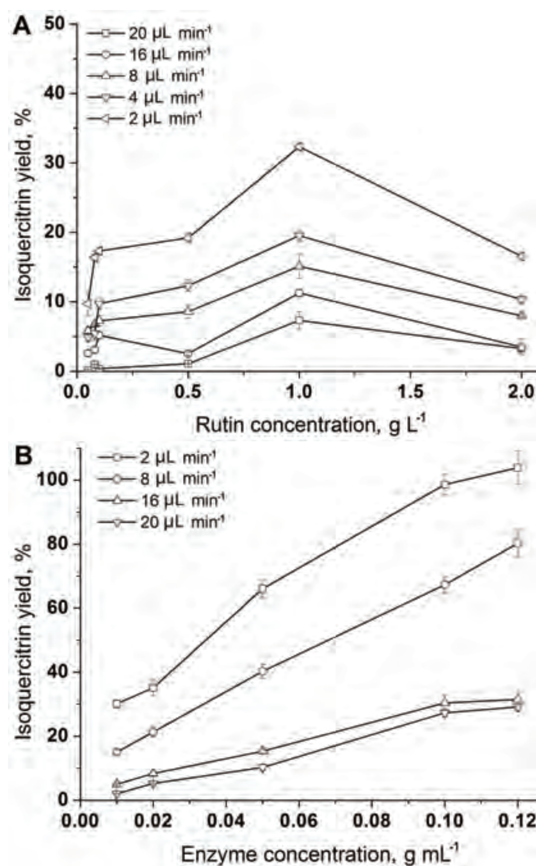

Fig. 5. A) Effects of rutin concentration and B) enzyme concentration on the isoquercitrin yield using the developed continuous-flow microreactor at different flow rates. Reaction temperature: 40 °C; A) hesperidinase concentration: 0.01 g mL^{-1}; B) rutin concentration: 1.0 g L^{-1}.

Available on line at www.shd.org.rs/JSCS/

(CC) 2015 SCS. All rights reserved.

approximately 34.5 % was obtained after 40 min. However, further increasing the inlet concentration to 2 g L^{-1} decreased the yield of isoquercitrin significantly. The reasons mainly include two aspects: 1) the hesperidinase concentration in this experiment was very limited, resulting in an enzyme that could not fully contact with the substrate; 2) substrate inhibition was present and enzyme denaturation was detected at a high substrate concentrations. In addition, higher substrate concentrations caused clogging in continuous flow reactors.[23] The inlet concentration of rutin used in the microreactor was in agreement with a previous study.[9] However, some studies reported that the inlet substrate concentrations used in a microreactor were 10 times lower than those in a batch reactor.[24]

Hence, the presented microreactor technology is economically feasible for the large-scale production of isoquercitrin without reducing the substrate concentration of rutin. Usually, substrate inhibition and product degradation were two possible reasons for the decrease in isoquercitrin conversion when the concentration of rutin was higher. Hypothetically, when the concentration of rutin is 1 g L^{-1}, a higher isoquercitrin yield should be obtained. However, if isoquercitrin is abundantly produced and the substrate concentration is appropriately increased, the yield and efficiency should be greatly improved. Thus, 1 g L^{-1} of rutin was chosen as a suitable substrate concentration for further study.

The effect of hesperidinase concentration

The effects of the hesperidinase concentration on isoquercitrin yield with different flow rates are shown in Fig. 5B. Under identical conditions of temperature and substrate concentration, the effect of the concentration of hesperidinase in the range of 0.01 to 0.12 g mL^{-1} on isoquercitrin yield at different flow rates was observed. An increase in the concentration of hesperidinase up to 0.1 g mL^{-1} had a positive effect on the isoquercitrin yield. When the concentration of hesperidinase was 0.1 g mL^{-1}, the yield of isoquercitrin was near 98.6 %. Further increases in the enzyme concentration up to 0.12 g mL^{-1} increased the yield of isoquercitrin only slightly. Hence, from an economic perspective, the best hesperidinase concentration was 0.1 g mL^{-1}.

The enzyme concentration plays an important role in enzymatic syntheses,[25] and the number of active sites in a particular enzyme can affect the reaction rate.[26] In addition, the separation of the product can be easily attained using triacetin as an organic phase to extract isoquercitrin from the reaction mixture after completion of the enzymatic reaction.[27] As expected, the yield under steady-state conditions increased with decreasing flow rate. A yield of approximately 98.6 % was achieved at an inlet rutin concentration of 1 g mL^{-1} and a flow rate of 2 μL min^{-1}. The aqueous phase with the hesperidinase and unreacted rutin could be recycled, which makes this process much more economic.

Available on line at www.shd.org.rs/JSCS/

(CC) 2015 SCS. All rights reserved.

The effect of flow rate and residence time

To investigate the effect of flow rate on the hesperidinase-catalyzed synthesis of isoquercitrin by the enzymatic hydrolysis of rutin, five different flow rates, *i.e.*, 2, 4, 8, 16 and 20 μL min^{-1}, were selected for testing. As shown in Figs. 3A, 4A, 5A and 5B, different isoquercitrin yields were obtained at various flow rates. In every figure, a flow rate of 2 μL min^{-1} resulted in a higher isoquercitrin yield than those obtained at the other rates. As the flow rate was further increased, the isoquercitrin yield was clearly reduced and reached the lowest yield at 20 μL min^{-1}. In particular, Fig. 5B shows that the isoquercitrin yield was the highest, up to 98.6 %, at a flow rate of 2 μL min^{-1}.

By using longer channels and reducing the flow rate, the residence time increases. Longer residence times allow a more complete, full contact between substrates and enzymes. However, increasing the channel length from 2 m to 4 m did not obviously change the isoquercitrin yield, possibly because the stability of hesperidinase was lower at longer channel lengths. In addition, the pressure would increase with a further reduction in the channel dimension, and this is disadvantageous to the actual operation.

Under different flow rates in a 4 m long microreactor with rectangular microchannels, the faster the flow rate, the longer was the residence time of the hesperidinase-catalyzed synthesis of isoquercitrin by enzymatic hydrolysis of rutin. In this microreactor, flow rates ranging from 2 to 20 μL min^{-1} were employed, and the corresponding residence time was decreased from 2.5 h to 15 min. The isoquercitrin yield increased as the residence time was extended, which was most likely due to the increase in the mass transfer of the substrate towards hesperidinase molecules because of the increase in the reaction time.[28,29] As the residence time increased, interactions between the substrate and enzyme increased, resulting in a higher yield of isoquercitrin. This result indicates that the flow rate is a vitally important factor for the operation of a microreactor with rectangular microchannels. At low flow rates, the increased residence time in the reaction system allowed for the completion of the enzymatic synthesis reaction.[30] At high flow rates, the residence time was most likely not long enough for the completion of enzymatic synthesis because the synthesis of isoquercitrin was not fast enough to complete the enzymatic reaction in such a short time.

Comparing the technology between the microreactor and the batch reactor

A maximum isoquercitrin yield of 98.6 % was obtained in the continuous-flow microreactor after 40 min, which was 16.1-fold faster than the yield of 91.4 % in the batch reactor (10 h, Table I). The calculated volume productivity at a rutin inlet concentration of 1 g L^{-1} was 3.23 μM min^{-1} at a residence time of 40 min, which was more than 1.2-fold higher than that reported within the batch reactor,[9] where the highest volume productivity was 2.49 μM min^{-1}. Thus, the

Available on line at www.shd.org.rs/JSCS/
(CC) 2015 SCS. All rights reserved.

volume productivity in the microreactor was 30 % higher than that in the batch reactor. Herein, although isoquercitrin production in a specific time was lower than that in a conventional reactor, the reaction time in the microreactor was 14 times lower than that in a batch reactor. Considering these two factors, biocatalysis technology in the microreactor could be a feasible industrial tool in the future.

TABLE I. Comparative results for the hesperidinase-catalyzed transformation of rutin to produce isoquercitrin in different reactors

Reactor	Substrate concentration, g L^{-1}	Enzyme (concentration) g mL^{-1}	Reaction time, h	Isoquercitrin yield, %	Volume productivity, μM min^{-1}	Ref.
Micro-reactor[a]	1	Hesperidinase (0.10)	0.67	98.60±3.25	3.23	This work
Batch reactor[b]	1	Hesperidinase (0.05)	10	91.46±0.55	2.49	Wang *et al.*[3]
Batch reactor[c]	0.92	Crude enzyme extract of *A. niger* (–)	4	–	–	You *et al.*[1]
Batch reactor[d]	0.46	Naringinase (0.05)	6	61	–	Vila-Real *et al.*[31]

[a]reaction conditions: rutin concentration 1 g L^{-1}, reaction temperature 40 °C, flow rate 2 μL min^{-1} for 40 min, [Bmim][BF_4]–buffer (pH 9.0), 10:90, *V/V*, as the reaction medium; [b]reaction conditions: rutin concentration 1 g L^{-1}, reaction temperature 40 °C, 120 rpm for 10 h, [Bmim][BF_4]–buffer (pH 9.0), 10:90, *V/V*, as the reaction medium; [c]reaction conditions: rutin concentration 0.92 g L^{-1}, reaction temperature 60 °C, 50 μL of crude enzyme extract of *A. niger* was resuspended in a reaction system; [d]reaction conditions: rutin concentration 0.46 g L^{-1}, reaction temperature 60 °C for 6 h in a 20 mmol L^{-1} citrate buffer system

As shown in Table I, You *et al.*[1] obtained isoquercitrin using a crude enzyme extract of *Aspergillus niger* in 4 h. The reaction time and the substrate concentration were 7.97-fold longer and 0.92-fold lower, respectively, than those in the microreactor. In addition, the results of Vila-Real *et al.*[31] indicated that isoquercitrin was obtained using the enzymatic hydrolysis of rutin with a production yield of 61 % under the conditions of 0.05 g mL^{-1} naringinase and 0.46 g L^{-1} rutin in 6 h. The isoquercitrin yield and rutin concentration were 37.6 % and 2.18-fold lower, respectively, than those in the microreactor, and the reaction time was much longer than that in the microreactor. This is due to the increased specific interfacial area and the reduced diffusion length in the microreactor, both improving the mass transfer rates in the reaction.[32] These results imply that to determine the suitable performance of a continuous-flow microreactor on an industrial scale, a numbering up approach with parallel microreactors could be adopted. Generally, the developed multi-channel microreactors and other similar multi-input microreactors are more inclined to the demands of industrial production.[33,34] The higher the number of channels, the greater the injection volume

Available on line at www.shd.org.rs/JSCS/

(CC) 2015 SCS. All rights reserved.

of the substrates. The volume productivity of products would be significantly increased.[35] Thus, a shorter reaction time would significantly decrease the overall production costs of preparing isoquercitrin.

Due to the rapid heat transfer and mixing in microreactors, the reactions could be performed significantly faster than those in batch modes, typically with increases in both yield and selectivity.[32] When examining rapid reactions that allowed for equivalent reaction times in flow and batch modes, the yield was improved.[36] In addition to high yields, microreactors provide environments for highly selective chemistry, most likely due to the precise temperature control. When compared to a batch reactor, the microreactor had a higher efficiency.[37] This improvement could be attributed to two factors:[38] 1) the surface-to-volume ratio in the microreactors is much higher than in batch reactors, which increases the enzyme active sites available to the reactants at any time; and 2) the volume of the microreactor is so limited that the reactants are forced to be in contact with enzyme active sites because the diffusion paths in microreactors are much smaller. Another advantage of using the developed microreactor is that the biocatalytic reaction could be performed continuously in the preparation of isoquercitrin. In summary, the developed biocatalysis method using a continuous-flow microreactor in a shorter time (40 min) could produce a comparable amount of isoquercitrin as in a batch reactor (10 h). Thus, the bioprocess is much more economical in the industrial preparation of isoquercitrin and other precious natural medicines.[39] Thus, the novel approach using a continuous-flow microreactor used to synthesize isoquercitrin through the enzymatic hydrolysis of rutin was both efficient and simple.

CONCLUSIONS

An efficient and rapid process for isoquercitrin production using the hesperidinase-catalyzed hydrolysis of rutin was successfully developed in a continuous-flow microchannel reactor. The maximum isoquercitrin yield of 98.6 % and a volume productivity of 3.23 μmol L^{-1} min^{-1} were obtained under the following optimum conditions: a flow rate of 2 μL min^{-1} (residence time of 40 min), a rutin concentration of 1 g L^{-1}, a hesperidinase concentration of 0.1 g mL^{-1}, and a temperature of 40 °C. The value of the activation energy E_a of the enzymatic reaction was 4.61 kJ mol^{-1} at 2 μL min^{-1}. The enzymatic reaction rate in the developed microreactor was approximately 26-fold higher than that in a batch reactor. Thus, the novel approach using a continuous-flow microreactor for the synthesis of isoquercitrin through the enzymatic hydrolysis of rutin was both efficient and simple. Moreover, this microtechnology could also be applied in the effective synthesis of other precious natural medicines.

Acknowledgements. This work was supported by the China Postdoctoral Science Foundation Funded Projects (2013T60505, 2012M510124), the Postdoctoral Science Foundation

Available on line at www.shd.org.rs/JSCS/

(CC) 2015 SCS. All rights reserved.

Funded Project of Jiangsu University (1143002085), and the Qing Lan Project of Jiangsu Province (2014).

ИЗВОД

БРЗА СИНТЕЗА ИЗОКВЕРЦИТРИНА ЕНЗИМСКОМ ХИДРОЛИЗОМ РУТИНА У МИКРОРЕАКТОРУ СА НЕПРЕКИДНИМ ПРОТОКОМ

JUN WANG[1,2], AN GONG[2], SHUANGSHUANG GU[2], HONGSHENG CUI[2] и XIANGYANG WU[1]

[1]*School of the Environment and Safety Engineering, Jiangsu University, Zhenjiang 212013, P R China и*
[2]*School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang 212018, P.R. China*

Изокверцитрин је ретки флавонолни гликозид широког опсега биолошких активности и кључни је интермедијер у синтези ензимски модификованог изокверцитрина. Да би се успоставио изузетно брз поступак за добијање изокверцитрина, коришћен је биореактор од стакла и полидиметилсилоксана са сталним протоком, а рутин је хидролизован хесперинидазом. Применом новог микроканалног реактора (ширине 200 μm, дубине 50 μm и дужине 2 m), са једним улазом T облика и једним излазом, максимални принос изокверцитрина (98,6 %) је добијен у кратком времену (40 min), под следећим оптималним условима: концентрација рутина 1 g L^{-1}, концентрација хесперинидазе 0,1 g mL^{-1}, реакциона температура 40 °C и проток 2 μL min^{-1}. Енергија активације за ензимску реакцију је била 4,61 kJ mol^{-1}, а брзина реакције и запремински принос су биле 16,1 пута, односно за 30 % веће него у стандардном реактору. Дакле, примена микрореактора са сталним протоком за синтезу изокверцитрина ензимском хидролизом рутина је ефикасан и једноставан поступак.

(Примљено 15. септембра, ревидирано 6. новембра, прихваћено 13. новембра 2014)

REFERENCES

1. H. J. You, H. J. Ahn, G. E. Ji, *J. Agric. Food Chem.* **58** (2010) 10886
2. K. Valentová, J. Vrba, M. Bancířová, J. Ulrichová, V. Křen, *Food Chem. Toxicol.* **68** (2014) 267
3. J. Wang, L. Zhao, G. Sun, Y. Liang, F. A. Wu, Z. Chen, S. Cui, *Afr. J. Biotechnol.* **10** (2013) 1460
4. L. Weignerová, P. Marhol, D. Gerstorferová, V. Křen, *Bioresour. Technol.* **115** (2012) 222
5. J. Tamayo-Ramos, M. Flipphi, E. Pardo, *Microb. Cell Fact.* **11** (2012) 26
6. D. Gerstorferová, B. Fliedrová, P. Halada, P. Marhol, V. Křen, L. Weignerová, *Process Biochem. (Oxford, UK)* **47** (2012) 828
7. J. Wang, M. Wang, F. Wu, Z. Chen, S. Cui, *J. Med. Plants Res.* **6** (2012) 1130
8. W. Lou, M. Zong, H. Wu, R. Xu, *Chin. J. Chem. Eng.* **12** (2004) 87
9. J. Wang, G. Sun, L. Yu, F. Wu, X. J. Guo, *Bioresour. Technol.* **128** (2013) 156
10. C. Wiles, P. Watts, S. Haswell, E. Pombo-Villar, *Tetrahedron* **61** (2005) 10757
11. F. Lévesque, P. H. Seeberger, *Angew. Chem. Int. Ed.* **51** (2012) 1706
12. G. Chen, J. Yue, Q. Yuan, *Chin. J. Chem. Eng.* **16** (2008) 663
13. A. Nagaki, D. Ichinari, J. Yoshida, *Chem. Commun.* **49** (2013) 3242
14. C. Wiles, P. Watts, S. Haswell, E. Pombo-Villar, *Lab Chip* **1** (2001) 100
15. M. P. Marques, P. Fernandes, *Molecules* **16** (2011) 8368
16. T. Inoue, K. Ohtaki, S. Murakami, S. Matsumoto, *Fuel Process Technol.* **108** (2013) 8
17. A. Pohar, I. Plazl, P. Žnidaršič-Plazl, *Lab Chip* **9** (2009) 3385
18. K. Kanno, H. Maeda, S. Izumo, M. Ikuno, K. Takeshita, A. Tashiro, M. Fujii, *Lab Chip* **2** (2002) 15

Available on line at www.shd.org.rs/JSCS/

(CC) 2015 SCS. All rights reserved.

19. B. Mason, K. Price, J. Steinbacher, A. Bogdan, D. McQuade, *Chem. Rev*. **107** (2007) 2300
20. J. Yang, F. Lu, L. Kostiuk, D. Kwok, *J. Micromech. Microeng*. **13** (2003) 963
21. K. Sakai-Kato, M. Kato, T. Toyo'Oka, *Anal. Chem.* **75** (2003) 388
22. B. Chen, H. Liu, Z. Guo, J. Huang, M. Wang, X. Xu, L. Zheng, *J. Agric. Food Chem*. **59** (2011) 1256
23. J. Lawrence, B. O'Sullivan, G. Lye, R. Wohlgemuth, N. Szita, *J. Mol. Catal., B* **95** (2013) 111
24. G. Stojkovič, P. Žnidaršič-Plazl, *Process Biochem. (Oxford, UK)* **47** (2012) 1102
25. A. Mimi Sakinah, A. Ismail, R. Illias, A. Zularisam, O. Hassan, T. Matsuura, *Sep. Purif. Technol.* **124** (2014) 61
26. G. Tian, S. Xiang, R. Noiva, W. Lennarz, H. Schindelin, *Cell* **124** (2006) 61
27. C. Stalikas, *J. Sep. Sci*. **30** (2007) 3268
28. G. Greenway, S. Haswell, D. Morgan, V. Skelton, P. Styring, *Sens. Actuators, B* **63** (2000) 153
29. Y. Wang, X. Zhang, A. Wang, X. Li, G. Wang, L. Zhao, *Chem. Eng. J*. **235** (2014) 191
30. S. Mohr, K. Fisher, N. Scrutton, N. Goddard, P. Fielden, *Lab Chip* **10** (2010) 1929
31. H. Vila-Real, A. J. Alfaia, M. R. Bronze, A. R. Calado, M. H. Ribeiro, *Enzyme Res*. (2011) 4760
32. V. Skelton, G. Greenway, S. Haswell, P. Styring, D. Morgan, B. Warrington, S. Wong, *Analyst* **126** (2001) 11
33. P. B. Wojciech, V. Willem, N. David, *Lab Chip* **7** (2007) 1717
34. J. Wagner, T. Kirner, G. Mayer, J. Albert, J. M. Kohler, *Chem. Eng. J.* **101** (2004) 251
35. W. Ehfreld, V. Hessel, H. Lowe, *Microreactors: New Technology for Modern Chemistry*, Wiley–VCH, Weinheim, Germany, 2000
36. S. Taghavi-Moghadam, A. Kleemann, G. Golbig, *Org. Process Res. Dev*. **5** (2001) 652
37. C. Basheer, F. S. Jahir Hussain, H. K. Lee, S. Valiyaveettil, *Tetrahedron Lett.* **45** (2004) 7297
38. J. Wang, S. S. Gu, H. S. Cui, X. Y. Wu, F. A. Wu, *Bioresour. Technol.* **158** (2014) 39
39. T. D. Zeng, D. Wheeler, A. V. Desai, B. Oenal, D. E. Reichert, P. A. Kenis, *Lab Chip* **24** (2010) 3387.

(CC) 2015 SCS. All rights reserved.