A novel water-water microfluidic droplet system enhances cyanidin-3-*O* glucoside content in red pigments from defective mulberry fruit

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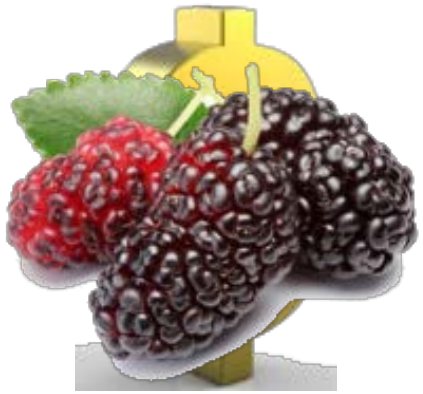
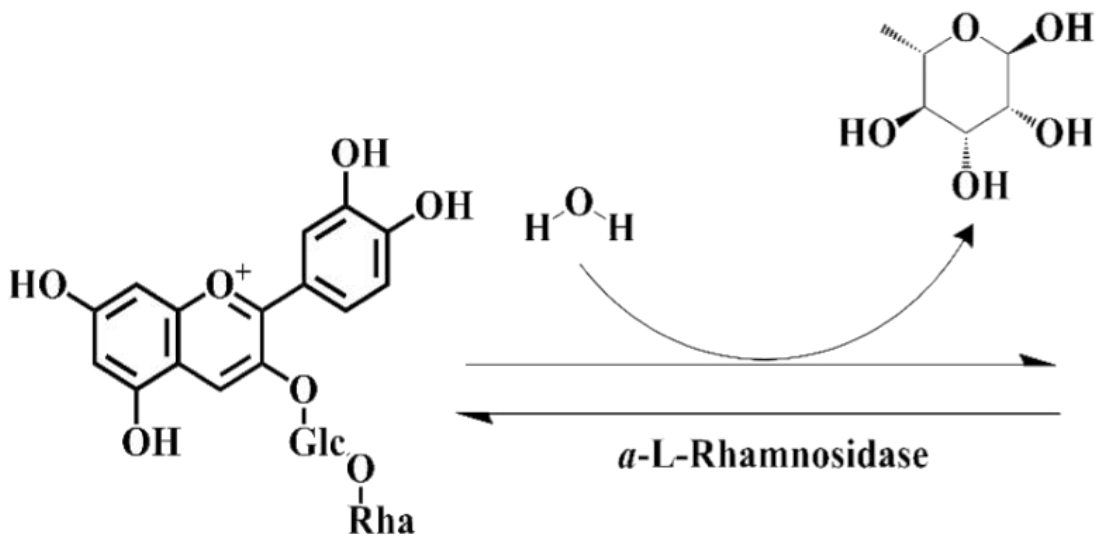
Introduce

Red Pigment of Mulberry —A Natural Food Pigment





90%

Cyanidin-3-*O*-rutinoside(C_3R)

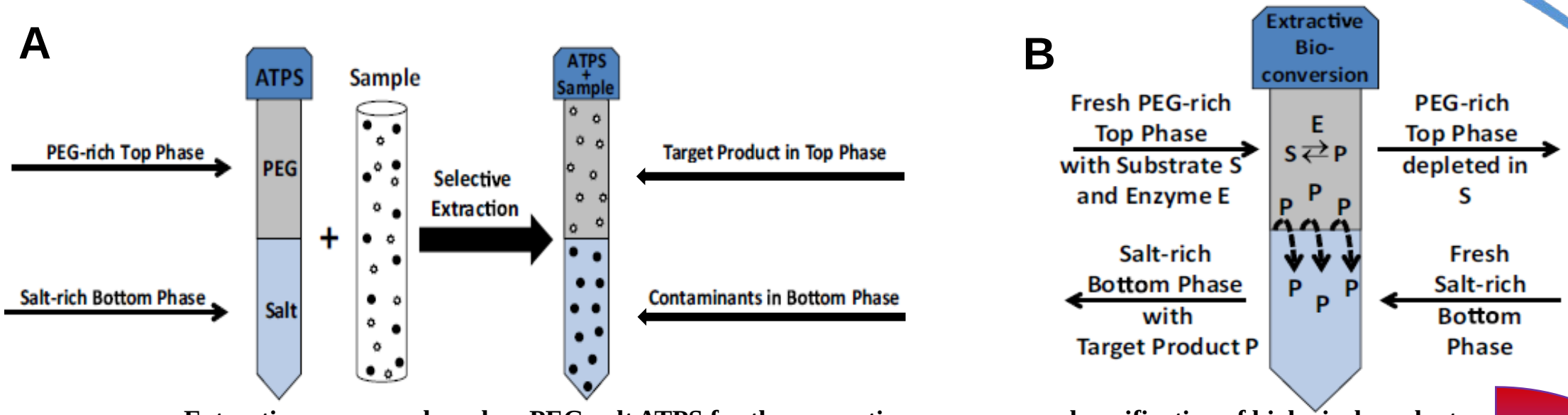
60%

Cyanidin-3-*O*-glucoside(C_3G)

30%

Song H, et al. *Nutrition Research*, 2016, 36(7): 710-718.

Two aqueous phase enzyme catalytic system



Extraction processes based on PEG-salt ATPS for the separation, recovery, and purification of biological products.

Advantage:
- Low cost
- Mild and non-toxic
- Reduce substrate inhibition

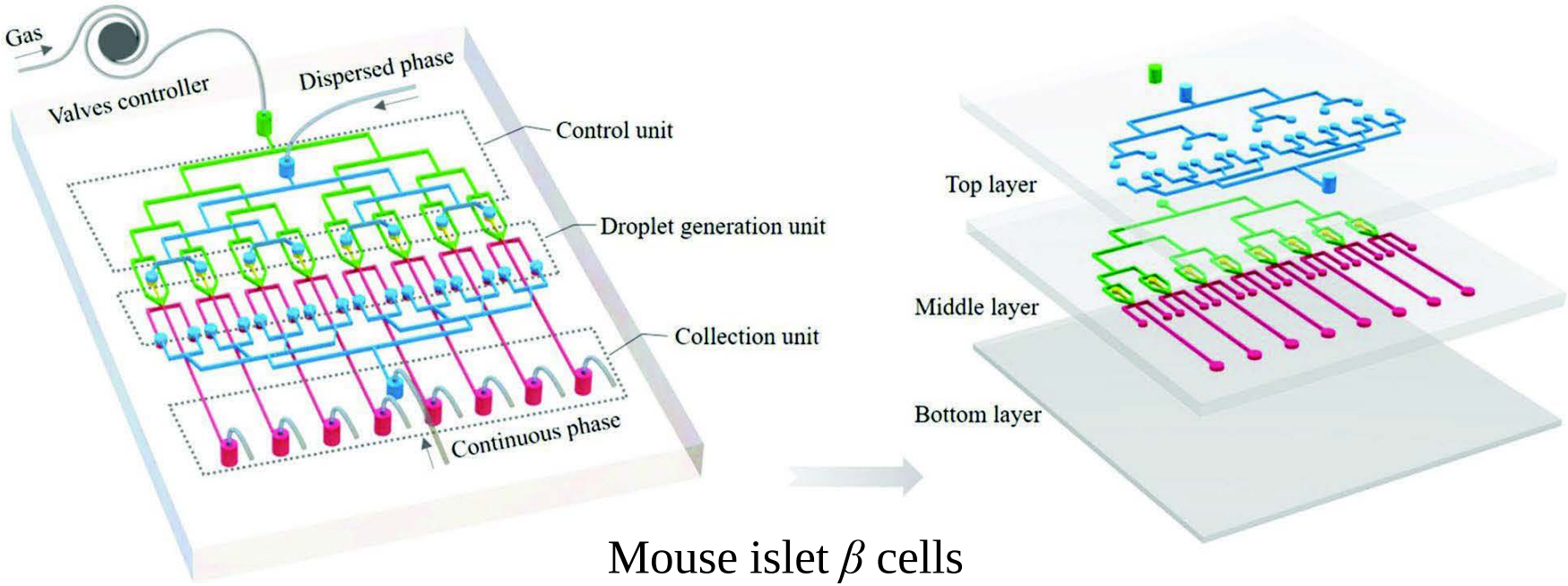
Disadvantage:
- Long separation time
- The reaction system is easy to emulsify
- Difficulty in separation of target product

Microfluidic technology

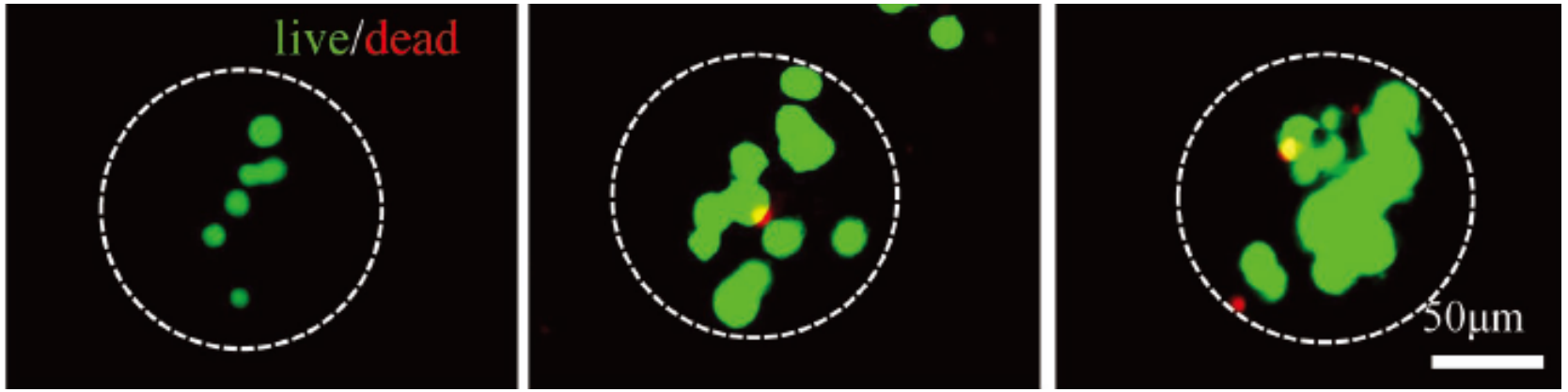
Integrate extraction, catalysis and separation

Glyk A, et al. *Applied Microbiology & Biotechnology*, 2015, 99(16): 6599-6616.
Krause J, et al. *Journal of Chromatography A*, 2015, 1391(1): 72-79.

W/W microfluidic droplet technology



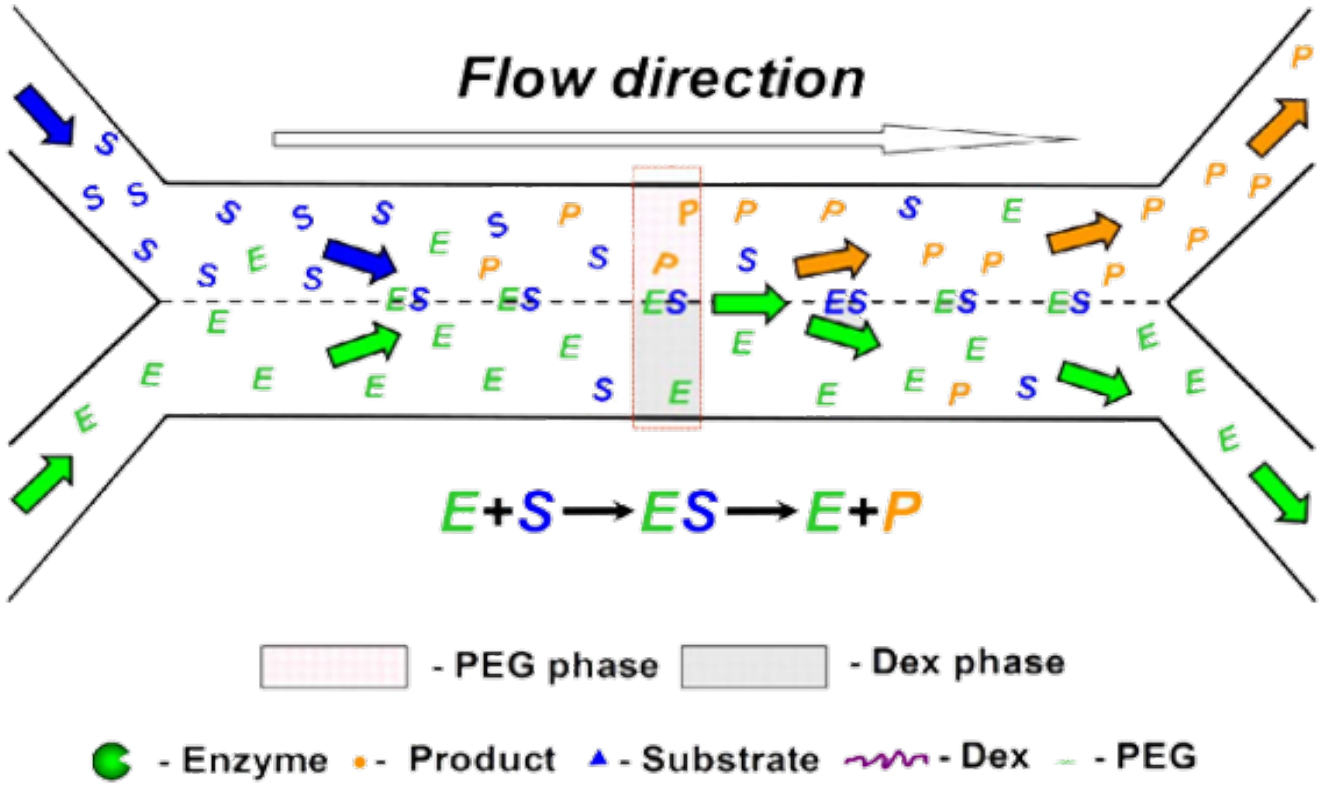
Mouse islet β cells

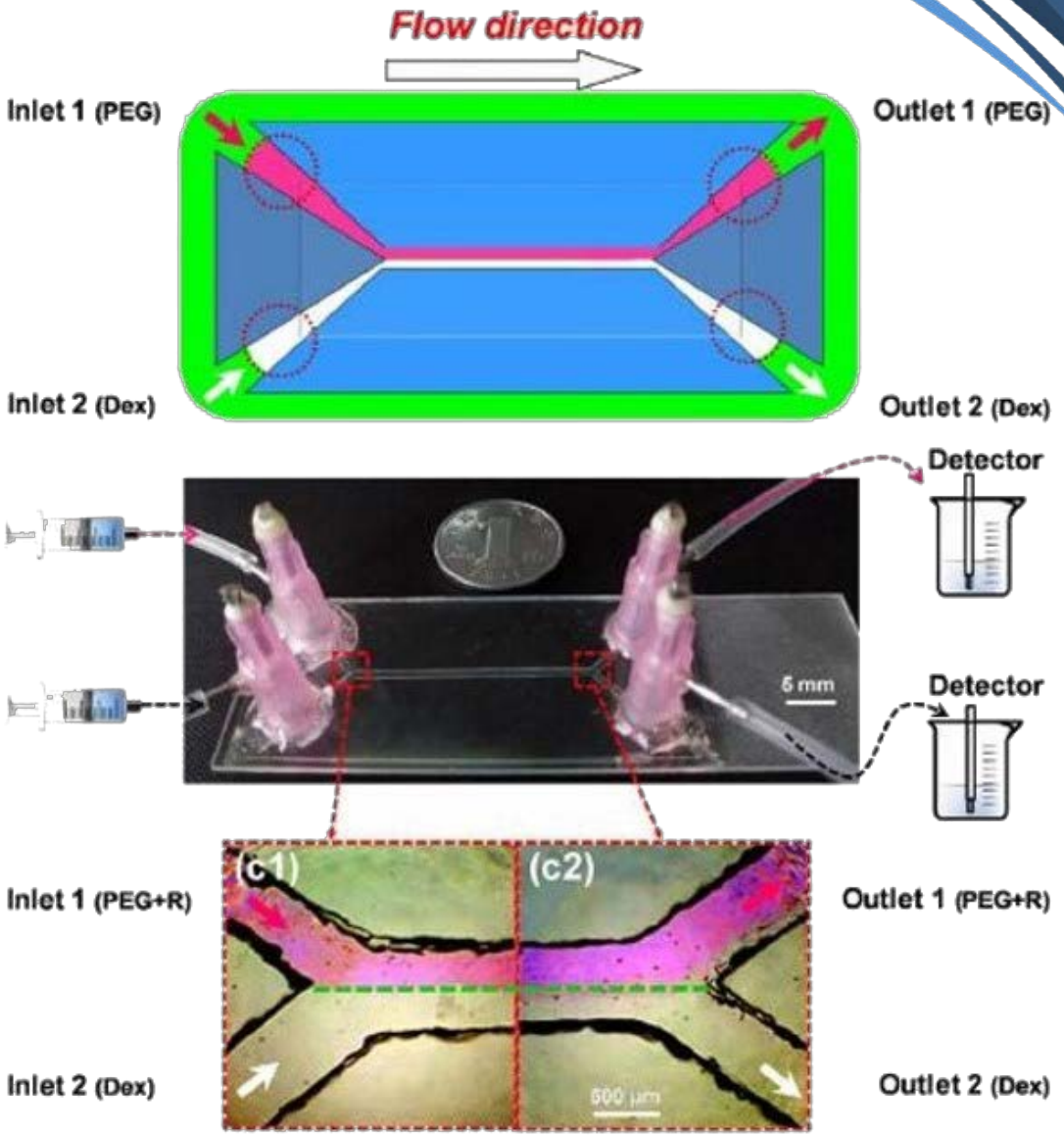


D1 **D4** **D7**

Liu H T, et al. *Small*, 2018, 14(36): e1801095.

Microfluidic two-phase enzyme catalysis system





500 times higher than a conventional reactor

Meng S X, et al. *Chemical Engineering Journal*, 2018, 335, 392-400.

Research content

1. Construction of biotransformation system and qualitative and quantitative analysis methods for mulberry red pigment
2. Separation and coupling preparation of mulberry red pigment by two aqueous phase immobilized enzyme catalysis
3. Modification of mulberry red pigment by W/W microdroplet enzyme catalysis
4. Modification of Mulberry Red Pigment by Microfluidic Double Aqueous Phase Immobilized Enzyme Transformation

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1 Construction of biotransformation system and qualitative and quantitative analysis methods for mulberry red pigment

C_3G 4.72±0.98 mg/100g

C_3R 7.86±1.33 mg/100g

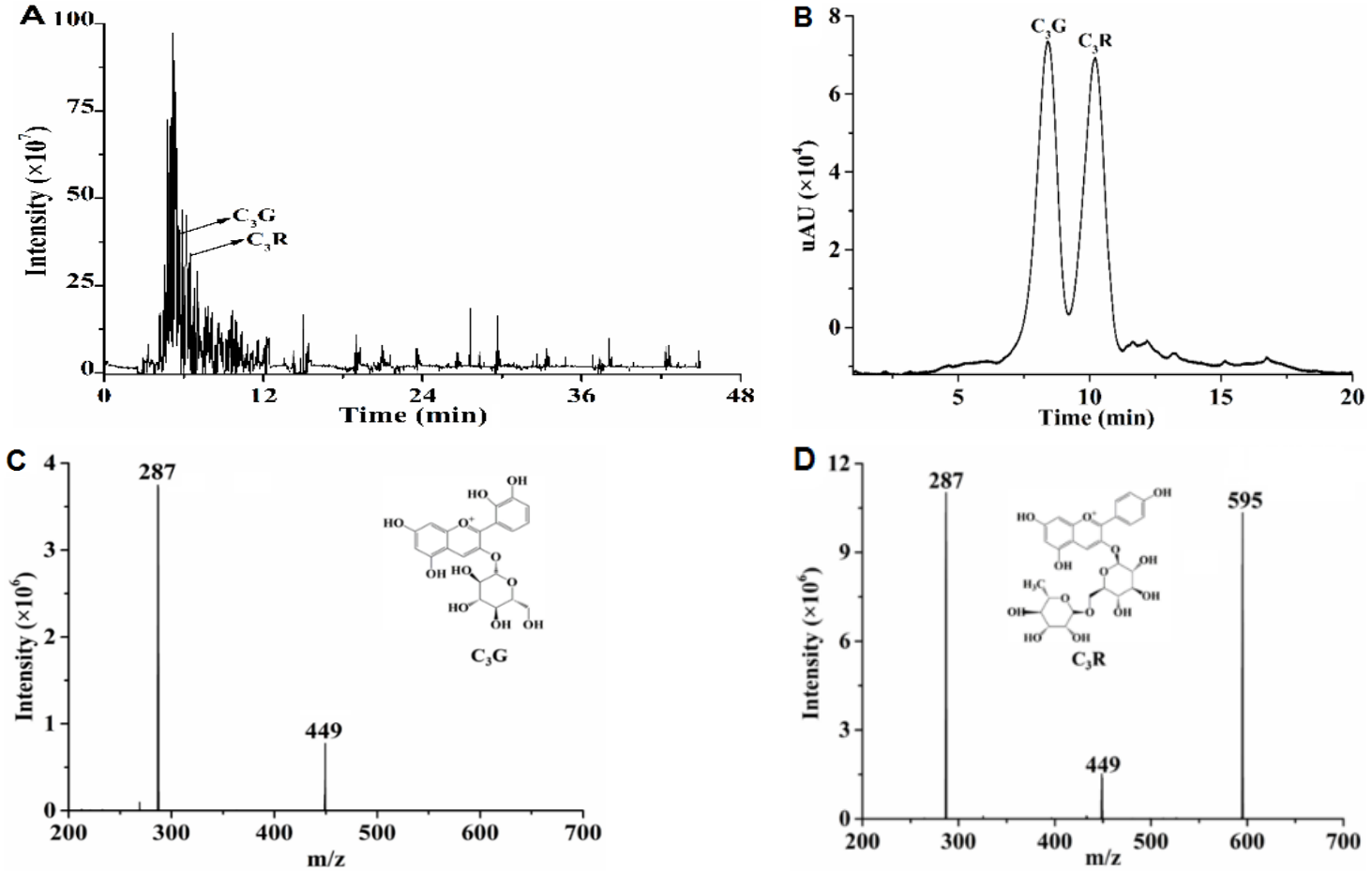

Fig. 1.1 HPLC-PDA-ESI-MS/MS and HPLC-UV chromatogram of mulberry

HPLC-PDA-ESI-MS/MS

1 Construction of biotransformation system and qualitative and quantitative analysis methods for mulberry red pigment

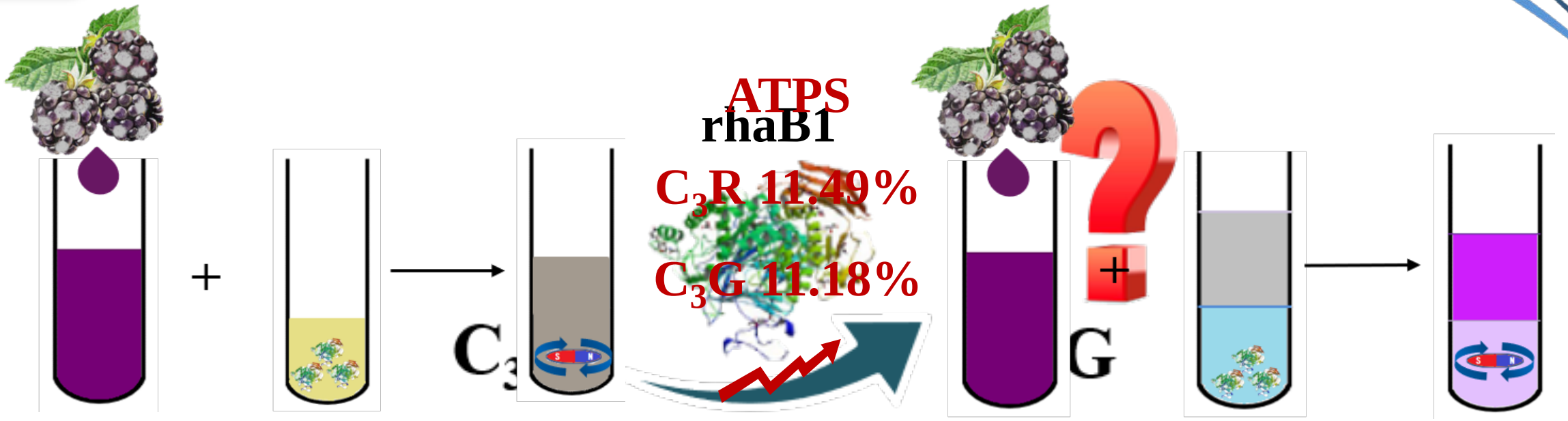

Table 1.1 Process comparison of homogeneous and biaqueous free enzyme systems

System	pH	Temperature（℃）	Concentration（mg/mL）	Conversion of C3R（%）	Purity of C3G （%）
Homogeneous free enzyme	5	45	0.086	62.92±0.79	75.29±0.78
Two aqueous phase free enzyme	5	45	0.11	74.41±0.85	86.47±1.49

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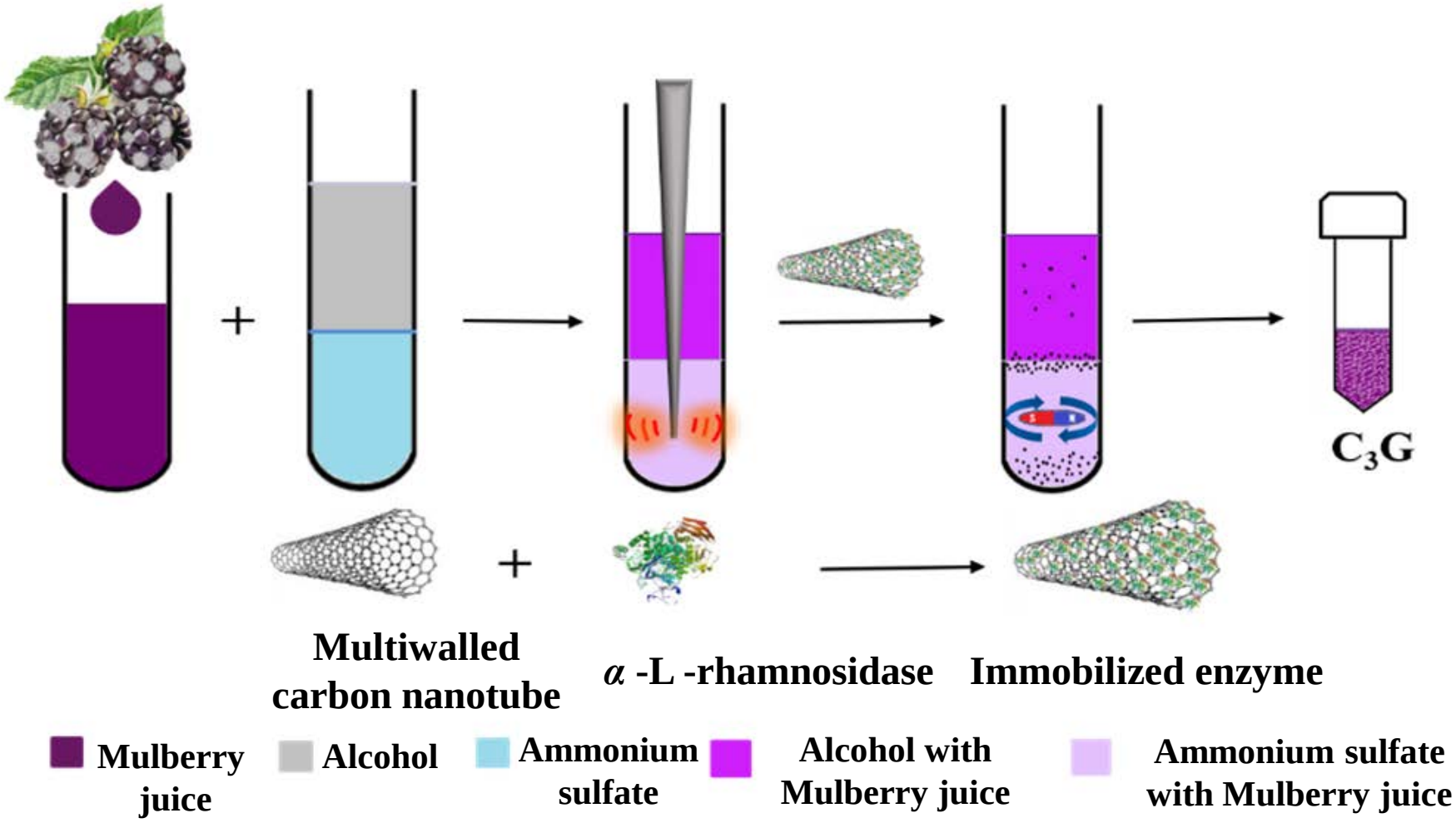
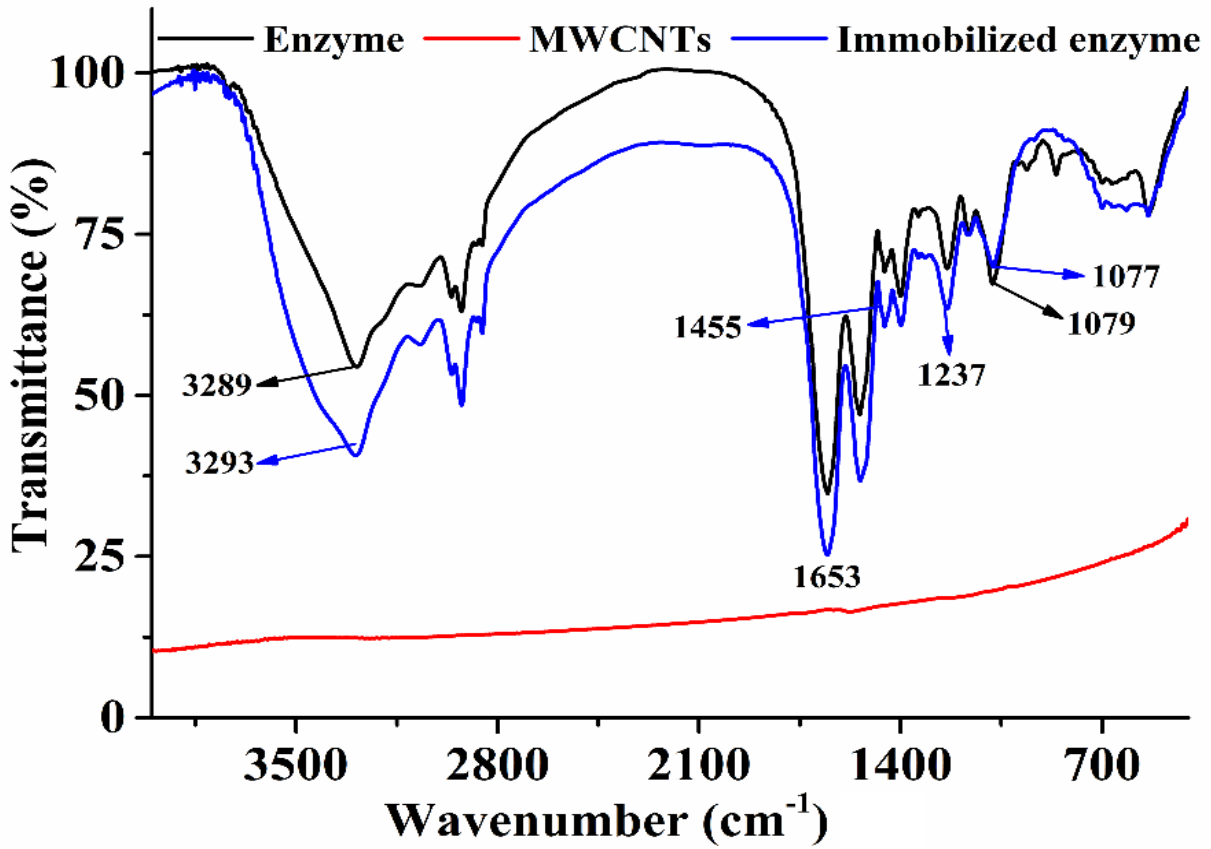


Fig. 3 Schematic diagram of two aqueous phase immobilized enzyme system

2 Separation and coupling preparation of mulberry red pigment by two aqueous phase immobilized enzyme catalysis

Characterization of immobilized α-L-rhamnosidase



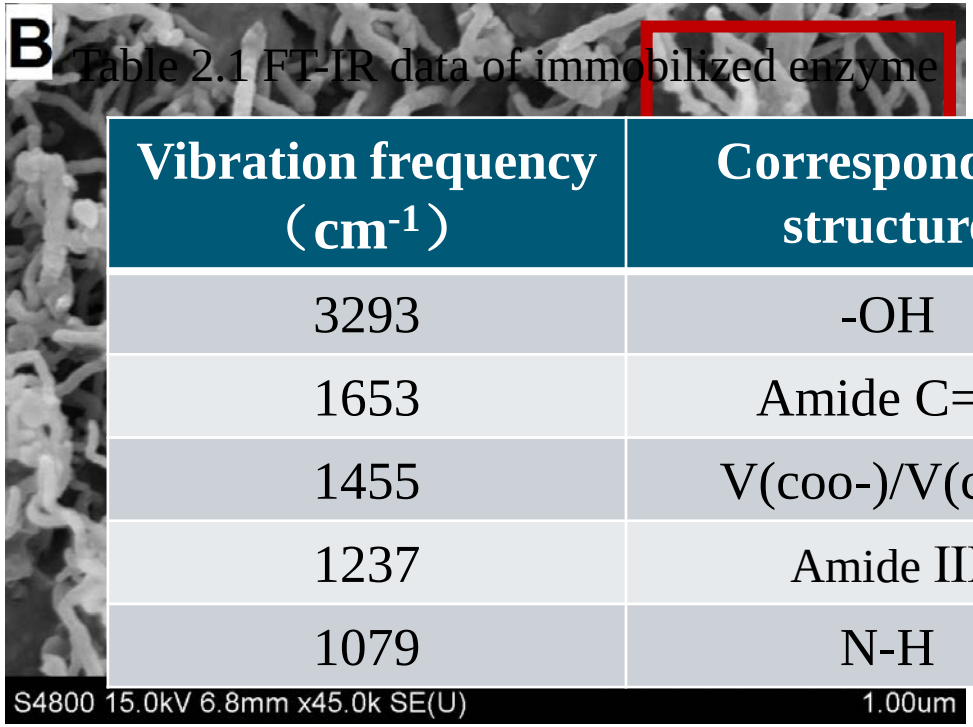


Table 2.1 FT-IR data of immobilized enzyme

Vibration frequency（cm^{-1}）	Corresponding structure
3293	-OH
1653	Amide C=O
1455	V(coo-)/V(c=c)
1237	Amide III
1079	N-H

mobilized α-L-rhamnosidase

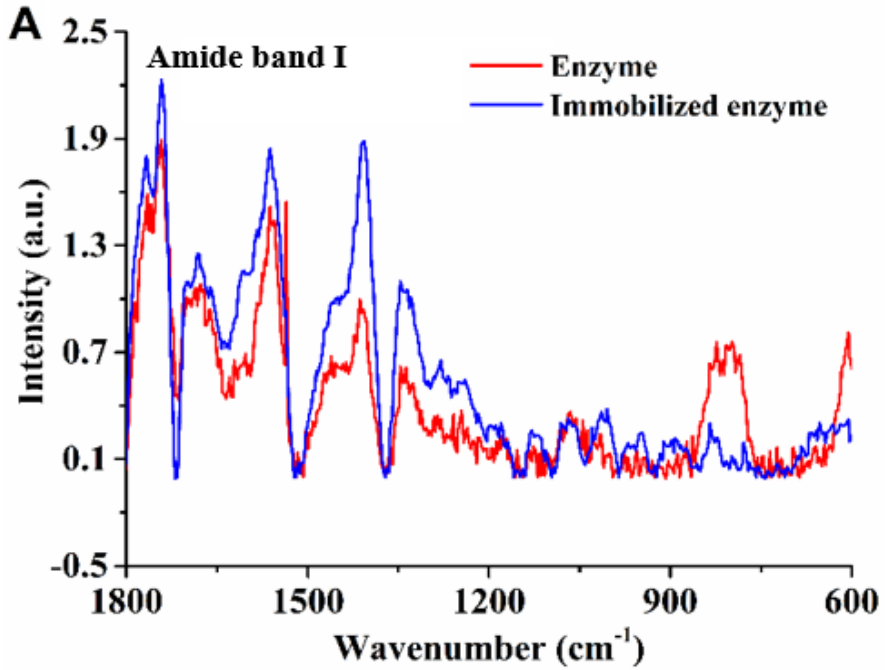
(A) multi-walled carbon nanotubes (MWNTs) (B) immobilized enzyme

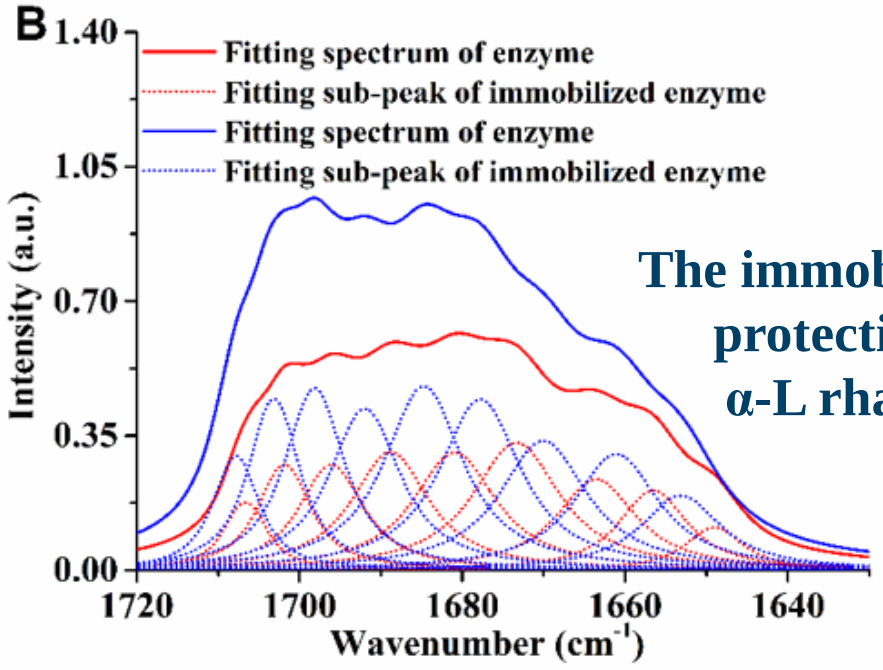
Fig. 2.2 FT-IR spectra of α-L-rhamnosidase, MWNTs, and immobilized enzymes

α-L-rhamnosidase has been successfully immobilized on multi-walled carbon nanotubes

2 Separation and coupling preparation of mulberry red pigment by two aqueous phase immobilized enzyme catalysis

Characterization of immobilized α-L-rhamnosidase





The immobilization had a protective effect on α-L rhamnosidase

Fig. 2.3 Raman and fitted Raman spectra of immobilized enzymes

Table 2.2 Protein secondary structure of α-L-rhamnosidase and MWNTs immobilized enzyme

Sample	α-spiral（%）	Random coil（%）	β-folding（%）	β-Angle（%）
α-L-rhamnosidase	9.79 ± 0.65[a]	21.88 ± 0.78[a]	29.14 ± 0.73[a]	39.19 ± 0.31[a]
Immobilized enzyme	14.39 ± 0.71[b]	23.99 ± 0.63[a]	27.59 ± 0.56[a]	34.03 ± 0.34[b]

2 Separation and coupling preparation of mulberry red pigment by two aqueous phase immobilized enzyme catalysis

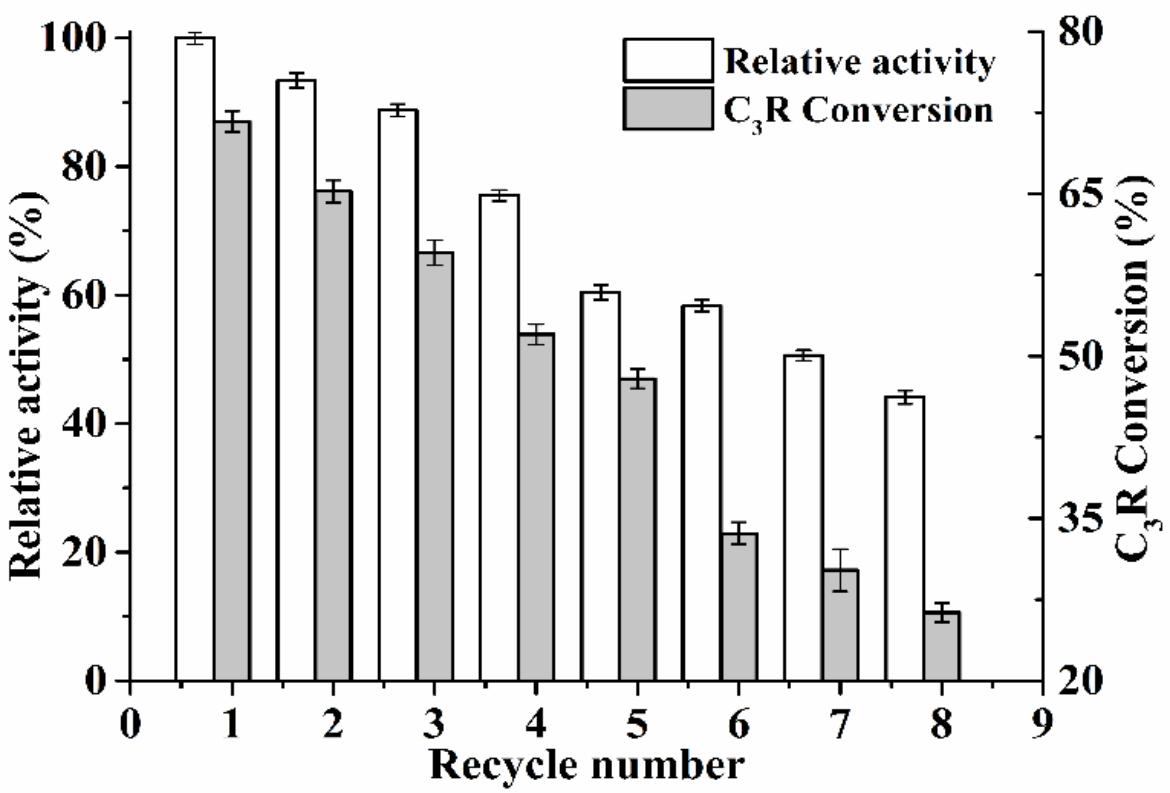


Fig. 2.4 Reuse of immobilized enzyme in liquid-solid three-phase system

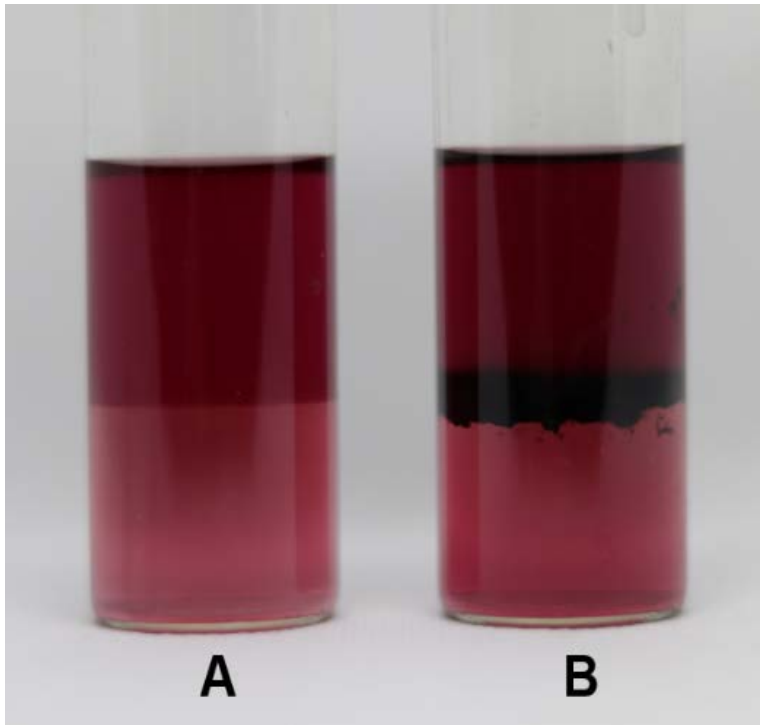


Fig. 2.5 Interface diagram of two aqueous phase system
(A) two aqueous phase free enzyme system;
(B) Two aqueous phase immobilized enzyme system

The immobilized enzyme can be reused for 7 times

Realize interfacial catalysis

2 Separation and coupling preparation of mulberry red pigment by two aqueous phase immobilized enzyme catalysis

Table 2.3 Process comparison of homogeneous and double aqueous phase systems

System	pH	Temperature（℃）	Concentration（mg/mL）	Conversion of C3R（%）	Purity of C3G（%）	pH
Homogeneous free enzyme	5	45	0.086	-	62.92±0.79[a]	75.29±0.78[a]
Two aqueous phase free enzyme	5	45	0.11	-	74.41±0.85[b]	86.47±1.49[b]
Two aqueous phase immobilized enzyme	5	45	0.11	7	71.68±0.94[c]	82.42±1.04[c]

1.28 times

ATPS-IE

C_3R 8.76%，C_3G 7.13%

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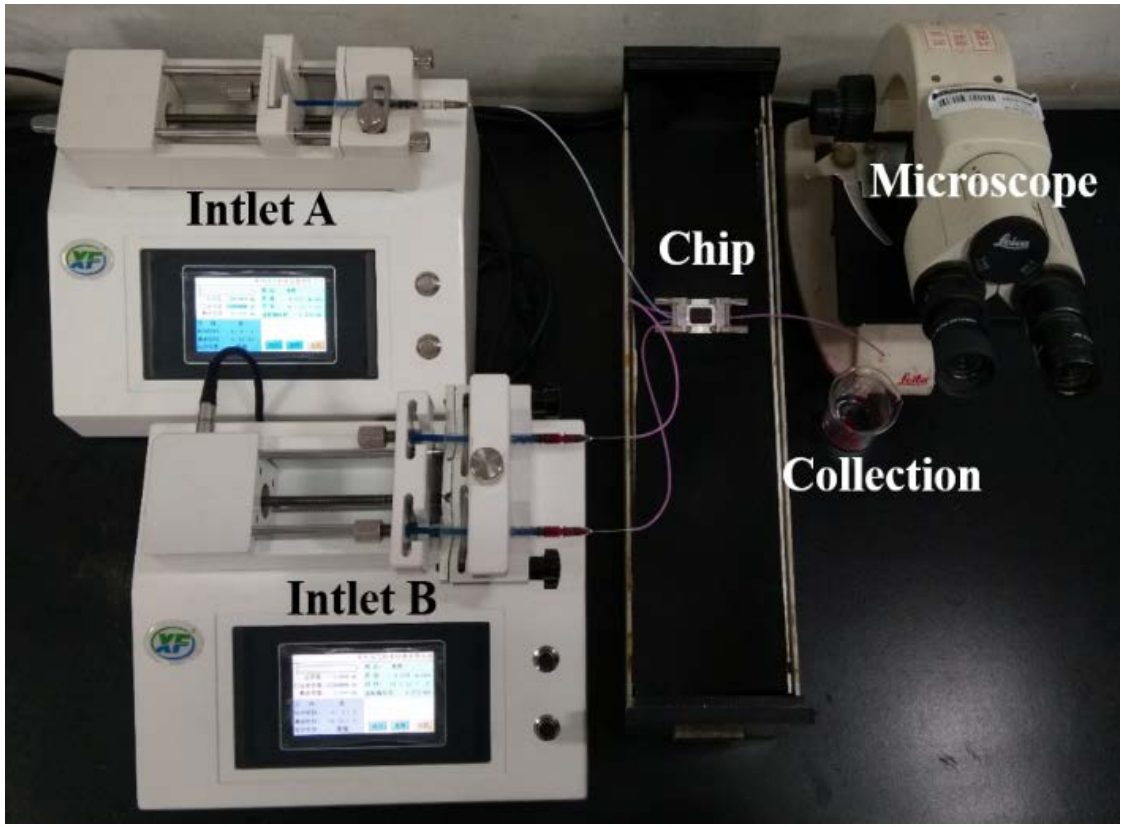

Fig. 3.1 Physical diagram of microdroplet device

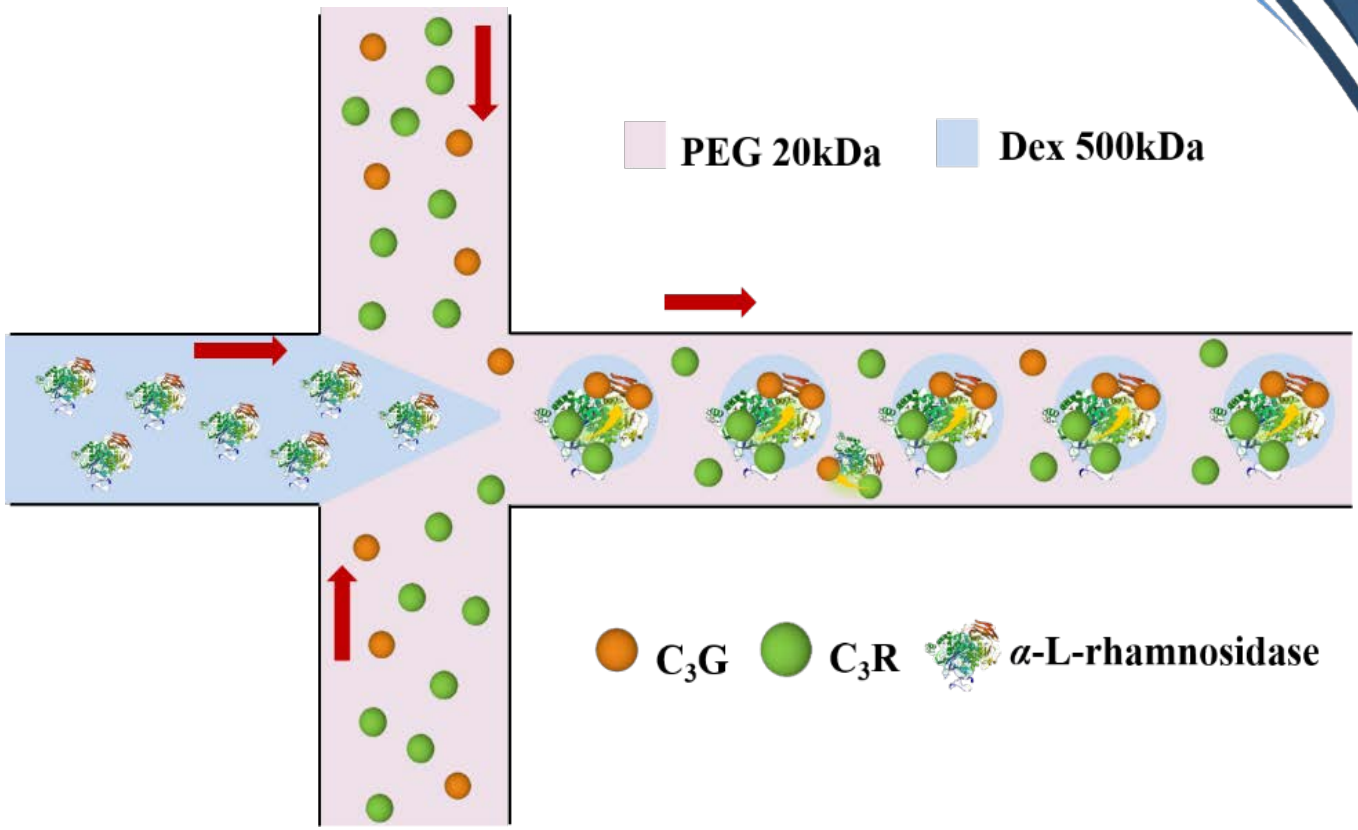

Fig. 3.2 Schematic diagram of W/W microfluidic system

3

Modification of mulberry red pigment by W/W microdroplet enzyme catalysis

Continuous phase 17%PEG flow rate: 0.2-1.0 μL/min, disperse phase 15% DEX flow rate: 0.058-0.09 μL/min

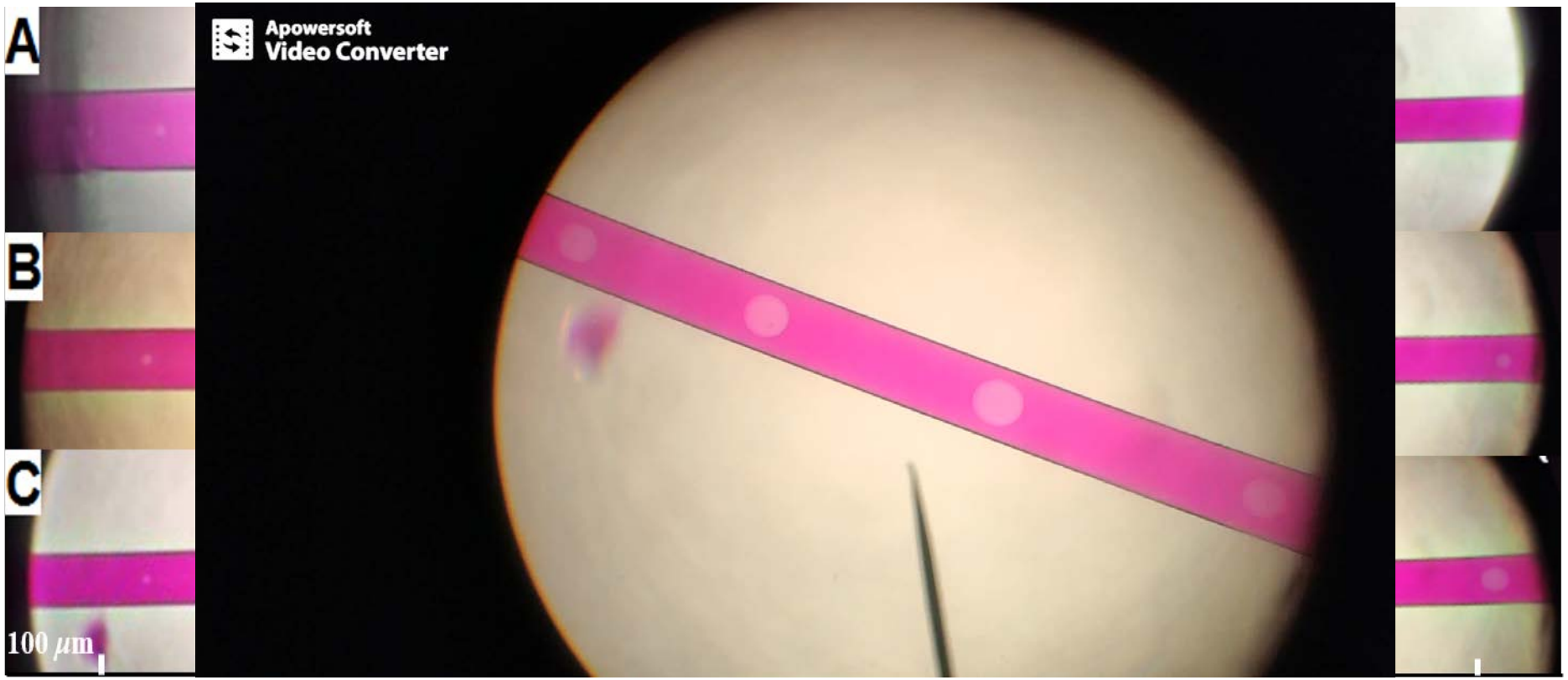

Fig. 3.3 When DEX is fixed at 0.058 L/min
Effect of PEG velocity on droplet formation in water
（A）1.1 μL/min；（B）0.2 μL/min；（C）1.0 μL/min

Fig. 3.4 PEG fixed at 0.4 L/min
Effect of DEX velocity on droplet formation in water
（A）0.1 μL/min；（B）0.058 μL/min；（C）0.09 μL/min

3 Modification of mulberry red pigment by W/W microdroplet enzyme catalysis

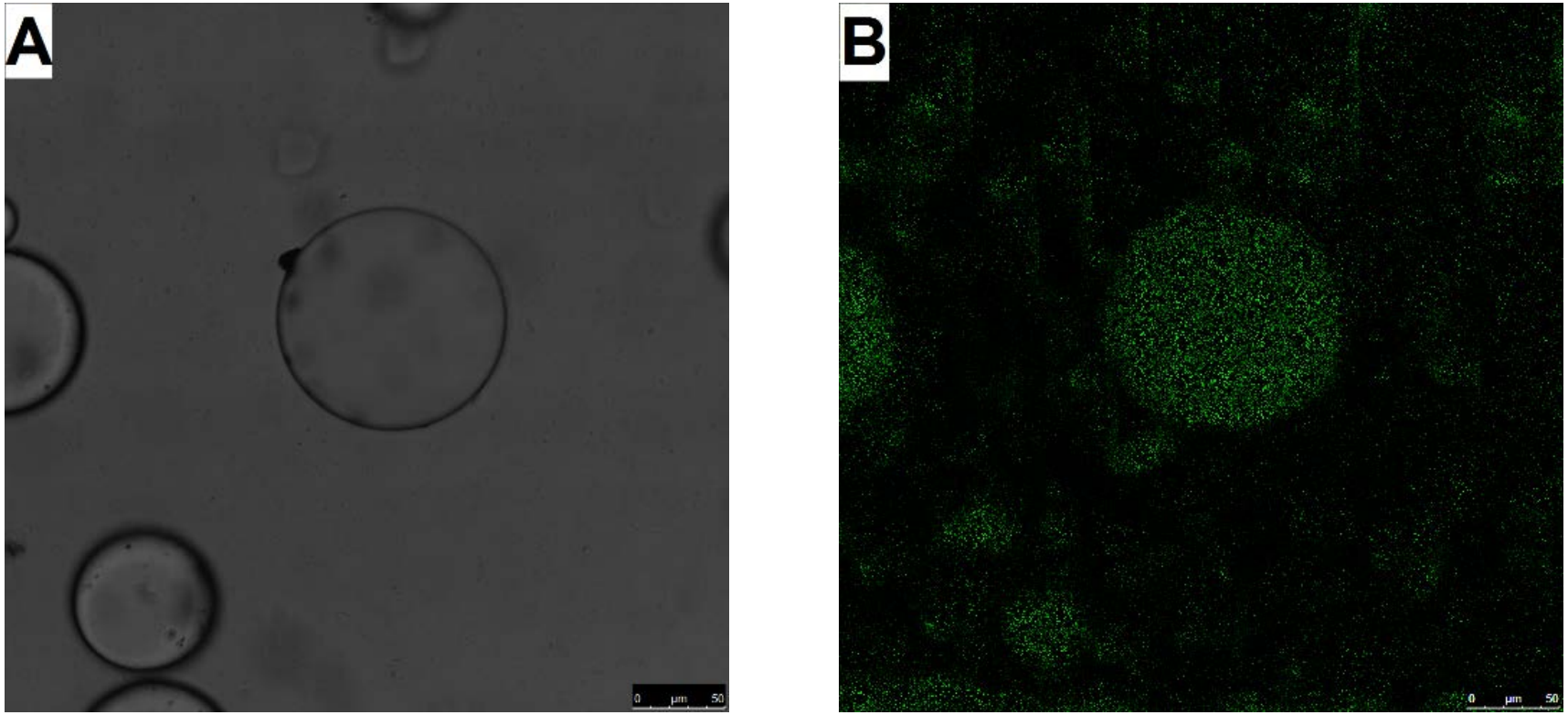

Fig. 3.5 Optical microscope image of W/W microdroplet (A) Laser confocal microscope image (B)

RHAB1-EGFP with green fluorescence
Successfully encapsulated inside W/W microdroplet

3 Modification of mulberry red pigment by W/W microdroplet enzyme catalysis

Table 3.1 Process comparison of homogeneous, aqueous two-phase and W/W micro-droplet systems

System	pH	Temperature （℃）	Substrate concentration（mg/mL）	Reuse（times）	Time（min）	Conversion rate of C_3R（%）	Purity of C_3G（%）
Homogeneous Free Enzyme	5	45	0.086	-	60	62.92 ± 0.79^{a}	75.29 ± 0.78^{a}
Two Aqueous Phase Free Enzyme	5	45	0.11	-	60	74.41 ± 0.85^{b}	86.47 ± 1.49^{b}
Two Aqueous Phase immobilized enzyme	5	45	0.11	7	60	71.68 ± 0.94^{c}	82.42 ± 1.04^{c}
W/W microdroplets	5	45	0.007	-	2.8	53.79 ± 0.98^{d}	68.14 ± 1.38^{d}

T 1/20

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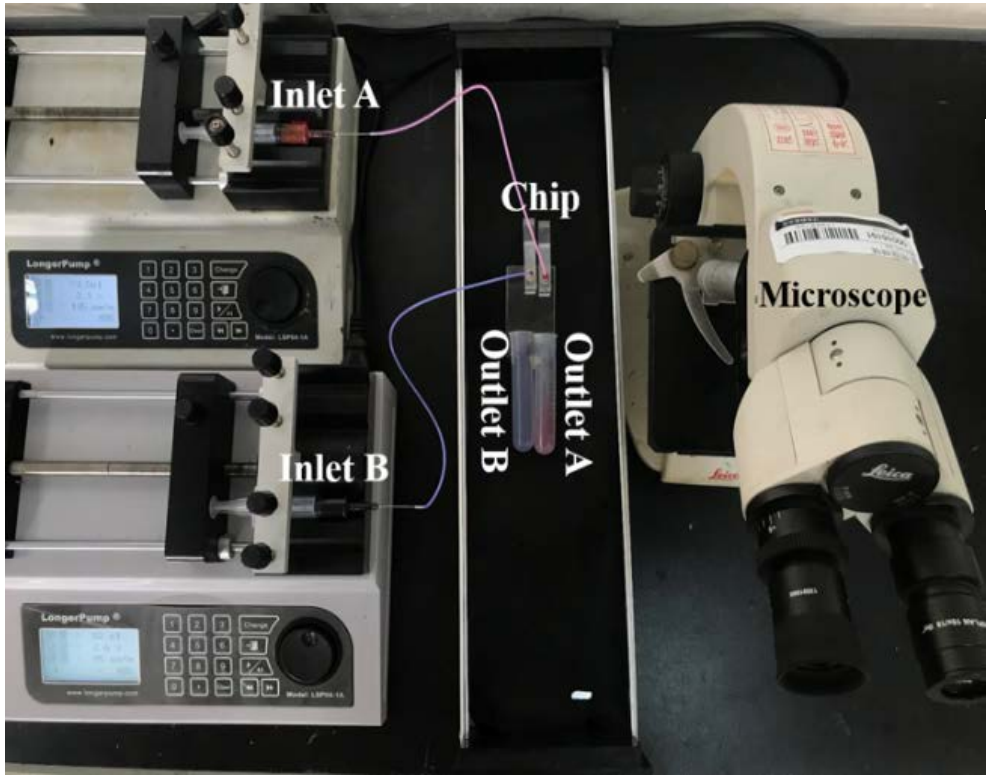

Fig. 4.1 Physical image of two aqueous phase microfluidic device

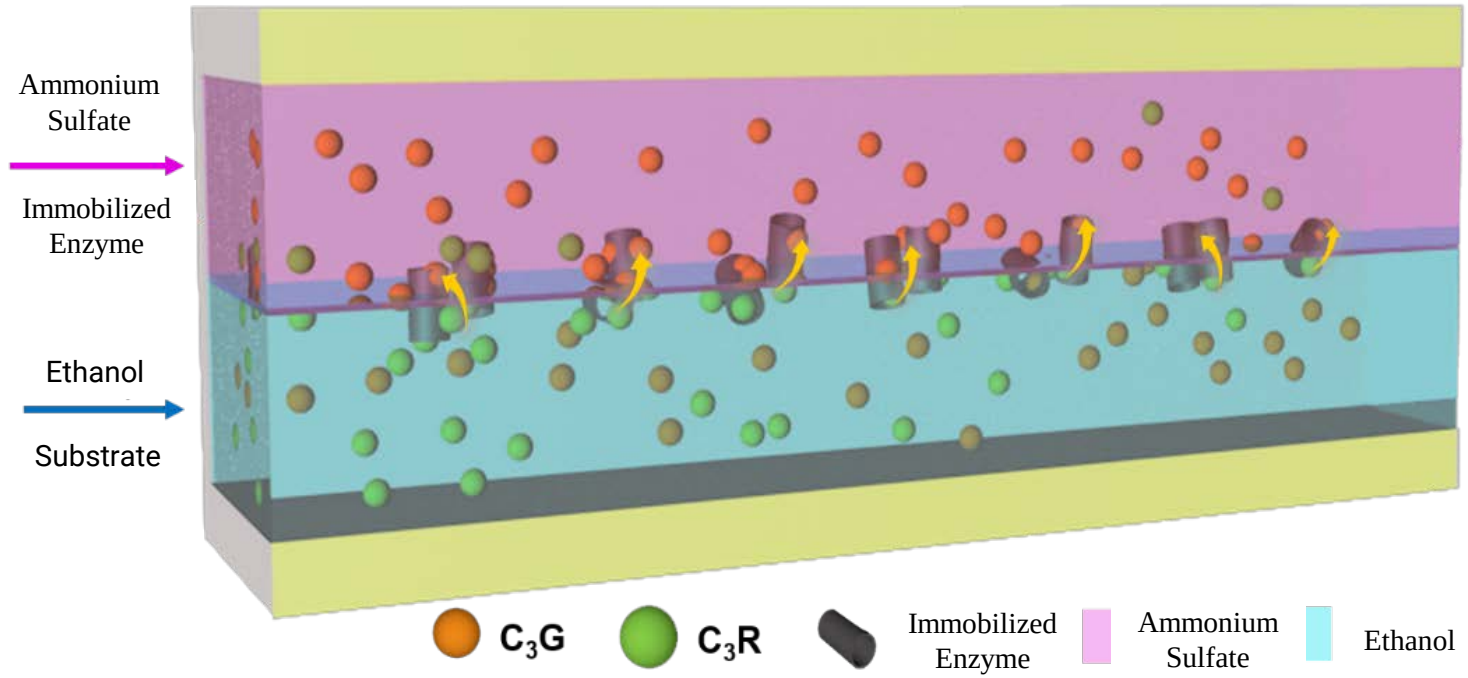

Fig. 4.2 Schematic diagram of microfluidic two aqueous phase immobilized enzyme system

4 Modification of Mulberry Red Pigment by Microfluidic Double Aqueous Phase Immobilized Enzyme Transformation

Flow rate of ammonium sulfate： 13.5-18 μL/min

Flow rate of ethanol： 8-12.5 μL/min

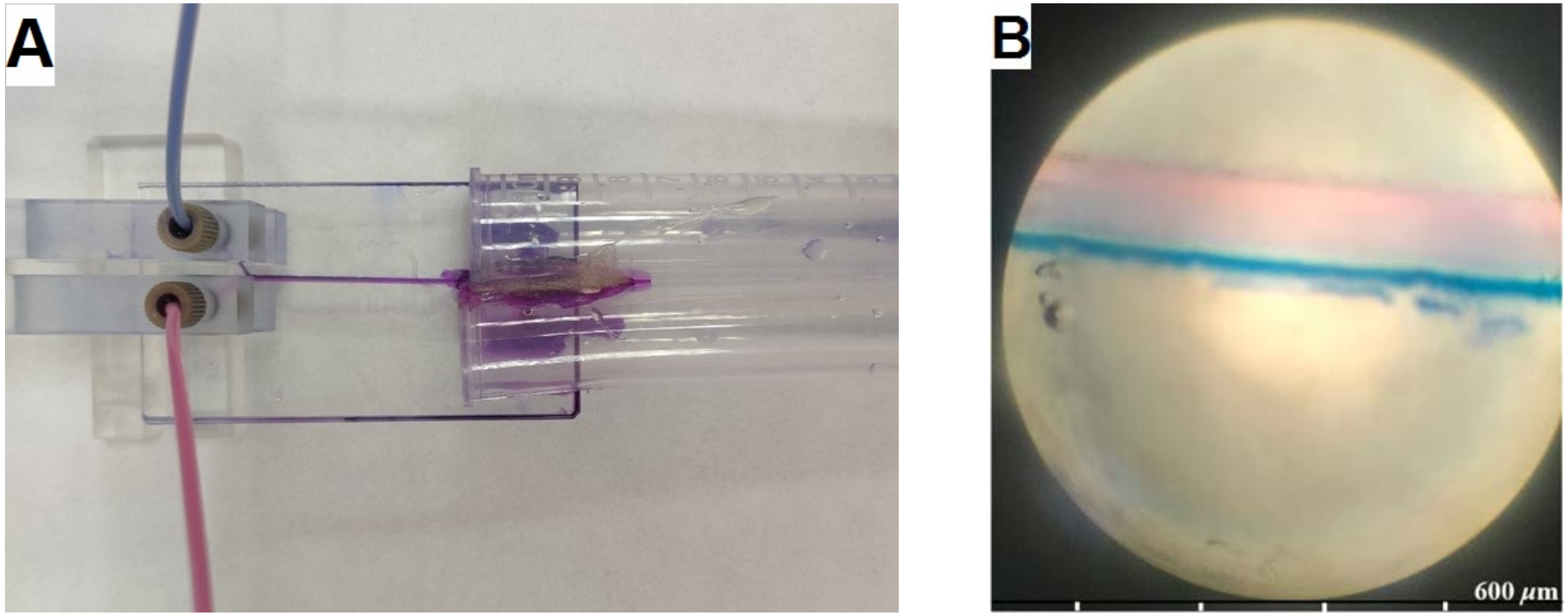

Fig. 4.3 Two aqueous phase microfluidic reactor
(A) Physical image of two aqueous phase microfluidic reactor；
(B) Micrograph of two aqueous phase microfluidic reactor

Parallel laminar flow was formed

4 Modification of Mulberry Red Pigment by Microfluidic Double Aqueous Phase Immobilized Enzyme Transformation

$$f[A_0] = \frac{C}{Q} + K_m ln(1-f)$$

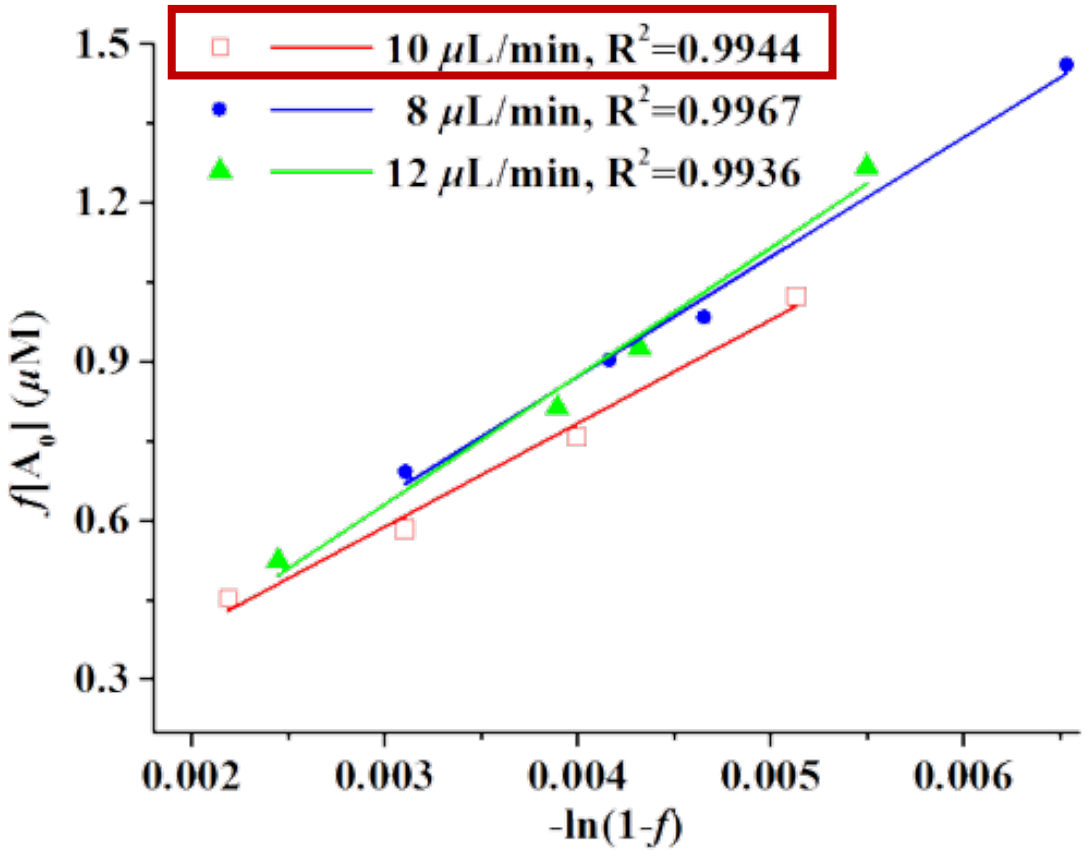

Fig. 4.4 Lilly-Hornby graph of immobilized enzyme in microchannel

The immobilized enzyme can be reused 9 times

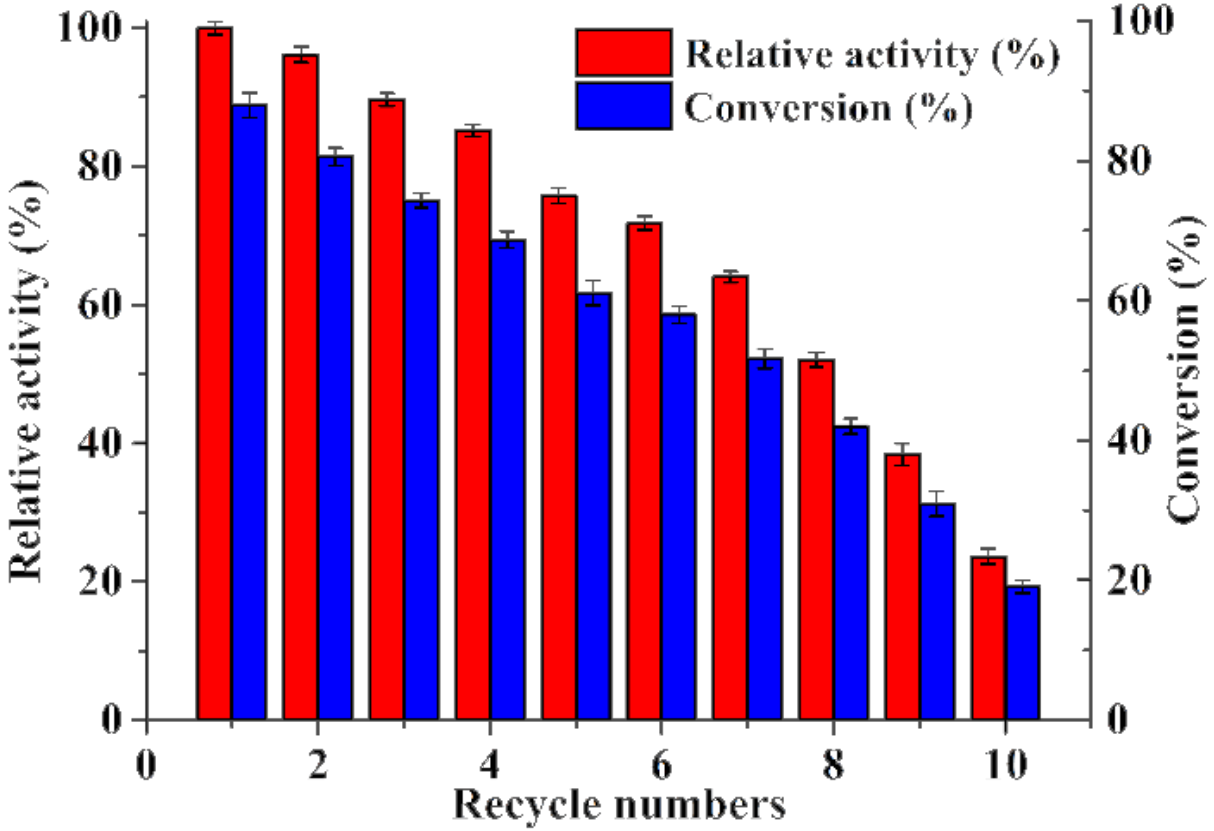

Fig. 4.5 Reuse of immobilized enzymes

4 Modification of Mulberry Red Pigment by Microfluidic Double Aqueous Phase Immobilized Enzyme Transformation

Table 4.1 Process comparison of different systems

System	pH	Temperature（℃）	Substrate concentration（mg/mL）	Reuse（times）	Time（min）	Conversion rate of C_3R（%）	Purity of C_3G（%）
Homogeneous Free Enzyme	5	45	0.086	-	60	62.92 ± 0.79^{a}	75.29 ± 0.78^{a}
Two Aqueous Phase Free Enzyme	5	45	0.11	-	60	74.41 ± 0.85^{b}	86.47 ± 1.49^{b}
Two Aqueous Phase immobilized enzyme	5	45	0.11	7	60	71.68 ± 0.94^{c}	82.42 ± 1.04^{c}
W/W microdroplets	5	45	0.007	-	2.8	53.79 ± 0.98^{d}	68.14 ± 1.38^{d}
Two Aqueous Phase Microfluidic	5	45	0.008	9	0.14	68.66 ± 1.43^{e}	80.78 ± 1.59^{e}

The C_3R conversion rate of MATPS is 5.74% higher than that of the homogeneous system.
The time-consuming is 7/3000 of the conventional reactor and 1/20 of the micro-droplet system.
The immobilized enzyme can be reused 9 times.

04 Conclusions

1. Homogeneous and "ethanol/ammonium sulfate" two aqueous phase free enzyme systems can catalyze the directional hydrolysis of C_3R to C_3G, and the conversion rate of aqueous two-phase is 11.49% higher than that of the homogeneous phase.

2. The "ethanol/ammonium sulfate/immobilized enzyme" three-phase system was successfully constructed. The optimum substrate concentration was 1.28 times that of the homogeneous phase, the conversion rate was 8.76% higher than that of the homogeneous phase, and the immobilized enzyme could be reused 7 times.

3. The W/W microdroplet system which the enzyme is located in the dispersed phase is successfully designed. The reaction time is 2.8 min, which takes only 1/20 of the conventional reactor.

4. Successfully created a microfluidic two aqueous phase immobilized enzyme system with parallel flow of "ethanol/ammonium sulfate". The reaction was only 8.6 seconds, the conversion rate was 5.74% higher than that of the homogeneous system, and the time was 7/3000 of the conventional reactor. 1/20 of the drop system, and the immobilized enzyme can be reused 9 times.

Thanks for your listening !