Recombinant *Escherichia coli* mutant strain producing GH78 *α*-L-rhamnosidase for microfluidic biofilm catalysis

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Abstract: In the present study, recombinant *Escherichia coli* mutant strain producing GH78 *α*-L-rhamnosidases was used to cultivate biofilms by segmentation flow for the biotransformation of isoquercitrin. A novel GH78 α-L-rhamnosidases from elephant feces were analysed, TM1 was the most active truncated mutant of rhaB1 compared to the other mutants, which was used to construct the recombinant *E. coli* BL21 plamisd. The recombinant mutant strain was used to cultivate biofilm in microfluidic reactors for the biotransformation. Our fndings might discover a promising alternative biocatalyst for biotransformation using microfluidic biofilm.
Keywords: biocatalysis; microfluidic biofilm; *α*-L-rhamnosidases; truncated mutants (TM); enzyme activity.

Introduction

Biofilms are formed by most species of bacteria, they are multi-layered communities of microbial cells embedded in extracellular matrixes which develop naturally at solid–liquid and air–liquid interfaces [1]. As a novel whole cell catalyst, biofilms are less susceptible to external hostile environments and antibiotic treatment and the robust nature makes them medicinally an attractive method for protecting and immobilising enzymes for biotransformation [2, 3]. In industry, whole cell biocatalysts predominate as they present a number of advantages for large scale processes. Preparation of whole cell biocatalysts is simple, not requiring lengthy protein purification procedures, and more complicated, multi-step syntheses that require multiple enzymes can all be contained inside single cells and expensive cofactors can be recycled by internal cellular mechanisms [4]. Meanwhile, cells can protect enzymes from harsh reaction conditions, which maintains the structure and activity of enzymes [5]. These advantages have shown biofilms are preferred to the planktonic cells and they are surface-adhered, so reactors can be operated in continuous-flow mode.

Microfluidics present high surface-area-to-volume ratios that enable efficient contact between the wall-adhered bioflm and the liquid phase, so that providing the environment for real-time studies of biofilm growth and kinetics of bio-catalytic conversion [6]. In addition, microfluidics has been used as one of the promising tools for evaluating and revealing the effects of factors, such as shear stress, surface topography, quorum sensing signals, temperature, nutrient concentration and so on in a quantitative way [7]. Many studies which have investigated the use of biofilms as catalysts in microreactors [8-10]. For example, the thermophilic anaerobic bacterium *Caldicellulosiruptor saccharolyticus* has been used for the generation of hydrogen [11]. However, there are currently very few reports of recombinant strains of bacteria being utilised to generate a specific enzyme and catalyse desired biotransformation reactions within biofilms. Also, the key strategy to achieve high biocatalytic activity is the identification of novel enzymes with kinetics optimized for organic synthesis [12]. Therefore, it is necessary to explore novel enzyme as a powerful catalyst with a beneficial reactor design to realize an efficient biotransformation.

The *α*-L-rhamnosidases (EC 3.2.1.40) are a wide spread and industrially important group of glycoside hydrolases, which are responsible for cleavage of terminal α-L-rhamnose residues from the nonreducing end of carbohydrates and their derivatives [13]. The *α*-L-rhamnosidases has been widely found in nature and has been reported from animals, plants, yeasts, fungi, and bacteria [14]. The majority of characterized bacterial α-L-rhamnosidases to date originate from Gram-positive bacteria, i.e. Bacillus, Lactobacillus, Staphylococcus, Clostridium, Streptomyces and Thermomicrobium species [15]. Most known bacterial-lrhamnosidases are classified into the GH 78 family (GH78) because of possessing the glycoside hydrolase (GH) motif. According to the CAZy database, GH 78 family contains 470 proteins, including 13 proteins from the Archaea, 394 proteins from the Bacteria, and 59 proteins from members of the Eukaryota [14]. Eighteen of these proteins are characterized biochemically and display the same type of enzymatic activity. For instance, up to now, two *α*-L-rhamnosidases were identified by activity assays in a comprehensive functional study on glycoside hydrolases (GHs) from cow rumen on the functional level [16]. Two crystal structures of *α*-L-rhamnosidases have been determined: α-L-rhamnosidase B (BsRhaB) from Bacillus sp. GL1 and the putative α-L-rhamnosidase BT1001 from Bacteroides thetaiotaomicron VPI-5482, which are both from GH78 family [16].The *α*-L-rhamnosidases are of interest for chemical industry, especially pharmaceutical and food industries [17]. The commercial enzyme preparations include naringinase and hesperidinase comprising *α*-L-rhamnosidase next to ß-d-glucosidase activity, which are applied for debittering and clarifying of fruit juices [18, 19]. The aroma optimization of wine is a further

potential field of application of α-L-rhamnosidases [20]. In addition, the derhamnosylating function of α-L-rhamnosidases can enhance the pharmacological properties of drugs like antibiotics as chloropolysporin, steroids as ruscin or gypenosides [21]. Therefore, the α-L-rhamnosidases are of considerable interest given their suitability in various applications within the food and pharmaceutical industries, which are worth looking insight into their structure and regulatory mechanism for further applications.

Flavonoids such as rutin and hesperidin mostly exist in the peel of plants such as grapefruit, pomelo, grape, lime and other rutaceae plants have anti-tumor, anti-inflammatory and other pharmacological effects, but these peels are easily discarded by people in daily life, resulting in a great waste of flavonoids. Isoquercitrin, a glycoside formed by quercetin and glucose, has many pharmacological effects such as antioxidation [22], also it is an intermediate of Enzymatically Modified Isoquercitrin (EMIQ). However, its content is extremely low, and it is difficult to extraction. It has found that rutin which has one more rhamnosyl than isoquercitrin in the molecular structure can be found aboundantly in nature [23], so that the isoquercitrin can be selectively prepared through its catalytic hydrolysis by rhamnosidase. Therefore, we synthesize isoquercitrin by extracting rutin from grapefruit peels in order to make use of resources and preserve flavonoids to a certain extent.

In the present study, recombinant Escherichia coli mutant strain producing GH78 α-L-rhamnosidases was used to cultivate biofilms by segmentation flow for the biotransformation of isoquercitrin. The formation and structure of biofilms in a microfluidic chip were investigated. The method highlights the potential of combining a powerful catalyst with a beneficial reactor design for biocatalytic transformations of compounds which are toxic, volatile, and have low solubility in water.

Materials and methods

Bacterial strains, plasmids and chemical reagents

The strains and plasmids used in this study are listed in Table I and primers are listed in Table 2. All organisms used have been cultivated in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) with appropriate antibiotics (kanamycin 50 μg/mL). *E coli* DH5α was used as the cloning strain. The plasmid pET28a and *E.coli* BL21 (DE3) were used as the gene expression vector and expression host strain, respectively.

Plasmid construction

The rhaB1 gene was provided by Professor Wolfgang Streit (Universität Hamburg, Germany). Based on its catalytic domain, five truncated mutants were designed. Recombinant plasmids harboring rhaB1 truncated mutant and *egfp* were constructed.

Protein expression and purification

The transformant *E.coli* BL21-rhaB1 truncation mutants were precultured overnight at 37 °C in LB medium. The expression cultures were inoculated with 1% of the resuspended cells and cultivated at 22 °C until they reached 0.6–0.8 OD600. The cultures were then transferred to 17 °C, and overexpression was induced with 400 μmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) for 16–20 h. The protein was harvested with a sonotrode (JY92-2D, Scientz, Ningbo, China) at 0.4–0.6 kW for 12–18 min. The crude lysate was then centrifuged at 12000 rpm for 30 min at 4 °C to remove cellular debris and purified using a Ni-TED 2000 packed column.

Biocatalytic assays

The α-L-rhamnosidase activity was determined with *p*NP-rham as a substrate. The enzymatic reaction was conducted by mixing 1 mM pNPR solution with phosphate buffer buffer (pH 7.4); the reaction was immediately initiated by adding 100 μL of properly enzyme solution and was incubated at 45 °C for 10 min. The reaction was stopped by the addition of 150 μL of 1 M Na2CO3. After 3 min centrifugation the absorptions were measured at 405 nm[10]. One unit of α-L-rhamnosidase activity was defined as the amount of enzyme required to release 1 μmol of p-nitrophenyl groups per minute at 45 °C and pH 7.4. The temperature dependent activity was determined between 10 °C and 70 °C. The pH dependency was analyzed from pH 3.7 to 4.5 in NaOAc, from pH 5.5 to 6.5 in MES, from pH 7.0 to 7.5 in PBS, and from pH 8.0 to 9.0 in Tris–HCl each at 50 mM buffer concentration with enzyme solutions. The initial enzyme activities were determined by hydrolyzing at 45 °C with 1 mM pNPR. The Michaelis constant (K_m) and the maximum velocity (V_{max}) were calculated from Lineweaver-Burk plots. These values were further used to calculate the turnover number (Kcat) and the catalytic efficiency (K_{cat} / K_m).

Biofilm cultivation in the segmented flow microfluidic chips

The reaction compartment consisted of a glass–PDMS microreactor with 800 μm (width) × 600 mm (depth) rectangular microchannels. The assembled set-up was sterilized by autoclaving at 121 °C for 20 min. During inoculation, the medium pump was stopped. After inoculation, the system was kept idle without medium supply

for 2 h to enable the initial attachment of the cells to the glass substratum. Medium feed by a peristaltic pump fitted at a flow rate of 10 μL/min. The system was operated in a single phase mode with only medium for 2–3 days and subsequently air segments were introduced at a flow rate of 10 μL/min. The reaction temperature was 32 °C. Product formation was analyzed using HPLC.

Characterization analysis

Proteins were detected using SDS-PAGE loading 10 mg of total protein/lane. The prepared samples of biofilms were characterized by XRD patterns on a SHIMADZU XRD-6000X diffractometer system (Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). FT-IR spectra were recorded on a BRUKER TENSOR27 (Bruker Instruments, Ettlingen, Baden-Württemberg, Germany). The morphology of biofilms was studied by ESEM (XL-30, Philips Ltd., Netherlands). The morphology of the recombination strain immobilized on the surface of the microchannel was studied using a fluorescence inversion microscope system (Nikon ECLIPSE TS100, Nikon Instruments Europe B.V., Kingston, Surrey, England) and CLSM. Three-dimensional (3D) image reconstructions, quantification of biofilms were obtained using the software packages IMARIS. Biofilms were imaged in situ non-invasively in real time at regular time intervals without interrupting the flow by exciting GFP (excitation at 488 nm and emission at 515 nm).

Enzymatic synthesis of isoquercitrin using microfluidic biofilm

The enzymatic reaction was performed in a microfluidic biofilm. The reaction temperatures were varied at 30, 35, 40, 45, 55 °C. The buffers tested in the rutin MES buffer (pH 5.5–6.5). The reaction time was maintained at 24 h.

Statistical analysis

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

Results and discussion

Expression, purifcation and identifcation of recombinant E. coli BL21-pET28a-TM-eGFP

As shown in Fig. 1(A), the specific band corresponded to the approximately 2808 bp gene construct. As shown in Fig. 1(B), the lanes 1, 2 and 3 were whole cell lysates of recombinant BL21-pET28a-TM, purified RhaB1-TM protein, and whole cell lysates of BL21-pET28a, respectively. Compared with lanes 1 and 3, the specific lane corresponding to the approximately 103 kDa protein RhaB1-TM was detected in the proteins obtained after IPTG induction (lane 2).

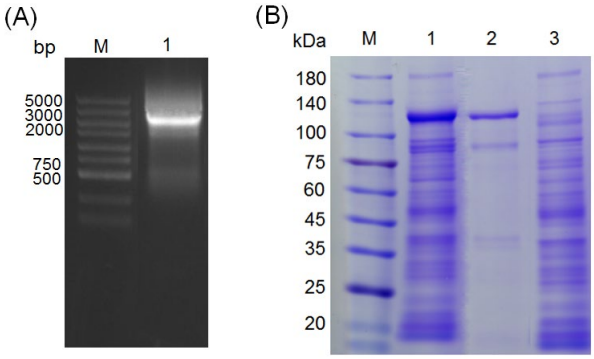

Fig 1. Confirmation of recombinant *E. coli* BL21-pET28a-TM-eGFP. (A) The recombinant gene TM-eGFP was extracted from transformed *E. coli* plamisd. Lines: M, 5 kb DNA marker; (B) SDS-PAGE of whole cell lysates. Lanes: M, protein size markers; 1, whole cell lysates of recombinant BL21-pET28a-TM; 2, purified RhaB1-TM protein; 3, whole cell lysates of BL21-pET28a.

Enzymatic properties of purifed recombinant RhaB1-TM-eGFP

All enzyme activities were assayed with pNP-rham as the substrate. As is shown in Fig 2, the purifed RhaB1-TM-eGFP displayed the optimal activity at pH 6.5. The optimal temperature was determined to be 35 °C.

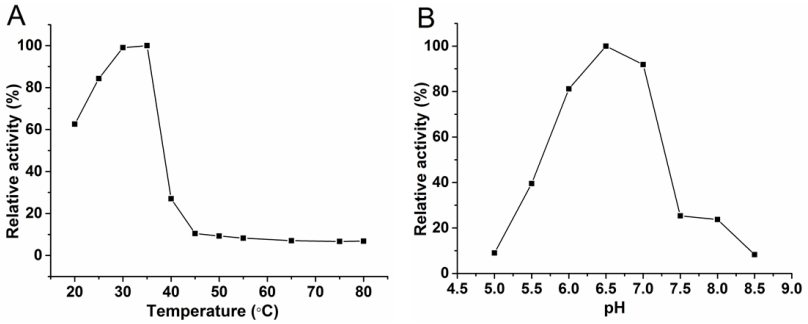

Fig 2. Enzymatic properties of *RhaB1-TM-eGFP*. (A) pH-dependent activity profles. (B) Temperature-dependent activity profles.

Aqueous-air segmented flow controls biofilm characteristics in microchip

Fig 3 A shows the biofilm cultivation under the single aqueous phase flow and aqueous-air segmented flow conditions for 72 h. At first, the structure of the biofilms was loose, while the biofilm was flat and compact under segmented flow, which may be affected by the fluid force generated by the aqueous phase [24]. Fig 4 A shows the different pH growth of biofilms under aqueous-air segmented flow. The optimal pH was determined to be 7.

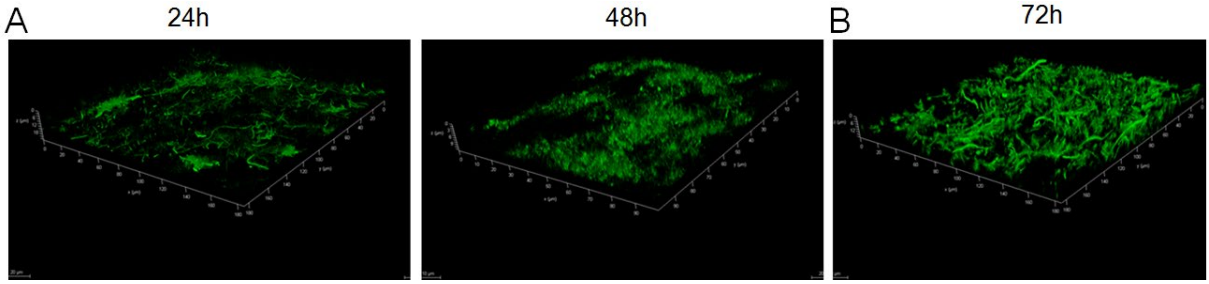

Fig 3. *E. coli* BL21*egfp* biofilm grown under single aqueous phase flow and aqueous-air segmented flow conditions. (A) Biofilm grown under single phase flow for 24 and 48 h; (B) Biofilm cultivation after air segments for 24 h.

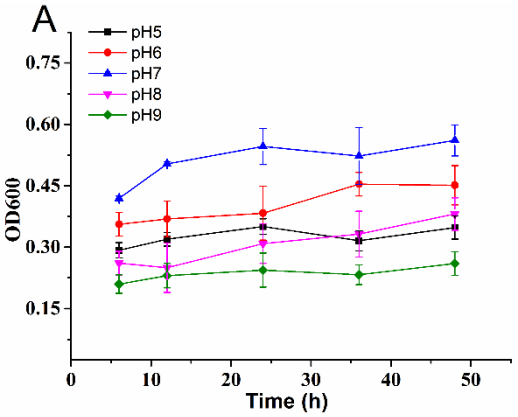

Figure 4. Effects of different pH growth of biofilms.

Conclusions

A microfluidic chip was used to rapidly colonize novel recombinant E. coli mutant strain and was used to cultivate biofilm by segmented flow. The experimental results showed that the optimum conditions were as follows. The optimal flow rate was 10 μL/min, the optimum pH was 7.0, and the culture time was 24 h.

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The authors have declared no conflicts of interest.

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