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Engineering of continuous bienzymatic cascade process using monolithic microreactors – In flow synthesis of trehalose

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ABSTRACT

Here, we present a two-step continuous flow enzymatic synthesis process in monolithic microreactors using basic sugars as substrates. In the first step UDP-glucose pyrophosphorylase (*Ta*GalU) catalyses the synthesis of uridine-diphosphate-glucose (UDP-Glc) using uridine triphosphate (UTP) and glucose-1-phosphate (Glc-1-P). This is followed by the trehalose transferase (mCherry-*Tu*TreT) catalysed reaction of UDP-Glc and Glc, to obtain trehalose. First, procedures for immobilisation of both enzymes on functionalised silica supports were studied and it was found that covalent bonding by amino groups using a glutaraldehyde linker gives highly active biocatalysts. Due to a drastic difference in temperature range of activity and stability of the immobilised enzymes a bi-reactor cascade was rationally the best solution. Depending on the applied flow rate and hence reaction (residence) time (1.5–10 min) the space-time-yield values varied, respectively, from 1.9 to 14.4 and 8.3 to 49.6 $g_{product}\cdot L^{-1}\cdot h^{-1}\cdot mg_{protein}^{-1}$, for UDP-glucose pyrophosphorylase and trehalose transferase catalysed reactions. Prolonged (100 h) continuous flow operation showed that the system is operationally stable, but owing to neutral pH, it is prone to microbiological infections. They can be eliminated applying an antibacterial/antifungal therapy or preventive actions by storing and washing the reactors with a NaN_3 solution. The presented process paves the way for the continuous in flow synthesis of natural and non-natural trehalose analogues and disaccharides.

1. Introduction

Trehalose is a non-reducing disaccharide, which serves not only as an energy source but also as an agent to protect the living cell against a variety of physical and chemical stress conditions. It is known for high water-retention activity and this makes it attractive in the development of additives and stabilizers that are useful in the food, cosmetic and pharmaceutical industries [1–5]. Traditional chemical routes to sugars usually suffer from poor regio-, enantio-, and stereoselectivity, tedious intermediates synthesis, work-up and purification as well as low overall yields. Therefore, enzymatic methods gradually take over this field, given their mild reaction conditions, high efficiency and stereo-selectivity and also environmental friendly features. Among several enzyme systems which have been found to be suitable for trehalose biosynthesis [3,6,7], the one which employs trehalose transferase and makes use of a widely available glucose (Glc) and a nucleotide diphosphate (NDP) sugar (e.g UDP-Glc) to produce trehalose, is of practical importance [2,8]. Furthermore, trehalose transferase from *Thermoproteus uzoniensis* (mCherry-*Tu*TreT) has been recently shown to have considerable potential to couple alternative sugars and nucleotide sugars to form trehalose analogues that are not possible to synthesize with the other trehalose producing enzymes [9]. Uridine-5′-diphosphate-glucose (UDP-Glc) which is a fundamentally important molecule in biology, as well as in food, pharmaceuticals and cosmetic chemistry, can also be obtained from an enzymatic pathway using several thermostable nucleotide-5′-diphosphate (NDP)-sugar pyrophosphorylases.

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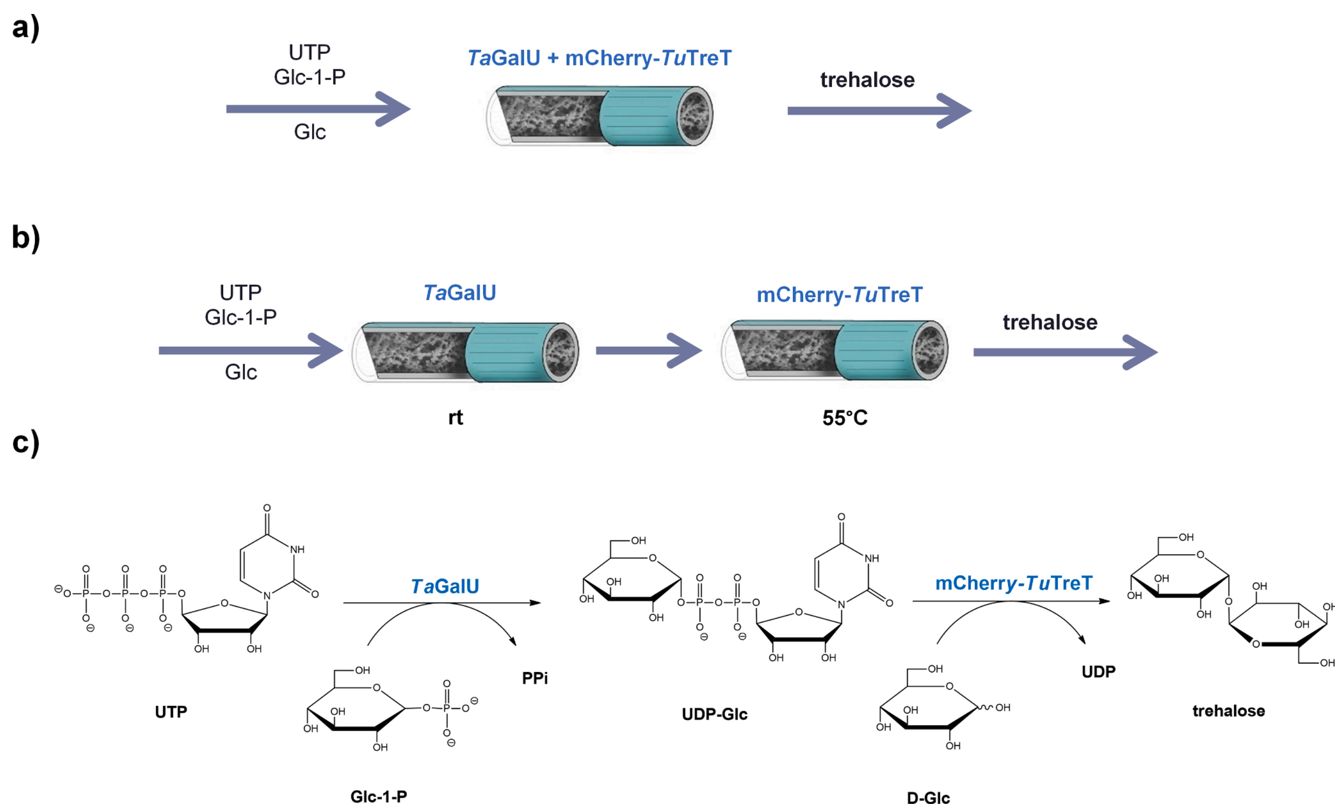
One of NDP-sugar pyrophosphorylases preferably producing UDP-Glc is UDP-glucose pyrophosphorylase from *Thermocrispum agreste* (*Ta*GalU) that converts uridine-5′-triphosphate (UTP) and glucose-1-phosphate (Glc-1-P) [10]. In this work these two enzymes were applied to form a continuous flow cascade process for trehalose synthesis from glucose using structured monolithic microreactors, which demonstrated huge potential in other enzyme catalysed reactions [11–14].

Enzymatic cascade reactions have been of great interest as they cut short conventional step-by-step synthesis, reduce waste, and they can be considered to be intrinsically green, while their in-flow implementations additionally boost performance [11,15]. However, as free enzymes in solution are relatively sensitive, often unstable and cannot be recovered and reused, they are usually immobilised using different techniques and carriers [16]. Most of the carriers used are fine particles, typically applied in batch (slurry) or packed bed reactors [17–19]. In agitated slurry systems a gradual abrasion or even disintegration of the particles adversely affects the process and hinders separation of the catalysts [17,18]. Application of low-volume flow reactors reduce these inconveniences and scaling up is resolved by numbering up a single reactor in parallel [20–26]. In most typical microreactors the working reaction volume is less than 1 mL, while the channels, usually capillaries 0.1–0.5 mm in diameter, intensify mass transfer and hence overall reaction kinetics. They also increase the surface-to-volume ratio to a value above 10^4 $m^2 \cdot m^{-3}$, which translates into an improvement in volumetric productivity (space–time yield, STY) and thus reduction in investment costs. Moreover, the risk and the energy consumption are reduced, and safety improved, in line with green chemistry principles [5,11–13,20–22,27,28]. An alternative, very attractive class of continuous reactors devised more recently by the Montpellier and Gliwice groups are small reactors made of silica rods/monoliths (up to 20 mm in dia., up to 10 cm length and 30 mL volume), which combine but also notably exceed the best specific features of capillary microreactors [11–13,29,30]. The monoliths feature very open macro-mesoporous hierarchical pore structure, with tens of thousands of irregular tortuous flow-through channels of micrometer sizes (e.g. 1–2, 6–8 or 20–50 μm, depending on the preparation protocol) and smaller mesopores present in a silica skeleton [13,29,30]. Due to these very fine tortuous channels the external mass transfer in the monolithic reactors is extremely intensive, and its limitation of reaction kinetics effectively eliminated, unlike in capillary reactors [11,12,31]. Moreover, the mesopores present in the silica skeleton expand the surface area to obtain the value of surface-to-volume ratio in the range of 10^6-10^7 $m^2 \cdot m^{-3}$ [11,14]. Finally from both an engineering and business perspective, due to a very large volume of voids (4 $cm^3 \cdot g^{-1}$), and hence much more open structure than in packed beds of beads, liquid reactant throughputs of several hundred $mL \cdot min^{-1} \cdot cm^{-2}$ can be applied in monolithic reactors at pressure drop of tens of $kPa \cdot cm^{-1}$, thus enabling multi $gram \cdot min^{-1}$ production scales [13,29]. In all these cases, there is no need to use HPLC pumps, even in systems that offer very high productivity, which is an additional advantage.

Since the aforementioned monolithic reactors proved to be very effective in the previous studies with immobilised enzyme catalysts [11–13,14], we considered it important to test their potential in the cascade reaction system.

Here we present the results of our studies of a two-stage process of enzymatic trehalose production using structured monolithic microreactors (Scheme 1). At first, we determined the conditions for the immobilisation of enzymes (UDP-glucose pyrophosphorylase from *Thermocrispum agreste* – *Ta*GalU, and *Thermoproteus uzoniensis* trehalose transferase - mCherry-*Tu*TreT) onto mesoporous cellular foams (MCF), and then the properties of the catalysts thus obtained were investigated. After that the enzymes were attached to the monolithic microreactors for mainstream experiments. Two variants of the process were considered: (i) a single reactor system, with both enzymes immobilised in the same monolith (Scheme 1a), (ii) a bi-reactor sequential system with each of the enzymes immobilised in separate reactors and these are connected in series (Scheme 1b).



Scheme 1. Trehalose synthesis by *Ta*GalU and mCherry-*Tu*TreT cascade: a single reactor system (a), bi-reactor sequential system (b), scheme of the reaction (c).

2. Materials and methods

Polyethylene glycol 35 000 (PEG), Pluronic P123, tetraethoxysilane (TEOS), precursors of functional groups: 3-aminopropyltrimethoxysilane (APTMS) and 3-glycidyloxypropyltrimetoxysilane (GPTMS), sodium azide (NaN_3) and 1,3,5-trimethylbenzene were purchased from Sigma Aldrich. Hexadecyltrimethylammonium bromide (CTAB) was from Acros Organics, whereas glutaraldehyde (GLA) as well as glucose-1-phosphate (Glc-1-P) and magnesium chloride, anhydrous were from Alfa Aesar. Tris(hydroxymethyl)aminomethane (Tris), HCl, ammonia solution (25%), ethanol (99,5%) and glucose (Glc) were obtained from Avantor. UDP-glucose (UDP-Glc) as well as uridine-5′-triphosphate (UTP) was purchased from Carbosynth.

2.1. Synthesis of mesostructured cellular foams (MCF)

The preparation of modified siliceous MCFs was described in detail earlier [32,33]. In brief Pluronic P123 was dissolved in 1.6 M HCl (5.3 mM) and then 1,3,5-trimethylbenzene (0.23 M) and NH_4F (8 mM) were added and stirred for 1 h at 40 °C. After that TEOS was added dropwise and stirred for 1 h at the same temperature, and the mixture of HCl:H_2O:P123:NH_4F:TMB:TEOS molar ratio 5.7:186:0.016:0.03:2:1 was stored at 40 °C for 20 h and then for 24 h at 100 °C. After that, the precipitate was filtered off, dried and calcined at 500 °C for 8 h.

2.2. Synthesis of silica monoliths (MH)

The procedure for the synthesis of monoliths was adapted from Szymańska et al. [12,13]. Briefly, PEG (8.67 g) was dissolved in 1 M nitric acid solution (100 mL). Then TEOS (82.8 mL) and CTAB (3.8 g) were added. The solution thus obtained was mixed, poured into the cylindrical mould, left to gel and aged for 10 days at 40 °C. Subsequently the silica monoliths (rods) were treated in 1 M ammonia solution for 9 h and 90 °C, washed with distilled water, dried at room temperature and calcined for 10 h at 550 °C.

2.3. Functionalisation of MCF with amino or epoxy groups

Briefly, 1 g of dry MCF were modified with amino groups using 0.27 mL APTMS (or to attach epoxy groups applying 0.34 mL GPTMS) dissolved in 25 mL dry toluene and gently stirred at 85 °C for 24 h. Then the supports/carrier were air dried.

2.4. MH functionalisation with amino groups

Briefly, 1 g of dry silica monoliths (rods 4 or 6 mm dia.) were modified with amino groups using 0.90 mL APTMS dissolved in 40 mL dry toluene. The solution containing the monoliths was gently stirred at 85 °C for three days. Then the monoliths were washed with ethanol at 50 °C for 5 h and air dried.

2.5. TaGalU activity assay

Details of ***Ta*GalU** production are given in SI. Activity of thus obtained enzymes was measured as the synthesis of UDP-glucose from uridine triphosphate (UTP) and glucose-1-phosphate (Glc-1-P) (Scheme 1c). Unless mentioned otherwise, UTP (2 mM) and Glc-1-P (2 mM) were dissolved in HEPES buffer (50 mM, pH 7.0) with the addition of $MgCl_2$ (4 mM). The substrate solution was first incubated at 30 °C, then the enzyme was added. The reaction was carried out at 30 °C. The amount of UDP-glucose obtained was determined by HPLC (Agilent 1200 series) using Accucore-150-Amide-HILIC Column (100 × 3 mm, particle size 2.6 μm) at 30 °C. Phase A was acetonitrile, whereas phase B was ammonium acetate solution (134 mM, pH 5.35). Separation was achieved using isocratic flow rate of 0.8 mL·min^{-1} and 30% B. Products were detected using UV absorbance at 260 nm. All the measurements were performed in triplicate.

2.6. mCherry-TuTreT activity assay

Production of mCherry-*Tu*TreT is detailed in SI**.** Its activity was measured as the rate of trehalose production (Scheme 1c). UDP-glucose (UDP-Glc, 10 mM) and glucose (Glc, 10 mM) were dissolved in HEPES buffer (50 mM, pH 7.0), with the $MgCl_2$ (20 mM) as cofactor. Before the enzyme was added, the reaction solution was incubated at 55 °C, and then the reaction was carried out at the same temperature. The amount of trehalose obtained was determined by HPLC using REZEX-ROA Organic Acid H+ (8%) column (300 × 7.8 mm) and 2.5 mM sulfuric acid as a mobile phase. A 10 μl aliquot of sample was injected on the HPLC operating at the flow rate of 0.5 mL·min^{-1}. All the measurements were performed in triplicate.

2.7. General procedure for TaGalU and mCherry-TuTreT immobilisation

Before immobilisation the supports (with amino or epoxy groups) were washed with ethanol, distilled water and phosphate buffer (Na_2HPO_4/KH_2PO_4, 0.1 M, pH 7.0). The carriers with amino groups were activated with glutaraldehyde before enzyme addition, by incubation with GLA solution (2.5% (v/v) in phosphate buffer (0.1 M, pH 7.0) for about 1 h. After that, the excess of GLA was eluted with distilled water and phosphate buffer (0.1 M, pH 7.0). Then the enzyme solution was added to the carrier (with amino or epoxy groups) and incubated for 3 h at the room temperature, then at 4 °C overnight. Excess of protein was removed by washing as described elsewhere [11–13]. Briefly, carriers were washed with phosphate buffer (0.1 M pH, 7.0), sodium acetate buffer (0.1 M, pH 4.5) and distilled water. In order to block unreacted active groups Tris-HCl buffer (0.5 M, pH 7.8) was used. The amount of immobilised enzyme was determined spectrophotometrically (at 280 nm) as the difference between the amount of enzyme given to immobilisation and amount of enzyme in solution after immobilisation.

2.8. General procedure for trehalose production in microreactors cascade

Before reaction, monoliths were washed with HEPES buffer (50 mM, pH 7.0) for 1 h (0.5 mL·min^{-1}). Reactions were then performed according to Scheme 1b. The first microreactor in cascade contained immobilised *Ta*GalU and was kept at room temperature whereas second microreactor contained immobilised mCherry-*Tu*TreT was incubated at 55 °C. Reaction mixture: UTP (10 mM), Glc-1-P (10 mM) and Glc (10 mM) in HEPES buffer (50 mM, pH 7.0), with $MgCl_2$ (20 mM) was pumped through microreactors with the flow rate 3 mL·h^{-1}. During the process samples were collected. Concentration of glucose and trehalose was determined by HPLC using Rezex column using the method described earlier.

3. Results and discussion

3.1. Immobilisation of TaGalU and mCherry-TuTreT onto the carrier

One of the most common and intensely studied techniques of enzyme immobilisation involves strong covalent bonding of the protein to the carrier. Clearly, the covalent bond must involve functional groups of the protein which are amenable to chemical modification, do not participate in the catalysis process and are not directly involved in the stabilization of the tertiary and quaternary enzyme structure [34,35]. The pH of the solution in which the immobilisation is carried out affects protein ionization and determines the availability of functional groups for the immobilisation process. For this reason, the pH impact on the immobilisation process was tested using enzyme solutions in a pH range of 6–8. Another parameter of fundamental importance for the effectiveness of the immobilisation process is the chemical nature of the applied carrier (type of functional groups on their surface). In this work, silica carriers

functionalised with epoxy (E) and amine (A) groups were used for both *Ta*GalU and mCherry-*Tu*TreT immobilisation. Our earlier studies [10] carried out for *Ta*GalU deposited on a carrier grafted with epoxy groups revealed low activity of the biocatalysts. However, when the amine-modified carrier was used, the biocatalysts proved to be both highly active and stable at pH 6.8. [10] A similar series of experiments was also performed for mCherry-*Tu*TreT. It was observed that the carriers functionalised with amine groups bound a similar amount of protein, regardless of the immobilisation pH (Fig. 1b), whereas those with epoxy groups bound slightly less protein, especially in a buffer of pH 7 (Fig. 1b). Thus, not surprisingly, the latter biocatalysts exhibited lower specific activity compared to the matrices that were functionalised with amine groups (Fig. 1a). But this lower activity could only partially be explained by a smaller amount of the bound enzyme, since the difference in the enzyme loading was max. 20%, whereas the drop in the catalytic activity could reach even 40%. Similar observations were also reported before for other enzymes, but the exact causes remain unknown [32,36]. Most likely, immobilisation of the enzyme on epoxy group-modified carriers results in its deformation or an excessive rigidity of the tertiary/quaternary protein structure, which hampers the structural and conformational flexibility that is required for optimal enzyme activity. Under the tested immobilisation conditions, the highest specific activity was exhibited by mCherry-*Tu*TreT immobilised on silica carrier grafted with amine groups (and activated with GLA) at pH 7 (Fig. 1a). GLA crosslinking tends to create a dimeric or trimeric GLA structures thus creating a longer spacer between the matrix and the protein surface [37]. Such microenvironments are not present in the carriers with epoxy groups, in which protein amine groups were attached close to the carriers' surface [32]. Additionally, biocatalysts immobilised via amino functionalised silica showed better storage stability than those that used epoxy groups (Fig. 1c). Low stability of mCherry-*Tu*TreT immobilised on carriers with epoxy groups was also observed by Mestrom et al. [38] and it was explained by the presence of the unreacted epoxide groups on the support surface. In our studies the unreacted epoxide groups were blocked by Tris-HCl buffer during the immobilisation process, but still a lower stability was observed.

Considerable similarity of optimal immobilisation conditions (type of carrier functional groups and immobilisation pH) for both enzymes involved in the cascade reaction paved the way for their simultaneous immobilisation in the same (micro)reactor. However, in addition to that, the compatibility of the process conditions had to be checked to determine if they allow for an effective and stable operation of both enzymes, ideally in close proximity. The process temperature is of particular significance in this respect; as a higher temperature increases the rate of the reaction, but if it is too high, protein denaturation and irreversible deactivation occurs. Therefore, we deemed it important to determine the optimal working temperature for the individual immobilised enzymes, as well as their long-term stability at selected temperatures. To this end, we have previously examined detailed characteristics of immobilised *Ta*GalU to find that the optimal working temperature is in the range of 50–55 °C [10]. However, after only 20 h at this temperature, the enzyme lost 90% of initial activity, and only when a temperature of 30–35 °C was applied, a high level of activity could be maintained for at least 100 h. The activity of mCherry-*Tu*TreT immobilised on an amine-modified silica carrier was tested in the temperature range of 35–80 °C (Fig. 2a). Initially, the rise of temperature caused the activity to increase very slowly, and the rapid increase in its value was observed only above 55 °C. No decrease in the enzyme activity was observed within the examined temperature range. In order to fully characterise the immobilised preparation, the long-term stability at 55, 65, and 75 °C was also examined (Fig. 2b). At 75 °C, the enzyme lost approximately 40% of the initial activity already after ca. 50 h, while after another 100 h it exhibited only 25% of the initial value. The first small loss of activity for an enzyme kept at 65 °C was observed only after approximately 40–50 h, while after further 170 h incubation, only 20% decrease in activity was observed. At 55 °C, however, the initial activity was maintained throughout the whole experiment, i.e. about 190 h. The thermal stability of the enzyme was expected as *Thermoproteus uzoniensis* is a hyperthermophilic archaeon with an optimal growth temperature of ca. 90 °C [39].

The results obtained for both enzymes indicate that *Ta*GalU is optimally stable and active at temperatures below 30–35 °C, whereas mCherry-*Tu*TreT is stable but only barely active at these temperatures. After 5 h of storage at 55 °C immobilised *Ta*GalU showed only 25% residual activity compared to storage at 30 °C [10]. These findings make the system in which both enzymes are immobilised in the same microreactor uneconomic (Scheme 1a). It would result in a low overall efficiency of the system when the temperature is below 30 °C (mCherry-*Tu*TreT activity limiting, less than 3% of maximal activity, Fig. 2a), or in a very short lifetime when using higher temperatures (*Ta*GalU activity limiting, low stability at temperature higher than 30 °C [10]). Thus, a rational solution is the use of two microreactors connected in series (Scheme 1b), with *Ta*GalU immobilised in the first one and mCherry-*Tu*TreT in the second one. In such a system each of the reactors operates at a temperature suitable for a given enzyme, and that ensures an optimal overall process performance in the long run. Additionally, if necessary, such a system would allow the collection and separation of the intermediate product of the process UDP-Glc. A similar solution was

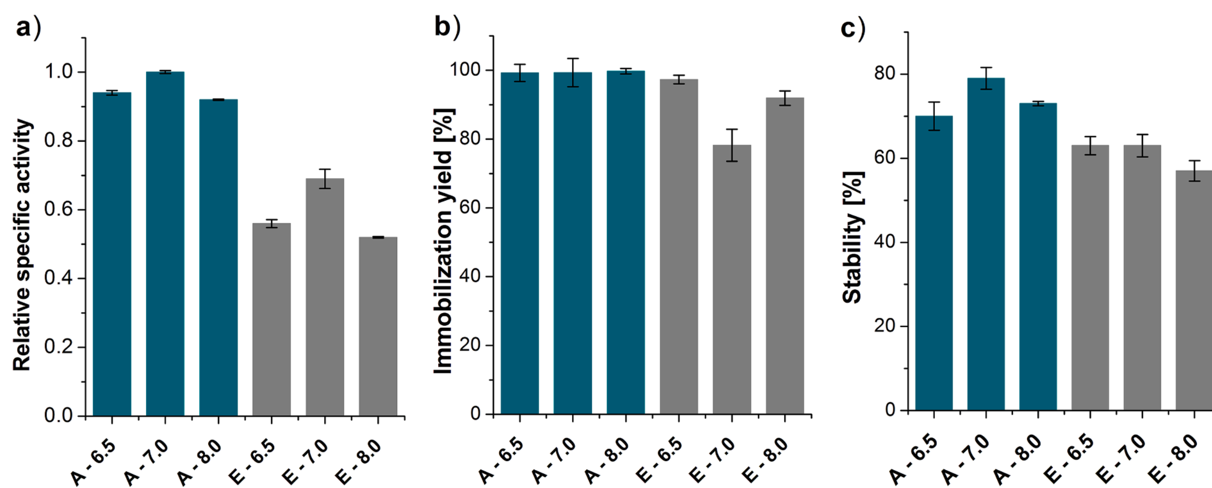


Fig. 1. Immobilisation of mCherry-*Tu*TreT on MCF functionalised with amino groups (A) and epoxy groups (E) at different pH (6.5, 7.0, 8.0). Relative specific activity of immobilised mCherry-*Tu*TreT (a), immobilisation yield (b), and stability after 1-month storage at 4 °C (c). *Conditions: mCherry-TuTreT immobilised on functionalised MCF, UDP-Glc (10 mM), Glc (10 mM), HEPES buffer (50 mM, pH 7.0), $MgCl_2$ (20 mM), activity measured at 55 °C.*

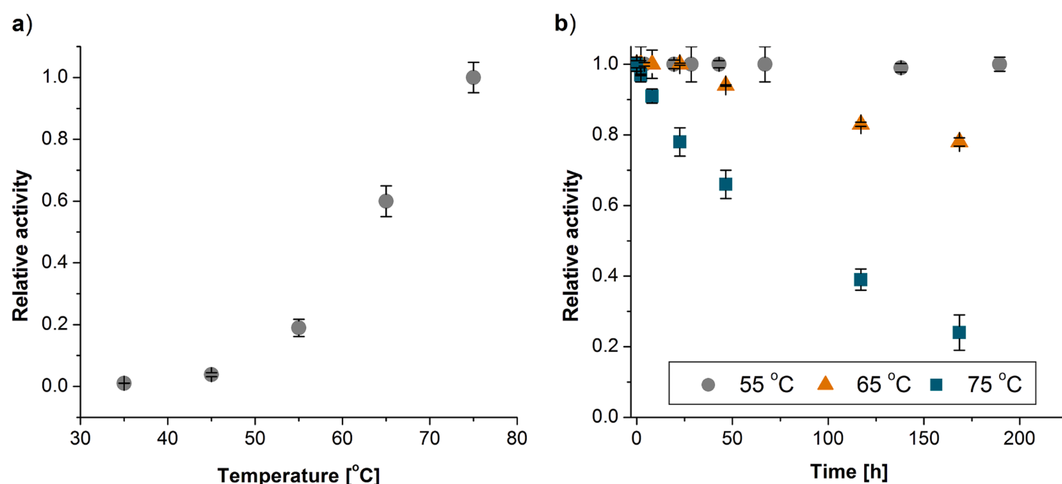


Fig. 2. Effect of temperature on immobilised mCherry-*Tu*TreT activity (a) and stability (b). *Conditions: mCherry-TuTreT immobilised on amino modified MCF, UDP-Glc (10 mM), Glc (10 mM), HEPES buffer (50 mM, pH 7.0), $MgCl_2$ (20 mM), (a) activity measured at 35–75 °C, (b) immobilised mCherry-TuTreT incubated at 55, 65 or 75 °C, and subsequently activity was measured at 55 °C.*

already proposed for in-flow nucleoside synthesis [40]. The advantage of this two-step setup would be the availability of a single reactor that could be used independently, either for "one-enzyme" synthesis or in different combinations in sequence.

3.2. Synthesis of trehalose in a two reactors sequential system

In light of these results, *Ta*GalU and mCherry-*Tu*TreT were immobilised in two monolithic reactors functionalised with amine groups. Then, their activity in the UDP-Glc (*Ta*GalU, Fig. 3a) and trehalose (mCherry-*Tu*TreT, Fig. 3b) synthesis reactions were specified for a range of flow rates, i.e. various mean (hydraulic) residence times of reactants in the reactor. As expected, the substrate conversion increased with the increase of the residence time (decrease in flow rate). Approximately 0.7 mM of UDP-Glc (35% substrate conversion) was obtained for a residence time of 1 min and protein load ca. 6 mg (Fig. 3a). A similar relation was obtained for the immobilised mCherry-*Tu*TreT, with a lower protein load of 1.4 mg; 30% conversion was obtained for a

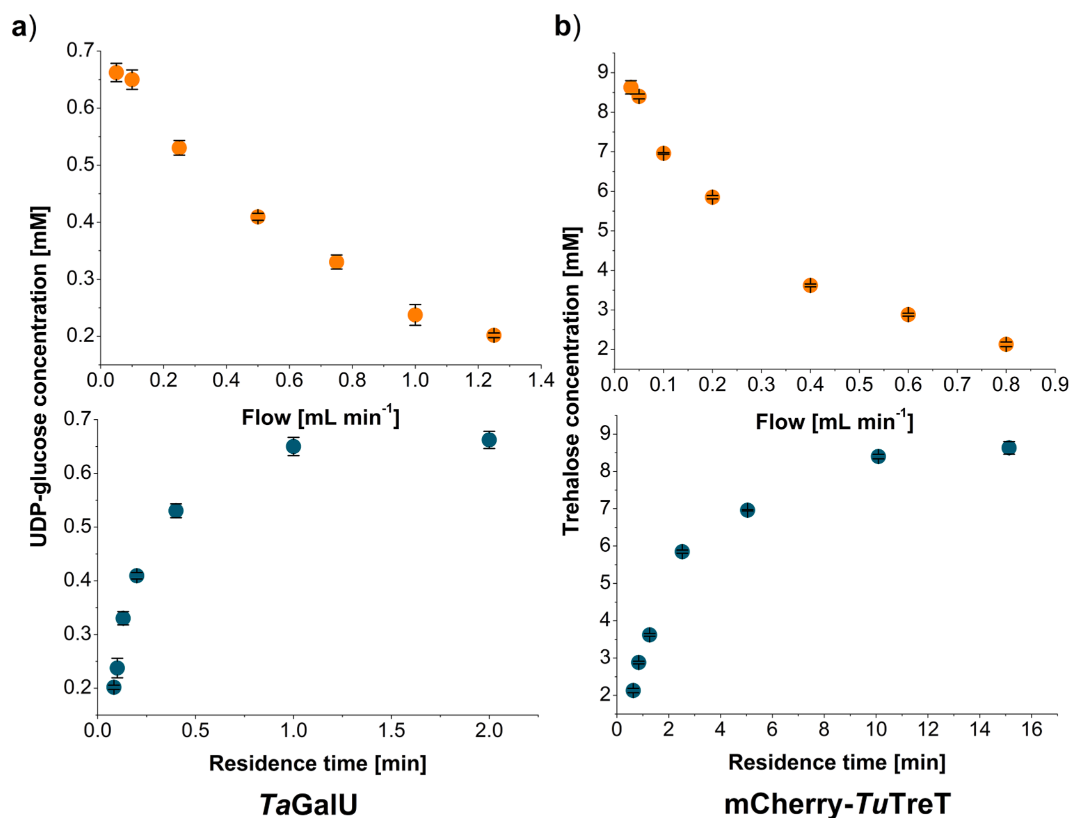


Fig. 3. In flow synthesis of UDP-Glc (a) and trehalose (b) by covalently immobilised *Ta*GalU (a) and mCherry-*Tu*TreT (b) on amino modified monoliths. *Conditions (TaGalU): TaGalU on amino group modified monolith (size: 4 × 10 mm, 6.3 mg of protein load) UTP (2 mM), Glc-1-P (2 mM), HEPES buffer (50 mM, pH 7.0), $MgCl_2$ (4 mM), 30 °C. Conditions (mCherry-TuTreT): mCherry-TuTreT on amino group modified monolith (size: 6 × 21 mm, 1.4 mg of protein load), UDP-Glc (10 mM), Glc (10 mM), HEPES buffer (50 mM, pH 7.0), $MgCl_2$ (20 mM), 55 °C.*

residence time of 1.2 min, whereas its extension to 10 min resulted in the conversion increase up to 90% (Fig. 3b). When the performance of the system was expressed in terms of STY, for *Ta*GalU it was in the range of 11.3–86.3 g $\cdot L^{-1} \cdot h^{-1}$ for the flow rates 0.05–1.25 mL$\cdot min^{-1}$, whereas for mCherry-*Tu*TreT it increased from 11.7 to 69.4 g $\cdot L^{-1} \cdot h^{-1}$ when the flow rate increased from 0.03 to 0.8 mL$\cdot min^{-1}$. Taking into account the enzyme content effect, the productivity values varied, depending on the applied flow rate, from 1.9 to 14.4 and 8.3 to 49.6 $g_{product} \cdot L^{-1} \cdot h^{-1} \cdot mg_{protein}^{-1}$, for *Ta*GalU and mCherry-*Tu*TreT, respectively.

Finally, two microreactors were connected in series to obtain trehalose from UTP, Glc-1-P and Glc (Fig. 4). In order to ensure complete conversion of UTP and Glc-1-P, the amount of *Ta*GalU immobilised inside the first microreactor was increased to 41 mg. The reactor with immobilised *Ta*GalU operated at room temperature, whereas that loaded with mCherry-*Tu*TreT, at 55 °C. Solution containing 10 mM of UTP, 10 mM of Glc-1-P and 10 mM of Glc in HEPES buffer was pumped through bimonolithic cascade with flow rate of 3 mL$\cdot h^{-1}$, i.e. residence time of 10 and 22 min, respectively in the reactor with *Ta*GalU and mCherry-*Tu*TreT. During the first 30 h of the process, the outlet concentration of trehalose remained stable at ca. 7 mM, and this gives the STY of 6.1 g$\cdot L^{-1} \cdot h^{-1}$. After this time, the concentration of glucose applied as a substrate, quite unexpectedly began to decline slowly. We attributed this to the invasion of microorganisms in the microreactor, as non-sterile conditions were used. Intensive rinsing with buffer resulted in a small improvement, and only after rinsing both reactors with a 5% penicillin/streptomycin/amphotericin B cocktail for 1 h followed by overnight incubation in the same solution, a significant improvement and return to the initial activity was observed. The concentration of both glucose and trehalose remained stable for the next 30 h. Yet, another decrease in the concentration of glucose in the tested reaction mixture was observed again after about 80 h of continuous operation, and it was large enough to hamper the production of trehalose. Again, the use of an antibacterial/antifungal cocktail restored the initial enzyme activity. These results clearly indicate that this action is effective, and not less importantly, that the immobilised enzymes are resistant to the penicillin/streptomycin/amphotericin B cocktail and retain catalytic activity. However, the procedure described only allows for a symptomatic treatment, without eliminating the source, and in each case its application resulted in breaks of minimum one day. Thus, it is hardly acceptable from a production point of view. In this aspect, preventive measures may include the storage of functionalised/activated microreactors in a sodium azide (NaN_3) solution, well known for its excellent microbial growth inhibition properties (Fig. 5). Because of its toxicity, monoliths with immobilised *Ta*GalU and mCherry-*Tu*TreT were stored only in a 0.05% NaN_3 solution at 4 °C. Before reuse, the reactors were washed with HEPES buffer and the enzyme activity was checked in standard reactions after 1 day, 1 week, 1 month and 2 months. Only a small decrease in the enzymes' activity was observed, which proves their good storage stability in the presence of 0.05% NaN_3. However, infections can occur not only during the storage of microreactors but also during the process itself. Therefore, the process stability of both enzymes in the presence of NaN_3 was controlled and they appeared to be stable for at least 100 h of continuous operation (see SI). The continuous use of the toxic NaN_3 additive necessitates its removal after the process, and this entails additional technological problems and costs. Thus, a rational solution may include short breaks in the process and the rinsing of reactors with a NaN_3 solution for disinfection. The results of such experiments are shown in Fig. 6. They indicate that technological breaks lasting 1 h per day are sufficient to ensure stable operation of the reactor cascade and eliminate the growth of microorganisms in the system. The other practical solution is to prevent bacterial/fungal infection by filtering all reactant solutions prior to use and keep sterile conditions, but this was not studied here.

The cascade described here is a modular system with high capacity to produce trehalose analogues with different sugar donors and acceptors. Most recently we have shown that the mCherry-*Tu*TreT is able to couple Glc with a wide range of D- and L-glycopyranose acceptors resulting in different α,α- and α,β-trehalose analogues [9]. Such trehalose analogues have great potential as alternative food additives. The presented modular approach should also prove effective if aglycan substrates or chemically labelled sugars are used as donor or acceptors for the synthesis of biosurfactants or molecular probes [8].

And no less importantly, the presented cascade can be further expanded which can ideally lead to the full synthesis of trehalose (and analogues) from Glc with only catalytic amounts of nucleotides. By complementing the cascade with a polyphosphate kinase, UTP can be regenerated at the expense of polyphosphate. Glc-1-P can be synthesized from Glc using hexokinase (using UTP as substrate) and phosphoglucomutase. Finally, a pyrophosphorylase can be included for a more favourable equilibrium of the Glc activation reaction. Such a complex six-enzyme cascade poses a considerable challenge, but the results presented here indicate that the core of this cascade is based on solid foundations.

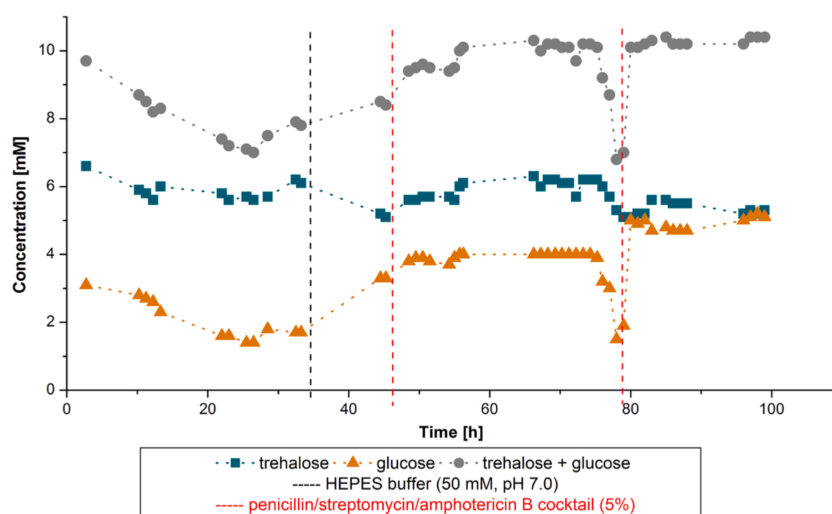


Fig. 4. Continuous flow trehalose synthesis in a bi-reactor cascade. *Conditions: TaGalU on amino group modified monolith (size: 6 × 21 mm, 41.1 mg of protein load), mCherry-TuTreT on amino group modified monolith (size: 6 × 46 mm, 9.8 mg of protein load), UTP (10 mM), Glc-1-P (10 mM), Glc (10 mM), HEPES buffer (50 mM, pH 7.0),* $MgCl_2$ *(20 mM), TaGalU – rt., mCherry-TuTreT – 55 °C, flow rate 3 mL*$\cdot h^{-1}$.

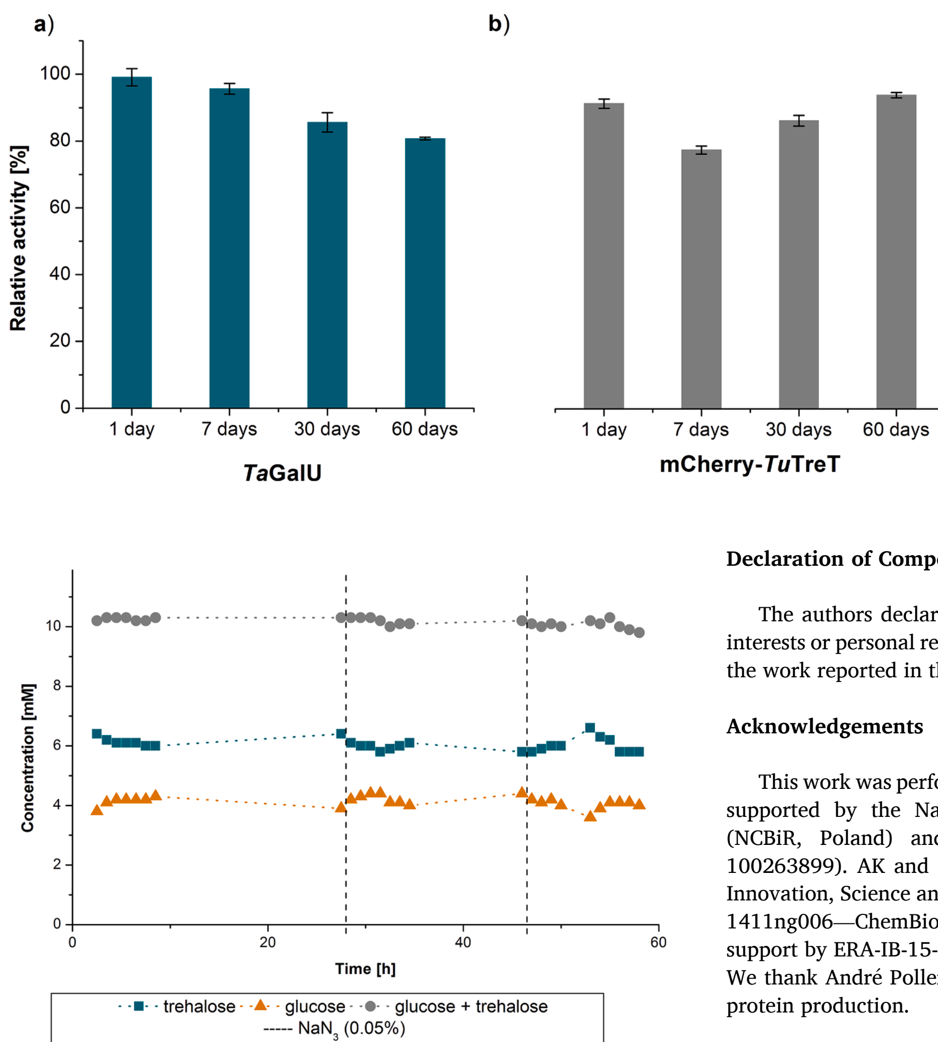


Fig. 5. Activity of *Ta*GalU and mCherry-*Tu*TreT immobilised inside microreactors after storage in 0.05% NaN_3 solution at 4 °C. *Conditions (TaGalU): TaGalU on amino group modified monolith (size: 6 × 21 mm, 41.1 mg of protein load), UTP (2 mM), Glc-1-P (2 mM), HEPES buffer (50 mM, pH 7.0),* $MgCl_2$ *(4 mM), 30 °C, flow rate 0.4 mL·min*$^{-1}$*. Conditions (mCherry-TuTreT): mCherry-TuTreT on amino group modified monolith (size: 6 × 21 mm, 1.4 mg of protein load), UDP-Glc (10 mM), Glc (10 mM), HEPES buffer (50 mM, pH 7.0),* $MgCl_2$ *(20 mM), 55 °C, flow rate 0.4 mL·min*$^{-1}$*.*

Fig. 6. Trehalose production in microreactors cascade with sodium azide solution washings. *Conditions: TaGalU on monolith with amino groups (size: 6 × 40 mm, 37.6 mg of protein load), mCherry-TuTreT (size: 6 × 46 mm, 10.0 mg of protein load), UTP (10 mM), Glc-1-P (10 mM), Glc (10 mM), HEPES buffer (50 mM, pH 7.0),* $MgCl_2$ *(20 mM), TaGalU – rt., mCherry-TuTreT – 55 °C, flow rate 3 mL·h*$^{-1}$*,* NaN_3 *(0.05%).*

4. Conclusions

*Ta*GalU and mCherry-*Tu*TreT were effectively immobilised on silica monolithic reactors via covalent bonding to pave the way to a continuous flow trehalose synthesis in two consecutive (cascade) reactions from substrates such as Glc, Glc-1-P and UTP. Considerable differences in temperature affected both activity and stability of each of the immobilised enzymes, prohibiting both reactions from running simultaneously in a single microreactor. The use of two monolithic reactors connected in series, operating at different but optimized temperatures, and the enzyme loads adjusted to compensate for differences in their specific activities, was shown to be a promising technology for trehalose production in a continuous process. Additionally, the proposed system allows for the synthesis of intermediates i.e. UDP-Glc, which in itself is a highly desirable compound. Emerging problems of microbiological infections can either be eliminated by using antibacterial/antifungal therapy or preventive actions by storing and washing the microreactors with a NaN_3 solution. The modular cascade to couple activated sugar donors and sugar acceptors can be further employed for the synthesis of natural and non-natural trehalose analogues and disaccharides.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2021.131439.

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