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Directed optimization of biocatalytic transglycosylation processes by the integration of genetic algorithms and fermentative approaches into a kinetic model

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ABSTRACT

Many biocatalysts exhibit strict stereospecificity and regioselectivity. However, their thermodynamically controlled equilibria often limit yields in industrial production processes. Herein, we describe the synthesis of fructooligosaccharides from sucrose by various fructansucrases. We previously demonstrated that transfructosylation to diverse acceptors yields D-glucose and the fructose-containing product along with diverse by-products. To streamline this reaction, we developed a procedure that allows the enhanced transfructosylation of diverse acceptors by different fructansucrases. By diverting the released glucose from the reaction via metabolism by living cells we limited the back reaction and forced the consumption of sucrose. The basic conditions for the resulting fermentation process were optimized by a genetic algorithm and integrated into a kinetic model. This strategy allows the prediction of optimal reaction parameters for the production of desired target compounds.

1. Introduction

Lactic acid bacteria produce fructans for extracellular carbohydrate storage. In addition, the exopolysaccharides function as stabilizers for cell membranes protecting the organisms against water stress caused by drought or temperature shifts [1]. Furthermore, it is known that fructans are important compounds for the growth and survival of many bacteria, and central to processes like phytopathogenesis and symbiosis [2]. The Gram positive model bacterium *Bacillus subtilis* secretes the levansucrase $SacB_{Bs}$ into its environment once large amounts of sucrose become available [3]. Levansucrases (E.C. 2.4.1.10) form the β(2→6) linked polyfructoside levan from sucrose. Recently, we demonstrated that the levansucrase (EC 2.4.1.162) from *B. subtilis* NCIMB 11871 ($SacB_{Bs}$) can be used for *in vitro* synthesis of sucrose analogues and fructooligosaccharides with good yields [4–6]. During this reaction, D-fructose, which is derived from sucrose, is transferred to an acceptor like galactose to form α-D-galactopyranosyl-1,2-β-D-fructofuranoside (Gal-Fru). The sucrose analogue Gal-Fru is also a donor substrate for the enzyme; consequently, the backward-reaction to sucrose is also catalyzed. This reaction is thermodynamically controlled and leads to a quasi-stationary state allowing the enzyme to produce fructans or to hydrolyze the disaccharides. The result is a product mixture consisting of glucose, galactose, fructose from sucrose hydrolysis, various oligosaccharides, sucrose, and Gal-Fru (Fig. 1). The separation of sucrose and Gal-Fru is very difficult due to their similar structures. Some experiments have been described that utilize intact microbial cells to influence the kinetic control of enzyme-based production of oligosaccharides [7–9]. In these experiments, the substrates are generated by heterologous genes or homologous cell metabolism, or they are imported into the cell. The enzymatic reaction occurs inside the cell and is under the kinetic control of the organism. In our system, the reaction takes place outside the cells. Live cells were added to selectively remove low concentrations of glucose from the substrate mixture and thus deprive the reaction of sucrose in order to avoid the quasi-stationary state and contamination of the product with sucrose.

To realize our conceptual system, a kinetic model was developed that is valid for two different enzymes and is presumed to be applicable to additional fructansucrases. The two enzymes are $SacB_{Bs}$ from *B. subtilis* and $SacB_{Bm}$ from *B. megaterium*. Both strains are widely used in industrial applications [29].

The kinetic model was implemented for reactions in the fermentation broth containing the sugar mixture as described above. The boundary conditions for the fermentative release of glucose from the mixture were defined by a genetic algorithm. Genetic algorithms originate from the analysis of biological

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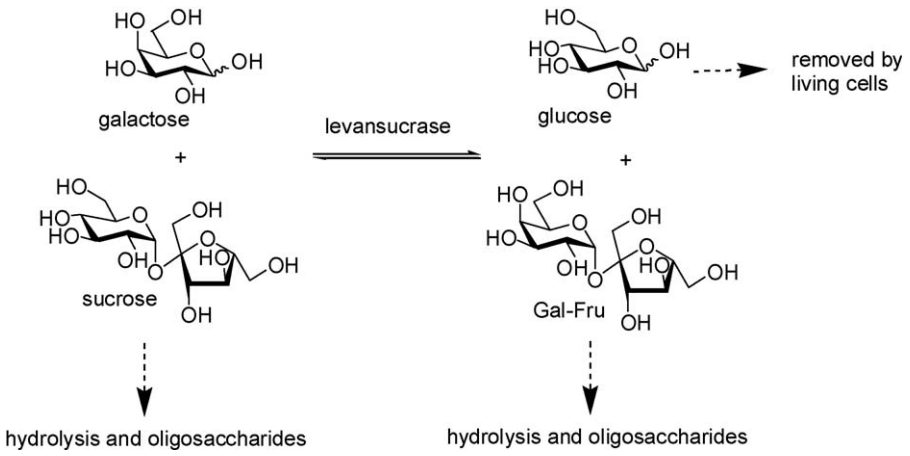

Fig. 1. Incubation of galactose and sucrose with the levansucrase SacB from *B. subtilis*: D-galactose and sucrose are converted to glucose and Gal-Fru. Gal-Fru and glucose are reconverted to D-galactose and sucrose if glucose is not removed by live cells.

systems [10,11]. They are function optimizers that do not necessarily yield optimal solutions, rather they generally serve to achieve high quality and efficient results in black box optimization problems [12]. They are simple data structures that represent the operating variables of different attributes – in our case, fermentation conditions – to be optimized. A set of different combinations of attributes is called a generation. One combination of attributes among many of a generation is called an individual. In this case, an individual is a fermentation approach that was tested on the desired functionality. After testing 39 individuals, we were able to define boundary conditions for the fermentation process. Here we show how the kinetic model allowed us to perform simulations of the Gal-Fru production process under boundary conditions with different levansucrases, and we succeeded in predicting the optimal reaction times for maximal yields.

2. Methods

2.1. Protein production and purification

2.1.1. B. megaterium $SacB_{Bm}$

The levansucrase $SacB_{Bm}$ was isolated from the supernatant of a sucrose-treated *B. megaterium* DSM319 culture and characterized as described previously [13]. For $SacB_{Bm}$ preparation, *B. megaterium* was cultivated aerobically at 37 °C in defined salt media (A5) supplemented with 0.5% (w/v) sucrose [14]. Under these conditions, the strain secretes $SacB_{Bm}$ into the fermentation broth. Within the first 3 h, the volumetric activity increased to 890 U L^{-1} and the cell dry weight (CDW) activity was 260 Ug_{CDW}^{-1}. $SacB_{Bm}$-containing broth was subsequently fractionated by centrifugation to a cell pellet and cell-free growth medium (2600 × *g*, 15 min, 4 °C). The enzyme was detected in the supernatant by activity staining after having been resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel (12% ProGel–Tris–Glycine-Gel, 1.0 mm, Nr.: TG12110) was obtained from Anamed (Germany). Samples were dissolved and denatured in 3.0 g L^{-1} Tris, 3.0 g L^{-1} SDS, 14.4 g L^{-1} glycine buffer and loaded onto the gel; samples were resolved on a Power-phor-N electrophoresis device (Anamed, Germany). The gel was washed three times in a wash buffer (50 mM phosphate buffer, pH 6.5; 340 μM calcium chloride; 0.1% (v/v) Triton X-100) after electrophoresis. Incubation for 24 h at 30 °C in washing buffer supplemented with 292 mM raffinose enables the *in situ* formation of polymers by the renatured active enzyme. Subsequent oxidation by periodic acid activates the polymer for staining with Schiff's reagent [15]. As described for *Lactobacillus reuteri* levansucrase, small amounts of Fe^{3+} inhibits the enzyme [16]. Consequently, 2.5 mL of supernatant was desalted using PD-10 columns (GE Healthcare, Uppsala, Sweden), and the enzyme was concentrated via ultra-filtration through BSA-saturated membranes. For this purpose, a 30,000 MWCO Vivaspin Concentrator (Vivaspin, Germany) was centrifuged 10 min at 3000 × *g* with a solution of 10 mg mL^{-1} BSA in water. The membrane was washed once with water before 20 mL of enzyme-containing supernatant were added. After 7 min of centrifugation at 3000 × *g*, approximately 0.5 mL retentate remained. The membrane was washed once with the retentate to recover any enzyme that had adsorbed to the membrane. Enzymatic activity was distributed between the retentate (approx. 80%) and the membrane (approx. 20%). Analyses of the optimal pH, optimal temperature, the substrate spectrum, kinetic parameters, and some sucrose consumption curves were performed with this concentrated enzyme preparation (1300 U L^{-1}). The fermentative approaches were performed with $SacB_{Bm}$-producing *E. coli* cells; the gene coding for $SacB_{Bm}$ was cloned into *E. coli* as described previously [13].

2.1.2. B. subtilis $SacB_{Bs}$

The levansucrase $SacB_{Bs}$ was produced by *E. coli* BL21(DE3) cells containing the $sacB_{Bs}$ expression plasmid p24FTF11871 as previously described (Seibel et al. [6]). The enzyme was purified using a 15 mL CM-Sepharose FF (GE Healthcare) column with a controlled pump mix system (Liquid Chromatography Controller LCC-500 Plus, LKB Pump P-500, Pharmacia) and a UV-detector (λ = 280 nm, Biotech UV-MII, Pharmacia). For this purpose, 6.25 mL cell-free extract in 50 mM phosphate buffer (pH 6.6) containing 13 mg mL^{-1} protein were loaded with a flow rate of 0.5 mL min^{-1}. For elution, a linear gradient was followed from 50 mM to 1 M phosphate buffer (pH 6.6) at a flow rate of 1 mL min^{-1}. Two milliliter fractions were collected (autosampler LKB Frac-100, Pharmacia) and tested by conversion of the sucrose substrate. Enzymatically active fractions were further analyzed by SDS-PAGE. The protein concentrations of the fractions were measured photometrically at λ = 280 nm (NanoDrop Spectrophotometer ND-100, peqLab Biotechnology). Fractions containing enzyme were desalted using PD-10 columns (GE Healthcare, Uppsala, Sweden). The heterologously produced enzymes were compared to the *B. subtilis* produced and purified enzymes in terms of optimal pH and sucrose consumption curves. No obvious differences were detectable. Hence, the determination of the kinetic parameters was performed with the enzymes produced in *E. coli*.

2.2. General description of the various levansucrase reactions

For the synthesis of levan, $SacB_{Bm}$ (1000 U L^{-1}) was incubated overnight with 2 M sucrose then denatured by incubating the reaction mixture for 10 min at 100 °C. Levan was precipitated and washed once with 70% (v/v) ethanol. The resulting levan pellet was freeze-dried and resuspended in hot water. The levan concentration was measured densitometrically against a standard of inulin (see Section 2.2.1).

Glucose and fructose concentrations were determined for the levan production experiments with the BioAnalyse glucose and fructose kit (R-Biopharm AG, Germany) according to the manufacturer's instructions.

For the measurement of the various acceptor activities and the determination of kinetic parameters, levansucrase $SacB_{Bs}$ (1300 U L^{-1}) was added to a reaction mixture containing different amounts of sucrose and acceptor in 50 mM phosphate buffer (pH 6) with 50 mg L^{-1} $CaCl_2$. The reaction temperature was maintained at 30 °C for indicated time intervals. To start the enzymatic reaction, 92.9 U L^{-1} of $SacB_{Bm}$ were added to a reaction mixture containing different amounts of sucrose and acceptor in 50 mM phosphate buffer (pH 6.5) containing 50 mg L^{-1} $CaCl_2$. The reaction temperature was maintained at 37 °C for various time intervals.

One unit of enzyme activity is defined as the release of 1 μmol of glucose from sucrose per minute. Product formation was analyzed in samples taken from the reaction mixture at suitable time intervals. Values for K_m, k_{cat}, and v_{max} were determined from the initial rate for 7 to 10 substrate concentrations by a direct fit of the data to the Michaelis–Menten equation employing the computer program Origin 7.0 (OriginLab Corporation). Three different initial concentrations of sucrose (36, 74 and 111 g L^{-1}) were incubated without acceptor substrate. One initial concentration of Gal-Fru (17.5 g L^{-1}) was incubated with 92.9 U L^{-1} $SacB_{Bm}$ without