Development of soluble and immobilized biocatalysts based on a recombinant thermostable \( \beta \)-fructosidase enabling complete sucrose inversion at pasteurization temperatures

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ABSTRACT

Biocatalysts for the industrial production of invert sugar are preferred to stably operate at high sucrose concentrations and pasteurization temperatures. Thermotoga maritima \( \beta \)-fructosidase (BfrA) is more thermostable and less susceptible to substrate inhibition than the current commercial invertase from Saccharomyces cerevisiae. In this research, the non-saccharolytic host Pichia pastoris was engineered for BfrA production. Fed-batch fermentation of the recombinant yeast for 72 h using cane sugar as a non-expensive energy source yielded cultures of cell densities over 100 g/L (dry biomass) with invertase activity exceeding 300 U/mL. BfrA was secreted to the cell periplasmic space and the culture medium as a fully active glycoprotein with unaltered thermostability. The extracellularly-released BfrA representing 85 % of the total proteins in the culture supernatant was either dried into powder to generate a soluble free enzyme biocatalyst (specific activity 15 000 U per gram of powder) or covalently immobilized on Glyoxyl-Sepharose CL 4B to generate an insoluble enzyme biocatalyst (specific activity 9249 U per gram of dry support) for reuse. As a third approach, the biomass bearing the periplasmic BfrA was submitted to a killing heat treatment and entrapped in calcium alginate beads to generate a reusable non-viable cell biocatalyst (specific activity 103 U per gram of dry beads). The three biocatalysts completely hydrolyzed cane sugar (70 %, w/v) in batchwise or continuous operation at 60 °C, offering alternative cost-effective options for the industrial manufacture of food-grade inverted sugar syrup. This research granted the 2013 Award of the Cuban National Academy of Sciences.

Keywords: invertase, invert syrup, immobilization, Thermotoga maritima, Pichia pastoris

RESUMEN

Desarrollo de biocatalizadores solubles e inmovilizados basados en una \( \beta \)-fructosidasa recombinante termoestable que permite la inversión total de la sacarosa a temperaturas de pasteurización. El empleo de biocatalizadores termoestables en la producción industrial de sirope invertido es aún una meta por lograr. La enzima \( \beta \)-fructosidasa (BfrA) de Thermotoga maritima es más termoestable y menos susceptible a la inhibición por sustrato que la invertasa comercial de Saccharomyces cerevisiae. En esta investigación, Pichia pastoris se modificó genéticamente para producir BfrA. La levadura recombinante alcanzó densidad celular superior a 100 g/L (biomasa seca) y actividad invertasa por encima de 300 U/mL en fermentaciones de 72 h con el empleo de sacarosa como fuente barata de carbono. BfrA se secretó al periplasma celular y al medio de cultivo en forma de una glicoproteína totalmente activa y termoestable. BfrA con pureza de 85 % en el sobrenadante de cultivo se secó hasta polvo para generar un biocatalizador de enzima soluble (actividad específica 15 000 U/g peso seco) o se inmovilizó de forma covalente al soporte Glioxil-Sepharosa CL 4B para generar un biocatalizador insoluble (actividad específica 9249 U/g peso seco) que permita reuso. En un tercer enfoque, la biomasa celular fue sometida a un tratamiento térmico y encapsulada en esferas de alginito de calcio para generar un biocatalizador reutilizable de células muertas (actividad específica 103 U/g peso seco). Los tres biocatalizadores hidrolizaron completamente el azúcar de caña (70 % p/v) en operación continua o discontinua a 60 °C, y ofrecen opciones alternativas rentables para la inversión enzimática del azúcar a escala industrial. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2013.

Palabras clave: invertasa, sirope invertido, inmovilización, Thermotoga maritima, Pichia pastoris

Introduction

An ideal biocatalyst for the enzymatic manufacture of invert sugar should optimally operate in a highly concentrated sucrose solution (70 %, w/v) to minimize the initial dilution and final concentration steps and at pasteurization temperatures (60-70 °C) to improve substrate diffusion and minimize microbial contamination. Saccharomyces cerevisiae has been for years the source of commercial invertase. The enzyme...
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A synthetic bfrA gene with codon usage modified for optimal host translation was fused to the alpha factor signal peptide of vector pGAPZαC (Invitrogen) and expressed in P. pastoris under the control of the constitutive GAP promoter and the AOX1 terminator. The gradual increase of the transgene dosage from one to four copies of the expression cassette resulted in the gradual increase of the transgene dosage from one to four copies of the expression cassette. The gradual increase of the transgene dosage from one to four copies of the expression cassette resulted in the gradual increase of the transgene dosage from one to four copies of the expression cassette. The gradual increase of the transgene dosage from one to four copies of the expression cassette resulted in the gradual increase of the transgene dosage from one to four copies of the expression cassette. The gradual increase of the transgene dosage from one to four copies of the expression cassette resulted in the gradual increase of the transgene dosage from one to four copies of the expression cassette.

PpBfrA(4×) showed high invertase activity in the cell periplasmic space (approximately 2000 U/g of dry undisrupted biomass) and the culture supernatant (approximately 150 U/mL) with a global volumetric productivity around 5700 U/L/h in fed-batch fermentation using cane sugar as the sole carbon source. The engineered yeast grew on sucrose by a respiratory route achieving average yield coefficient of 0.60 g dry biomass per gram of sucrose consumed. The average cell density after fermentation for 72 h exceeded 100 g/L (dry biomass).

BfrA purified either from the cell periplasm or the culture supernatant migrated as two bands on SDS-PAGE with estimated molecular masses of 53 and 58 kDa (Figures 1 and 2). A treatment with endoglycosidase H₁ produced a single protein band with the expected size of 51 kDa. This result indicates that BfrA is secreted to the periplasmic space as a glycoprotein with two different glycosylation degrees and then it is released to the external medium without further modification. The presence of N-linked oligosaccharides did not alter the optimal activity, thermal stability, substrate specificity, and kinetic properties of recombinant BfrA, which hydrolyzed sucrose with a catalytic efficiency (kcat/Km) of 5.6 × 10^4 M⁻¹ s⁻¹ at pH 5.5 and 75 °C. This value is similar to that reported for the unglycosylated enzyme produced in Escherichia coli [3, 4].

Development of a soluble BfrA biocatalyst and batch hydrolysis of cane sugar

The fully active N-glycosylated BfrA represented 85 % of the total amount of proteins released to the external medium by PpBfrA(4×) at the end of the fed-batch fermentation. PpBfrA was first used to develop a thermostable soluble biocatalyst following the steps described below. The yeast cells remaining in the culture supernatant migrated as two bands on SDS-PAGE with estimated molecular masses of 53 and 58 kDa (Figures 1 and 2). A treatment with endoglycosidase H₁ produced a single protein band with the expected size of 51 kDa. This result indicates that BfrA is secreted to the periplasmic space as a glycoprotein with two different glycosylation degrees and then it is released to the external medium without further modification. The presence of N-linked oligosaccharides did not alter the optimal activity, thermal stability, substrate specificity, and kinetic properties of recombinant BfrA, which hydrolyzed sucrose with a catalytic efficiency (kcat/Km) of 5.6 × 10^4 M⁻¹ s⁻¹ at pH 5.5 and 75 °C. This value is similar to that reported for the unglycosylated enzyme produced in Escherichia coli [3, 4].

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Results and discussion

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Figure 1. N-glycosylation analysis of BfrA purified by IMAC from the culture supernatant of the recombinant Pichia pastoris strain PpBfrA(4×). Proteins were reacted or not with endoglycosidase H₁, separated in a SDS-polyacrylamide gel (12.5 %) and revealed by Coomassie Blue staining. A) Lanes: 1, PageRuler prestained protein ladder (Fermentas); 2, culture supernatant (10 µg of total proteins); 3, purified BfrA (10 µg). B) Lanes: 1, broad-range protein marker (BIO-RAD); 2, untreated BfrA (5 µg); 3, endoH-treated BfrA (5 µg).
in the culture supernatant after centrifugation were removed in two consecutive microfiltration steps using filters with pore size of 0.45 and 0.2 µm, respectively. The cell-free filtrate was concentrated with continuous dialysis against 50 mM sodium acetate buffer (pH 5.5) by ultrafiltration using a 30 kDa cut-off membrane and submitted to a drying rotoevaporation process at 50-60 ºC. The resulting BfrA powder with average specific invertase activity of 15 000 U/g and protein purity above 90 % was fully stable at the assayed storage temperatures of 4 and 30 ºC for at least 10 months.

Soluble BfrA was reacted with sucrose in the 1-70 % (w/v) concentration range at pH 5.5 and 60 ºC in a 1-L batch reactor with stirring at 250 rpm (Figure 3). Initial velocity measurements show that hydrolysis occurred gradually faster as the substrate concentration was driven from 1 to 10 % (w/v). The enzyme suffered from slight inhibition at sucrose levels above 20 % (w/v). Under these conditions, the velocity of sucrose hydrolysis decreased and 1-kestose \( \alpha \text{glu}(1,2)\beta \text{fru}(1,2)\beta \text{fru} \) was synthesized as a result of the transfructosylating side reaction of the enzyme. At the highest assayed substrate concentration (70 %, w/v), the overall BfrA activity (hydrolysis plus transferase) was 1.6-fold reduced and 1-kestose represented 19 % of the total products at initial reaction rates. As the reactions proceeded, sucrose and the synthesized trisaccharide were completely hydrolyzed (data not shown).

Time-course analyses of batch sucrose hydrolysis by the soluble BfrA biocatalyst were then conducted in the temperature range of 50-70 ºC using an enzyme dose of 5 U/g of cane sugar at an initial concentration of 70 % (w/v) and constant pH 5.5 (Figure 4). Due to its high specific activity, the BfrA powder added negligible mass (4.2 g; 3500 U) to the reaction volume (1 L). The complete substrate inversion took 9, 11 and 14 h for the reactions at 70, 60 and 50 ºC, respectively. The average productivity in the reaction at 70 ºC was 15.8 g of substrate hydrolysed/enzyme unit/hour. The highest productivity value of 20.3 was reached at the reaction time interval between 4 and 5 h when half the mean values was below 10 %.

of the initial substrate content had been transformed. The incubation at 70 °C promoted the occurrence of Maillard browning reactions, while the products solution remained colourless in the experiments at 60 and 50 °C.

The thermostable soluble BfrA biocatalyst can have a prolonged reuse in batch reactions at 60 °C if it is operated in a membrane bioreactor. For this purpose, the tank outlet port is recommended to be coupled to a cartridge-type ultrafiltration membrane with 30-kDa cutoff to retain the enzyme inside the reactor after discharging the product solution.

Rational immobilization of BfrA on Glyoxyl-Sepharose CL 4B

Recombinant BfrA containing the C-terminal polyhistidine tag was purified by Ni affinity chromatography from the culture supernatant and optimally bound to Glyoxyl-Sepharose CL 4B using the Rational Design of Immobilized Derivatives (RDID) strategy. Multi-point-covalent attachment of the N-glycosylated BfrA onto the activated support at pH 10 allowed total recovery of the loaded enzyme and its activity. The immobilization process caused no variation in the catalytic properties of the enzyme and further enhanced the thermal stability. The immobilized enzyme biocatalyst showed average specific activity around 9249 U/g (dry weight).

Complete inversion of cane sugar (70 %, w/v) in a batch stirred tank reactor at 60 °C was achieved with a productivity of 22.2 g of substrate hydrolysed/g of biocatalyst/h (Figure 5) [13].

Calcium alginate entrapment of heat-killed cells with periplasmic BfrA activity

P. pastoris PpBfrA(4×) cells bearing periplasmic BfrA activity were submitted to a heat-killing treatment (30 min at 70 °C) prior to calcium alginate entrapment to prevent the occurrence of sucrose fermentation, an undesirable process which causes substrate loss and the formation of the by-products ethanol and carbon dioxide. The invertase activity of the non-viable cells (3092 U/g, dry weight) increased almost 1.2-fold in comparison to the untreated biomass (2603 U/g, dry weight) due to an improved diffusion of the substrate and products through the yeast cell wall. Maximal values of immobilization yield (99.6 %) and specific invertase activity (103 U/g of dry beads) were achieved with biomass loading of 300 g/L (wet weight). The immobilization of more concentrated biomass (400 g/L) restricted the internal diffusion of the substrate sucrose resulting in calcium-alginate beads of lower specific activity (90.7 U/g dry beads).

Batch hydrolysis of cane sugar (70 %, w/v) by the entrapped cells was conducted in a laboratory-scale stirred reactor operating at 60 °C. The complete sucrose inversion took 10 h with average productivity of 4.37 g of substrate hydrolysed/g of dry beads/h (Figure 5). The beads retained 47 % of its original invertase activity after recycling in a stirred tank reactor for 15 days. The half-life of BfrA in the entrapped cells during batchwise operation at 60 °C was 14 days [14].

Thermal stability of the immobilized biocatalysts during continuous production of invert sugar

The operational stability of BfrA immobilized on Glyoxyl-Sepharose CL 4B and the heat-killed yeast cells entrapped in calcium alginate gel was evaluated for continuous hydrolysis of sugar cane (70 %, w/v) at pH 5.5 and 60 °C (Figure 6). The conversion efficiency of the covalently immobilized enzyme dropped to 96.7 % and 45.2 % after operation with constant feeding flow of 5 mL/h for 1 and 6 days, respectively. The entrapped cells were fed at a higher constant flow rate (30 mL/h) and retained 48 % of the initial BfrA activity after 21 days of reuse. The half-life values of the invertase activity of the immobilized enzyme and the entrapped cells with continuous reuse in the fixed-bed reactors at 60 °C were estimated to be 5 and 20 days, respectively [13, 14]. We do not exclude the possibility that the support with

Figure 5. Batch hydrolysis of cane sugar at 60 °C by BfrA immobilized on Glyoxyl-Sepharose CL 4B (GSBfrA) and heat-killed yeast cells entrapped in Ca-alginate beads (PpABfrA). The time-course reactions were conducted in a stirred tank reactor using 4 g of GSBfrA or 100 g of PpABfrA (wet weights) per L of 70 % (w/v) substrate in 50 mM sodium acetate buffer (pH 5.5). The hydrolysis rate (%) refers the amount of cane sugar converted into reducing sugars. The experiment was replicated three times. Standard deviation of the mean values was below 10 %.

Figure 6. Operational stability of BfrA immobilized on Glyoxyl-Sepharose CL 4B (GSBfrA) and heat-killed yeast cells entrapped in Calcium alginate gel (PpABfrA). Continuous reactions were conducted using 70 % (w/v) cane sugar in 50 mM sodium acetate buffer (pH 5.5) at 60 °C. A 1-mL column was packed with 0.35 g of GSBfrA (wet weight) and fed at constant flow rate of 5 mL/h. A 250-mL column was packed with 150 g of PpABfrA (wet beads) and fed at constant flow rate of 30 mL/h. The hydrolysis rate (%) refers the amount of cane sugar converted into reducing sugars. The experiment was replicated three times. Standard deviation of the mean values was below 10 %.
the covalently bound enzyme may have partially escaped through the column filter during operation.

Relevance of the study

An ideal immobilized biocatalyst for the industrial scale production of invert sugar should allow the complete hydrolysis of the substrate sucrose at high concentrations with stable operation under pasteurization conditions. Current soluble or immobilized biocatalysts based on the thermolabile invertase from *Saccharomyces cerevisiae* are operated at temperatures not exceeding 50 ºC and exhibit substrate inhibition kinetics. *T. maritima* β-fructosidase (BfA) is the most thermoactive and thermostable sucrose-hydrolysing enzyme so far identified and it is less sensible to substrate inhibition than the commercial yeast invertase.

In this research, we have developed three thermostable biocatalysts using BfA produced as a fully active glycoprotein in a recombinant *P. pastoris* strain. The biocatalysts consisting of a soluble enzyme powder, a covalently immobilized enzyme, or heat-killed yeast cells entrapped in Ca-alginate beads fully hydrolyzed cane sugar (70 %, w/v) to an equimolar mixture of glucose and fructose in a resulting solution which was free of by-products and microbial contamination. The highly concentrated and colorless invert syrup does not require downstream processing prior to use in the food industry. The soluble BfA powder adds negligible volume to the stirred batch reactor and allows short-time reactions due to its high specific activity and the absence of diffusional barriers between the substrate and the free enzyme. The immobilized enzyme is most appropriate for continuous sucrose hydrolysis in a fixed-bed reactor, while the entrapped non-viable cells can be extensively reused either in batchwise or continuous operation at 60 ºC. The three thermostable biocatalysts developed in this research offer alternative operational options for the cost-effective manufacture of food-grade inverted sugar syrups at industrial scale.