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ORIGINAL PAPER

Production of a thermophilic maltooligosyl-trehalose synthase in *Lactococcus lactis*

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Abstract The thermoacidophilic archaeon *Sulfolobus solfataricus* MT4 encodes a maltooligosyltrehalose synthase (MTS), that catalyzes an intramolecular transglycosylation process converting the glycosidic linkages at the reducing end of dextrins from α-1,4 into α-1,1. In this research the gene encoding MTS was cloned and expressed in *Lactococcus lactis* NZ9000 using the so-called NICE system. Growth conditions of the recombinant strain were optimized in flask experiments in relation to enzyme production. Batch experiments in 2 L-fermenters were performed on the best identified semidefined medium and 256 U L^{-1} of recombinant MTS were produced. Purified recombinant MTS shows its optimal activity at 70 °C and pH 5.5, prefers maltoheptaose and maltohexaose as substrates, and demonstrates minimal side hydrolytic activity.

Keywords Intramolecular transglycosylation · Nisin induction · Recombinant *Lactococcus lactis* · Thermozymes · Trehalosyl-dextrins

Introduction

Enzymes isolated from thermophiles have distinctive qualities that make them more attractive and better suited than mesophilic enzymes for use in industrial processes. Not only do they have optimal activity at extreme temperatures, but they generally exhibit greater tolerance to changes in pH, exposure to solvents and denaturating agents, and to pressure. Three enzymes from *Sulfolobus solfataricus* MT4 were expressed by our group at high yields in *Escherichia coli* [strain RB-791 and BL21(DE3)] and exploited in immobilized cell bioprocesses to convert maltodextrins to trehalose, glucose, and their corresponding non-reducing derivatives, at high temperature [5, 7, 17].

More recently we focused on the production of trehalosyl–dextrins, namely linear dextrins containing trehalose as an end unit. Since these sugars do not undergo the Maillard reaction in the presence of proteins and peptides they represent an interesting alternative to the conventional dextrins commonly utilized in food preparations, pharmaceuticals and cosmetics.

The enzyme maltooligosyltrehalose synthase (MTS) transforms starch and dextrins into the corresponding trehalosyl derivatives through an intramolecular transglycosylation process that converts the glycosidic linkages at the reducing end of the chain from α,1–4 into α,1–1. Nakada and collaborators [13] first reported the purification of MTS from *Arthrobacter* sp. Q36, and, subsequently, the corresponding thermophilic proteins were isolated from *S. acidocaldarius*, *S. shibatae* and *S. solfataricus* [4, 8, 14].

Cloning of the gene encoding MTS and its expression in *E. coli* has so far been achieved by several groups [6, 10, 14, 16] providing significant information on the behavior of the enzyme although not shortening the route to industrial applications.

We wished to increase the availability of MTS, to further the development of an industrial process. In this study *Lactococcus lactis* was selected as host for the expression of the biocatalyst since its genetics have been extensively characterized enabling easy handling; moreover lactic acid bacteria (LAB) are widely used in the food industry.

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In order to achieve a high inducible expression of the recombinant *L. lactis* MTS (*Ll*MTS) enzyme we utilized the nisin-controlled expression system (NICE, developed at NIZO Food Research, Ede, The Netherlands) which is based on the combination of the *nisA* promoter, fully activated from subtoxic amounts of nisin, and the *nisRK* regulatory genes [3].

Our group has recently achieved success applying this strategy to the expression of an α-glucosidase isolated from *S. solfataricus* in *L. lactis* [7].

Here we report on the cloning of the MTS gene from *S. solfataricus* and on the optimization of the expression and fermentation procedures with the aim of increasing enzyme yields in *L. lactis*.

Materials and methods

Construction of the expression vector pNZ8148-MTS and recombinant strain

The recombinant plasmid was constructed by isolating the entire MTS gene from the Ptrc99A vector, previously expressed in *E. coli* RB-791 by our group [2], as a 2.2 kb 5′ *Nco*I–3′ *Spe*I fragment. The fragment was inserted into the pNZ8148 vector (kindly provided from Nizo Food Research, Ede, The Netherlands) [11] previously linearized with the same restriction enzymes resulting in a recombinant plasmid indicated as pNZ8148-MTS.

Lactococcus lactis NZ9000 [11] competent cells (kindly provided by NIZO Food Research, Ede, The Netherlands) were electroporated [18] using a Gene Pulser (Bio-Rad Laboratories) with the pNZ8148-MTS expression vector and positive clones were selected on medium containing 0.005% w/w of chloramphenicol.

Restriction enzymes and T4 DNA-ligase were purchased from Invitrogen (Carlsbad, California).

Media and growth conditions

Lactococcus lactis was grown at 30 °C in complex medium M17 (Difco Laboratories) and in various alternative semidefined media (Table 1) supplemented with 0.5% w/w glucose and 0.005% w/w chloramphenicol. The key parameters for enzyme production were studied in liquid M17 medium in static cultivation.

The production kinetics of the enzyme were first evaluated by inducing the cells at increasing optical densities (OD_{600} equal to 0.25, 0.5, 1, 1.5) with 1 ng mL^{-1} of nisin. In a second set of experiments increasing concentrations of nisin (1–8 ng mL^{-1}) were tested either in relation to the OD of the sample or at fixed optical density (0.5 OD_{600}). Cells were harvested at different time intervals after induction and tested for MTS expression by enzymatic assays on maltodextrins.

Production experiments

Batch fermentation experiments were performed in a Biostat CT bioreactor (2 L working volume) [15]. The recombinant *L. lactis* strain was grown without aeration at 30 °C, pH 6.5, 200 rpm on M17, and on SDM4 (as described in Table 1). Samples were withdrawn every hour to follow growth and enzyme production. Induction was performed at OD_{600} equal to 0.5, using nisin (1 ng mL^{-1}) with a repeat induction 4−5 h later.

Cell disruption and enzymatic assays

Cells harvested by centrifugation throughout fermentation were resuspended to OD_{600} = 20 in Tris–HCl 10 mM pH 8

Table 1 Composition of semidefined media tested in flask experiments and production of *Ll*MTS

Components (g L^{-1})	SDM1	SDM2	SDM3	SDM4	SDM5
Yeast extract	2		2	2.5	2.5
Pancreatic digest	10	10			
Casein hydrolysate		2	5	5	5
Soy peptone			5	5	5
Yeast nitrogen base				5	5
Disodium glycerophosphate				19	19
Milk peptone					5
Mineral salts[a]	√	√	√	Only $MgSO_4$	Only $MgSO_4$
Enymatic activity (U L^{-1})	1.08 ± 0.02	1.44 ± 0.03	0.36 ± 0.01	15.66 ± 0.54	15.48 ± 0.46

Values are the mean ± SD of three experiments

[a] Ammonium citrate (2 g L^{-1}), sodium acetate (5 g L^{-1}), magnesium sulfate (0.1 g L^{-1}), manganese sulfate (0.05 g L^{-1}), potassium phosphate (2 g L^{-1})

and mechanically disrupted by vigorous shaking with 0.1–0.3 cm diameter glass beads (ten cycles of 1 min with 30 s interval on ice).

Enzymatic assays were performed with maltohexaose (M6) 10 mM as substrate in sodium-acetate 50 mM pH 5.5 for 10 min at 75 °C (unless otherwise indicated). All the reactions were stopped by cooling the mixtures in an ice-water bath. One unit was defined as the amount of enzyme which produces 1 μM min^{-1} of trehalosyl-maltotetraose (TM4).

Protein purification and characterization

The purification protocol consisting of four steps of thermoprecipitation followed by a size exclusion chromatographic step was a slight modification of the procedures described by de Pascale et al. [2]. The protein content of all extracts was determined according to [1].

A pool of purified recombinant enzyme was used to study the influence of pH, temperature and substrate specificity. For the determination of the optimal pH *Ll*MTS was incubated at 75 °C for 30 min with maltohexaose (M6) 5 mM. The following 50 mM buffers were used in different pH ranges: citrate–phosphate buffer (pH 3.5–6.5), phosphate buffer (pH 7), Tris–HCl buffer (pH 9).

The effect of temperature was analyzed by incubating the enzyme at temperatures ranging from 40 to 90 °C for 30 min with M6 5 mM as substrate, in sodium-acetate 50 mM pH 5.5.

In order to establish the most suitable substrate for the transglycosylation reaction *Ll*MTS was assayed at 70 °C for 30 min with maltooligosaccharides (5 mM) characterized by different numbers of repetitive units, starch and starch derivatives (2.5 mg mL^{-1}). In addition, the hydrolytic activity of *Ll*MTS was assayed both in optimal conditions (70 °C, 50 mM sodium acetate buffer, pH 5.5) and using the procedure described by Fang and collaborators [6] (75 °C, 50 mM citrate–phosphate buffer, pH 5). The quantitative determinations of substrate and products in the reaction mixture were performed with a Dionex chromatograph as previously described [17].

Results

Flask experiments on engineered *L. lactis* NZ9000

The MTS gene, previously isolated from *S. solfataricus* MT4, was cloned into the expression vector pNZ8148 and introduced in NZ9000 competent cells. A series of flask experiments on M17 demonstrated that best results were obtained by inducing cells with nisin 1 ng mL^{-1}at OD_{600} equal to 0.5 (data not shown); induction with the same amount of inducer but at later growth stages (e.g. $OD_{600} \geq 0.75$) resulted in a decrease of enzyme production (data not shown). Increasing concentrations of nisin in relation to the OD_{600} of the sample were also tested and, as shown in Fig. 1, the concentration of inducer needed to reach a maximum *Ll*MTS production (23 ± 4 U L^{-1}) is positively correlated to the cell density. However, inducing the culture later with higher nisin concentration did not affect the yield significantly.

The ability of the recombinant *L. lactis* to grow on a variety of complex nitrogen sources (Table 1) and produce the MTS enzyme was tested in flask experiments in order to define a medium composition suitable for large scale cultivation. The enzymatic activity attained for each experiment is also shown in the table; strain behavior was comparable on SDM4 and SDM5. The former, containing casein hydrolysates, together with bacteriological and vegetal peptones instead of meat extracts, was chosen for further fermenter experiments.

Production of the recombinant *Ll*MTS

Fermenter experiments in batch mode were carried out with glucose as the main carbon source. The process led to a fourfold higher biomass production compared to growth in flasks, reaching a final OD_{600} value equal to 12, and a corresponding increase in enzymatic activity from 15 to 256 U L^{-1}. Productivity (Φ = U $(L\ h)^{-1}$) obtained was 10 U $(L\ h)^{-1}$. Assays on samples withdrawn at different time intervals indicate that *Ll*MTS is actively produced

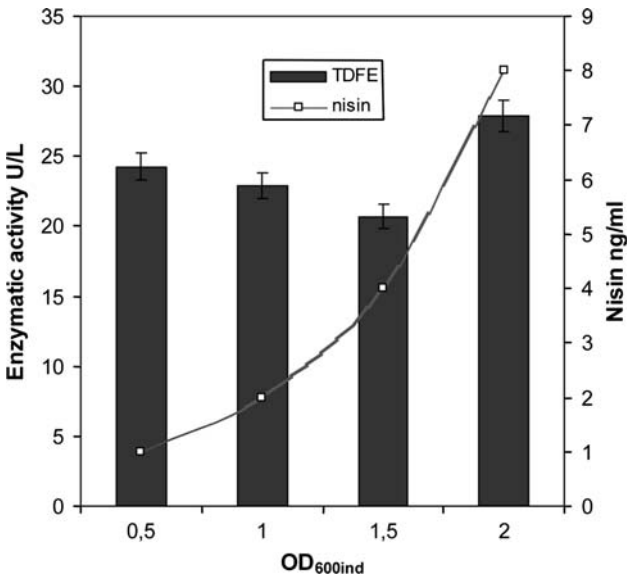

Fig. 1 Enzymatic activity resulting from induction with a concentration of nisin that increased in relation to the optical density of the sample. Mean values and SD are calculated based on three different sets of experiments. Experiments were performed on M17 medium

during the whole growth phase. Lactic acid concentration increased up to toxic levels during the course of the fermentation, reaching 40 g L^{-1}.

Purification and characterization of the enzyme

Thermal treatment and size exclusion chromatography resulted in a 45-fold purification of the enzyme, a specific activity of 14.4 U mg^{-1} and a final yield of 19.2%. Analysis of the purified MTS in SDS-PAGE (12%) shows a single band corresponding to the expected molecular mass of about 87 kDa (data not shown). The enzyme showed an optimal activity at 70 °C and pH 5.5. Thermoactivity, thermostability, and optimal pH were analogous to the natural enzyme. *Ll*MTS proved stable at 75 °C for more than 24 h, with a half life of about 50 h.

*Ll*MTS used maltoheptaose and maltohexaose as preferential substrates for the transglycosylation reaction (data not shown). Starch, amylose and amylopectin were not good substrates for the enzyme. Side hydrolytic activity of the enzyme, that is detrimental in foreseen biotechnological applications, was investigated with assays either in the identified optimal conditions or according to the protocol described by Fang et al. [6]. As illustrated in Table 2, *Ll*MTS was not active on maltotriose and also showed a low hydrolytic activity both at 70 and at 75 °C. Higher affinity for substrates was observed on maltotetraose and maltoheptaose demonstrating a side activity of 2.97 and 0.89%, respectively, at the optimal temperature.

Discussion

The employment of MTS, isolated from *S. solfataricus*, in the development of innovative industrial processes is very promising especially for the production of non-reducing carbohydrates such as trehalosyl-dextrins. In fact, it is very important to perform carbohydrate biotransformations at high temperatures in order to couple reduced contamination risk during the process with the lower viscosity of the highly concentrated solutions of starch or dextrins used as starting material [2, 4].

Since the use of MTS is mostly hampered by high production costs related to fermentation of the natural host, different strategies have been used to reach higher titers of MTS in *E. coli* [10, 14, 16]. Fang et al. [6] recently achieved a high yield of active MTS. However, since certain industrial applications require the development of GRAS (generally recognized as safe) expression systems, the use of *E. coli* as a host is limited due to the production of endotoxins that can contaminate the desired recombinant protein.

Therefore, in the present study, we investigated *L. lactis* as a host and expressed MTS isolated from *S. solfataricus* with a nisin inducible expression system. *L. lactis* is a GRAS microorganism commonly used in the manufacturing of dairy products and recently proposed as a new probiotic bacterium [9].

The correlation between inducer concentration and cell density of the culture at the moment of induction was examined in order to improve *Ll*MTS expression. Results demonstrate that the addition of linearly increasing amounts of nisin at a fixed optical density does not lead to proportionally growing enzyme yields; an eightfold increase of inducer concentration is necessary to reach higher MTS titers. Moreover, as also reported by Mierau and colleagues [12], it seems that increasing the concentration of inducer proportionally to the OD_{600} of the culture yields similar amounts of enzyme even when the addition is performed between the late exponential and early stationary phase (Fig. 1).

A major point to take into consideration in order to facilitate certification and production is medium design. Regulatory agencies prefer industrial media free from animal derived ingredients. Among various semidefined media tested, the one containing yeast extract, casein hydrolysate,

Table 2 Analysis of the transglycosylation and hydrolytic activity of the recombinant MTS

Substrate (14 mM)	Trehalosyl dextrin formation rate (μM min^{-1})		Glucose formation rate (μM min^{-1})		Relative reactivity				Ratio of *H*/*T*[c](%)	
					Transglycosylation (%)		Hydrolysis (%)			
	70 °C[a]	75 °C[b]	70 °C[a]	75 °C[b]	70 °C[a]	75 °C[b]	70 °C[a]	75 °C[b]	70 °C[a]	75 °C[b]
Maltotriose	0	0	0.91	0.85	0	0	11.60	12.90	+∞	+∞
Maltotetraose	5.04	3.15	0.15	0.24	70.77	51.86	2.07	3.96	2.97	7.60
Maltoheptaose	7.81	6.30	0.07	0.09	82.37	68.60	0.77	1.01	0.89	1.43

[a] Reactions performed in optimal conditions: 70 °C, in 50 mM sodium acetate buffer (pH 5.5). The concentration of substrates (M3–M4–M7) was 14 mM

[b] Assays performed in the conditions described by Fang and colleagues [6]: 75 °C, in 50 mM citrate–phosphate buffer (pH 5), the concentration of substrates was 14 mM

[c] Ratio of hydrolysis to transglycosylation

soy peptone, and yeast nitrogen base (SDM4) was the best for supporting enzyme expression. In fact despite the difficulties previously encountered in attaining MTS overexpression, the enzyme yield in fermentation experiments with the recombinant *L. lactis* was similar to that of *E. coli* RB-791 transformed with the pTrc99AMTS plasmid, which corresponds to a 100-fold increase in production compared with the natural host.

Enzyme characterization showed that *Ll*MTS has properties similar to those of other recombinant or natural MTSs. Assays evaluating the side hydrolytic activity of the enzyme in the presence of three different substrates (M3–M4–M7) showed that interestingly the ratio of hydrolysis to transglycosylation of M7 was negligible. These results are in agreement with those of Fang and collaborators [6], suggesting that the enzyme has great potential.

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