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ORIGINAL PAPER

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Evaluation of a high temperature immobilised enzyme reactor for production of non-reducing oligosaccharides

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Abstract There is interest in the production of non-reducing carbohydrates due to their potential application in various industrial fields, particularly the food industry. In this paper, we describe the development of an immobilised cell bioprocess for the synthesis of non-reducing maltodextrins at high temperatures. The trehalosyl-dextrins-forming enzyme (TDFE) isolated from the thermoacidophilic archaeon *Sulfolobus solfataricus* (strain MT4), was recently expressed at high yields in *Escherichia coli* (strain Rb-791). Here, we evaluate different matrices, such as polyacrylamide gel, crude egg white, chitosan and calcium alginate for their effectiveness in immobilising whole recombinant *E. coli* cells subjected to prior thermal permeabilisation. Calcium-alginate based gels formed a solid biocatalyst with a good activity yield and the best enzymatic stability at the operating temperature (75°C). Therefore, these beads were used to pack a glass column reactor to perform the bioconversion of interest. Optimal operating parameters were defined in relation to the substrate stream flow-rate and the substrate-to-biocatalyst ratio. The production of trehalosylmaltotetraose from maltohexaose reached equilibrium with a constant of about 2.6 at 75°C. The bioreactor was exploited for production of trehalosylmaltodextrins from a commercial mixture of maltodextrins, achieving a productivity of 106.5 mg ml^{-1} h^{-1} (g biocatalyst)$^{-1}$ with ~40% conversion when using a 30% (w/v) solution.

Keywords Thermophilic enzymes · Permeabilised recombinant cells · Trehalosyl-dextrins production · Packed bed bioreactor

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Introduction

Trehalosyl-dextrins are linear dextrins containing trehalose as end units. Due to the absence of reducing activity, these sugars do not undergo the Maillard reaction in the presence of proteins and peptides. These compounds therefore represent interesting and innovative alternatives to the conventional carbohydrates generally utilized in food preparations, pharmaceutics and cosmetics. The trehalosyl-dextrins-forming enzyme (TDFE) transforms starch and dextrins to the corresponding trehalosyl derivatives with an intramolecular transglycosidation process that converts the glycosidic linkages at the reducing end from α-1,4 into α-1,1. The enzyme that catalyses this biotransformation was found in a number of mesophilic and thermophilic microorganisms. Nakada et al. [12] first reported the purification of TDFE from *Arthrobacter* sp. Q36, and, more recently, the corresponding thermophilic proteins were isolated from *Sulfolobus acidocaldarius*, *Sulfolobus solfataricus* and *Sulfolobus shibatae* [6, 13]. There are advantages for the application of thermophilic enzymes in place of their mesophilic counterparts for carbohydrate processing. In fact, the biotransformation of starch and polysaccharides has traditionally been conducted at high temperatures in order to lower viscosity and avoid contamination. In spite of these motivations, there are very few bioprocesses exploiting extremophilic enzymes. The application of these peculiar biocatalysts is hampered mainly by high production costs, which are not compatible with the frequent enzyme replacement typical of biotransformation by free enzymes.

Kato [10] reported the production of trehalose from starch using free enzymes with a yield of 81.5%; a result that is quite favourable for industrial applications. However, the glycosyltransferase activity from *S. solfataricus* KM1 decreased to 90% in only 6 h at 70–80°C. Bringing a thermophilic biocatalyst to the market requires: (1) overexpression of the gene encoding the activity of interest in a suitable mesophilic host, (2) development

of fermentation strategies to improve enzyme yield and/or productivity, (3) design of easy and low-cost downstream procedures leading to high recovery of pure enzyme, and (4) the possible use of immobilised biocatalyst to increase stability at operating conditions. Biotransformations based on immobilised enzymes are largely preferred in the biochemical and food industry because they provide the opportunity to improve volumetric productivity by achieving a high concentration of catalytic biomass in a controllable form [6, 18].

Several large-scale industrial processes, such as the production of high fructose syrup, and the manufacture of L-amino acids, already employ immobilised-enzyme biocatalysts [1, 18]. Our group successfully exploited a bioreactor based on recombinant immobilised cells for the production of trehalose at high temperature [7]. In the framework of this research we evaluated the possibility of employing a similar technique for the production of non-reducing dextrins. In particular, we focused our research interest on the development of a high-temperature bioprocess based on permeabilised recombinant *Escherichia coli* cells containing TDFE from Sulfolobales.

Materials and methods

Materials

Maltohexaose, alginic acid sodium salt, SDS and glutaraldehyde were from Sigma Aldrich (St. Louis, Mo.). Acrylamide and polyacrylamide were from Gibco BRL (Grand Island, N.Y.). Chitosan (40% acetylation grade, 37–100 μm) was purchased from Chitobios (Ancona, Italy). Commercial dextrins were kindly offered by Roquette (Services Technique et Laboratories, Lestrem, France), and contained glucose(G), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose (M5), maltohexaose (M6) maltoheptaose (M7), and dextrins with higher polymerisation degrees (up to 3–5% w/w). Calcium chloride dihydrate was from Serva (Feinbiochemica, Heidelberg, Germany). All other chemicals and reagents used were analytical grade products.

Microorganisms and cultivation

E. coli Rb791 competent cells were transformed with a pTrcTDFE expression vector as previously described [3] and grown overnight in Luria-Bertani medium at 37°C on a shaker at 200 rpm. The resulting culture was inoculated into a Biostat CT fermenter (2 l working volume; Braun, Melsungen, Germany) and cells were grown in batch or microfiltration mode in a semi-defined medium, in some cases with a modified membrane bioreactor developed by our group as previously described [15, 16]. The biomass was separated from the supernatant by centrifugation and re-suspended in a buffer solution before the immobilisation procedure.

Immobilisation protocols

E. coli cells (5 g wet weight) were suspended in 5 ml 50 mM sodium acetate buffer (pH 5.5) and permeabilised by incubating for 1 h at 75°C with stirring in a thermostatically controlled bath. After incubation cells were assayed for TDFE activity.

Permeabilised cells (5 g), suspended in 5 ml 50 mM sodium acetate buffer pH 5.5 (final volume 10 ml), were entrapped using different supports. Polyacrylamide-entrapped cells (PEC), egg-white-entrapped cells (EWEC) and chitosan-entrapped cells (CEC) were prepared following previously described methods [2, 5]. Ca-alginate gel beads [0.3 ± 0.1 cm diameter, 2% (w/v) final gel concentration] were prepared by the method of Smidsrod and Skjak-Braek [17].

Intact biocatalyst beads, or semi-spheres appropriately cut to permit viewing of cells in the interior, were dehydrated with increasing ethanol concentrations, critical-point dried (Polaron Thermo PC) and spatter coated with gold before observing them by scanning electron microscopy (SEM; Philips 505).

Enzyme assay

TDFE activity in the cytosol and cellular suspension was assayed under standard conditions at 75°C, in 50 mM sodium acetate buffer (pH 5.5) using M6 as substrate at a concentration of 0.67 mM. No similar activity in the temperature range 65–85°C was ever detected in wild-type *E. coli* before its transformation with the plasmid of interest. In the standard mixture, M6 was combined with 1–10 μg free enzyme or 5 μl 10-fold diluted cellular suspension for 30 min. The reaction, linear for at least 2 h, was stopped in an ice-water bath and the amount of product—trehalosylmaltotetraose (TM4)—formed was determined by high performance anion exchange chromatography (HPAEC).

TDFE activity in immobilised cells was determined at 75°C, with stirring in a water bath equipped with a temperature control system, using 1.125 ml standard reaction mixture together with either 5–10 mg wet PEC, 2–4 mg wet EWEC, 3–5 mg CEC, or two Ca-alginate beads (4–5 mg wet weight). Substrate and products were quantified by HPAEC. One unit was defined as the amount of enzyme that produces 1 μmol TM4/min.

Thermostability studies

The immobilised cell biocatalysts were tested for thermostability by incubating several vials containing 5 mg PEC (0.03 U TDFE), 4 mg EWEC (0.028 U TDFE),or 3 mg CEC (0.017 U TDFE) in 1.125 ml 50 mM sodium acetate buffer (pH 5.5) at 75°C. Ca-alginate beads (15 mg, 0.143 U TDFE) were mixed with 1.125 ml 50 mM calcium acetate buffer (pH 5.5), to avoid matrix damage, and incubated at 75°C. Samples were withdrawn at different times and residual activity determined under standard conditions.

High performance anion exchange chromatography

Quantitative determination of substrate and products in the reaction mixtures was performed using a Dionex Chromatograph DX-500 (Dionex, Sunnyvale, Calif.), equipped with a pulsed amperometric detector (PAD), using a Carbopac PA 100 column, as previously described [9]. Elution was carried out with the following gradient: NaOH 160 mM (Buffer A) and sodium acetate 300 mM (Buffer B), ($t = 0$ min 0% Buffer B; $t = 6$ min 0% Buffer B; $t = 36$ min 60% Buffer B). Elution of carbohydrates (M7 to G), at a flow-rate of 1 ml/min, was completed within 40 min. Detection was accomplished by triple-pulsed potential using a gold electrode.

Bioreactor

Two glass columns (5 and 50 ml working volume; Microglass, Naples, Italy) jacketed by a coaxial glass cylinder connected to a thermostatic bath to maintain the temperature at 75°C, were packed with the biocatalyst beads. Packing was improved by using glass beads of a similar diameter ($\varnothing = 0.1$–0.3 cm) at the inlet and outlet of the column, where the jacket converges resulting in poor heat transfer. The substrate solution (either M6 or dextrin mixtures dissolved in 50 mM calcium acetate buffer at pH 5.5) was fed into the bioreactor using a peristaltic pump (Model M312; Gilson,

Villiers-le-Bel, France) at a specific flow rate. Once the solution passed through the catalytic bed it was collected either manually (when operating the 5 ml reactor) or by a second pump (50 ml bioreactor) and sometimes recycled. It has to be considered that because the void fraction of the packed bioreactors was about 30%, the volume of substrate solution generally had to be 5- to 10-fold of that required to fill the catalytic bed [11].

Determination of the apparent kinetic parameters

A very small amount of biocatalyst (about 0.4 g) was used to assemble a differential reactor (2 cm long). Its residence time was adjusted in order to have a very low extent of conversion; the concentration of substrate along the catalytic bed could therefore be considered approximately constant. Solutions with increasing concentration of M6 were pumped through the reactor and collected and analysed after one cycle (about 5 s residence time); data were standardised with respect to biocatalyst amount and residence time. The inverse of the reaction rates with respect to the inverse of M6 concentration were plotted following the Lineweaver-Burk equation to evaluate the apparent parameters of the immobilised enzyme.

Evaluation of bioreactor performances

Several experiments were carried out to define the optimal operating conditions using the smaller bioreactor (15 cm length). First, conversions were performed at a constant substrate concentration and a fixed bed length to establish the reaction-limiting step (external mass transfer vs intrinsic kinetic), increasing the flow rate from 0.2 to 1.6 ml/min. Using a fixed flow rate, the substrate ratio of M6 to biocatalyst was progressively changed.

Several experiments were carried out using dextrin mixtures at high concentration (10, 20 and 30% w/v) as substrate in order to evaluate the bioreactor performance with regard to industrial application. The bioreactor was operated in total recycle mode and samples were withdrawn at pre-defined intervals, always when an integral number of cycles was completed (e.g. 1, 5, 10, 20, etc.). Each cycle corresponded to a residence time of about 10.6 s.

Evaluation of the equilibrium constant

The equilibrium constant at 75°C was evaluated, averaging the product-to-substrate ratio when a plateau was reached in many conversion experiments (over 30). However, an experiment was set up with the aim of confirming that the reaction we were studying did in fact reach an equilibrium stage. A typical bioconversion using a 30 mM M6 solution as substrate, 4 g biocatalyst, $T = 75$°C and pH = 5.5, was completed, and when the plateau was established (e.g. two successive samples showed the same concentration of substrate and product) a spike of M6 was added to the recycled stream to restore the initial M6 concentration (30 mM). The reaction was then followed by sampling at exactly the same intervals as the previous step. The volume was maintained constant between the first and the second part of the experiment.

Results and discussion

Immobilisation of permeabilised *E. coli* cells containing TDFE

In a previous paper, we demonstrated that thermal permeabilisation of recombinant cells containing the enzyme of interest increased the specific activity in *E. coli* cells to 83% of the cell-free extract [9]. This permeabilisation method can be applied because TDFE is thermostable, thus allowing selective denaturation of mesophilic proteins. A further advantage of this method is that it avoids the use of organic solvents, which are frequently exploited for breaking down the selective barrier of the cell membrane [4, 14, 17]. The high recovery of TDFE activity permitted the immobilisation of whole cells instead of purified free enzyme, thereby avoiding cumbersome and expensive downstream procedures (e.g. cell disruption, enzyme purification).

The yields of recombinant *E. coli* cells entrapped in different natural polymeric matrices, in terms of residual TDFE activity, are shown in Table 1. PEC-entrapped cells exhibited the highest enzyme activity, while the other immobilised biocatalysts showed residual activities of between 40% and 24%.

A key parameter in the selection of the most appropriate immobilisation technique for the development of an applied bioprocess is the stability of the resulting biocatalyst. For this reason we investigated the stability of TDFE in each immobilised biocatalyst at 75°C, the process temperature. The results are shown in Fig. 1; PEC exhibited the lowest stability (23% after 4 h of incubation), while EWEC and CEC showed half-lives of 5 h and 19 h, respectively. In the same figure it can be observed that Ca-alginate gel beads retained 100% of initial activity. At longer incubation times, the thermostability in the latter support was very much higher than

Table 1 Enzyme yields after completing the different procedures for permeabilized *Escherichia coli* cells entrapedin the various matrices. *PEC* Polyacrylamide entrapped cells, *EWEC* egg white-entrapped cells, *CEC* chitosan-entrapped cells

Samples	Yield (%)
Cell free extract	100
PEC	81
EWEC	35
CEC	40
Ca-alginate	24

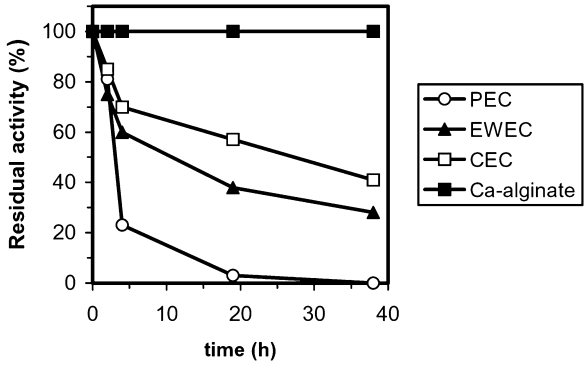

Fig. 1 Residual trehalosyl-dextrins-forming enzyme (TDFE) activity detected at 75°C in whole cells immobilised in different supports

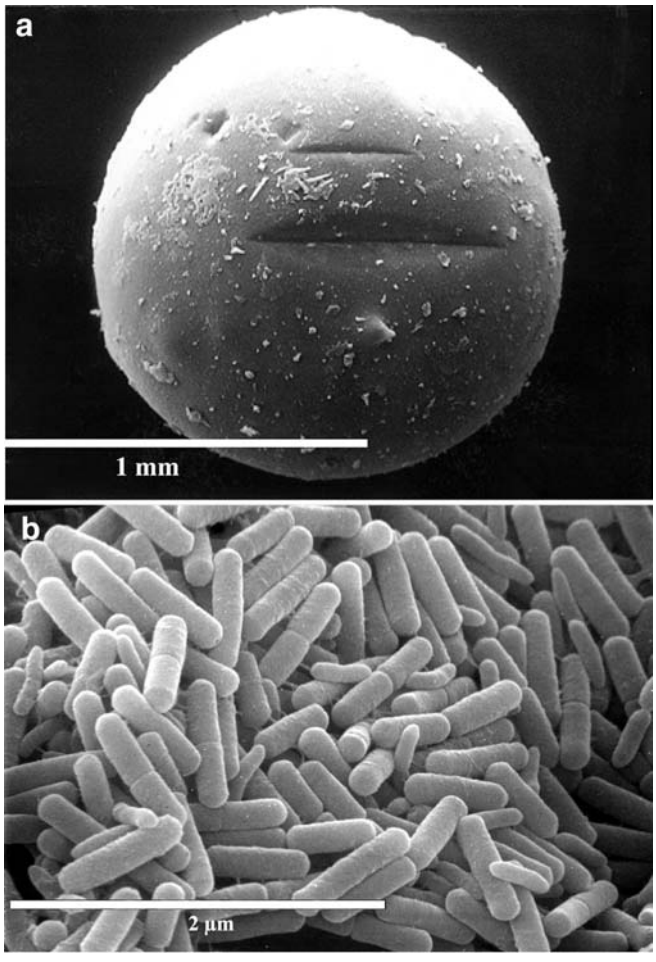

Fig. 2 Scanning electron micrographs of Ca-alginate beads (**a**) entrapping recombinant *Escherichia coli* cells containing TDFE (**b**)

with the other immobilised biocatalysts (> 65% activity after 2 months of incubation at 75°C). Figure 2 shows SEM of *E. coli* cells immobilised in Ca-alginate. Since their structural integrity was preserved, cells were not affected by the permeabilisation treatment.

Equilibrium constant determination

Complete conversion of substrate into products was never achieved. The data confirmed that, regardless of modification of flow rate, enzyme amount or initial substrate concentration, production of TM4 always reached a plateau at a degree of conversion of less than 100%. The kinetic constant was evaluated by averaging all the data generated during optimisation experiments, and a value of 2.6 ± 0.1 was obtained. In addition, an experiment was performed in which substrate was added to the reaction mixture after the plateau was reached. This modified the equilibrium concentrations by increasing the direct reaction rate, as expected, but the new equilibrium was restored after a brief period of time (Fig. 3).

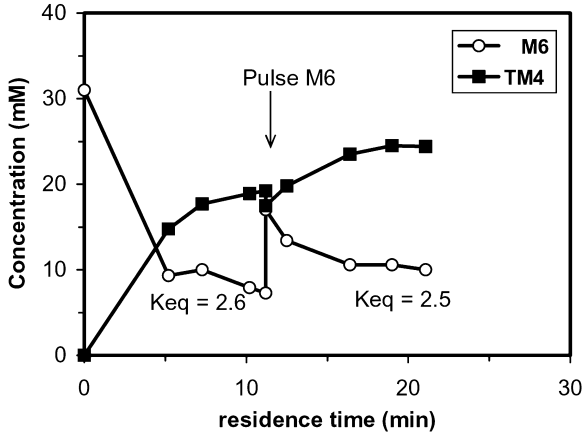

Fig. 3 Determination of the equilibrium constant (*Keq*) of the conversion of maltohexaose (*M6*) into trehalosylmaltotetraose (*TM4*) catalysed by TDFE

Immobilised cell packed bed bioreactor

Our process exploited recombinant permeabilised *E. coli* cells that were not metabolically active at the operating temperature, therefore there was no need for the addition of oxygen or nutrients. The pH did not change during the reaction, thus the bioreactor set up was very simple. Early observation of the kinetics of biotransformation established that the immobilised enzyme was following typical Michaelis-Menten kinetics. This in turn suggested that a plug flow tubular reactor (PFTR) would give a greater conversion than a continuous stirred tank reactor of the same volume [1].

Before establishing the experimental set up, the intrinsic kinetic parameters were measured exploiting a differential PFTR, and assuring a conversion lower than 10% for each cycle. This permitted the assumption of constant concentration along the biocatalyst bed. The V_{max} and K_m for the immobilised enzyme were 7.5 mM min^{-1} (g biocatalyst)$^{-1}$ and 5.4 mM, respectively. The apparent K_m value was almost twice that reported for free TDFE [6] or glycosyltransferase from *S. solfataricus* KM1 [9, 10], as predicted for an immobilised biocatalyst. However, the affinity of the entrapped enzyme for M6 could still be considered as quite good.

Evaluation of optimal operating conditions

Once the set up of the bioreactor was completed, we studied the influence of enzyme amount on the biotransformation rate, keeping both flow rate (0.2 ml/min) and substrate concentration (10 mM) constant. Using 1.8 U enzyme a conversion of 45% was reached in 2 min, while by doubling the enzymatic units we achieved 57% conversion in a residence time of 1.4 min. Using 14.4 U immobilised enzyme (normally contained in 4 g biocatalyst) we obtained a conversion of 65–70% within a 3 min residence time (data not shown).

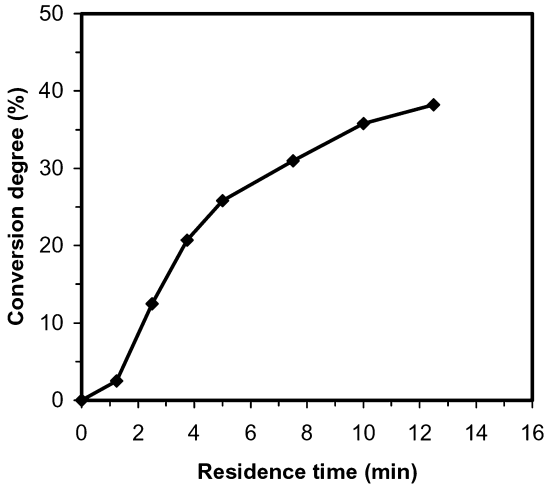

Fig. 4 Experiment on maltodextrins mixture (30% w/v). The graph reports the conversion degree obtained by dividing the amount of non-reducing dextrins [trehalosylmaltopentaose (TM5), TM4, trehalosylmaltotriose (TM3), trehalosylmaltose (TM2)] formed by the total dextrins [(maltoheptaose (M7), M6, maltopentaose (M5), maltotetraose (M4), maltotriose (M3)] in the inlet stream

Another set of experiments was carried out to explore the influence of substrate concentration on reaction rate, whilst keeping both the catalytic bed length (using 1.022 g biocatalyst) and the flow rate (1.6 ml/min) constant. A higher concentration of M6 led to a higher initial rate of reaction. In particular, the TM4 formation rate was 2.5 mM min^{-1}·(g biocatalyst)$^{-1}$ using an inlet stream at 10 mM M6, while this increased to about 7 mM min^{-1}·(g biocatalyst)$^{-1}$ when a 30 mM M6 solution was fed (Table 2).

Successive experiments were performed to establish whether the external mass transfer or the intrinsic kinetic was limiting in the biotransformation rate. The key parameter in these experiments was the stream linear velocity. Conversion curves were completed at increasing flow-rates from 0.2 ml/min to 1.6 ml/min, revealing the latter to be optimal in relation to the biotransformation rate. In fact, the productivity increased from 6 mM min^{-1}·(g biocatalyst)$^{-1}$ to 9 mM min^{-1}·(g biocatalyst)$^{-1}$. Data on productivity exploiting different reaction parameters showed that a flow rate of 1.6 ml/min gave the best results, especially when coupled with a high concentration of M6 in the inlet stream (Table 2).

Overall, the best conversion profile was obtained at a flow rate of 1.6 ml/min using 0.5 ± 0.05 U immobilised enzyme per 1 mM M6. No diffusion hindrance was noticed when the linear velocity was increased up to 30–35 cm/min. In fact, conversion kinetics were inherent to the sole enzymatic activity, with no apparent delay in conversion due to substrate transport within the biocatalyst beads.

Activity was totally retained, even after a whole set of experiments (i.e. after 10 days). The beads were spherical and their diameter was quite homogeneous, indicating that the beads retained their shape, and the apparent absence of cell debris or proteins in the outlet stream is a good indicator that the compressibility of the matrix was very low at the operational flow-rate. During our work we constructed an operating stability curve using the bioreactor in successive bioconversion cycles of 24 h/day with 30 mM M6 as substrate, at a flow-rate of 1.6 ml/min, finding a half-life of 20 days (data not shown).

Experiments of industrial interest

The bioprocess was optimised using a relatively pure substrate (M6), which normally contains only maltopentaose (M5) as an impurity and which, therefore, is extremely suitable for precise rate definition. However, to prove this biocatalyst useful for industrial applications, we investigated the performance of the packed bed reactor in the production of non-reducing saccharides from a commercially available maltodextrin mixture. All data obtained using the pure substrate helped to set up the experiments correctly. In this case, the flow-rate was set at 1.6 ml/min because, with all maltodextrins having between four and seven glucose residues being substrates for the enzyme, we expected an overall conversion rate that was an average of the rates for all these substrates. Kato reported differential affinity of the glycosyltransferase from *S. solfataricus* KM1 for maltodextrins with increasing polymerization degree (DP 3–7), showing that the optimal substrate was M5, with M6 and M7 having relative activities of 80% and 88%, respectively [10]. In our experience, the TDFE from *S. solfataricus* MT4 had optimal activity on M6, with very similar affinity for both M5 and M7; both enzymes are less active on M4, and almost inactive on M3 [3, 6]. Figure 4 shows the degree of conversion into non-reducing dextrins. The percentage was obtained by dividing the total amount of trehalosyl-maltodextrins formed (mmol) by the total influx of substrates M7, M5, M6, M4 (mmol). Finally, we obtained a productivity of 106.5 mg ml^{-1}·

Table 2 Trehalosylmaltotetraose (TM4) productivity in relation to bioprocess parameters. Experiments were performed in a 15 cm glass column reactor (i.d. = 1.2 cm)

Flow rate (ml/min)	Biocatalyst amount (g)	Maltohexaose (mM)	Productivity [mM min^{-1} (g biocatalyst)$^{-1}$]	Conversion (%)
0.2	0.5	10	2.8	56%
0.8	0.5	10	3.9	66%
0.8	4	30	6.0	40%
1.6	4	30	9.0	60%

h^{-1}·(g biocatalyst)$^{-1}$ of trehalosyl-maltodextrins when using a 30% w/v solution.

Conclusions

The aim of developing a novel biocatalyst for the production of non-reducing sugars at high temperatures (75°C) was successfully achieved. Immobilisation of whole permeabilised cells permitted a substantial simplification of the downstream flow. Furthermore, the well known entrapment method allowed easy manufacturing of the biocatalyst. Biocatalyst performance was evaluated in relation to the simplicity of the protocols, availability of materials and, more important, stability of the matrices.

In spite of the lowest activity yield, we determined that Ca-alginate-immobilised enzyme has an unforeseen stability, supporting selection of this material to produce entrapped cell biocatalyst suitable for packing the bioreactor. Several operating conditions were screened to determine the rate-limiting step of the bioconversion and define the optimal parameters. Finally, we exploited the packed bed reactor for biotransformation of maltodextrins. A substrate concentration compatible with industrial processes (10–30% w/v) was used, confirming the potentiality of this bioprocess, based on an innovative thermophilic biocatalyst, to convert these oligosaccharides into non-reducing derivatives of great potential interest to the food industry.

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