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Trehalose production at high temperature exploiting an immobilized cell bioreactor

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Abstract The enzymatic production of trehalose from dextrins was studied as a series reaction in a packed bed reactor containing immobilized recombinant *Escherichia coli* cells, expressing either the *Sulfolobus solfataricus* (strain MT4) trehalosyl-dextrin forming enzyme (TDFE) or the trehalose-forming enzyme (TFE). The cells, subjected to thermal treatments to increase cell permeability and to inactivate the unwanted host proteins, were entrapped separately or together in a calcium alginate polymeric matrix. The biocatalyst beads were used to pack a tubular glass reactor that was operated in a recycle mode. The performances of a bioreactor containing alternate layers of *Ec*TFE and *Ec*TDFE alginate beads were evaluated and compared with the performance of the co-immobilized biocatalysts. The latter showed a superior throughput, therefore the bioreactor packed with the co-entrapped biocatalysts was tested for the production of trehalose from concentrated dextrin solutions (10%–30% w/v) and a conversion up to 90% was obtained. This conversion corresponded to a production of 127 g trehalose h^{-1} kg^{-1} of biocatalyst. The results obtained suggest that the bioprocess described may be of interest in the development of a large-scale industrial process for trehalose production at high temperature.

Key words Trehalose production · Thermozymes · Recombinant cell entrapment · Co-immobilization · Packed bed bioreactor

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Introduction

Trehalose is a non-reducing disaccharide consisting of two α-1,1-linked glucose molecules. This non-reducing sugar acts as carbohydrate reserve and, in many organisms, plays an important role as a protecting agent against environmental stresses (Arguelles 2000). In addition, trehalose can be used as a sweetener, stabilizer for dried and frozen foods, moisture retainer in medicines and cosmetics, and as a drug preservative (Roser 1991; Portmann and Birch 1995; Paiva and Panek 1996). Recently, many studies have shown that trehalose protects proteins against drying or freezing (Xie and Timasheff 1997; Singer and Lindquist 1998; Simola et al. 2000) and improves the survival of cryopreserved mammalian cells, thus opening new perspectives for tissue engineering (Eroglu et al. 2000; Guo et al. 2000). Trehalose was initially produced by extraction from plants and baker's yeast grown on glucose, but both processes were extremely expensive. In order to lower the production costs, a novel biotechnological process was developed by Hayashibara Biochemical Laboratories (Okayama, Japan; USP06017899; USP05922580; USP0535636). This process is based on the use of two enzymes, isolated from the mesophilic microorganism *Arthrobacter* sp. Q36, able to convert starch and dextrins into trehalose (Nakada et al. 1995a, b). The first enzyme, trehalosyl dextrin-forming enzyme (TDFE), acts as an intramolecular transglycosidase, which converts the α-1,4 glycosidic linkage, at the reducing end of dextrins, in the α-1,1 glycosidic bond. The second enzyme, trehalose-forming enzyme (TFE), hydrolyses the α-1,4 linkage adjacent to the α-1,1 bond of trehalosyl dextrins, giving the complete transformation of the substrate, in an equimolecular mixture of trehalose and low-molecular-weight dextrins. However, further improvements in trehalose production could be obtained by using a process at high temperatures exploiting recently isolated thermophilic enzymes (Nakada et al. 1996a, b; Di Lernia et al. 1998; Kato 1999). In particular, the thermophilic and thermostable TDFE and TFE, able to form trehalose from starch and dextrins at high temperature, have been isolated by our group from the thermoacidophilic archaeon *Sulfolobus shibatae* (Di Lernia

et al. 1998). Since the production of *Ss*TDFE and *Ss*TFE from the wild-type strain was very expensive due to the low biomass yield and the severe fermentation conditions, the two enzymes were expressed into *Escherichia coli* (De Pascale et al. 2001) and produced at high yield in a microfiltration bioreactor (Schiraldi et al. 2001).

In this paper we report on the performance of bioreactors for trehalose production containing immobilized recombinant *E. coli* cells. The recombinant cells were first heat-treated to improve membrane permeability, and to inactivate the unwanted host proteins, and then entrapped in alginate. With this method, the downstream purification of enzymes was avoided and the advantages of immobilized cell and enzyme biotransformations, such as thermal and operational stability, were exploited (Klimacek et al. 1999; Arcos et al. 2000; Nigam 2000; Briante et al. 2000).

Materials and methods

Materials

Maltohexaose (M6), maltoheptaose (M7), alginic acid sodium salt, and Triton X-100 were purchased from Sigma Aldrich (St. Louis, MO, USA). Commercial dextrins were kindly provided by Roquette (Services Technique et Laboratories, Lestrem, France). The mixture contained: M7, M6, maltopentaose (M5), maltotetraose (M4), maltotriose (M3), maltose (M2) and glucose (G). Calcium chloride dihydrate was obtained from SERVA (Feihbiochemica, Heidelberg, Germany). Trehalosylmaltotetraose (TM4) was produced in our laboratory using recombinant TDFE.

Microorganisms and cultivation

The recombinant *E. coli* Rb-791 strains expressing either *Ss*TDFE (r*Ec*TDFE) or *Ss*TFE (r*Ec*TFE) were kindly provided by D. De Pascale (Federico II University, Naples, Italy). The cells were cultivated as previously described (De Pascale et al. 2000; Schiraldi et al. 2001) and harvested by centrifugation before starting the immobilization procedure.

Immobilization

Centrifuged cells were added to 50 mM sodium acetate buffer pH 5.5, in a ratio 1 : 1 (wet weight : volume), and subjected to thermal treatment at 75°C. r*Ec*TDFE was incubated for 1 h while r*Ec*TFE had to be treated for 2 h in the presence of Triton X-100 (0.5%, w/v).

The permeabilized whole-cell suspension was added to an equivalent volume of a sodium alginate solution with a final concentration of 2% w/v and the mixture was extruded as small drops by means of a syringe into a stirred solution of calcium chloride at room temperature, where the gel beads (0.3 ± 0.1 cm diameter) were formed (Smidsrod and Skjak-Braek 1990). The co-immobilization was performed using the same amounts of the two enzymes. Due to the diverse expression level, different quantities of r*Ec*TDFE and r*Ec*TFE were used. The specific activity of the beads containing r*Ec*TDFE and r*Ec*TFE co-entrapped was generally in the range between 0.6 and 1 U/g biocatalyst.

Enzyme assay

According to a previously described protocol (Di Lernia et al. 1998; De Pascale et al. 2000), TDFE and TFE activities were assayed in the cytosol and in the cellular suspension at 75°C, in 50 mM sodium acetate buffer pH 5.5 using M6 and TM4 (0.67 mM) as substrate, respectively. TDFE activity in the Ca-alginate beads was determined, at the same temperature and pH, by incubating one or two beads (4–5 mg wet weight) in the standard reaction mixture (1.125 ml) for 15 min. TFE activity in the Ca-alginate beads was determined by adding one or two beads (4–5 mg wet weight) to 0.750 ml of standard reaction mixture and incubating in the same conditions for 15 min. The activity of the cell beads containing the co-immobilized biocatalyst was also assayed under standard conditions using one or two beads, M6 as substrate, and incubating for 20 min. All the reactions were stopped by cooling the mixtures in an ice-water bath. One unit was defined as the amount of enzyme that produces 1 μmol/min of TM4 and 1 μmol/min of trehalose, respectively.

High-performance anion exchange chromatography

The quantitative determinations of the substrates and products in the reaction mixtures were performed with a Dionex chromatograph (Dionex, Sunnyvale, CA, USA), equipped with pulsed amperometric detector (PAD). Baseline separation of carbohydrates was achieved within 10 min using Carbopac PA-100 guard and analytical column. The mobile phase consisted of two buffers, 160 mM sodium hydroxide (Buffer A) and 300 mM sodium acetate (Buffer B), used to obtain a linear gradient. The sample loop volume was 50 μl and the eluent flow rate was 1.0 ml/min. Detection was accomplished by triple-pulsed amperometry using a gold electrode.

Bioreactor

Two different tubular glass reactors, 5 ml and 125 ml internal volume, respectively, were designed by our group and built by Microglass (Naples, Italy). Temperature was regulated by connecting an external water-jacket, fourfold the internal volume of the catalytic bed, to a thermostatic bath. The 5 ml reactor, 15 cm length and 0.7 cm internal diameter (i.d.), was packed with 4 g of biocatalyst while the 125 ml reactor, 40 cm long, 2 cm i.d., was packed with 125 g of beads; the corresponding void fraction was about 35% in both cases. The packing was completed by using glass beads (ϕ = 0.3 ± 0.1 cm) at the inlet and outlet of the column,

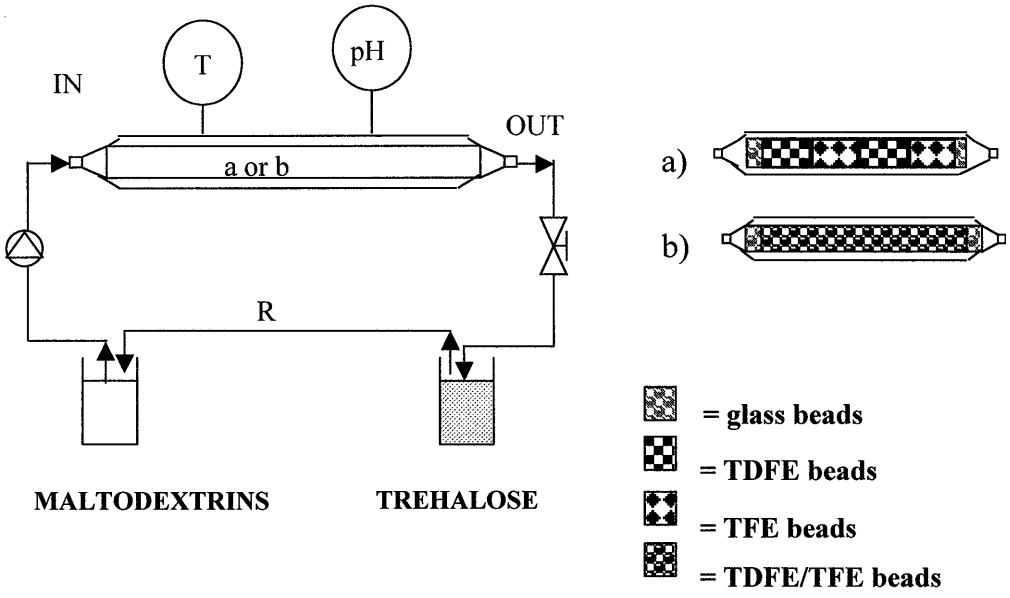

Fig. 1. Schematic view of the bioprocess: *a* series bioreactor, 5 ml; *b* co-immobilized cell bioreactor (5 ml, 125 ml)

where the jacket convergence resulted in a worse heat transfer. With this method we also improved the stream spreading over the whole inlet section before reaching the biocatalyst. The inlet of the bioreactor was connected to a peristaltic pump (Model M312; Gilson, Villiers-le-Bel, France), which was regulated in order to keep the substrate solution flux at the desired value (linear velocity 30 cm/min). The bioreactor is schematically drawn in Fig. 1.

Operating protocol

All the experiments were performed at 75°C, which coincides with the optimal temperature for r*Ec*TDFE (De Pascale et al. 2001; Schiraldi et al. 2001) and permitted longer stability of both enzymes. The comparative evaluation of the alternate layers (1 : 1 enzyme ratio) and the co-immobilized cell bioreactors was performed on a 5 ml glass column (Fig. 1). The substrate for these experiments was M6 in a concentration range of 10–50 mM, dissolved into 50 mM calcium acetate buffer at pH 5.5. Conversions of a commercial mixture of dextrins were performed in the 5 ml and the 125 ml bioreactors packed with the co-entrapped *E. coli* cells. The 5 ml bioreactor was operated at a flow rate of 1.6 ml/min, corresponding to a linear velocity of 30 cm/min. Each cycle corresponded to a residence time of 0.177 min. The 125 ml reactor was operated at the same linear velocity (flow rate/cross section area) as the 5 ml bioreactor. The residence time was 1.34 min per cycle. The biotransformation of a 20% dextrin solution was performed. In all experiments, samples were withdrawn at defined residence time in order to investigate the conversion kinetics. The reactors were also operated in a total recycle mode in order to keep a high linear velocity in order to overcome transport resistance while increasing residence time.

Results and discussion

In this study we report the immobilization of recombinant *E. coli* cells expressing thermophilic enzymes able to convert polysaccharides into trehalose. Immobilization procedures were performed using Ca-alginate because this polymeric matrix, easily acquirable and previously described in a number of studies (Smidsrod and Skjak-Braek 1990), was stable at process temperature (75°C), showing no cell leakage for prolonged and repeated operations. We compared the performance of an alternate-layer or co-entrapped cell bioreactor on trehalose final yield and productivity. A first scaling-up attempt was completed by operating a glass bioreactor with a 125-ml catalytic bed (40 cm length).

Comparisons on TDFE and TFE activity in the cell-free extract, in whole cells, and in Ca-alginate-entrapped cells

The activity of recombinant TDFE and TFE, determined in whole cells of *E. coli*, corresponded to only 20% and 25% of the corresponding cell-free extracts, as shown in Table 1. This was probably due to the low permeability of the cell membranes, since a similar behavior was demonstrated by De Riso et al. (1996) for rhodanese contained in *Bacillus stearothermophilus*, *Bacillus acidocaldarius*, and *Sulfolobus solfataricus* whole cells. However, after the thermal treatment of the cellular suspensions of r*Ec*TDFE, the specific activity increased and reached 83% of the activity measured in the cell-free extract. In order to increase activity of r*Ec*TFE, the cell suspension was subjected to thermal treatment in the presence of a detergent (Triton X-100, 0.5%

w/v) (Table 1). Only when the two treatments were combined 75% of the cell-free extract activity was recovered; this result was comparable with that achieved for TDFE after the sole thermal treatment. These permeabilization methods also determined the denaturation of the mesophilic proteins. The high recovery of the enzyme activity permitted us to immobilize whole cells instead of free enzymes, thus avoiding expensive steps for downstream processing, such as cell disruption and enzyme purification. The efficiency of the immobilization process was evaluated in terms of residual activity. In particular, *Ec*TDFE and *Ec*TFE cell-free extracts (100% activity) were compared to the corresponding entrapped cells, showing a residual activity of 24% and 15%, respectively. The activity of *E. coli* cells co-entrapped in Ca-alginate was 15% of the activity determined in the combined cell-free extracts. Even if the recovery values were quite low, we decided to exploit the Ca-alginate gel beads because of their high stability at the operational temperature; in fact no leakage was observed (spectrophotometric measurements) during repeated experiments.

Table 1. Comparison between TDFE and TFE activity in *Escherichia coli* crude extracts, intact and permeabilized cells

Samples	TDFE specific activity (Yield) U/g cells wet weight (%)	TFE specific activity (Yield) U/g cells wet weight (%)
Cell-free extract	36 (100)	132 (100)
Cellular suspension	7 (20)	33 (25)
Cellular suspension in Triton X-100 (0.5% w/v)	27 (75)	40 (30)
Cellular suspension 75°C	30 (83)[a]	58 (44)[b]
Cellular suspension in Triton X-100 (0.5% w/v) 75°C	30 (83)[a]	99 (75)[b]

[a] The incubation time for TDFE was 1 h
[b] The incubation time for TFE was 2 h

A co-immobilization process was carried out, employing different amounts of recombinant cells containing TDFE or TFE because of their very diverse expression yields and the different values of enzyme recovery after entrapment. This procedure guaranteed that each Ca-alginate bead contained a roughly equivalent concentration of the two enzymes. In fact, Kobayashi et al. (1997) reported that the highest trehalose production in a batch system was obtained when the concentration ratio of the two enzymes was approximately 1. Co-entrapment, allowing the physical proximity of the two enzymes, led to faster rates of sequential enzyme reaction, by facilitating transfer of reaction intermediates to the catalytic sites of the next enzyme. In fact, the substrate and reaction intermediates did not need to diffuse into the beads twice.

Thermostability of TDFE and TFE

The stability at operative temperature (75°C) of free enzyme, whole-cell suspension and Ca-alginate-entrapped cells was compared for both enzymes (Fig. 2). The data showed that Ca-alginate gel beads containing *Ec*TDFE retained more than 60% of initial activity after 2 months of

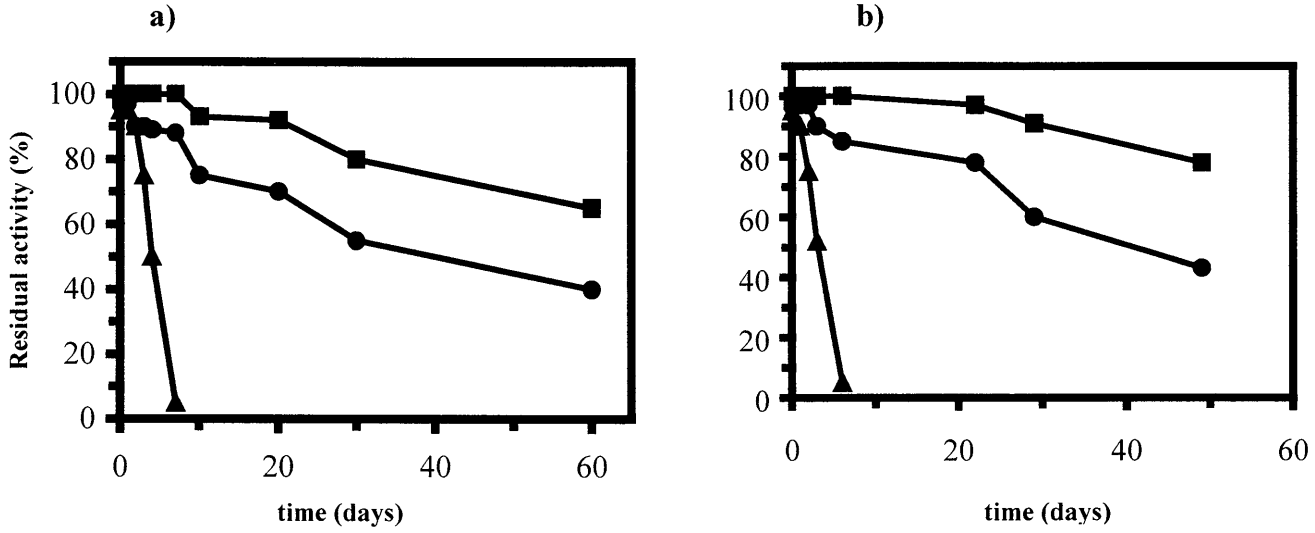

Fig. 2. Thermostability at 75°C of TDFE (**a**) and TFE (**b**). Comparison between free enzymes (*triangles*), cellular suspensions (*circles*), and Ca-alginate-entrapped cells (*squares*). **a** Purified enzyme (1 U/ml) in 50 mM sodium acetate buffer pH 5.5. Whole-cell suspension 0.200 ml (30 U/ml) in 50 mM sodium acetate buffer pH 5.5. Ca-alginate beads (15 mg, 0.143 U) were incubated in different vials containing 1.125 ml 50 mM sodium acetate buffer pH 5.5. **b** 0.500 ml (0.5 U/ml) of purified enzyme in 50 mM sodium acetate buffer pH 5.5. Whole-cell suspension (0.500 ml; 108 U/ml) in 50 mM sodium acetate buffer pH 5.5. Ca-alginate beads (5 mg, 4 U/g of support) in different vials containing 0.750 ml 50 mM sodium acetate buffer pH 5.5. Samples were withdrawn at different times and the residual activity determined under standard conditions

incubation, while free enzyme and whole-cell suspension showed a half-life of 4 days and 1 month, respectively. As shown in Fig. 2, the beads containing *Ec*TFE retained about 80% of initial activity after 50 days of incubation, while free enzyme and whole-cell suspension showed a half-life of 3 and 40 days, respectively. These results are extremely valuable when compared with those from previous studies. In fact, De Riso et al. (1996) reported that *B. acidocaldarius* cells entrapped in Ca-alginate lost their rhodanese activity after a few minutes at 40°C. Furthermore, storage stability experiments were performed at 4°C; the biocatalysts retained their initial activity for 1 year.

Bioreactor experiments

The biotransformation of M6 into trehalose was studied on an alternate-layers bioreactor. The latter was built by packing *Ec*TDFE and *Ec*TFE beads alternately (Fig. 1), so that the sequential reaction could proceed in the same reactor. Bioconversion experiments were carried out using a 25-mM and a 50-mM M6 inlet stream, which was recycled to the reactor to obtain an overall residence time of 32 min. Figure 3 shows (bioconversion with 25 mM M6) concentrations of substrate (M6), intermediates (TM4) and product (T) at increasing residence times. The sensitivity to substrate concentration was confirmed by a higher conversion kinetic at 50 mM (data not shown) than at 25 mM. However, in both cases, we were not able to completely convert the initial M6 into trehalose. We observed the presence of undesirable side reactions, giving glucose and maltotriose as main products, as previously reported (Kato 1999; Gueguen et al. 2001). In our opinion the double barrier that the substrate had to pass to reach the catalytic site, first for M6 to *Ec*TDFE and then for TM4 to *Ec*TFE, was responsible for low reaction rates, thus leading to the competition with normally slower side reactions. In order to overcome this limitation, we decided to use co-entrapped cells to pack another tubular reactor, so that each bead contained the two catalytic activities. Experiments were performed with M6 concentrations varying from 10 to 30 mM. Figure 4 shows substrate and product concentrations at increasing residence times using 13 mM M6. In this experiment M6 was completely consumed, giving trehalose as main product (90% of the theoretical yield), and maltotriose and glucose as secondary products. In addition, the productivity was enhanced from 6 mg/ml·h in the alternate-layers bioreactor to 18 mg/ml·h in a co-entrapped cell bioreactor employing the same enzymatic units. Comparing the conversion curves of the two bioreactor configurations, we found that no intermediate TM4 could be detected during the process in the co-entrapped cell bioreactor, suggesting that TM4 was immediately converted into trehalose. Further experiments were completed in order to better characterize the bioreactor potentialities and, in particular, the bioconversion of highly concentrated dextrin mixtures was performed. Figure 5 shows the conversion kinetic of substrates into products using a commercially available dextrin solution (20% w/v). The conversion (%) was calculated as the ratio of final trehalose versus the maximal achievable concentration. Because dextrin mixtures include oligosaccharides with different numbers of residues (i.e., 2–7), the stoichiometry has to be considered in the maximal theoretical yield calculation. In particular, two trehalose molecules can be obtained from M7 and M6, one from M5 and M4, while none could be obtained by using M3, because the enzymes were not active on this substrate. This experiment resulted in an 80% conversion and a corresponding trehalose productivity of 127 mg/h·g biocatalyst.

To further demonstrate the feasibility of our bioreactor for industrial biotransformations, we decided to scale up our process by packing a 125-ml tubular reactor. A biotransformation was performed using the same linear velocity and 10% w/v dextrin solution as substrate. In 16 min we

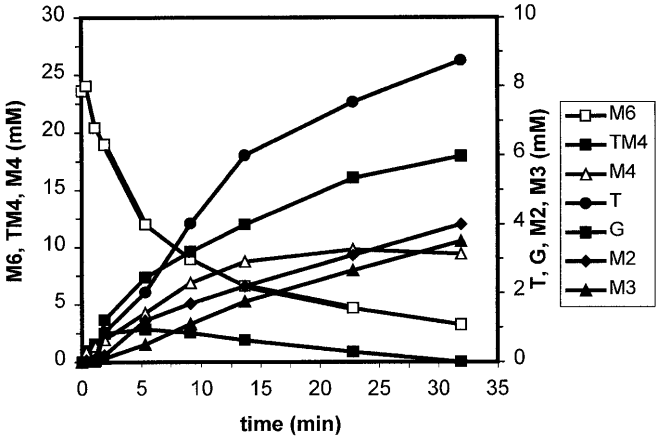

Fig. 3. Bioconversion of maltohexaose *M6* (25 mM) exploiting a 5-ml stage bioreactor packed alternately with 7-cm TDFE beads (1.4 g, 5 U) and 4-cm TFE beads (0.9 g, 5 U). The linear velocity was 30 cm/min. Biotransformation intermediates and products are: trehalosylmaltotetraose (*TM4*), maltotetraose (*M4*), trehalose (*T*), glucose (*G*), maltose (*M2*), maltotriose (*M3*)

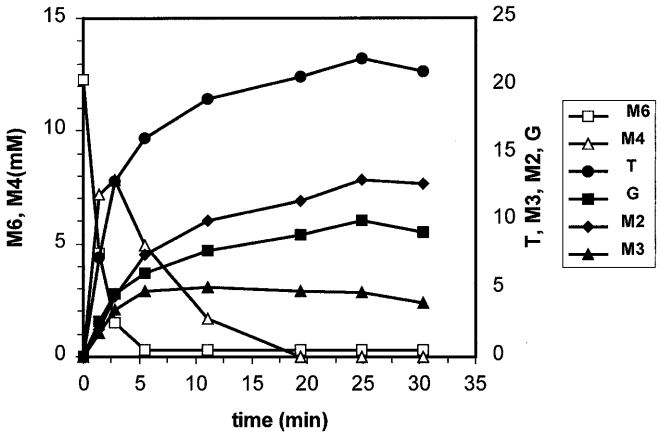

Fig. 4. Bioconversion of maltohexaose *M6* (13.5 mM) exploiting a 5 ml co-entrapped bioreactor packed with 4.55 g biocatalyst (5 U) using a linear velocity of 30 cm/min. Biotransformation intermediates and products are: maltotetraose (*M4*), trehalose (*T*), glucose (*G*), maltose (*M2*), maltotriose (*M3*)

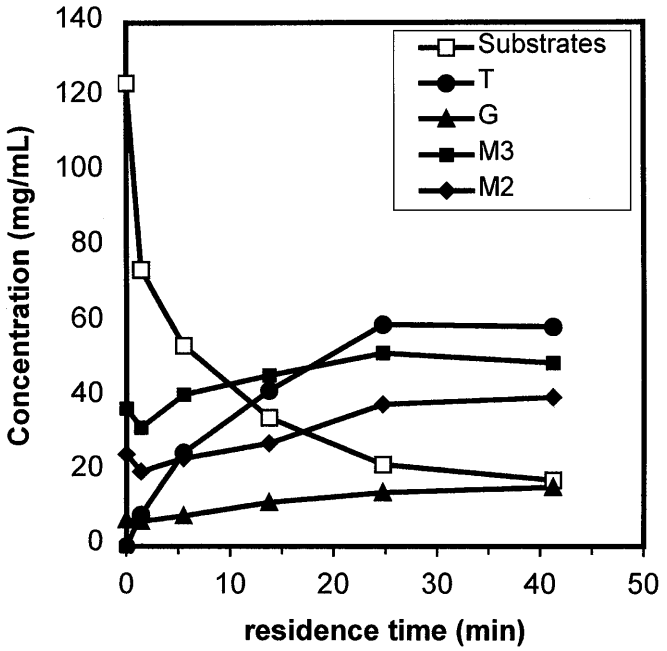

Fig. 5. Bioconversion of dextrins (20% w/v; relative initial composition M7 = 16%, M6 = 18.3%, M5 = 15%, M4 = 15%, and saccharides with 1–3 glucose residues) exploiting a 5-ml co-entrapped bioreactor packed with 4.55 g biocatalyst (5 U), using a linear velocity of 30 cm/min. Final composition was M7 = 0%, M6 = 3.0%, M5 = 0%, M4 = 7.0%, M3 = 27%, M2 = 22.0%, T = 32.3%, and G = 8.7%

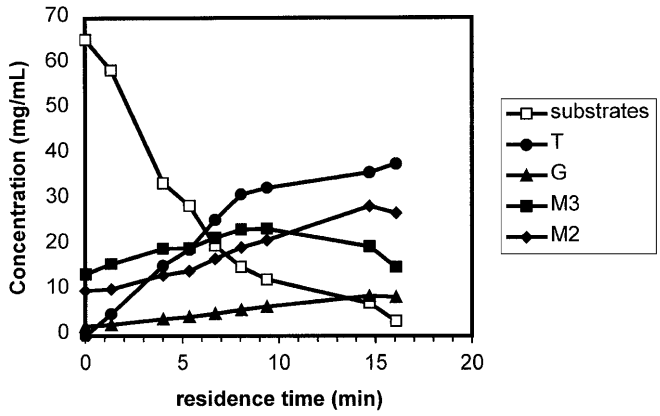

Fig. 6. Bioconversion of dextrins (10% w/v; relative initial composition M7 = 28%, M6 = 18%, M5 = 14.8, M4 = 11.2%, and saccharides with 1–3 glucose residues) exploiting a 125-ml co-entrapped bioreactor packed with 125 g biocatalyst (77 U) using a linear velocity of 30 cm/min. Final substrate composition was M7 = 0%, M6 = 0.4%, M5 = 0%, M4 = 3.2%, M3 = 29.5%, M2 = 16.5%, T = 41.2%, and G = 9.2%

achieved 92% conversion, corresponding to a trehalose productivity of 113 mg/h·g biocatalyst (Fig. 6).

These results are very encouraging in relation to the maintained productivity level; in fact by keeping constant the linear velocity, the bioprocess was very reproducible, always giving a final conversion over 80%. These conversion values were higher than those reported by Kobayashi et al. (1997), which exploited the two thermophilic free enzymes in a batch system, reaching a yield of 78% when using liquefied starch at a concentration of 25% w/v. A similar conversion was reported by Klimacek et al. (1999), when fungal trehalose and starch phosphorylases were co-immobilized and exploited in a plug-flow reactor. However, these authors reported a trehalose productivity of 2.6 g/l·h at 50°C, lower than 140 g/l·h that we obtained in our process.

As already mentioned, side products such as maltotriose, and especially glucose, were produced by hydrolytic reactions catalyzed by both enzymes (Gueguen at al. 2001). This explains why the final conversion was always lower than the theoretical yield. These undesirable side hydrolytic activities could possibly be reduced by modifying the catalytic sites of both enzymes through protein engineering, thus improving the trehalose production.

The operational stability was studied using 20% w/v dextrin solution, and the bioreactor completed several biotransformations, maintaining the maximal activity with a half-life greater than 10 days. During the operational stability experiments we were able to obtain a total production of 100 g of trehalose per gram of co-entrapped wet cells.

Conclusions

The development of novel biotechnological processes for trehalose production is very challenging in relation to its widening spectrum of applications. In this research project, the possibility of producing trehalose at high temperatures, exploiting a packed bed reactor, was studied. Recombinant *E. coli* cells expressing TDFE and TFE were immobilized in Ca-alginate, since this matrix represents an inexpensive support and its mechanical properties are suited to prolonged operation at high temperatures. The performances of an alternate-layers bioreactor and a co-entrapped cell bioreactor were compared, suggesting that co-immobilization improved both trehalose yield and productivity. To our knowledge, this is the first time that recombinant *E. coli*, containing thermophilic enzymes catalyzing sequential reactions, has been co-immobilized, thus improving bioprocess design. The entrapped biocatalysts showed very high stability at 75°C (1–2 months) when incubated in buffer at pH 5.5. The half-life of the co-entrapped biocatalyst in operating conditions similar to industrial processes was found to be 10 days. The biocatalysts retained their initial activity after storage at 4°C for 1 year. These results are encouraging for the development of a high-temperature process for trehalose production, thus avoiding product contamination and improving high-molecular-weight carbohydrate solubility.

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