Trehalose production: exploiting novel approaches

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Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a unique sugar capable of protecting biomolecules against environmental stress. It is a stable, colorless, odor-free and non-reducing disaccharide, and is widespread in nature. Trehalose has a key role in the survival of some plants and insects, termed anhydrobionts, in harsh environments, even when most of their water body is removed. The properties of these types of organisms drove attention towards the study of trehalose. Since then, it proved to be an active stabilizer of enzymes, proteins, biomasses, pharmaceutical preparations and even organs for transplantation. Recently, trehalose has been accepted as a safe food ingredient by the European regulation system following approval by the US Food and Drug Administration. The wide range of applications of this sugar has increased the interest of many research groups into the development of novel and economically feasible production systems. This article provides a comprehensive review of the current achievements in the biotechnological production of trehalose.

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Trehalose was first described in the early 19th century as a component of the ergot of rye [1], later it was discovered in a great variety of species, most notably the anhydrobionts [2–4]. These unusual organisms, which include the so-called 'resurrection plant' (*Selaginella lepidophylla*), certain brine shrimps, nematodes and baker's yeast, have the incredible property of surviving for years to dehydration. When 99% of their body water is removed, they still resume life promptly after rehydration. At first, trehalose was thought to serve as a means of carbohydrate storage but recently it was shown to be produced in response to stress. In yeast, the most ancient actor of biotechnological transformation, trehalose was found to accumulate, in certain physiological conditions, up to 10–15% of cell dry weight [5].

Trehalose is among the most chemically unreactive sugars and its strong stability is a result of the very low energy (1 kcal mol^{-1}) of the glycoside oxygen bond joining the two hexose rings. In comparison, another non-reducing disaccharide, sucrose, has an energy bond of 27 kcal mol^{-1} [6]. Therefore, trehalose does not dissociate into two reducing monosaccharidic constituents unless exposed to extreme hydrolytic conditions or to the action of trehalase.

The interesting properties related to trehalose accumulation in organisms suggest a wide range of applications in several fields including cosmetics, pharmaceuticals and the agri-food sector [7,8]. The mechanism for biomolecule stabilization was first studied using Fourier transform infrared spectroscopy (FTIR) to characterize the interactions between proteins and sugars [9]. Infrared (IR) and Raman spectroscopy were then used to find that the most important role of the disaccharide was to concentrate the remaining water next to the protein [10]. The protein stabilization capacities of trehalose suggest several potential uses, such as moisturizers, preserving agents and chaperones (chaperones assist in preventing protein aggregation [11] – the main cause of illness such as Creutzfeld-Jacob disease, cystic fibrosis and amyloid disorders).

The most interesting applications of this non-reducing disaccharide include the stabilization of vaccines during storage at room temperature, the preservation of enzymes and protection of mammalian cells from damage in freeze-drying [12,13]. Roser and Colaco [14] predicted that health care in developing countries would be much improved if the majority of vaccines could be kept stable at room temperature. In fact, encouraging results were obtained when oral polio vaccine dried in the presence of trehalose was demonstrated to be stable at 45°C compared with the liquid solution chilled at 4°C [14]. Emerging areas of applications of α-α-trehalose are in cosmetics as a liposome stabilizer and in food stuff as a stable sweetener. In fact, it is only 45% as sweet as sucrose and so it could be used, in combination with other bulk sweeteners, to allow full flavour of the product to be appreciated. In addition, unlike other disaccharides, trehalose does not engage chemical reactions with amino acids or proteins, thus preventing the browning of the product during processing (Maillard reaction) [15]. Especially because of the foreseen applications in the food industry (see Table 1) a novel and economical method for trehalose production was needed, other than extraction from plants and brewery-spent yeast.

In the early 1990s the cost of 1 kg of commercialized trehalose could reach US$ 700 [6]. Since then many other processes have been proposed to reduce the production costs. A group of Brazilian researchers developed a method to overproduce trehalose in *Saccharomyces cerevisiae*, in response to stress conditions and using this approach they reached a content of 20% of sugars on cell dry weight [16]. Other groups studied the physiology of yeast when immobilized on a solid matrix [17,18], showing an increase in final trehalose content.

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Table 1. Predicted application of trehalose in the food manufacturing

Type of food	Examples
Dried food	Egg, vegetables, fish, meat, cereal, beans, curd, powder milk, fruits, soup and so on
Frozen food	General cooked food
Confection	Candy, gum chocolate and so on
Confectionery	Cake, baked confectionary, jam, cream, sweet been jam and rice cake (oriental food)
Beverage	Coffee, tea, fruits juice and dairy beverage
Fermented food	Bread and yogurt
Concentrated	Juice
Tablet	Tablet food and medical supplies
Others	Ice cream, sauce, sweetener and seasoning

Because trehalose has mild sweetness, high solubility, low cariogenicity, low hygroscopicity and it is stable during processing and storage it can be used in several food preparations.

These achievements already contributed to the price of trehalose decreasing by 50%. Although the cost was now affordable for very specific and high added value applications, for example in the pharmaceutical industry, it was not compatible with emerging uses in the food industry. Because of the promising applications in the development of novel foods and of new strategies for preservation, research continued and novel technologies were suggested. The most interesting have been filed for patents and successfully exploited in production processes leading to a major reduction in the commercial price of trehalose to 5–6 US$ kg^{-1}.

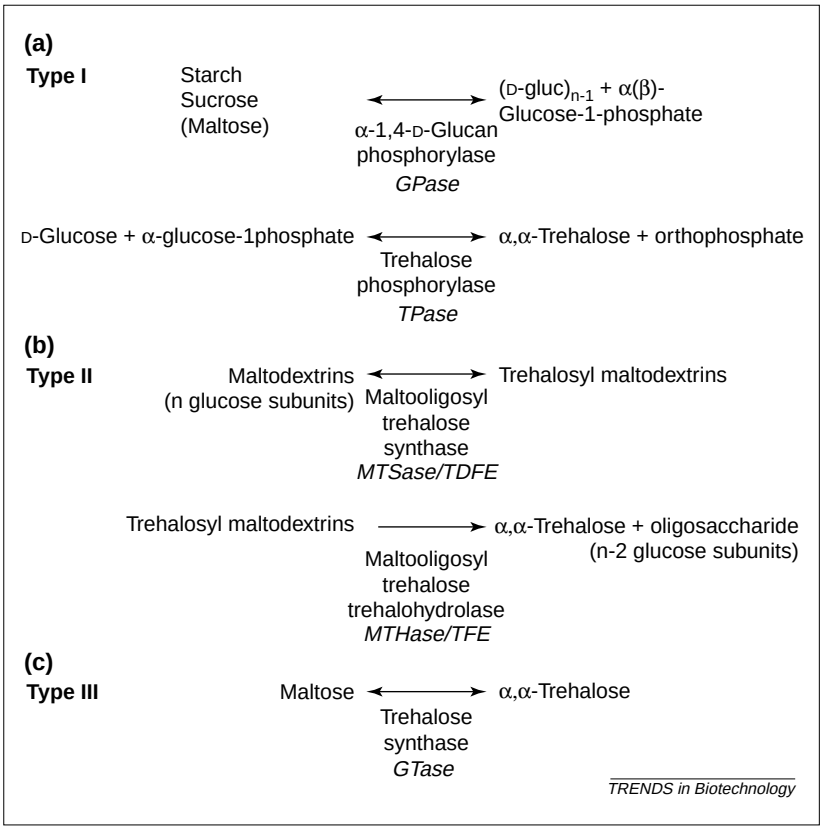

Fig. 1. Biosynthesis of trehalose, major mechanisms of bioconversion of different substrates into the non-reducing disaccharide: (a) two-step bioconversion using sequential action of glucose(maltose)phosphhorylase and trehalose phosphorylase; (b) conversion mechanism found in bacteria and archaea, maltodextrins are converted into trehalose by two sequential steps catalysed by MTSase and MTH; (c) maltose is converted into trehalose in a single reaction, following an intramolecular transglucosylation mechanism, GTase was found in mesophilic and thermophilic microorganisms. Abbreviations: GP, glucose/glucan phosphorylase; GT, glucosyltransferase; MTH, maltooligosyl trehalose trehalohydrase; MTS, maltooligosyl trehalose synthase; TDFE, trehalosyl dextrin forming enzyme; TFE, trehalose forming enzyme.

The novel approaches of trehalose production had a common ratio that was privileging enzymatic biotransformation rather than extraction from biomasses. Three main enzymatic routes for trehalose synthesis were discovered (Fig. 1): a phosphorilase system found in fungi and yeast; a glycosyltransferase–hydrolase system that occurs in mesophilic bacteria such as *Arthrobacter* sp. but also, more interestingly, in extremophilic microorganisms; and a trehalose synthase catalyzing an intramolecular transglycosilation from maltose, (*Pimelobacter* sp., *Thermus* ssp., etc.). These routes have been intensely discussed in the literature and few biotechnological processes were developed exploiting these enzymatic systems, which are the objects of several patents.

The phosphorylase system – two step conversion from starch

In the conventional preparation of trehalose, microorganisms (yeast) are grown. After cultivation, cells are disrupted and standard extraction procedures with heating and alcohol reflux are used as disclosed in a Japanese patent [19]. Biosynthesis of trehalose in yeast is occurring following the pathway in Fig. 1 (type I). Another possibility was proposed, based on the use of maltose phosphorylase (EC 2.4.1.8) and trehalose phosphorylase (EC 2.4.1.64) catalyzing the conversion of maltose into trehalose (the coupling reaction is similar to the one described for type I given that β-glucose 1-phosphate is produced in place of α-glucose 1-phosphate [20,21].

The Japanese method, however, is not suitable for industrial-scale preparations because the content of trehalose present in microrganisms as a starting material is usually ≤15% w/w on a dry solid basis and the extraction and purification steps are complicated. However, use of phosphorylases has some drawbacks. First, because trehalose is formed via glucose 1-phosphate, maltose as substrate could not be used at a relatively high concentration. Second, the yield of trehalose is relatively low owing to the reversibility of the enzymatic reaction systems of the phosphorylases. Third, it is substantially difficult to retain the stability of the reaction systems and to smoothly continue their enzymatic reaction. Thus these two processes have not yet been realized on a large scale.

The use of metabolic engineering improved the production of trehalose in the culture of microorganisms belonging to the genus *Candida*, in particular a trehalose-assimilating defective mutant. Trehalose was produced in the cytosol and subsequently recovered. The process was filed for patent in Japan in 1995 [22].

More recently, a trehalose phosphorylase (TPase) that catalyses the synthesis of trehalose from D-glucose and α-D–glucose 1-phosphate from *Grifola frondosa*, a basidiomycete, was discovered, and cloned into *Escherichia coli* for overexpression. The recombinant cells produced 50% of the protein as insoluble fraction (i.e. inclusion bodies). The TPase obtained (fusion protein) was purified and used to produce trehalose up to 35 mol% (moles of trehalose/total moles) whereas the enzyme from wild-type converted substrate up to 65 mol% [23].

A two step conversion to trehalose

In 1994, two patents were issued by Hayashibara Biochemical Laboratories Inc. (Okyama, Japan) disclosing a novel non-reducing saccharide forming enzyme (maltooligosyl trehalose synthase, MTSase; EC 5.4.99.15) [24] and a trehalose releasing enzyme (maltooligosyl trehalose trehalohydrolase, MTHase; EC 3.2.1.141) [25] obtainable from the culture of microorganisms such as *Rhizobium* sp. M-11 and *Arthrobacter* sp. Q 36 (Fig. 1, type II). The first enzyme acts on reducing maltodextrins having a polymerisation degree (DP) higher than three and catalyses the conversion of the α-1,4 linkage at the reducing end to an α-1,1, the second enzyme specifically acts on the α-1,4 linkage adjacent to the α-1,1 liberating trehalose and a lower molecular weight maltooligosaccharide.

Maruta *et al.* reported that this novel enzymatic system, after completion of growth in a fermenter on a medium containing also complex components, presented biocatalytic activity (complete reaction) in the culture broth (0.9 U ml^{-1}) as well as in the microbial cells (0.6 U ml^{-1}). The crude enzyme solutions have been purified, following conventional procedure, and fully characterized. The enzymatic synthesis was proposed to be a novel biosynthesis of trehalose involving two reactions (Fig. 1, type II). The conversion kinetic and the product formation were studied using maltodextrins containing up to seven glucose residues showing that a very poor activity could be detected on maltotriose [26–28].

The enzymatic synthesis of trehalose is currently obtained using the MTSase and the MTHase described above by Hayashibara Biochemical Laboratories Inc. However, research has been focused on the isolation of similar biocatalysts from thermophilic sources. In fact, the use of thermophilic enzymes in the biotransformation of carbohydrates could deliver benefits such as the improvement in solubility of substrates (starch or high molecular weight dextrins), the lowering of viscosity thus leading to a better access for enzymatic attack, and reduce risk of contamination. In 1991 Lama *et al.* first reported on the existence of a biosynthetic pathway for trehalose synthesis in the thermoacidophilic archaebacterium *Sulfolobus solfataricus* [29]. The mechanism was clarified later when a trehalosyl dextrin forming enzyme and a trehalose releasing enzyme were purified from *Sulfolobus acidocaldarius* ATCC33909 [30–32], the enzymes were characterized with respect to kinetic parameters and optimal environmental conditions for conversion. Successively, the trehalose biosynthesis genes, encoding maltooligosyltrehalose trehalohydrolase, glycogen debrancing enzyme and maltoolygosyltrehalose synthase, called *treZ*, *treX* and *treY* respectively, have been cloned from *Sulfolobus acidocaldarius*, and the amino-acid sequences was deduced and compared with the corresponding enzymes from *Arthrobacter* sp. Q36 and of other microorganisms from *Sulfolobales* [33–35]. In addition, recombinant MTSase obtained in *E. coli*, was crystallized and the structure analysed using X-ray diffraction after reductive methylation of lysine residues to improve crystal quality [36].

Successively, *Sulfolobus solfataricus* KM1 cell homogenates showed an amylolytic activity to convert soluble starch to trehalose [37,38], the enzymes demonstrated a high thermostability and also an efficient method for enzymatic activity determination was developed using maltopentaose and maltotriosyltrehalose as the substrate for glycosyltransferase and α-amylase, respectively. The enzymes were purified, their chemico-physical properties, substrate specificity and reactivity were studied and seemed virtually the same as others found previously, the author thus claimed that these enzymes are unique. The genes encoding trehalose-producing enzymes glycosyltransferase and α-amylase from *Sulfolobus solfataricus* KM1 were cloned and expressed in *E. coli* JM109 yielding 10 U g^{-1} wet cells and 2822 U g^{-1} wet cells, respectively [39]. In addition, the α-amylase was expressed and produced at higher yield in the food yeast *Candida utiliz* [40].

In our laboratory, enzymes responsible for trehalose synthesis were purified from *Sulfolobus shibatae* DSM 5389. They were named trehalosyl dextrins forming enzyme (TDFE) and trehalose forming enzyme (TFE) and were characterized from a chemico-physical and a kinetic viewpoint. They were found to be very similar to the ones isolated from other *Sulfolobales*. In addition, the kinetic of conversion of maltoheptaose to trehalose was investigated, determining intermediates and products using HPLC [41]. Table 2 consists of a comparison of the characteristics of the enzymes.

Almost contemporary, similar biocatalytic activities were found in *Sulfolobus solfataricus* MT4 (optimal growth temperature, 87°C). These enzymes showed a high thermostability and seemed suitable to develop a novel bioprocess for trehalose production. To make feasible the industrial application of these enzymes it was necessary to develop a production process at low cost and to improve the stability of biocatalysts. The production of TDFE and TFE from the wild-type strain was very

Table 2. Comparison of chemico-physical properties of the enzymes responsible for trehalose production

Microorganism	Enzyme	T_{OPT} (°C)	pH_{OPT}	Km (mM)	Production	Trehalose yield (%)	Ref.
Fungi and yeasts							
	TPS-ase (1st step) TPPase (2nd step)	50	6.8	28(MD)	1–1.2 U $g_{biomass}^{-1}$	n.r.	[53]
	GPase TPase	25	6.9	_	_	40	[21]
Bacteria							
Pimelobacter R48	GTase	45	7.0	n.r.			[45]
Pseudomonas putida		37	10				
Thermus aquaticus	GTase	65	6.5	n.r.	31.4 U $g_{biomass}^{-1}$	81 (40°C) 71 (60°C)	[46–48]
Arthrobacter sp. Q36	MTS	40	7.0	1.4 (M6)	16.3 U $ml_{culture}^{-1}$	92	[27]
	MTH	40	7.0	7.0 (MT4)	25.1 U $ml_{culture}^{-1}$		[28]
Brevibaterium helvolum	MTS	35	6.5	_	8 U $ml_{culture}^{-1}$	90	[50]
	MTH				12 U $ml_{culture}^{-1}$		
Rhizobium sp. M-11	MTS	40	7.0	_	1.5 U $ml_{culture}^{-1}$	60	[24]
	MTH	45	7.0		2.0 U $ml_{culture}^{-1}$		[25]
Archaea							
Sulfolobus acidocaldarius	MTS	75	5.5–6	5.7 (M6)	1.2 U $g_{biomass}^{-1}$	82	[30–32]
	MTH	75	5–5.5	3.7(MT4)	3.4 U $g_{biomass}^{-1}$		
Sulfolobus solfataricus KM1	MTS	70–80	5.0–6.0	2.7 (M6)		80–83	[37]
	α-amylase	70–85	4.5–5.5	_			[38]
Escherichia coli JM109 expressing	MTS			n.r.	10 U $g_{biomass}^{-1}$		[39]
Sulfolobus solfataricus KM1 enzymes	α-amylase			n.r.	2822 U $g_{biomass}^{-1}$		
Sulfolobus shibatae	TDFE	70	4.5–5.5	2.0(M6)	1.2 U $g_{biomass}^{-1}$	75–80	[41]
	TFE	85	4.5–5.5	1.0(MT4)	3.5 U $g_{biomass}^{-1}$		
E. coli Rb-791 expressing	TDFE	75	5	_	36 U $g_{biomass}^{-1}$	80–85	[42]
Sulfolobus solfataricus MT4 enzymes	TFE	85	5	_	726 U $g_{biomass}^{-1}$		

GP, glucose/glucan phosphorylase; GT, glucosyltransferase; M6, maltohexaose; MD, maltodextrins; MT4, trehalosylmaltotetraose; MTH, maltooligosyl trehalose trehalohydrase; MTS, maltooligosyl trehalose synthase; TDFE, trehalosyl dextrin forming enzyme; TFE, trehalose forming enzyme; TPase trehalose phosphorylase; TPP, trehalose phosphate phosphatase; TPS, trehalose phosphate synthase.

expensive owing to the low biomass yield typical of extremophilic microorganism cultivation, therefore they were expressed into mesophilic host [42] and produced at high yield in a microfiltration bioreactor [43]. By exploiting the thermostability of the enzymes of interest a quick two-step purification procedure was used consisting of a heat treatment that caused the selective precipitation of the host proteins followed by a chromatographic step on a sephacryl S-200 column.

Recently, to improve the coupling of the two catalytic activities of interest, the coding sequences of TFE and TDFE were fused to obtain a new chimeric protein able to convert dextrins to trehalose at high temperature (75°C) by sequential enzymatic steps [44].

Glucosyltransferase catalyzing an intramolecular transglycosylation to produce α,α-trehalose

A trehalose synthase (EC 5.4.99.16) was purified from a cell-free extract of *Pimelobacter* sp. R48 [45]. The enzyme catalyzed a one step conversion of maltose into trehalose by intramolecular transglucosylation as well as the reverse reaction but was inactive on the other saccharides (Fig. 1, type III) [46]. Its optimum pH was 7.5 and optimum temperature was 20°C. The conversion kinetic of maltose was independent of its concentration when this was higher than 5% (w/w) and the maximal yield in trehalose decreased from 81% to 76% when temperature increased from 5°C to 25°C. In 1996, Nishimoto *et al.* reported the purification and characterization of a thermostable trehalose synthase [47], from *Thermus aquaticus* ATCC 33923, and it was more thermostable and thermoactive than the correspondent biocatalysts isolated from *Pimelobacter* sp. R48 and *Pseudomonas putida* H262. The optimal parameters for conversion were pH 6.5 and a temperature of 60°C. The substrate specificity was demonstrated by the authors confirming the previous studies on the mesophilic counterpart, the biotransformation resulted in a 70% conversion at substrate concentration ranging from 2.5 to 40% (w/v) within 72 h, whereas at 40°C the maximum yield was 82%. Trehalose synthase had a weak hydrolytic activity, which increased as the temperature increased. Therefore, at higher

temperatures the conversion of maltose into trehalose decreased. Nevertheless the equilibrium was established to lie in direction of the trehalose synthesis. The trehalose synthase gene from *Pimelobacter* sp. R48 [45] and the corresponding gene from *Thermus aquaticus* ATCC 33923 were cloned and expressed [48,49].

Bioprocesses for trehalose production

Most of the biocatalysts described here have been used to develop novel bioprocesses that proved suitable for large-scale production of trehalose. In Table 2 the chemico-physical characteristics, kinetic parameters and yields of trehalose of these methods were compared. Here, we describe the most interesting results achieved in the past few years.

Batch processes with enzymes in solution

The enzymes isolated from *Arthrobacter* Q36, MTSase and MTHase were exploited in a process using pre-treated corn starch as the substrate. The starch hydrolysates had a degree of glucose polymerization (DP) of 10–35 were converted by the two enzymes at 40°C, the resulting solution contained mainly trehalose (≥66% w/w), and in lower amount reducing oligosaccharides, glycosyltrehalose and glucose. Trehalose yield was discovered to depend on the degree of substrate polymerization [25].

Trehalose synthesis was also accomplished by exploiting MTS and MTH from *Brevibacterium helvolum*. By coupling the activity of the recombinant enzymes with another α-amylase, which helped in debranching natural starch, the production of trehalose from this natural source was improved [50].

The two enzymes isolated from *Sulfolobus solfataricus* KM1, glycosyltransferase and α-amylase, were used for trehalose production from a variety of maltooligosaccharides of DP 3–7, yielding 36–67% trehalose. The conversion was improved to 83% when amylose with a higher polymerisation degree (DP 17) was used. Similar to the enzymes previously studied, higher yields corresponded to longer glucose chains in substrate, and on using starch as substrate, and pullulanase as the debranching enzyme, the yield increases to 80–81% showing KM1 to produce trehalose from starch by a new pathway through the joint action of glycosyltransferase and α-amylase. A process for trehalose production from starch was also developed exploiting the recombinant glycosyltransferase and the α-amylase from *Sulfolobus solfataricus* – the optimal ratio between the two enzymes was shown to be 1:1. Next, starch and amylose were used for conversion at 60°C, eventually using a debranching enzyme before incubation with the trehalose forming enzymes. The yield was ~80% trehalose on a dry solid basis, whereas the theoretical yield was calculated to be 106% [51,52].

Immobilized cell bioprocesses

Trehalose phosphorylase from *Schizophyllum commune* BT2115 and starch phosphorylase from *Corynebacterium callunae* DSM 20147 were produced and purified [53]. Both enzymes were immobilized by adsorption and were used in a packed bed configuration to produce trehalose from starch. The substrates were maltodextrins, glucose and phosphates. The authors reported that trehalose productivity was constant at 2.6 g l^{-1} h^{-1} for 72 h of continuous process.

A trehalose synthase from the basidiomycete *Grifola frondosa* able to form trehalose from α-D-glucose 1-phosphate and D-glucose, was used in combination with sucrose phosphorylase to obtain the non-reducing disaccharide in high yield from sucrose. Trehalose was generated at 18 mmol h^{-1} with a final yield of 90 mol % [23].

We evaluated the possibility of producing trehalose at high temperature exploiting the enzymes from *Sulfolobus solfataricus* MT4. The design of a novel bioprocess was based on using permeabilized recombinant *E. coli* cells, containing TDFE and TFE activities, entrapped in calcium alginate gel beads. The biocatalysts obtained were used in a packed bed bioreactor. We compared the performance of a stage reactor with that of a co-immobilized bioreactor in converting maltohexaose into trehalose. Using the stage bioreactor, maltohexaose was converted into a carbohydrate mixture containing trehalosylmaltodextrins, trehalose and glucose, the co-immobilized cell bioreactor resulted in a faster biotransformation of substrate into the non-reducing disaccharide without evidencing the accumulation of intermediates. This improvement can be ascribed to the physical proximity of the two enzymes that resulted in minor transport barriers. In addition, to support the possibility of the development of a novel industrial process, the co-entrapped cell bioreactor was exploited in the biotransformation of a 30% w/v dextrin mixture obtaining an 83% conversion with respect to the theoretical trehalose production. The corresponding productivity was 140 g l h^{-1} [54].

Conclusions

Trehalose was chosen by Mother Nature to protect life and in the past decade several studies elucidated the stabilization mechanisms both *in vivo* and *in vitro*. The results overcame predictions, showing the potential role that trehalose could have as a powerful biotechnological tool in many applications and products, ranging from the aforementioned uses in pharmaceutical preparation, or as living tissue preserver, to that of moisturizer in cosmetic preparation to that of key element in food preparations subject to drying processes or concentration. The actual possibility of using trehalose in so many fields became feasible because of the intense effort made in the field of enzymology for the discovery of suitable biocatalyst to be used in the

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production process. It was shown that new production technology brought to the dramatic decrease in trehalose price (e.g. from US$ 700 to US$ 6), it can be predicted that, with the development of suitable expression system (e.g. GRAS hosts), the application of the extremophilic biocatalysts could further improve the process and further decrease the cost of the disaccharide.

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