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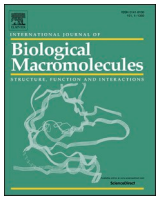

Review

Co-production of levan with other high-value bioproducts: A review

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ABSTRACT

Levan is a homopolysaccharide of fructose that has both scientific and industrial importance, with various applications in health, pharmaceutical, cosmetic and food industries. Despite its broad spectrum of uses, there are only a limited number of commercial levan sources due to the high costs related to its production. To make production economically viable, efforts have been concentrated on the selection of levan-producing microorganisms, the genetic manipulation of new strains, and the use of inexpensive agro-industrial byproducts as substrates. Another efficient strategy involves the concomitant synthesis of other products with high market value and as such, the successful co-production of levan was demonstrated with fructooligosaccharides, ethanol, sorbitol, poly-ε-lysine, poly-γ-glutamic acid and polyhydroxyalkanoates. This paper offers a systematic review of important aspects regarding recent strategies involving the simultaneous synthesis of levan and other bioproducts of aggregate value reported to date and discusses the challenges and opportunities for its large-scale production and applications.

1. Introduction

Fructans occur naturally in numerous plants, bacteria and archaea as well as certain fungi. These fructose-based homopolysaccharides are synthesized by fructosyltransferase (FT) enzymes from sucrose-based substrates. Fructans are generally formed by fructose residues joined by β-2,1 or β-2,6 linkages to form the polysaccharides inulin and levan, respectively, as well as fructooligosaccharides [1]. Among the fructan polysaccharides, inulin has long received the greatest attention due to its health benefits. Recently, however, there has been a growing number of studies conducted with levan in different biotechnological fields [1–3].

Levan-type fructans have a majority of β-2,6 linkages in their backbone (Fig. 1) and they are extracellularly produced from sucrose by various microorganisms including species of the genera *Azotobacter*, *Bacillus, Erwinia, Gluconobacter, Halomonas, Microbacterium, Pseudomonas* and *Zymomonas* [4]. Due to its distinct properties, such as solubility in both water and oil, low intrinsic viscosity, good compatibility, film-forming capacity, strong adhesivity, stability in the presence of heat and acid-alkaline media, compatibility with salts and surfactants and high retention capacity for water and chemical products, levan is an attractive molecule with numerous applications in the biomedical, pharmaceutical, food and cosmetic industries [5–8].

Levan has considerable potential as an additive in functional foods and feed, source of prebiotic fiber, cryoprotectant agent, emulsifier, stabilizer, thickener, encapsulating agent, osmoregulator, plasma substitute, extender of drug activity, as an antitumor, antidiabetic and antihyperlipidemic agent [6–11]. This vast spectrum of properties and applications makes levan an industrially important biomaterial. However, its availability is limited due to drawbacks associated with its commercial-scale production, which remains economically unviable [4,12]. The microbial production of levan is mainly performed using submerged fermentation. Various production systems designed to increase yields via different microbial systems and specific culture conditions have been reviewed [4,13]. However, the observed yield in microbial production processes is generally <58 % of the theoretical yield and there are no viable standard protocols for its production and purification [4,10].

One option for reducing production costs is the use of systems involving the simultaneous synthesis of two or more microbial products [12]. Thus, a successful strategy for diminishing levan production costs is the co-production of other fructan products, such as fructooligosaccharides [14] and/or non-fructans, such as poly-γ-glutamic acid [15], α-amylase [16], poly-ε-lysine [17], sorbitol [18] and ethanol [19]. In silico and in vitro studies on the microbial *co*-production of levan and polyhydroxyalkanoates by *Halomonas smyrnensis* cultures were also reported [12,20].

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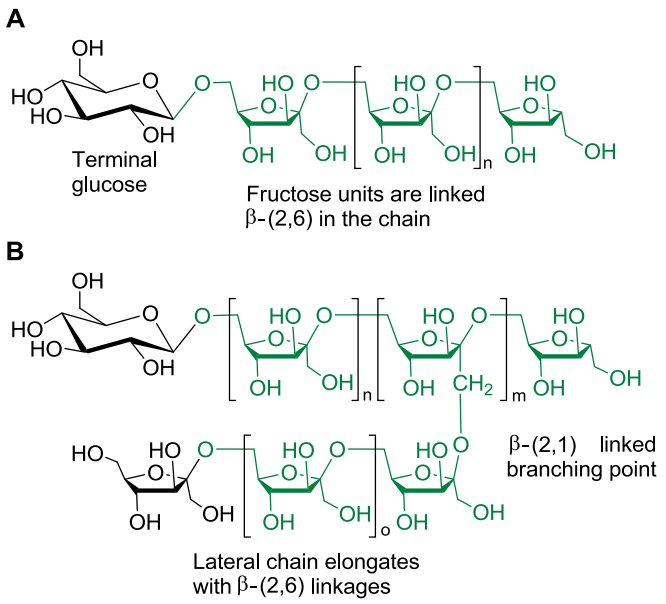

Fig. 1. Chemistry structures of linear (a) and branched (b) levan.

Although levan has been widely documented in recent years [1,4,8,10], there are no reports specifically focused on its co-production with other microbial products. Hence this review aimed to provide an up-to-date snapshot of the literature regarding the simultaneous and sequential production of bacterial levan and other compounds.

2. Co-production of levan with fructooligosaccharides

Fructooligosaccharides (FOS) have well established health promoting functional and physiological properties that made them one of the most prominent ingredients in food industry. Besides their low glycemic and caloric indices (2 kcal/g), they also have prebiotic properties promoting the growth of Bifidobacteria and Lactobacilli. FOS also lower the relative risk of colon cancer, improve the intestinal absorption of Ca^{2+} and Mg^{2+} and reduce lipid levels in the blood [21].

The joint synthesis of levan and FOS occurs through the transfructosylation reaction catalyzed mainly by levansucrase (EC 2.4.4.10), which, according to the Carbohydrate-Active Enzymes database (CAZY, http://www.cazy.org/), is a multifunctional enzyme group in the glycosyl hydrolase 68 family [4,14]. GFn formula FOSs (n = 1–10) are formed by the sequential transfructosylation of a fructosyl residue in a sucrose molecule. Levansucrase synthesizes FOS with a predominance of β-(2,6) linkages, generating 6-kestose and other FOS of the 6F series. However, some levansucrases also produce FOS of the 1F series (e.g., 1-kestose) and 6G series (e.g., neokestose). The hydrolytic activity of levansucrases also gives rise to free fructose, which accepts fructosyl units to produce levan oligomers (levanbiose, levantriose, etc.) (Fig. 2) [2,21].

The successful simultaneous production of levan and FOS using different sources of sugars has been described with several bacterial strains. For instance, Bekers et al. [22] used the remaining biomass from the co-production of levan and ethanol as well as the cell-free broth in the production of FOS by *Zymomonas mobilis*. Under optimized synthesis conditions, while levan concentration reached up to 47 %, FOS content was 18.2 % of total carbohydrates and was comprised of only 1-, 6- and neokestoses and nystose. The presence of ethanol (7.0 %) in the culture medium diminished the activity of the FOS-forming enzyme by 24 % in the first 24 h of incubation.

The use of lactose as substrate for *Bacillus amyloliquefaciens* levansucrase was reported for the co-synthesis of levan and FOS [23]. In 0.8 M of sucrose, levansucrase from *B. amyloliquefaciens* synthesized mainly levan (47 %, *w*/w), with a low FOS content (3 %, w/w) while in the presence of lactose, levansucrase generated lactosucrose as the major product, with a maximum mass fraction of 60 %. The maximum accepting capacity for levansucrase from *Geobacillus stearothermophilus* was also studied in the joint production of levan and FOS using hexoses, raffinose and glycerol [23]. Compared to glucose, fructose, glycerol and raffinose, the enzyme was most effective in the presence of sucrose producing 35 % levan and 6.3 % FOS by mass.

A pioneering study investigated the co-synthesis of FOS and levan by *B. amyloliquefaciens* in reaction systems involving maple syrup at degrees Brix (°Bx) of 15, 30 and 60 [25]. With 30°Bx maple syrup, levan with degree of polymerization (DP) between 10 and 30 were synthesized as major products (>80 %). With 66°Bx maple syrup, levan products with DP of 10–30 and DP ≥ 30 were the most abundant products at 30 °C and 8 °C, respectively. The supplementation of 15°Bx and 30°Bx maple syrup with lactose generated lactosucrose as the major product (Table 1), after a 24-h reaction time. In the reaction system with 15°Bx maple syrup, the lactosucrose produced served as an acceptor for the polymerization of levan, as demonstrated by the levan yields, which were only 5.42 % at 12 h and then increased considerably up to ten-fold at 48 h. In the reaction system with 30°Bx maple syrup, the accumulation of levan was low (<10 % at 48 h) and lactosucrose formation was favored at high sucrose concentrations.

The concentration of sucrose exerts a significant influence on the proportions of biosynthesized levan and FOS. The effects of sucrose concentration and temperature on the formation of levan, production of FOS and hydrolysis of sucrose by levansucrase from *Z. mobilis* was investigated [21]. The highest mass fraction of levan (25 %) was achieved using a sucrose concentration of 100 g/L and temperature of 4 °C. The increase in the concentration of sucrose (to 600 g/L) and temperature (to 40 °C) led to a reduction in the concentration of levan and an increase in the FOS content (by 40 %). This effect can be used to shift the activity of levansucrase for the production of either levan or FOS.

Recently, Bersaneti et al. [26] demonstrated that it was possible to produce FOS (nystose) and levan simultaneously with *Bacillus subtilis natto* under different reaction conditions. The highest nystose production in test tubes (69.7 g/L) was obtained from 350 g/L of sucrose at 35 °C after 36 h (Table 1). However, the production of levan increased significantly in the bioreactor (63.6 g/L). In a bioreactor, the greatest production of FOS was 41.3 g/L under the same conditions. The enzymatic levan yields reached 86.9 g/L under similar conditions as those used for the synthesis of FOS. Hence enzymatic co-production of FOS and levan can be considered as an effective approach to provide an economically viable and innovative alternative to the functional food industry.

Rational mutagenesis studies have also been reported as a strategy to suppress the production of levan and increase the yield of short-chain FOS. For instance, Kang et al. [27] isolated and characterized the gene of levansucrase from *Leuconostoc mesenteroides* B-512 (MIFT). The M1FT enzyme was able to convert sucrose into 18 % levan, 17 % 1-kestose, 11 % nystose and 7 % 1,1,1-kestopentaose while releasing glucose. Moreover, M1FT produced erlose as an acceptor product with maltose.

Bacillus sp. are a group rich in levansucrases, so they have been widely used in the synthesis of levan and FOS [28–30]. Possiel et al. [31], created a levansucrase library for *Bacillus megaterium* and focused on the synthesis of inulin-type FOS and levan. The authors made modifications at three amino acid positions of the oligosaccharide elongation pathway and found out that native R370 reduced the ability of the enzyme to synthesize levan-type oligosaccharides, directing its specificity towards neoFOS and 1-kestose. The R370Q mutants produced mainly 1-kestose and neokestose, while K373H or K373L formed 6-kestose and 6-nystose as major products (Fig. 2).

Homann et al. [32] identified a novel levansucrase in the supernatant of a cell culture of *B. megaterium* DSM319, which was expressed in heterologous form in *Escherichia coli*. The enzyme produced a levan with a molecular weight of 2711 kDa as well as a set of FOS, including 1-

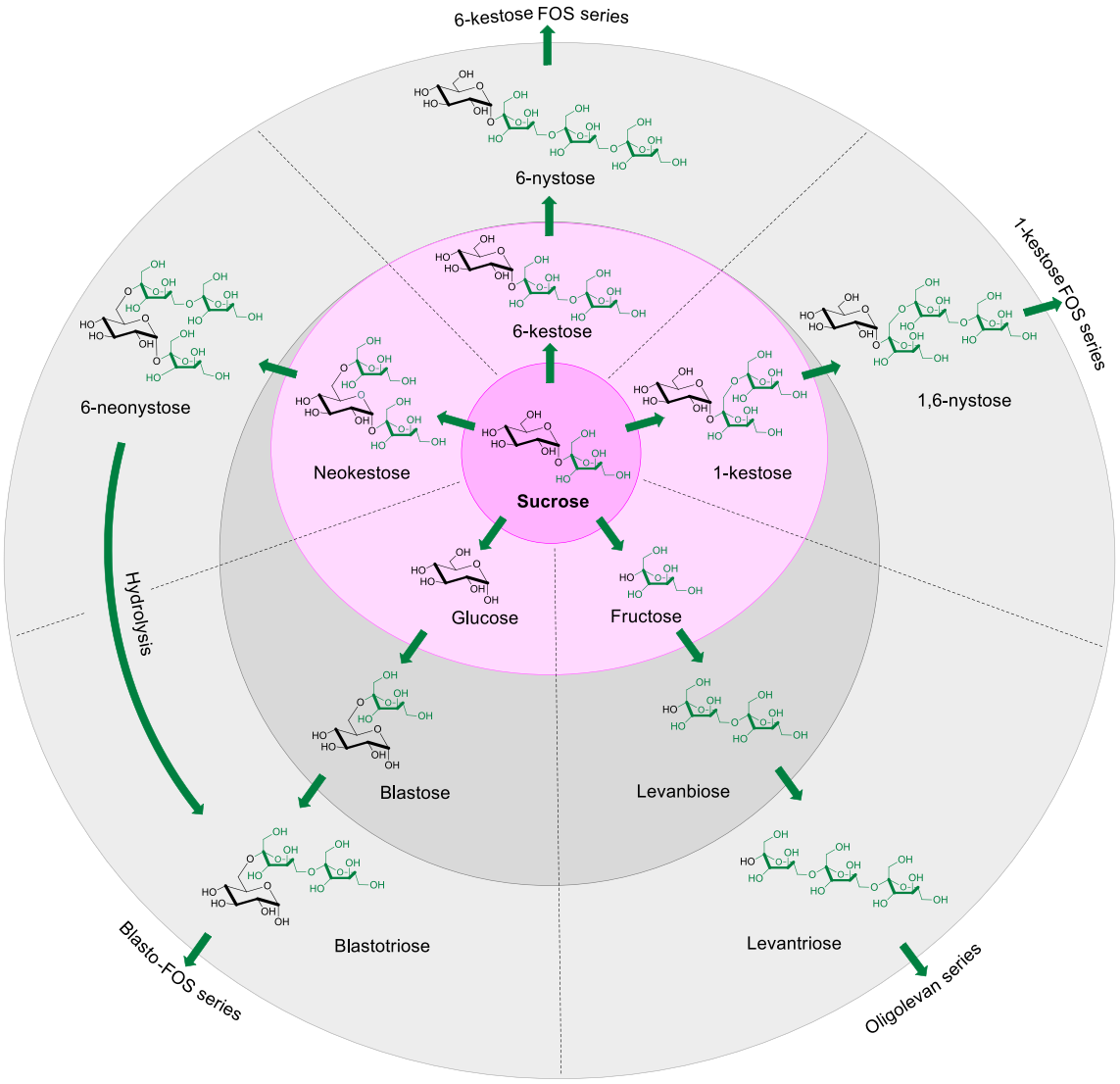

Fig. 2. Routes for obtaining fructooligosaccharides and oligolevan series synthesis. In the initial stage, sucrose hydrolysis and neokestose, 6-kestose and 1-kestose are synthesized. These products are elongated in the second stage to produce 6-neokestosis, 6-nystose and 1,6-nystose, respectively. These intermediates are then augmented by the enzyme to achieve low and/or high molecular weight levans. In the third stage, blastosis, levanbiosis and inulobiosis are synthesized. Finally, in the final stage, neocestosis and 6-neocestosis are hydrolyzed and their products incorporated into the synthetic pathway that results in the Blasto-FOS series [2].

kestose, 6-kestose, nystose, neokestose and blastose (Fig. 2). Levansucrase from *Lactobacillus panis* TMW1.648 was also isolated from fermented dough and the gene identified was expressed in heterologous form in *E. coli*, producing a high molecular mass levan and kestose from sucrose [33].

A hybrid system of enzymes (LevB1SacB) was designed from the fusion of a levansucrase from *B. subtilis* (SacB) and an endolevanase from *Bacillus licheniformis* (LevB) for the co-production of levan and levan-type FOS with DP 2–10 [34]. The simultaneous synthesis and hydrolysis reactions yielded 97 % levan-type FOS when 1.0 U/mL of LevB1 reacted with 100 g/L of levan produced by SacB from *B. subtilis*. Using low molecular weight levan (8.3 kDa) at a concentration of 150 g/L, levan-type FOS were obtained with DP 2–8 (6-kestose, levanbiose and blastose), the main product of which was levanbiose. The same LevB1SacB system recently converted 82–87 % of sucrose into 64.3–82.1 g/L of high molecular weight levan (> 2200 kDa) with a L-FOSs yield of 35 % [35].

A β-fructosidase (BfrA) with the capacity to synthesis FOS, levan and β-(2,6) neolevan was purified from *Aspergillus oryzae* FS4 and expressed in both *E. coli* and *Pichia pastoris* [36]. BfrA produced FOS that consisted of a mixture of GF2, GF3 and GF4 (Table 1). Two types of GF2 levanoligosaccharides were detected and identified as 6-kestose and neokestose. Moreover, 6-kestose accounted for 19 % of all products. GF3 was identified as levan-type 6-nystose and the GF4 has not yet been confirmed. Levan-type 6-kestose and the neolevan type (neokestose, Fig. 2) exhibited greater prebiotic activity than the inulin-type FOS.

The gene from L. *mesenteroides* MTCC 10508 levansucrase (TrLmLEVS) was cloned and expressed in *E. coli* and tested with different sugar sources [36]. High-performance liquid chromatography (HPLC) analyses showed that the extract from the stalk of sweet sorghum (containing 114 g/L of sucrose) yielded about 40 g/L of levan and short-chain FOS (DP 3–7), such as 6-kestose and 6-nystose (Table 1). From pure sucrose (10 %) and TrLmLEVS (100 mg/L), the production of levan and FOS was 31 g/L and 2 g/L, respectively, at 5 h. After this time, a reduction in the levan:FOS ratio occurred. The use of table sugar produced about 303 g of levan per kg of sugar and FOS of DP 3–7. Similar results were found when using brown sugar, which generated about 305 g of levan per kg of sugar; however, the formation of FOS was not mentioned. The use of molasses (~50 % *w*/w sugars) yielded around 5 g of levan per liter of molasses.

The substitution of Y237S with serine in wild-type *B. amyloliquefaciens* KK9 (LsKK9) was designed to increase the

Table 1
Examples of fructooligosaccharides co-produced with levan.

Microbial strain	Medium and culture conditions	Product A	Product B	Reference
Rahnella aquatilis	Sucrose (200 g/L), pH 6.0, 17–30 °C, 24 h	Levan (27 g/L)	Oligosaccharides (10–11 g/L)	[38]
Zymomonas mobilis 113 "S"	Sucrose (100 and 150 g/L) and Syrup sucrose (with 70 % of sucrose), inoculum (10 %), pH 4.9, 30 °C, 24 h.	Levan (46.6 %)	FOS (18.2 %): 1-Kestose, 6-Kestose, Neokestose and Nystose.	[22]
Leuconostoc mesenteroides B-512	Sucrose (50–150 mM), levansucrase (100 U/mg), pH 6.2, 30 °C.	Levan (18 %)	1-Kestose (17 %), Nystose (11 %), 1,1,1-Kestopentaose (7 %)	[27]
B. amyloliquefaciens	Sucrose (10 g/L), yeast extract (10 g/L), pH 6.0, 35 °C, 150 rpm, 11 h.	Levan (47 %, *w*/w)	FOS (3 %, w/w): Lactossacarose (60 %), (majority).	[23]
Geobacillus stearothermophilus	Fructose (1 %, *w/v*), glucose (1 %, *w/v*), glycerol (1 %, w/v), raffinose (1 %, w/v), sucrose (0.25 to 2 %, w/v), and mixture of each, pH 6.0–6.5, 47 °C, 6 h.	Levan (35 % w/w)	FOS (6.3 % w/w)	[24]
Aspergillus oryzae FS4 (BfrA) expressed in *E. coli* and *Pichia pastoris*	Sucrose (33 %), 50 mM KPB (pH 7.0). 50 °C, 5 h.	Levan (19 %)	FOS (56 %): GF_2 (26.2 %), GF_3 (20.4 %), GF_4 (9.2 %).	[36]
B. subtilis (SacB) and *B. licheniformis* (LevB).	Sucrose and levan (100 g/L), 35 °C, for SacB. Reactions were carried out using 1 U/mL of LevB1 for 5 h.	Levan de 8.3 kDa (100–150 g/L)	FOS (97 %, DP 2–8): Levanbiose (majority).	[34]
Z. mobilis	Sucrose (100–600 g/L), levansucrase (5 U/mL), pH 5.0 e 5.4 (50 mM acetate buffer), 4–40 °C, 48 h.	Levan (25 %)	FOS (40 %): 1-kestose, Blastose, 6-kestose, Neokestose, Nystose, Neonystose and ^{1}F- Fructosylnystose.	[21]
B. amyloliquefaciens	Maple syrups (°Brix: 15, 30 e 60), levansucrase (72 U/mL), pH 6.0, 30 °C, 48 h.	Levan (DP ≥30): 55.31 % (42.85 g/L), Levan (10 < DP ≤ 30):> 80 %	FOS (2 < DP ≤10):	[25]
B. licheniformis ANT 179	Erlenmeyer flasks (250 mL): Sugarcane juice (20 %, *v*/v), Cane molsses (20 %, v/v) and sucrose (50 %, w/v), pH 6.0, 35 °C, 200 rpm, 72 h.	Levan (50.3 g/L)	FOS-type inulin: Kestose and Neokestose (Yield, n.r.*)	[39]
B. subtilis natto CCT7712	Erlenmeyer flask and bioreactor. Sucrose (50–350 g/L), pH 6.0, 30–50 °C, 12–36 h.	Levan (63.6–86.9 g/L	Nystose (41.3–69.7 g/L)	[52]
Leuconostoc mesenteroides MTCC 10508 (LmLEVS) in *E. coli*	Sucrose (10 %), sweet sorghum juice (114 g/L), TrLmLEVS (100 mg/ L), pH 6.0, 30 °C, 5 h.	Levan (30–34 %): 31.1–40 g/L	FOS (DP 3–7): 2 g/L (6-Kestose and 6-Nystose)	[36]
B. amyloliquefaciens KK9	Sucrose (20–40 %), pH 6.0, 37 °C.	Levan (12–20 %)	FOS (12–20 %)	[37]
Bacillus aryabhattai GYC2–3	Sucrose (250 g/L), pH 8.0, 30 °C, 150 rpm, 120 h.	Levan (26 g/L)	FOS: 1-Kestose, 6-Kestose, Neokestose and Nystose (Yield, n.r.*)	[29]
Z. mobilis ZM4 (ATCC 31821)	Bioreactor of 400 mL (Box-Behnken): Sucrose (350 g/L), pH 5/6, 30 °C, 100 rpm, 72 h.	Levan (8.0 g/L)	FOS (156.5 g/L): 1-Kestose (66.9 g/L), 6-Kestose (67.8 g/L) e Nystose (21.8 g/L).	[40]
Z. mobilis ZM4 pB1-sacB	Bioreactor (400 mL): Sucrose (350 g/L), pH 5/6, 30 °C, 100 rpm, 72 h.	Levan (9.7 g/L)	FOS (73.4 g/L): 1-Kestose (38.23 g/L), 6-Kestose (15.95 g/L) e Nystose (17.90 g/L).	[41]
B. amyloliquefaciens	Maple syrup (30 and 66°Brix), U/mL (3.73 and 5), pH (6.6 and 6.04), 23.12 and 29.92 h.	Levan (147.09 g/L)	FOS (109.20 g/L)	[30]

* Not reported.

production of short-chain levan type FOS (LFOS) and levan [37]. The concentration of the products (levan/FOS) produced by wild-type LsKK9 increased by 20 % (*w/v*) in a medium containing 50 % (w/v) sucrose (Table 1). In the case of Y237S, the concentration of levan/FOS increased and leveled off when ≥40 % (w/v) of sucrose was used. LFOS produced by the Y237S variant exhibited greater prebiotic activity than the wild type.

In turn, Kim et al. [38] evaluated the use of water-miscible organic solvents to increase the formation of levan and oligosaccharides in *Rahnella aquatilis*. The maximum concentration of levan (27 g/L) occurred at an optimal temperature of 17–30 °C, resulting in 57 % of the theoretical yield (Table 1). The oligosaccharides were selectively accumulated in a high concentration of substrate with a maximum content of 10–11 g/L in the entire temperature range analyzed (4–50 °C).

Xavier & Ramana [39] evaluated the maximum production of levan and FOS by *B. licheniformis* ANT 179 using sugarcane juice, cane molasses and sucrose with respective maximum levan yields of 50.3, 26 and 43.0 g/L, respectively. Regarding the production of FOS, a FOS-type inulin (kestose and neokestose) was reported, but its rate was not reported.

Recently, it has been shown that *Zymomonas* strains were capable of co-producing not just two, but up to four bioproducts. Using a strain of *Z. mobilis* ZM4 and the Box-Behnken design approach, Braga et al. [40] obtained four different bioproducts of levan, ethanol, sorbitol and FOS where the FOS was composed of 1-kestose, 6-kestose and nystose. In another work, the same authors constructed recombinant strains of *Z. mobilis* ZM4 (pB1, pB1-L196 and pB1-sacB) mutated from *Schwanniomyces occidentalis* in order to control the relative composition of FOS [41]. The best rates were obtained with *Z. mobilis* ZM4 pB1-sacB which produced 9.7 g/L of levan, 92.1 g/L of ethanol, 35.2 g/L of sorbitol and 73.4 g/L of FOS. FOS presented rates of 38.2 g/L, 16 g/L and 17.9 g/L for 1-Kestose, 6-Kestose and Nystose, respectively (Table 1 and Table 2).

3. Co-production of levan with other bioproducts

Besides FOS, non-fructan products of high economic value, such as ethanol, sorbitol, poly-γ-glutamic acid, α-amylase, poly-ε-lysine and polyhydroxyalkanoates [12], have been obtained simultaneously with levan either in flasks or a bioreaction and either in a batch or fed batch system (Fig. 3).

3.1. Ethanol

The ethanol production process is in constant evolution and ethanol biorefineries need to reduce the overall production cost in order to become economically viable. The strategies for increasing the mass fraction of ethanol in fermentation are related to sources of cheaper raw materials [42]. Moreover, co-production strategies with high-value products have been reported with *Z. mobilis*, which is an ethanol-producing bacterium that also produces levan through the action of levansucrase [43].

The general fermentation standards for the co-production of levan and ethanol by strains of *Z. mobilis* (27 and Z10) were established using

Table 2
Examples of other high-value bio-products co-produced with levan.

Microbial strain	Medium and culture conditions	Product A	Product B	Reference
Zymomonas mobilis (27 e Z10)	Sucrose (50–200 g/L), pH 6.0, 30 °C, fermenter (1 L).	Levan (~7.5 g//L, from unmetabolized fructose)	Ethanol (13.2–19.8 g/L, maximum rate: 1–3 g/L.h)	[44]
Z. mobilis 113	Sucrose (150 or 300 g/L), Batch and continuous processes. pH 5.5, 30 °C, 24 h.	Levan (35–64.2 g/L)	Ethanol (0.17–0.31 g/g)	[45]
Z. mobilis 113 "S"	Reused culture liquid (65 %) and syrup without additives (10–15 %, with control).	Levan (~27 g/L)	Ethanol (~48 g/L)	[46]
Z. mobilis 113 "S"	Sucrose (100 g/L), Inoculum (10 %), 30 °C, pH 4.9. Cells immobilized to stainless steel wire spheres (WS) and Al_2O_3 granules.	Levan (1.3–1.5 %)	Ethanol (2–3 %)	[49]
Z. mobilis 113 "S"	Commercial sucrose syrup (100 g) with 50–60 % (*w*/w). Batch and fed batch, 45 °C, 24 or 48 h..	Levan (40.14 g/L)	Ethanol (35.0 g/L)	[22]
Z. mobilis CT2	Sucrose (50–250 g/L), Inoculum (10 %), Batch fermentation, pH 5.0, 25–35 °C, 24 h.	Levan (27.2 g/L)	Ethanol (46.4 g/L)	[43]
Z. mobilis ZAG-12	Sucrose (100–160 g/L), Yeast extract (5.0 g/L), pH 6.0, 30 °C, 24 h.	Levan (3.90 g/L)	Ethanol (9.36 g/L)	[51]
Z. mobilis CCT 4494	Cells immobilized on alginate and chitosan beads. Sucrose (350 g/L), pH 4.0, 30 °C, 200 rpm, 24 h.	Levan (21.11 g/L)	Ethanol (93.4 g/L)	[49]
Z. mobilis CCT4494	Immobilization of cells for adsorption in loofa sponge and sugarcane bagasse. Glucose (20 g/L), 30 °C, 150 rpm, 24 h.	Levan (32.13 g/L)	Ethanol (148.2 g/L)	[50]
Z. mobilis ZM4 (ATCC 31821)	Bioreactor of 400 mL (Box-Behnken): Sucrose (350 g/L), pH 5/6, 30 °C, 100 rpm, 48–72 h.	Levan (8.0 g/L	Ethanol (86.7 g/L)	[40]
Z. mobilis ZM4 pB1-sacB	Bioreactor (400 mL): Sucrose (350 g/L), pH 5/6, 30 °C, 100 rpm, 72 h.	Levan (9.7 g/L)	Ethanol (92 g/L)	[41]
Z. mobilis/B. subtilis (natto) Takahashi	Sucrose (250 g/L) and Glucose, pH 5.5, 30 °C, 175 rpm, 120 h	Levan (56.0 g/L)	Bioethanol (21.1–26.5 g/L)	[19]
Z. mobilis (VTT-E-78082 e CP 4)	Sucrose (150 g/L), Yeast extract (2.5 g/L), 30 °C, pH 5.0, 200 rpm	Levan (8 % wt), or 10 % of the sucrose used.	Sorbitol (11 % wt)	[18]
Z. mobilis CP 4	Sucrose (150 g/L), pH 5.0, 25 °C, 20 h.	Levan (40.14 g/L)	Sorbitol (11.41 g/L)	[52]
Z. mobilis ZM4 (ATCC 31821)	Bioreactor of 400 mL (Box-Behnken): Sucrose (350 g/L), pH 5/6, 30 °C, 100 rpm, 48–72 h.	Levan (8.0 g/L	Sorbitol (45.6 g/L)	[40]
Z. mobilis ZM4 pB1-sacB	Bioreactor (400 mL): Sucrose (350 g/L), pH 5/6, 30 °C, 100 rpm, 72 h.	Levan (9.7 g/L)	Sorbitol (35.2 g/L)	[41]
Bacillus licheniformis 4071 – ΔepsAB Δdgp ΔpgsBCAE Δgdh	Bioreactor (7.5 L), fed-batch cultivation with intermittent sucrose feeding (50 g/L), pH 7.0, 180–550 rpm, 30–45 °C, 72 h	Levan (71.0 g/L)	2 R,3 S- butanediol (88.6 g/L)	[58]
B. subtilis (natto) Takahashi	Sucrose (50 g/L), L-glutamate (15 g/L), Batch/single stage, pH 7.0, 37 °C, 150 rpm, 21 h.	Levan (5.04 g/L)	γ-PGA (6.96 g/L)	[56]
B. amyloliquefaciens (cepas NK-L, NK-1 e NK-ΔLP)	Culture in flask (24 h) and bioreactor (5 L), pH 6.0, 37 °C, 250 rpm, 48 h.	Levan (13.9 g/L e 22.6 g/L)	γ-PGA (3.53 e 3.61 g/L)	[15]
B. amyloliquefaciens NK-Q-X	Soluble starch (10 g/L), shaked flasks (100 mL), pH 7.0, 37 °C, 180 rpm, 48 h.	Levan (31.1 g/L)	γ-PGA (2.19–4.62 g/L) e α-Amylase (96 %, 0.66 U/mL)	[16]
B. subtilis/ Streptomycesal bulus	Sucrose (250 g/L), Batch sequential (2 stage), pH 6.0, 37 °C, 175 rpm, 55 h.	Levan (60.0 g/L)	Poly(ε-Lysine) (4.37 g/L)	[17]
Halomonas smyrnensis AAD6^T	Sucrose (50 g/L), glucose (20 g/L), Shake flask, pH 7.0, 37 °C, 180 rpm, 120 h.	Levan (15.3 g/L).	PHB (1.34 g/L, 45.8 %)	[12]
H. smyrnensis BAE2	Sucrose (50 g/L), Shake flask, pH 7.0, 37 °C, 180 rpm, 120 h.	Levan (17.15 g/L)	PHB (3.15 g/L)	[20]
Bacillus thuringiensis HA1	Erlenmeyer flasks (250 mL) containing sucrose (20 (g/L),	Levan (16.5 mg/L)	PHB (0.058 g/L)	[63]
Suhomyces kilbournensis HD1	37 °C, 72 h, 150 rpm.	Levan (86 mg/L)	PHB (0.148 g/L)	

sucrose concentrations ranging from 50 to 200 g/L [44]. At the highest concentration of sucrose (200 g/L), the 27 strain had a high hydrolysis rate (65.4 g/L.h), but only 18 % of the hydrolyzed sucrose was converted into ethanol. When cultivated in 150 g/L of sucrose, the same stain had a hydrolysis rate of 60.3 g/L.h, yielding 76 % ethanol. In the discontinuous culture of sucrose at a concentration of 50 g/L, nearly all the sucrose was hydrolyzed after 26 h, with the formation of approximately 13.2 g/L of ethanol. The 210 strain had a slow specific growth rate, with a 72 % yield of ethanol, reflecting a longer fermentation time in comparison to the 27 strain. Maximum ethanol production rates ranged from 1 to 3 g/L for both strains cultivated with 50 to 200 g/L of initial sucrose, respectively. The yield of levan (not presented) could come from half of the 7.5 g//L of non-metabolized fructose (Table 2).

Two biotechnological processes (batch and continuous) were designed to obtain levan and ethanol simultaneous from sucrose by *Z. mobilis* 113 [45]. The transfer of carbon from the substrate in the batch and continuous processes was 66.7 % and 61 %, respectively. In the batch process using sucrose at 15 % for 24 h, the maximum concentration of levan was 35 g/L, with a yield of 0.24 g/g and productivity of 0.90 g/L.h. The yield of ethanol was 0.31 g/g and productivity was 1.1 g/L.h. In the continuous process, the conversion of carbon from the substrate into levan was achieved at pH 4.8, generating 64.2 g/L of levan, with a yield of 0.22 g/g and productivity of 3.2 g/L.h. The yield of ethanol was 0.17 g/g and productivity was 2.5 g/L.h.

In sucrose medium, *Z. mobilis* 113 "S" is capable of producing about 40–70 g/L of ethanol while producing levan [46]. Alternative sources of sucrose such as beet diffusion juice (no additives) and its syrup were used as raw material in the co-production of levan and ethanol by batch fermentation. In this work, concentrations of levan and ethanol of 27 and 48 g/L, respectively, were obtained.

Through batch and fed batch processes, Bekers et al. [22] evaluated the use of sugarcane juice and sucrose as substrates in the production of levan and ethanol and investigated the interaction between these substrates in a complete factorial design. The batch process improved the production of levan, which reached 40.14 g/L. Supplementation with sugarcane juice did not have a significant effect on the generation of levan but promoted the production of biomass and ethanol. The production of levan was more substantial in the batch process with 150 g/L of sucrose. After the second stage, the culture broth contained 2.0 g/L of biomass, 30 g/L of levan (in wet weight) and 35.0 g/L of ethanol

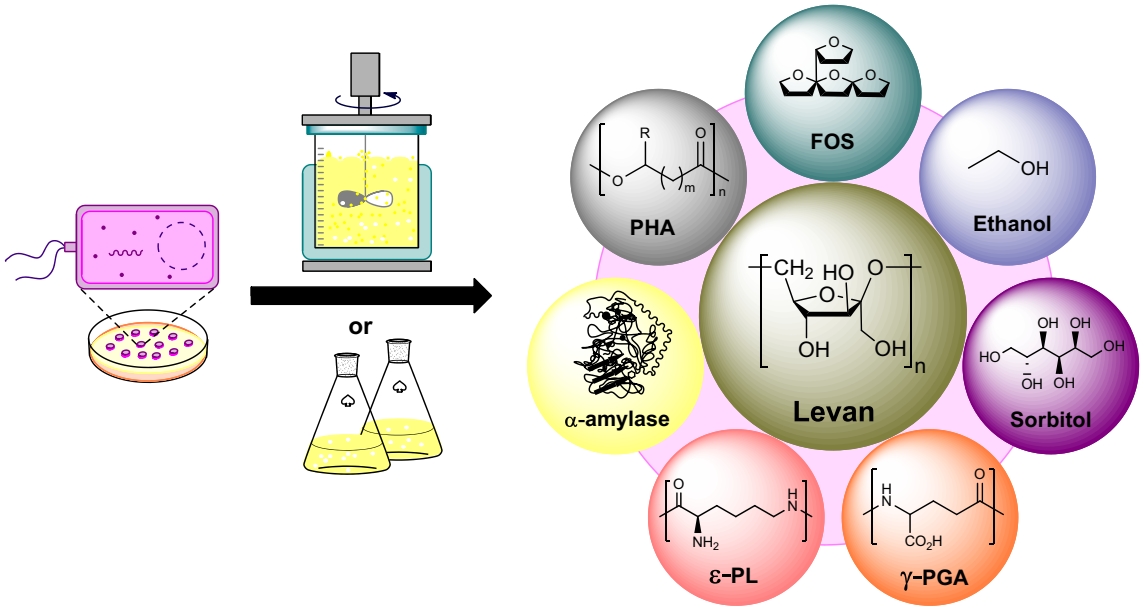

Fig. 3. Co-production of levan with other bio-products of high market value. The co-production of levan with other compounds can be carried out in batch or batch fed in shaker flasks or bioreactor.

(Table 2).

In another work, Bekers et al. [47] used biomass and culture liquid (cell-free) from the co-synthesis of ethanol and levan to obtain FOS by *Z. mobilis* 113 "S". In the second fermentation stage, the cell-free supernatant showed high activity for FOS synthesis (31 % of total carbohydrates). At this stage, levansucrase activity for levan production was approximately 30–40 % of the total activity.

A tandem system was designed for the production of levan and ethanol through microbial fermentation using sucrose as substrate [49]. The tandem process involved the production of levan by *B. subtilis* (natto) Takahashi in a sucrose medium, the separation of levan by ultrafiltration to obtain glucose as a byproduct and the fermentation of the remaining glucose by *Z. mobilis* to produce ethanol. The fermentation of *B. subtilis* (natto) Takahashi in a medium containing 250 g/L of sucrose yielded 56.0 g/L of levan in a 48-h period. After the removal of the cells, the fermented broth with the remaining glucose was incubated with *Z. mobilis* at 30 °C, pH 5.5 and 175 rpm for 120 h, generating 21.1–26.5 g/L of bioethanol. The tandem process presented in this work has an ecological advantage, as the entire substrate was used without wasting any of the byproducts in the process. Moreover, the volume of alcohol necessary for the isolation of levan could be reduced by one-quarter of the volume normally used in conventional precipitation.

The joint production of levan and ethanol has also been investigated in fermentation processes with bacteria on different immobilization supports. Bekers et al. [48] analyzed the production of levan and ethanol by *Z. mobilis* linked to stainless-steel wire spheres (WS) and granules of Al_2O_3 in comparison to cells trapped in calcium alginate. No significant differences were found in the production of levan and ethanol between the two fermentation systems. The maximum concentration of ethanol and levan at the end of the second and third fermentation cycles reached rates of 2.3–2.7 % and 1.3–1.5 %, respectively. WS and Al_2O_3 bacterial immobilization systems can improve the supply of inoculum for the fermentation of sucrose and consequently produce levan and ethanol with greater efficiency compared to the suspended cell-free biomass.

Santos and Cruz [49] studied the co-synthesis of levan and ethanol by *Z. mobilis* immobilized on alginate and chitosan granules and evaluated the capacity of the granules for reuse in sequential fermentations. The chitosan support achieved higher levan yields, whereas alginate resulted in higher ethanol titers (Table 2). In the sequential batch fermentation recycling the immobilization support, better results were achieved with the alginate support, which was efficient in maintaining cell viability on the support to produce 21.11 g/L of levan and 87.21 g/L of ethanol. The chitosan support proved to be inadequate for sequential batches, as the granules dissolved after the second cycle.

In another work, Santos and Cruz [50] designed two experiments for the joint production of levan and ethanol by *Z. mobilis* using luffa and sugarcane bagasse as immobilization supports. The reuse capacity of the supports was evaluated during 12 days of recycling in subsequent fermentations. The results revealed that the luffa was the better immobilization support, presenting 2.58 g/L of immobilized cells and 19.13 g/L of levan. The supported sugarcane bagasse yielded a higher quantity of ethanol (103.82 g/L). In the recycling experiment, the bagasse support was more promising, maintaining cell viability up to the last fermentation cycle. This support also reached higher concentrations for immobilized biomass, levan and ethanol with efficient sucrose consumption (92.64 %) (Table 2).

Strains deficient in extracellular proteases can exhibit a higher production of extracellular protein and, consequently, increase the production of levan and ethanol [16]. For instance, besides levansucrase (*sac*A), *Z. mobilis* produces another extracellular enzyme (*sac*C) and the two sucrases were investigated for the co-production of levan and ethanol [43]. A mutant strain of *Z. mobilis* CT2 with *sac*C from altered sucrase designed prior to the deletion of the gene resulted in a greater quantity of levan (27.2 g/L) compared to that achieved with the parental B14023 strain (15.5 g/L) (Table 2) using 200 g/L of sucrose at 25 °C and pH 5.0. The increase in the fermentation temperature from 25 to 35 °C increased the concentration of ethanol from 17.8 to 46.4 g/L due to the increase in the hydrolysis rate of the sucrose, but the transfructosylation activity was reduced from 165 to 55 U/mL. Supplementation with glucose or fructose to the culture medium significantly diminished the production of levan due to the inhibition of levansucrase activity.

The identification of differentially expressed proteins in the production of levan and ethanol by *Z. mobilis* was determined by proteomic analysis to provide a global profile of regulating proteins [51]. Most proteins detected were found to participate in the metabolism of carbohydrates (57 %), serving as enzymes that catalyze fundamental reactions in several metabolic pathways for the biosynthesis of ethanol, levan and other compounds. The levan and ethanol yields as well as the amount of sucrose consumed changed with the temperature where highest levels were achieved at 20 °C (Table 2).

3.2. Sorbitol

Sorbitol is a naturally occurring sugar alcohol used in the biobased synthesis of polyurethane, as an excipient of formulations for several medications and as an osmoprotectant [52,53]. The co-formation of levan and sorbitol was reported with strains of *Z. mobilis* in a fermentation medium containing sucrose, glucose and fructose [18,52].

Starting with a fermentation medium containing 150 g/L of sucrose and 2.5 g/L of yeast extract with *Z. mobilis* VTT-E-78082 isolated as a beer contaminant and *Z. mobilis* CP 4, Viikari [18] obtained levan and sorbitol at quantities of 8 % and 11 % of the original sucrose content, respectively (Table 2). For the first 6 h of fermentation, constant fructose and glucose concentrations suggested comparable rates of sucrose utilization and ethanol/levan co-production. The concentration of ethanol was slightly superior to that of sorbitol when using the VTT-E-78082 strain. The ethanol yield from the fermentation of both strains of *Zymomonas* was small due to the formation of levan and sorbitol. According to the authors, the formation of sorbitol can be performed in concert with the production of ethanol, as its maximum production (17 g/L) occurred with the greatest concentration of ethanol.

Borsari et al. [52] used a complete factorial design (2^3) to obtain responses on the production of levan, biomass, ethanol and sorbitol by *Z. mobilis* CP4, analyzing the fermentation conditions, type of substrate, concentrations and fermentation processes (batch and fed batch). The greatest concentration of levan (40.14 g/L) was achieved with 150 g/L of sucrose in the batch fermentation process. The addition of sugarcane juice to the fermentation medium favored bacterial growth and, consequently, increased the formation of sorbitol and ethanol. The best conversion coefficient into ethanol was 73.64 % and 71.48 % in the batch and fed batch systems, respectively, using 50 g/L of sugarcane juice. Regarding sorbitol, the best production was 17.36 g/L, with 41.71 % conversion efficiency. The greatest concentration of sorbitol was 11.32 and 11.41 g/L, with conversion efficiency of 20.17 % and 19.62 %, respectively.

3.3. Poly-γ-glutamic acid

Like levan, poly-γ-glutamic acid (γ-PGA) is a viscous, high molecular weight substance that is soluble in water and physiologically active. It has recognized effects on the increase in thrombus and immunological function [16,54]. Recently, it has been verified that a diet (5 %) of the mixture of PGA and levan (6:4 *w*/w) was significantly reduced epididymal fat ratio and fat cell size in C7BL/6 mice after 12 weeks [54]. Moreover, γ-PGA participates in the formation of bacterial biofilm, reticulating with other compounds to finalize the biofilm [16]. The co-production of levan and γ-PGA has mainly been reported with strains of *Bacillus* [55].

The joint production of levan and γ-PGA from *B. subtilis* (natto) Takahashi was achieved in a medium containing 5 % (*w*/w) sucrose and 1.5 % (w/w) L-glutamate [56]. Under these conditions, around 4–5 g/L (42 %, w/w) of levan and 6.9 g/L (58 %, w/w) of γ-PGA were obtained in a single batch fermentation in 21 h at pH 6.0 and a temperature range of 25 to 40 °C (Table 2). When cultivated in a medium containing 20 % (w/w) sucrose, *B. subtilis* (natto) Takahashi selectively produced levan but without L-glutamate.

B. amyloliquefaciens NK-1 is another strain with the potential for the joint production of levan and γ-PGA [15]. The creation of the NK-ΔLP strain (deficient in γ-PGA) from *B. amyloliquefaciens* NK-1 yielded only 1.97 g/L of levan. The optimization of the culture conditions using the response surface methodology (RSM) yielded 13.9 g/L and 22.6 g/L of levan in a culture flask and bioreactor, respectively (Table 2). The purity of the levan produced by the NK-ΔLP strain reached 92.7 % in 48 h. The two strains achieved comparable γ-PGA production. The mass fraction of γ-PGA was 3.53 g/L and 3.61 g/L for the NK-L and NK-1 strains, respectively. Moreover, the NK-L strain exhibited increased purity of the γ-PGA product (91.2 %), which was higher than that achieved with the NK-1 control stain (79.5 %).

The *B. amyloliquefaciens* NK-ΔLP strain is capable of producing levan of high purity but with a relatively low yield [15,16]. To improve levan production, gene encoding for the TasA biofilm-forming protein, pgsBCA cluster harboring genes of the γ-PGA synthesis pathway as well as six extracellular protease genes were deleted from NK-1. Among the mutant strains, NK-Q-7 and NK-Q-1 strains produced 31.1 g/L and 17.5 g/L levan in flasks, which were 103 % and 14.4 % higher than the NK-ΔLP strain. However, not all mutant strains were that efficient and in fact, NK-Q-4 and NK-Q-5 strains led to 21.3 % and 32.4 % lower levan yields, respectively [16].

When the wild type and mutant strains were compared for their γ-PGA production, only the NK-P-5 strain showed an increase in comparison to NK-1 and lower yields were observed for strains with deleted nprE and aprE (Table 2). The dry biomass of the NK-P-7 strain was also lower than that of the other strains [16].

Regarding the results related to the fermentation of levan and α-amylase with *B. amyloliquefaciens* NK-ΔLP and the NK-Q-X mutant strains, the NK-Q-7 strain reached higher levan yields, with 102 % and 78 % increases in comparison to the NK-ΔLP and NK-Q-1 strains, respectively. Likewise, the NK-Q-7 strain achieved higher amylase yields and enzyme activities when compared to NK-Δ LP and NK-Q-1 strains and thus could be used as a biofactory for the production of secreted proteins or the synthesis of products related to these proteins [16].

Stains of *B. subtilis*, including the starter of fermented soybean (natto), produce capsular polymers consisting of γ-PGA and levan [57]. Capsular polymers are known to protect cells from infection by phages. However, the bacteriophage ϕNIT1 carries a γ-PGA hydrolase gene (pghP), which helps neutralize the protection strategy of the host cell. A comparative genomic analysis revealed the diversity among ϕNIT1 phages and *Bacillus* carrying genes similar to these hydrolases and hence provided a new perspective to understand their acquisition mechanism.

Recently, Song, Kwon & Song [58] applied CRISPR-Cas9 mediated engineering to the natural soil isolate *Bacillus licheniformis* 4071 strain for simultaneous and selective production of exopolymers and polyols. Exopolymers were exopolysaccharides (EPS), levan synthesized by sacB levansucrase and γ-PGA produced by γ-PGA synthase encoded by pgsBCAE operon. When *epsAB* genes for tyrosine kinases controlling EPS synthesis were deleted from the 4071 strain, the produced exopolymers were higher in levan and lower in EPS and γ-PGA content compared to wild type strain that was attributed to a tightly linked regulatory control of pgsBCAE and epsA-O operons reported for biofilm formation and authors suggested that deletion of *epsAB* genes as a key strategy for selective production of levan. For the polyols, glycerol, (2 *R*,3 *S*)- butanediol and (2 *R*,3 *R*)- butanediol production were controlled by deleting their respective genes *dgp*, *budC* and *gdh*. From the several combinations, the most efficient and selective co-production was achieved with 71.0 g/L of levan and 88.6 g/L of (2 *R*,3 *S*)-butanediol by fed-batch fermentation (Table 2).

3.4. Poly-ε-L-lysine

Poly-ε-L-lysine (ε-PL) is an atypical biopolymer composed of L-lysine connected between α-carboxyl and ε-amine groups. It has excellent properties, such as solubility in water, stability in the presence of heat, biodegradability and non-toxicity. Due to its properties, ε-PL has been used as a conservative in foods and cosmetics, a drug carrier and a gene carrier in gene therapy [59]. Recent studies have concentrated on biotechnological production and the understanding of the biosynthetic mechanisms of microbial ε-PL [60].

Shih et al. [17] developed a strategy to make the production of ε-PL economically viable using residues from the fermentation of levan by *Streptomyces albulus*. Sequential fermentation for the production of the two biopolymers began with the production of levan by *B. subtilis* (natto) in a medium containing sucrose. The product and cells were separated by ultrafiltration and the remaining broth composed of small sugar

molecules was used for the production of e-PL by *S. albulus*. After the second stage of the process, 60 g/L of levan and 4.37 g/L of e-PL were obtained over a 72-hour period. The work points to a current approach for the reduction in the use of solvent for biopolymer recovery and the use of the fermentation residue from the first stage to produce a second valuable product. This process is considered ecologically correct an in line with the concept of biorefinery.

3.5. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are linear polyesters formed by hydroxy acid monomers and synthesized by a broad gamut of bacteria and accumulate intracellularly as a carbon and energy reserve. While PHAs are considered an alternative to petroleum-based polymers due to their biodegradability, the high production cost limits their acceptance by industries [61,62]. The co-production of exopolysaccharides (EPSs, such as levan) and PHA is a frequently observed characteristic among the halophile family Halomonadaceae.

Tohme et al. [12] studied the co-production of levan and PHB by *Halomonas smyrnensis* under illimited culture conditions using simple carbon sources, such as glucose or sucrose. Fermentation was performed in shake flasks using 137.2 g/L of sea salt from the Çamaltı region in Turkey at pH 7.0 and a temperature of 37 °C. Using 20 g/L of glucose, concentrations of PHA reached a maximum after 120 h, with 1.34 g/L (45.8 %). In the presence of glucose, no levan was detected or recovered from the fermentation medium after the removal of the cells. When the cultures were cultivated in 50 g/L of sucrose, the greatest concentration of PHA was 0.48 g/L (27 % PHA in biomass) after 144 h. In parallel with PHB, 15.3 g/L of levan were synthesized in the presence of sucrose.

In a more recent study by the same group, *H. smyrnensis* AAD6^T mutants with increased levan and PHB productivity were generated through sequential UV irradiation and chemical mutagenesis by ethyl methanesulfate (EMS) treatment and their whole genomes were comparatively analyzed to identify gene candidates linked to enhanced productivity [20]. *H. smyrnensis* BAE2 mutant strain stood out with the highest levan and PHB (3.15 ± 0.18 g/L) yields and a three-fold higher levan productivity when compared with the AAD6 wild type strain. Experiments with different salt concentrations in the fermentation medium revealed that for both the wild type and mutant strains, the optimum salt concentration was the same for levan production as well as for PHB accumulation. Hence this was particularly significant because two valuable biopolymers, levan and PHB, could be efficiently co-produced under the same conditions. Another interesting aspect of this study was the comparative genome and metabolic pathway analysis that was done in order to investigate the potential molecular mechanisms associated with the observed differences over the growth, substrate utilization and PHB/levan production profiles between the wild type strain and mutants. For this, deletions, insertions and single/multi nucleotide variations within the genome sequences of mutants were identified and lack of any change in the levansucrase gene sequence of mutants suggested the possible indirect effect of any other factor that could change the expression and secretion of the enzyme. On the other hand, there were some mutations accumulated within several tripartite tricarboxylate transporter genes and TCA enzymes which in turn pointed to the central role of TCA cycle-associated genes in the observed significant changes in the mutant's physiology reflected in their growth and levan production profiles.

Besides these, there were also interesting changes in genes associated with osmoregulatory mechanisms. Especially the mutations of the glutamate, choline and glycine betaine transport proteins may have led to a dysfunctioning uptake mechanisms of these key cytoplasmic osmoprotectants. Hence as a physiological response to alleviate the osmotic stress, these mutants with impaired osmoregulation mechanism were suggested to protect themselves with a thick levan exopolysaccharide matrix, which in turn is a well-known role of EPSs in nature [20].

Vega-Vidaurri et al. [63] isolated three bacterial strains co-producing PHB and EPS (levan and inulin types). The strains identified as *Bacillus thuringiensis* HA1 and *Suhomyces kilbournensis* HD1 were able to synthesize PHA and levan, whereas an AJ1 isolate (unidentified) was able to produce PHA and inulin. After 72 h of submerged fermentation, *B. thuringiensis* HA1 produced 0.058 g/L of PHA and 16.5 mg/L of levan, while *S. kilbournensis* HD1 synthesized 0.148 g/L and 86 mg/L of PHA and levan, respectively (Table 2). In turn, the AJ1 isolate produced 0.044 g/L of PHA and 15 mg/L of EPS of the inulin type.

4. Conclusions and future perspectives

The state of the art of simultaneous production strategies clearly shows that there is a high potential in such microbial and enzymatic processes and within the last decade, the spectrum of products increased from only a few like FOS and ethanol to diverse compounds involving popular biodegradable polymers like γ-PGA and PHB. Moreover, integrative approaches for the co-production of these bioproducts are in line with the concept of biorefinery, the aim of which is a zero-waste policy. Hence a single-cell biorefinery can compensate for the limited availability of levan by generating a byproduct with high value for the market. The selection of microbial strains that generate high mass fractions of bioproducts and the use of inexpensive carbon sources will assist in designing sustainable manufacturing systems for large-scale production.

Also, the use of halophilic cultures as cell factories may open new frontiers in this aspect by allowing co-production at high salt conditions that in turn will enable the use of open microbial production systems with very low risk of contamination. This will further reduce the production costs via subsiding the costs of sterilization and preventing contamination. With the use of systems engineering principles, the producer strains can further be improved in their productivity. Studies with halophilic systems led to some interesting viewpoints worth to mention here. While there are many studies focused on engineering the levansucrase enzyme sequence or optimization of the substrate composition of the production medium, systems-based studies pointed to the importance of other metabolic pathways that may interplay with levan production. In an early study, mannitol was determined as a critical metabolite for levan production by metabolic systems analysis and supplementing the medium with this osmolyte increased levan titers up to two-fold along with a higher sucrose utilization rate [64]. A subsequent study pointed to the possible regulatory role of Quorum Sensing (QS) phenomenon where boric acid supplementation was found to improve both sucrose utilization and levan production [65]. Then, through genome-based metabolic systems engineering approach, in silico simulations of our genome scale metabolic model for *H. smyrnensis* AAD6T led to a list of gene knockout strategies which emphasized the vital role of the fructose uptake mechanism and pointed out the fructose-specific phosphotransferase system (PTS_{fru}) as the most promising target for further metabolic engineering studies. The new strain BMA14 was constructed by insertional mutagenesis of PTS_{fru} and triparental mating and it further improved the levan titers [66]. The importance of osmoregulatory mechanisms and transferase systems were again verified by a recent mutagenesis study where comparative genome analysis and metabolic pathway analysis pointed to significant differences between the genes related to transport reactions and osmolytes [20]. These studies clearly suggested that for future studies, a “pull strategy” by targeting the transport reactions could be adopted rather than a “push strategy” where cells are exposed to increased substrate concentrations. Moreover, since survival under stress is very much related to the adaptive remodelling of gene expression profiles, transcriptome studies revealing the transcriptional regulatory mechanisms may provide further insights on the suggested interplay between osmoregulation, quorum sensing and levan biosynthesis and lead to novel targets for metabolic engineering strategies.

Another elegant approach is the CRISPR-Cas9 mediated engineering

of *B. licheniformis* for selective production of EPS, levan and γ-PGA along with glycerol and butanediol isomers [58]. The constructed strains hold high industrial value since their product spectrum can easily be controlled by supplying the respective substrate to the fermentation medium.

Besides these cultivation-based strategies, enzymatic routes leading to multiple products along with levan also show great potential for future applications. One interesting example is the bioconversion of levan to difructose anhydrides (DFAs), which are tricyclic fructo-disaccharides having several health benefits such as decreasing body fat, acting as immunomodulator, anti-pathogenic and anti-cancerous agents and prebiotic sucrose substitutes, inducing calcium absorption as well as wound healing in the intestines [67]. In this context, multi-enzyme cascade reactions using glycoside hydrolase 32 (GH32) family levan degrading enzymes of levanase (EC 3.2.1.65), levanbiohydrolase (EC 3.2.1.64) and levan fructotransferase (EC 4.2.2.16) is a very promising strategy to obtain levan oligosaccharides, levanbiose along with DFA IV [68]. While one-pot DFA IV production from sucrose through co-fermentation of two recombinant *Saccharomyces cerevisiae* strains excreting these enzymes was reported [69], an efficient one-pot enzymatic process to obtain highly valuable DFAs from common table sugar still calls for further research.

Overall, holistic approaches where omic data are obtained faster at lower costs will also open new frontiers to levan producers in terms of the search for overproducer strains and optimization of production conditions. There are numerous tools for analyzing and integrating omic data in designing bioprocesses and in near future, they will become a crucial mandatory part in every knowledge-based production strategy. For levan production, this systems-based approach is still at its infancy and limited to few studies. Even a standard genome analysis may reveal interesting new byproducts for a cell factory or novel enzymes for bioconversion. There is no doubt that artificial intelligence and machine learning will soon lead to more accurate and cheaper gene editing as well as to more precise structural predictions and hence better strategies for engineering enzymes and strains more effectively.

Generally, the ever-increasing interest in levan polysaccharide produces a huge pressure on the industry for coping up the demand at affordable prices and the spectrum of co-products are expected to increase following this course. Also consumer demand towards natural products made from renewable resources and the positive impact of the pandemic on the markets for immune boosting formulations and functional foods will serve as additional forces to make levan production reach economic feasibility on a commercial scale. On this way, in silico assisted knowledge is expected to lead this very challenging race of providing levan for high value applications at affordable prices.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] J. Combie, E.T. Öner, From healing wounds to resorbable electronics, Levan can fill bioadhesive roles in scores of markets, Bioinspir. Biomim. 14 (1) (2019) 011001.

[2] M.E. Ortiz-Soto, J.R. Porras-Domínguez, J. Seibel, A. López-Munguía, A close look at the structural features and reaction conditions that modulate the synthesis of low and high molecular weight fructans by levansucrases, Carbohydr. Polym. 219 (2019) 130–142.

[3] O. Kırtel, E.T. Öner, Levan polysaccharide for biomedical applications, in: Soft Matter for Biomedical Applications, 2021, pp. 134–154.

[4] E.T. Öner, L. Hernández, J. Combie, Review of Levan polysaccharide: from a century of past experiences to future prospects, Biotechnol. Adv. 34 (5) (2016) 827–844.

[5] S.K. Gupta, P. Das, S.K. Singh, M.S. Akhtar, D.K. Meena, S.C. Mandal, Microbial Levan, an ideal prebiotic and immunonutrient in aquaculture, World Aquac. (2011) 61–64.

[6] S.A. Kang, K.H. Jang, J.W. Seo, K.H. Kim, Y.H. Kim, D. Rairakhwada, M.Y. Seo, J. O. Lee, S.D. Ha, C.H. Kim, S.K. Rhee, Levan: applications and perspectives, in: Microbial Production of Biopolymers and Polymer Precursors: Applications and Perspectives, 2009, pp. 145–161.

[7] A.D. Sezer, H.K. Sarılmiser, E. Rayaman, A. Cevikbaş, E.T. Öner, J. Akbuğa, Development and characterization of vancomycin-loaded Levan-based microparticular system for drug delivery, Pharm. Dev. Technol. 1–8 (2015).

[8] E.C. Siqueira, J.S. Rebouças, I.O. Pinheiro, F.R. Formiga, Levan-based nanostructured systems: an overview, Int. J. Pharm. 580 (119242) (2020) 1–11.

[9] S.J. Kim, P.K. Bae, B.H. Chung, Self-assembled Levan nanoparticles for targeted breast cancer imaging, Chem. Comm. 51 (1) (2015) 107–110.

[10] Á. González-Garcinuño, A. Tabernero, Á. Domínguez, M.A. Galán, E.M.M.Del Valle, Levan and levansucrases: Polymer, enzyme, micro-organisms and biomedical applications, Biocatal. Biotransform. 36 (3) (2017) 1–12, 2422.

[11] E.C. Siqueira, B. Doboszewski, J.S. Rebouças, I.O. Pinheiro, E.T. Öner, F. R. Formiga, Molecular modification of Levan and biotechnological applications of its derivatives, in: Microbial Exopolysaccharides: Current Research and Developments, 2019, pp. 247–294.

[12] S. Tohme, G.G. Hacıosmanoğlu, M.S. Eroğlu, C. Kasavi, S. Genç, S.G. Can, E. T. Oner, Halomonas smyrnensis as a cell factory for co-production of PHB and Levan, Int. J. Biol. Macromol. 18 (PtA) (2018) 1238–1246.

[13] O. Ates, Systems biology of microbial exopolysaccharides production, Front. Bioeng. Biotechnol. 3 (200) (2015) 1–16.

[14] A. Hill, F. Tian, S. Karboune, Synthesis of Levan and fructooligosaccharides by levansucrase: catalytic, structural and substrate-specificity properties, Curr. Org. Chem. 21 (2) (2017) 149–161.

[15] J. Feng, Y. Gu, L. Han, K. Bi, Y. Quan, C. Yang, W. Zhang, W. Cao, S. Wang, W. Gao, Y. Sun, C. Song, Construction of a bacillus amyloliquefaciens strain for high purity Levan production, FEMS Microbiol. Lett. 362 (11) (2015) 1–7.

[16] J. Feng, Y. Gu, Y. Quan, W. Zhang, M. Cao, W. Gao, C. Song, C. Yang, S. Wang, Recruiting a new strategy to improve Levan production in bacillus amyloliquefaciens, Sci. Rep. 5 (13814) (2015) 1–12.

[17] I.L. Shih, T.C. Wang, S.Z. Chou, G.D. Lee, Sequential production of two biopolymers-Levan and poly-ε-lysine by microbial fermentation, Bioresour. Technol. 102 (4) (2011) 3966–3969.

[18] L. Viikari, Formation of Levan and sorbitol from sucrose by zymomonas mobilis, Appl. Microbiol. Biotechnol. 19 (1984) 252–255.

[19] I.L. Shih, L.D. Chen, T.C. Wang, J.Y. Wu, K.S. Liaw, Tandem production of Levan and ethanol by microbial fermentation, Green Chem. 12 (7) (2010) 1242–1247.

[20] B.A. Erkorkmaz, O. Kırtel, G. Abaramak, E. Nikerel, E.T. Öner, UV and chemically induced Halomonas smyrnensis mutants for enhanced Levan productivity, J. Biotechnol. 356 (2022) 19–29.

[21] P. Santos-Moriano, L. Fernandez-Arrojo, A. Poveda, J. Jimenez-Barbero, A. O. Ballesteros, F.J. Plou, Levan versus fructooligosaccharide synthesis using the levansucrase from zymomonas mobilis: effect of reaction conditions, J. Mol. Catal. B Enzym. 119 (2015) 18–25.

[22] M. Bekers, J. Laukevics, D. Upite, E. Kaminska, A. Vigants, U. Viesturs, L. Pankova, A. Danilevics, Fructooligosaccharide and Levan producing activity of zymomonas mobilis extracellular levansucrase, Process Biochem. 38 (5) (2002) 701–706.

[23] F. Tian, S. Karboune, Enzymatic synthesis of fructooligosaccharides by levansucrase from bacillus amyloliquefaciens: specificity, kinetics, and product characterization, J. Mol. Catal. B Enzym. 82 (2012) 71–79.

[24] L. Inthanavong, F. Tian, M. Khodadadi, S. Karboune, Properties of Geobacillus stearothermophilus levansucrase as potential biocatalyst for the synthesis of Levan and fructooligosaccharides, Biotechnol. Prog. 29 (6) (2013) 1405–1415.

[25] M. Li, S. Seo, S. Karboune, Bacillus amyloliquefaciens levansucrase-catalyzed the synthesis of fructooligosaccharides, oligolevan and Levan in maple syrup-based reaction systems, Carbohydr. Polym. 20 (133) (2015) 203–212.

[26] G.T. Bersaneti, N.C. Pan, B. Cristiani, M.A.P.C. Celligoi, Co-production of fructooligosaccharides and Levan by levansucrase from Bacillus subtilis natto with potential application in the food industry, Appl. Biochem. Biotechnol. 184 (3) (2018) 838–851.

[27] H.K. Kang, M.Y. Seo, E.S. Seo, D. Kim, S.Y. Chung, A. Kimura, D.F. Day, J.F., Robyt, cloning and expression of levansucrase from Leuconostoc mesenteroides B-512 FMC in Escherichia coli, Biochim. Biophys. Acta - Gene Struct. Express. 1727 (1) (2005) 5–15.

[28] K.S. Belghith, I. Dahech, H. Belghith, H. Mejdou, Microbial production of levansucrase for synthesis of fructooligosaccharides and Levan, Int. J. Biol. Macromol. 50 (2) (2012) 451–458.

[29] A. Nasir, F. Sattar, I. Ashfaq, S.R. Lindemann, M.H. Chen, W. Van den Ende, E. T. Öner, O. Kirtel, S. Khaliq, M.A. Ghauri, M.A. Anwar, Production and characterization of a high molecular weight Levan and fructooligosaccharides from a rhizospheric isolate of bacillus aryabhattai, LWT - Food Sci. Technol. 123 (109093) (2020) 1–8.

[30] S. Karboune, S. Seo, M. Li, A. Waglay, L. Lagacé, Biotransformation of sucrose rich maple syrups into fructooligosaccharides, oligolevans and levans using levansucrase biocatalyst: bioprocess optimization and prebiotic activity assessment, Food Chem. 382 (132355) (2022) 1–9.

[31] C. Possiel, M.E. Ortiz-Soto, J. Ertl, A. Münch, A. Vogel, R. Schmiedel, J. Seibel, Exploring the sequence variability of polymerization-involved residues in the production of Levan- and inulin-type fructooligosaccharides with a levansucrase, Sci. Rep. 9 (7720) (2019) 1–11.

[32] A. Homann, R. Biedendieck, S. Götze, D. Jahn, J. Seibel, Insights into polymer versus oligosaccharide synthesis: mutagenesis and mechanistic studies of a novel levansucrase from bacillus megaterium, Biochem. J. 407 (2) (2017) 189–198.

[33] F.W. Waldherr, D. Meissner, R.F. Vogel, Genetic and functional characterization of Lactobacillus panis levansucrase, Arch. Microbiol. 190 (4) (2008) 497–505.
[34] J.R. Porras-Domínguez, M.E. Ávila-Fernández, M.E. Rodríguez-Alegría, A. Miranda-Molina, A. Escalante, R. González-Cervantes, C. Olvera, A. López-Munguía, Levan-type FOS production using a bacillus licheniformis endolevanase, Process Biochem. 49 (5) (2014) 783–790.
[35] J.R. Porras-Domínguez, M.E. Rodríguez-Alegría, Á. Ávila-Fernández, S. Montiel-Salgado, A. López-Munguía, Levan-type fructooligosaccharides synthesis by a levansucrase-endolevanase fusion enzyme (LevB1SacB), Carbohydr. Polym. 177 (1) (2017) 40–48.
[36] J.S. Jadaun, L.K. Narnoliya, N. Agarwal, S.P.S. Singh, Catalytic biosynthesis of Levan and short-chain fructooligosaccharides from sucrose-containing feedstocks by employing the levansucrase from leuconostoc mesenteroides MTCC10508, Int. J. Biol. Macromol. 127 (2019) 486–895.
[37] P. Phengnoi, T. Charoenwongpaiboon, K. Wangpaiboon, M. Klaewkla, S. Nakapong, W. Visessanguan, K. Ito, R. Pichyangkura, K. Kuttiyawong, Levansucrase from Bacillus amyloliquefaciens KK9 and its Y237S variant producing the high bioactive Levan-type fructooligosaccharides, Biomol. 10 (5) (2020) 1–13.
[38] M.G. Kim, J.W. Seo, K.B. Song, C.H. Kim, B.H. Chung, S.K. Rhee, Levan and fructosyl derivatives formation by a recombinant levansucrase from rahnella aquatilis, Biotechnol. Lett. 20 (4) (1998) 333–336.
[39] J.R. Xavier, K.V. Ramana, Optimization of Levan production by cold-active bacillus licheniformis ANT 179 and fructooligosaccharide synthesis by its levansucrase, Appl. Biochem. Biotechnol. 181 (2017) 986–1006.
[40] A. Braga, D. Gomes, C. Amorim, S.C. Silvério, J. Alves, J. Rainha, B. Cardoso, J. L. Rodrigues, L.R. Rodrigues, One-step production of a novel prebiotic mixture using zymomonas mobilis ZM4, Biochem. Eng. J. 183 (108443) (2022) 1–8.
[41] A. Braga, D. Gomes, J. Rainha, B.B. Cardoso, C. Amorim, S.C. Silvério, M. Fernandez-Lobato, J.L. Rodrigues, L.R. Rodrigues, Tailoring fructooligosaccharides composition with engineered zymomonas mobilis ZM4, Appl. Microbiol. Biotechnol. 2022b, 106(12) (2022) 4617–4626.
[42] S. Nagamatsu, N. Coutouné, J. José, M.B. Fiamenghi, G.A.G. Pereira, J.V. C. Oliveira, M.F. Carazzolle, Ethanol production process driving changes on industrial strains, FEMS Yeast Res. 21 (1) (2021) 1–10.
[43] V. Senthilkumar, P. Gunasekaran, Influence of fermentation conditions on Levan production by zymomonas mobilis CT2, Indian J. Biotechnol. 4 (2005) 491–496.
[44] E. Lyness, H.W. Doelle, Fermentation pattern of sucrose to ethanol conversions by zymomonas mobilis, Biotechnol. Bioeng. 23 (7) (1981) 1449–1460.
[45] M.J. Beker, J.E. Shvinka, L.M. Pankova, M.G. Laivenieks, I.N. Mezhbarde, A simultaneous sucrose bioconversion into ethanol and Levan by zymomonas mobilis, Appl. Biochem. Biotechnol. 24 (25) (1990) 265–2774.
[46] M. Bekers, R. Linde, A. Danilevich, E. Kaminska, D. Upite, A. Vigant, R. Scherbaka, Sugar beet diffusion juice and syrup as media for ethanol and levan production by Zymomonas mobilis, Food Biotechnol. 13 (1) (1999) 107–119.
[47] M. Bekers, D. Upite, E. Kaminska, D. Grube, J. Laukevics, I. Vina, A. Vigants, P. Zikmanis, Fructan biosynthesis by intra- and extracellular zymomonas mobilis levansucrase after simultaneous production of ethanol and Levan, Acta Biotechnol. 23 (1) (2003) 85–93.
[48] M. Bekers, J. Laukevics, A. Karsakevich, E. Ventina, E. Kaminska, D. Upite, I. Vina, R. Linde, R. Scherbaka, Levan-ethanol biosynthesis using zymomonas mobilis cells immobilized by attachment and entrapment, Process Biochem. 36 (10) (2001) 979–986.
[49] V.A. Santos, C.H.G. Cruz, Ethanol and Levan production by sequential bath using zymomonas mobilis immobilized on alginate and chitosan beads, Acta Sci.-Technol. 38 (3) (2016) 263–271.
[50] V.A. Santos, C.H.G. Cruz, Zymomonas mobilis immobilized on loofa sponge and sugarcane bagasse for Levan and ethanol production using repeated batch fermentation, Braz. J. Chem. Eng. 34 (2) (2017) 407–418.
[51] D.R. Cavalcanti, C.B. Malafaia, T.D. Silva, B.S. Santos, G.M.T. Calazans, M.V. Silva, Protein extraction method for the proteomic study of zymomonas mobilis during production of ethanol and levans, Genet. Mol. Res. 14 (4) (2015) 14406–144421.
[52] R.R.J. Borsari, M.A.P.C. Celligoi, J.B. Buzato, R.S.S. Silva, Influence of carbon source and the fermentation process on Levan production by zymomonas mobilis analyzed by the surface response method, Ciênc. Tecnol. Aliment. 26 (3) (2006) 604–609.
[53] J. Xiang, Y. Saisai, J. Zhang, J. Wu, Y. Shao, Z. Wang, M. Yang, The preparation of sorbitol and its application in polyurethane: a review, Polym. Bull. 79 (2021) 2667–2684.
[54] K.H. Jang, M.J. Kim, A mixture of poly-γ-glutamic acid and Levan ameliorates obesity in high fat diet-induced mice, Food Sci. Biotechnol. 31 (3) (2022) 349–356.
[55] I.L. Shih, Y.T. Yu, C.J. Shieh, C.Y. Hsieh, Selective production and characterization of Levan by Bacillus subtilis (Natto) Takahashi, J. Agric. Food Chem. 53 (21) (2005) 8211–8215.
[56] I.L. Shih, Y.T. Yu, Simultaneous and selective production of Levan and poly (gamma-glutamic acid) by Bacillus subtilis, Biotechnol. Lett. 27 (2005) 103–106.
[57] T. Ozaki, N. Abe, K. Kimura, A. Suzuki, J. Kaneko, Genomic analysis of Bacillus subtilis lytic bacteriophage ϕNIT1 capable of obstructing natto fermentation carrying genes for the capsule-lytic soluble enzymes poly-γ-glutamate hydrolase and levanase, Biosci. Biotechnol. Biochem. 86 (1) (2016) 135–146.
[58] C.W. Song, M. Kwon, H. Song, Simultaneous and selective production of exopolymers and polyols by metabolically engineered bacillus licheniformis strains, Biochem. Eng. J. 181 (108381) (2022) 1–11.
[59] L. Wang, C. Zhang, J. Zhang, Z. Rao, X. Xu, Z. Mao, X. Chen, Epsilon-poly-L-lysine: recent advances in biomanufacturing and applications, Front. Bioeng. Biotechnol. 28 (9) (2021) 1–21, 748976.
[60] Z. Xu, Z. Xu, X. Feng, D. Xu, J. Liang, H. Xu, Recent advances in the biotechnological production of microbial poly(ε-L-lysine) and understanding of its biosynthetic mechanism, Appl. Microbiol. Biotechnol. 100 (15) (2016) 6619–6630.
[61] T.M.M.M. Amaro, D. Rosa, G. Comi, L. Iacumin, Prospects for the use of whey for polyhydroxyalkanoate (PHA) production, Front. Microbiol. 10 (2019) 1–12.
[62] A.A. Alves, E.C. Siqueira, M.P.S. Barros, P.E.C. Silva, L.M. Houllou, Polyhydroxyalkanoates: a review of microbial production and technology application, Int. J. Environ. Sci. Technol. 1–12 (2022).
[63] J. Vega-Vidaurri, F. Hernandez-Rosas, M.A. Rios-Corripio, J.M. Loeza-Corte, M. Rojas-Lopez, R. Hernandez-Martinez, Coproduction of polyhydroxyalkanoates and exopolysaccharide by submerged fermentation using autochthonous bacterial strains, Chem. Pap. 76 (2022) 2419–2429.
[64] O. Ates, K.Y. Arga, E.T. Öner, The stimulatory effect of mannitol on Levan biosynthesis: lessons from metabolic systems analysis of halomonas smyrnensis AAD6T, Biotechnol. Prog. 29 (6) (2013) 1386–1397.
[65] H.K. Sarilmiser, O. Ates, G. Ozdemir, K.Y. Arga, E.T. Öner, Effective stimulating factors for microbial Levan production by halomonas smyrnensis AAD6T, J. Biosci. Bioeng. 119 (4) (2015) 455–463.
[66] B. Aydin, T. Ozer, E.T. Öner, K.Y. Arga, The genome-based metabolic systems engineering to boost Levan production in a halophilic bacterial model. OMICS, J. Integr. Biol. 22 (3) (2018) 198–209.
[67] H. Hang, Recent advances on the difructose anhydride IV preparation from Levan conversion, Appl. Microbiol. Biotechnol. 101 (20) (2017) 7477–7486.
[68] W. Zhang, W. Xu, D. Ni, Q. Dai, C. Guang, T. Zhang, W. Mu, An overview of Levan-degrading enzyme from microbes, Appl. Microbiol. Biotechnol. 103 (19) (2019) 7891–7902.
[69] H. Ko, J.H. Bae, B.H. Sung, M.J. Kim, S.H. Park, J.H. Sohn, Direct production of difructose anhydride IV from sucrose by co-fermentation of recombinant yeasts, Sci. Rep. 9 (1) (2019) 15980.