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Review

Review on production, characterization and applications of microbial levan

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ABSTRACT

Levan is a homopolymer of fructose naturally obtained from both plants and microorganisms. Microbial levans are more advantageous, economical and industrially feasible with numerous applications. Bacterial levans are much larger than those produced by plants with multiple branches and molecular weights ranging from 2 to 100 million Da. However levans from plants generally have molecular weights ranging from about 2000 to 33,000 Da. Microbial levans have wide range of applications in food, medicine, pharmaceutical, cosmetic and commercial industrial sectors. With excellent polymeric medicinal properties and ease of production, microbial levan appear as a valuable and versatile biopolymer of the future. The present article summarizes and discusses the most essential properties of bioactive microbial levan and recent developments in its production, characterization and the emerging applications in health and industry.

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1. Introduction

Levan is a homopolysaccharide contains fructose and this backbone makes the levan a unique carbohydrate polymer (Cerning, 1990). It has been produced by a broad range of micro-organisms as exopolysaccharides (EPS) and by a limited number of plant species as non-structural storage carbohydrates (Ebskamp, Smeekens, & Weisbeek, 1999). In microbes, these polymers contribute to the formation of the EPS matrix and help in microbial biofilm formation (Franken, Brandt, Tai, & Bauer, 2013). Studies on the collection, biosynthesis and production of levan started between 1870 to 1940 in Germany, France and Britain (Hendry & Wallace, 1993). Commercial marketing of polysaccharide polymers started in the year 1930 in the United States of America (Donot, Fontana, Baccou, & Schorr-Galindo, 2012). Insights on the microbial production of levan started as early as in 1930 (Murphy, 1952).

Along with the general properties like bio-compatibility, bio-degradability, renewability, flexibility, and eco friendliness, levan also offers some important biomedical properties such as anti-oxidant, anti-inflammatory, anti-carcinogenic, anti-AIDS, hyperglycaemic inhibitor etc. (Dahech et al., 2011b). It is a natural adhesive and surfactant (Crooks, Gabbianelli, Gunn, & Shanmuganandamurthy, 2009; Kang et al., 2009) and also widely used in cosmetic industry in the preparation of skin creams, moisturizers etc. (Küçükaşik et al., 2011). This wide range utilization of levan in numerous industrial sectors made it a versatile polymer and its cost effective production on large scale is much required to meet the demands all over the world. In this review, we summarize the recent developments in identification, characterization, and commercial production, biomedical and industrial potential of microbial levan.

2. Biosynthesis of levan

Levan is diversely distributed in plants, yeasts, fungi and bacteria (Jang et al., 2003) and its biosynthesis is usually carried out by an enzyme levansucrase (EC 2.4.1.10) also known as sucrose 6-fructosyltransferase (Hernández et al., 1999). A wide range of bacterial strains produce levansucrase including *Lactobacillus johnsonii* and *Lactobacillus gasserii, Bacillus subtilis*, *Aerobacter levanicum*, *S. salivarius* etc. (Han, 1990). Interestingly, *B. subtilis* is able to produce both in inducible and constitutive extracellular levansucrase. Whereas *A. levanicum* is constitutive and endocellular (Abdel-Fattah, Mahmoud, & Esawy, 2005). The main function of levansucrase is to catalyse the transfer of D-fructosyl residues from fructose to yield levan by trans-fructosylation (Donot et al., 2012; Han, 1990).

Levansucrase first gets accumulated in the periplasm, where it adopts its final confirmation and then excreted into the outer environment. In general, two types of excretion mechanisms of levansucrase can be found in microorganisms. In gram-positive bacteria like *Bacillus stearothermophilus*, *B. subtilis, Bacillus amyloliquefaciens*, it is a two-step process *i.e.*, cleavage of signal peptide followed by protein folding. The presence of metallic ions of iron or calcium or a change in the outer environmental pH completes the signal for levansucrase excretion (Donot et al., 2012; Hernández et al., 1999). In gram-negative bacteria like *Erwinia amylovora*, *Glycinea*, *Rahnella aquatilis*, *Zymomonas mobilis* and *P. syringae pv. Phaseolicola*, a signal peptide pathway initiates the secretion of levansucrase into the outer environment (Ben Ammar et al., 2002; Donot et al., 2012; Hestrin, Feingold, & Avigad, 1956).

Levansucrase catalysed microbial biosynthesis of levan is a step by step process starts with transfer of a hexosyl group from a donor molecule to a growing acceptor molecule (Hestrin et al., 1956). Single fructofuranosyl units are added to the C_6 hydroxyl of the non-reducing fructose terminal unit of a growing levan polysaccharide chain (Meng & Fütterer, 2003).

$$\text{Fructose} \sim \text{R} + \text{Enzyme} \square (\text{Fructose} \sim \text{Enzyme})\text{complex} + \text{R}$$

$$(\text{Fructose} \sim \text{Enzyme})\text{complex} + \text{Acceptor} \rightarrow (\text{Fructose} < \text{Acceptor}) + \text{Enzyme}$$

The (Fructose < Acceptor) molecule continues to grow as levan polysaccharide. But, the presence of minimal amount of levan in levan synthesizing system accelerates the rate of polymerization, increases the final yield and aids in the production of homogenous and high molecular weight levan (Hestrin et al., 1956). The symbol "∼" represents Lipmann's symbol for the high-energy bond. This symbol describes the linkage of fructofuranose (fr) to carbonyl of aldose (R) as in the donor (fr ∼ R) or to enzyme (enz) and where "<"represents the linkage of fructofuranose to acceptor, which may be water or the carbinol site in a saccharide (Hestrin et al., 1956). Fig. 1 shows detailed biosynthesis of microbial levan.

Microbial levansucrase is formed and secreted in to the extracellular environment at an acidic pH (Venugopal, 2011). The enzyme levansucrase acts highly on sucrose and has low activity on other sugars like fructose, mannose, raffinose, maltose etc. Studies on the activity of levansucrase on different sugars and sugar derivatives showed that a few sugars and sugar alcohols inhibit the action of this enzyme. But the activity of the enzyme is relatively increased when used with inhibitory sugars in the presence of polyethylene glycol (Avineri-Shapiro & Hestrin, 1945; Han, 1990).

3. Levan in plants

Few plant grasses namely *Agropyron cristatum*, *Dactylis glomerata* and *Poa secunda* produce levan in the form of carbohydrate store houses. These are usually present in stems and leaf sheaths. In growing seasons they get degraded and provide the plant with required carbohydrates for grain filling. Wheat and barley also contain levan in little quantities (Gupta, Das, Singh, Akhtar, Meena, & Mandal, 2011).

4. Chemical structure and properties of levan

4.1. Structure

Carbohydrate polymers continue to attract much attention in scientific research due to their high potential applicability, structural variety and complexity (Dumitriu, 2012). Levan is a nat-

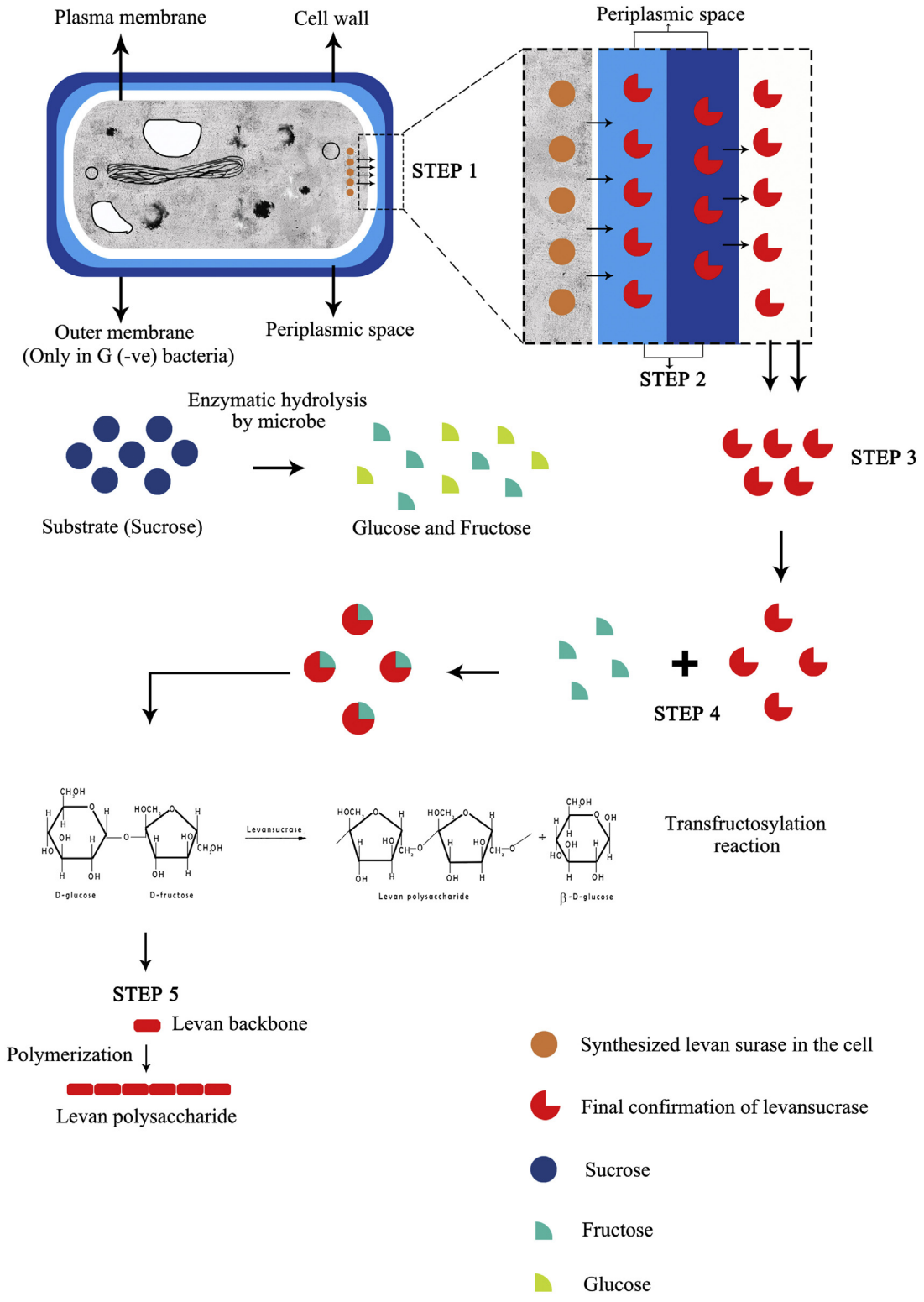

Fig. 1. Schematic representation of biosynthesis of Levan from sucrose. Step 1: Biosynthesis of Levansucrase occurs in the cell. Step 2: Levansucrase gets accumulated into the periplasmic space where it adopts its final confirmation. Step 3: Levansucrase is excreted out of the cell into the surrounding environment either by the cleavage of signal peptide followed by protein folding or by the mechanism initiated through a signal peptide pathway itself. Step 4: Levansucrase acts on the donor molecules (substrates) and the synthesis of Levan starts by transfrucosylation reactions. Step 5: Levansucrase continues adding fructose subunits to the growing Levan polysaccharide chain and finishes the synthesis of Levan.

ural homopolysaccharide composed of D-fructo-furanosyl residues joined together by β-(2,6) linkages and β-(2,1) linkages in core and branch chains respectively and carry a D-glucosyl residue at the end of the chain as shown in Fig. 2. β-(2,1) Linkages contribute to a total of 12% branching in the levan polysaccharide chain (Venugopal, 2011). Levans produced by enzyme systems usually contain 10 to 12 monomeric units but the levan produced by whole cell systems constitute several million residues (Marshall

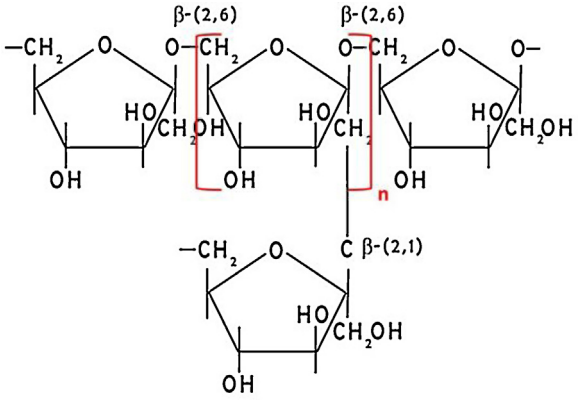

Fig. 2. Levan structure showing the fructane backbone along with β-(2,6) linkages and β-(2,1) branching.

& Weigel, 1980; Ullrich, 2009). Organisms depend on different substrates to produce levans with similar structures. But the degree of polymerization and branching of the repeating unit varies from one to another. The furanose ring structure of levan gives enhanced flexibility to the polysaccharide molecule and the β-(2,6) linked levan polysaccharide molecules tend to be left-hand twist (Gupta et al., 2011; Han, 1990).

Levan is also known as 2,6-β-D-fructan, (2,6-β-D-fructosyl)*n*, (2,6-β-D fructosyl)*n* + 1, 2,6-β-δ-fructan, (2,6-β-δ-fructosyl)*n*, (2,6-β-δ-fructosyl)*n* + 1, and poly fructane with the IUPAC name (2*R*,3*S*,4*S*,5*R*)-5-[[(2*R*,3*S*,4*S*,5*R*)-5-[[(2*R*,3*S*,4*S*,5*R*)-3,4-dihydroxy-2,5-bis(hydroxymethyl)oxolan-2-yl]oxymethyl]-3,4-dihydroxy-2-(hydroxymethyl)oxolan-2-yl]oxymethyl]-2-(hydroxymethyl) oxolane-2,3,4-triol.

4.2. Solubility

Levan is a water soluble carbohydrate polymer that is soluble both in water and oil due to its β-(2,6) linkage. But it does not readily settle out in a water suspension and also possess varying solubility in cold water but is completely soluble in hot water (Gupta et al., 2011). Levan is insoluble in all most all organic solvents like methanol, ethanol, isopropanol, *n*-propanol, acetone, methylethylketone, toluene, ethyl lactate, methyl caprylate/caprate, methylpalmitate/oleate, D-limonene, propylene carbonate, ethylene carbonate, 1-vinyl-2-pyrrolidone, methoxypolyethylene glycol, polyethylene glycol, kerosene, gasoline, dimethyl formamide, ethoxyethyl acetate, acetic anhydride and furfuryl alcohol. Unlike most other polymers levan does not swell in water (Ullrich, 2009).

4.3. Tensile strength

The entangled branches of levan contribute to its cohesive strength and the large number of hydroxyl groups help to form adhesive bonds with a variety of substrates. In general levan is referred as "green" adhesive and dried levan adhesives can be easily removed with water. Levan's tensile strength on aluminium and excellent shear strength on plastics made levan superior to many petrochemical based adhesives. On aluminium, levan has a tensile strength up to 10.3 MPa (1500 psi) depending on the surface finish and preparation method (Rehm, 2009; Ullrich, 2009).

4.4. Viscosity

This fructane is an unusual polysaccharide having low intrinsic viscosity compared to the other high molecular weight molecules. Intrinsic viscosity [η] is a measure of solute's contribution to the viscosity η of a solution. Previous studies have found intrinsic viscosities for levan in water ranging from 0.07 to 0.18 dL/g for levans with molecular weights ranging from 16 to 24 million Da (Ehrlich et al., 1974; Newbrun & Baker, 1968; Stivala & Bahary, 1978)

The viscosity of *Z. mobilis* levan solutions was found to be stable at pH ranging from 4 to 11 at room temperature (Harada, 1965), but are slightly affected by increased salt concentrations and temperatures up to 70 °C (Vina, Karsakevich, Gonta, Linde, & Bekers, 1998). It resembles like chewing gum at 50% solid concentration (Ullrich, 2009). Low viscosity offered by levan encouraged its use in many pharmaceutical applications in the making of capsules and coatings and in several therapeutic applications too.

4.5. Other properties

Other important properties of levan are non-toxic and ocular non-irritant. Levan is fairly heat stable with a melting point temperature of 225 °C (Ullrich, 2009) and is strictly non reducing, not hydrolysed by the action of amylases as well as invertases secreted by the yeast (Gupta et al., 2011) These remarkable properties of levan made it superior in many commercial industrial sectors as standard film forming agent and as a stable polymer.

5. Production and estimation of microbial levan

5.1. Fermentative production of microbial levan

Microbial production of exopolysaccharides is mostly carried out using submerged fermentation techniques (Han, 1990). Cultural conditions required for both the growth of microorganism and the production of polysaccharide is unique for each and every microbe and for the polymer too. Environmental conditions such as pH, temperature, aeration and agitation must be established and optimized for each organism for increased yields. The production of levan can also be done *in vitro* using the enzyme levansucrase with medium containing sucrose as energy source (Abdel-Fattah et al., 2005). Practically the yield of observed microbial levan is less than 58% of the theoretical yield (Rhee et al., 2005).

Different production strategies employ different organisms and organism specific production conditions for better and enhanced yield. Concentration of carbon, nitrogen source and other minor nutrients play an important role in the growth of the microorganism and as well as the production of microbial levan. A comprehensive list of different production strategies is given in Table 1. The microbial production as well as recovery of levan is schemed in Fig. 3.

5.2. Biotransformation of fructose to levan

Apart from the conventional whole cell systems for levan synthesis, cell free systems are also now being widely employed to eliminate the risks of contamination. Levan sucrase, the enzyme responsible for the synthesis of levan is isolated from levan producing microbial strains. The isolated levansucrase can be used in the free or immobilized form depending upon the production requirements (Lim, Lee, Lee, & Song, 2001). However, the yield of levan by cell free systems is comparatively lower than whole cell systems (Abdel-Fattah et al., 2005; Hestrin, Avineri-Shapiro, & Aschner, 1943)

Levan can be qualitatively and quantitatively determined by tests generally used for the estimation of carbohydrate polymers. The hydrolysed levan sample can be estimated by DNS assay for reducing sugar content. The total sugar content could be computed from phenol sulphuric acid assay with sucrose as standard (Poli et al., 2009) and through anthrone test (Dawes & Ribbons, 1966).

Table 1
Microbial Levan production strategies.

S. no.	Organism	Substrate	Method (SmF/SSF)	Production conditions	Reference
1	*Bacillus subtilis*	Sucrose	SmF	Duration: 4 days Temperature: 30 °C	Hestrin et al. (1943)
2	*Bacillus polymyxa*	Sucrose	SmF	Duration: 10 days Temperature: 30 °C	Han (1989)
3	*Bacillus polymyxa*	Sucrose, Sugarcane juice and Beet molasses	SmF	Duration: 10days Temperature: 30 °C Rotary shaker speed: 150 rpm	Han and Watson (1992)
4	*Zymonas mobilis*	Sucrose	SmF	Fermentation at room temperature	Jang et al. (2001a)
5	*Arthrobacter ureafaciens*	Sucrose	SmF	Temperature: 55 °C	Lim et al. (2001)
6	*Zymomonas mobilis*	Sucrose	SmF	Agitation: 200 rpm pH: 6	Alegre, Wendt, and Rigo (2009)
7	*Bacillus subtilis*	Sucrose and L-glutamic acid	SmF	Temperature: 25 to 40 °C for 21 h	Shih and Yu (2005)
8	*Bacillus subtilis*	Sucrose	SmF	Temperature: 37 °C Shaker speed: 150 rpm Time:21 h and pH: 7	Shih, Yu, Shieh, and Hsieh (2005)
9	*Zymomonas mobilis*	(a) Sucrose (b) Yeast extract	SmF	Volume: 300 mL in Pyrex flasks Duration:72 h; Inoculum: 10%(v/v) Agitation: 100 rpm	Melo, Pimentel, Lopes, and Calazans, (2007)
10	*Bacillus subtilis*	Sucrose	SmF	Temperature of 32 to 37 °C for 16 hrs at 150 rpm.	Szwengiel, Czarnecka, Roszyk, and Czarnecki (2004)
11	*Halomonas sp.*	(a) sugar beet molasses (BM) (b) starch molasses (SM)	SmF	Temperature: 37 °C Duration:24 hr Orbital shaker:180	Küçükaşik et al. (2011)
12	*Bacillus subtilis*	Sucrose	SmF	Temperature: 37 °C; pH:6 Agitation speed: 175 rpm Duration:48 hrs	Shih et al. (2010)
13	*Bacillus* sp.	Sucrose	SmF	Optimum pH: 5–10 Temperature: 40 °C	Belghith, Dahech, Belghith, and Mejdoub (2012)
14	*Microbacterium laevaniformans*	Date syrup and sucrose	SmF	Temperature: 37 °C, duration: 48 h, speed: 240 rpm	Moosavi-Nasab, Layegh, Aminlari, and Hashemi (2010)
15	*Pseudomonas fluorescens*	Sucrose	SmF	Fermentation at 40 °C pH 5.2	Jathore et al. (2012)
16	*Gluconacetobacter diazotrophicus*	Sucrose	SmF	Temperature: 30 °C, Reactor volume: 1.5 L pH: 6	Molinari and Boiardi (2013)
17	*Bacillus licheniformis*	Sucrose	SmF	Batch mode fermentation	Arab, Mahmoud, and Higgins (2007)
18	*Bacillus subtilis*	Sucrose and Glucose	SmF	Temperature:30°C	Abdel-Fattah et al. (2005)
19	*Geobacillus stearothermophilus*	Sucrose, fructose glucose, glycerol and raffinose	SmF	pH: 6.0–6.5 Temperature: 47 °C	Inthanavong, Tian, Khodadadi, and Karboune (2013)
20	*Microbacterium laevaniformans*	Sucrose	SmF	pH: 7.0–7.2 Duration: 3 days Temperature: 37 °C	Bae, Oh, Lee, Yoo and Lee (2008)
21	*Bacillus subtilis*	Sucrose	NA	NA	Esawy et al. (2013)
22	*Bacillus subtilis*	Sucrose	SmF	Temperature: 25 to 40 °C Duration: 48 h; Agitation speed: 150–200 rpm; pH: 6	Shih et al. (2005)
23	*Bacillus licheniformis*	Sucrose	SmF	Temperature: 40₀C; pH: 6.4; Agitataion speed: 200 rpm; Duration:36 hrs	Dahech et al. (2013a–c)
24	*Bacillus sp.*	Glucose, fructose, sucrose	SmF	Temperature: 50 °C pH: 6.5	Belghith et al. (2012a)
25	*Microbacterium laevaniformans*	Date syrup (25%) Sucrose (20%)	SmF	pH:6.0 Duration: 48 h Temperature: 37 °C	Moosavi-Nasab et al. (2010)
26	*Zymomonas mobilis*	Sucrose	SmF	NA	de Paula, Pinheiro, Lopes, and Calazans (2008)
27	*Halomonas sp.*	Sugar beet molasses and starch molasses	SmF	Molasses is pre-treated with sulfuric acid, Tricalcium phosphate and activated charcoal treatment	Küçükaşik et al. (2011)

28	*Bacillus subtilis*	Sucrose	SmF	Duration: 21 h pH: 6.0; Temperature: 25–40 °C	Shih and Yu (2005)
29	*Bacillus licheniformis*	Sucrose	NA	NA	Arab et al. (2007)
30	*Zymomonasmobilis* B-14023	Sucrose	SmF	Duration: 42.3 h pH: 6.0	Silbir, Dagbagli, Yegin, Baysal, and Goksungur (2014)
31	*Erwiniaherbicola*	Sucrose	SmF	pH: 7.2; Temperature: 25 °C Duration: 8 days	Keith et al. (1991)
32	*Pseudomonas fluorescens*	Sucrose	SmF	Shaker speed: 180 rpm Temperature: 28 ± 2 °C	Jathore et al. (2012)
33	*Bacillus licheniformis*	Sucrose	SmF	Temperature: 40 °C Shaker speed: 200 rpm Duration: 36 h; pH: 7.4	Dahech et al. (2013a–c)
34	*Bacillus subtilis* (natto)	Sucrose and fructose	SmF	Temperature: 37 °C pH:6.0; agitation speed:175 rpm Duration:55 h	Ing-Lung, Tsaur-Chin, Shou-Zoo, and Gen-Der (2011)
35	*Zymomonas mobilis*	Sucrose, molasses and sugar cane syrup	SmF	Temperature: 25 °C	de Oliveira, da Silva, Buzato, and Celligoi (2007)
36	*Zymomonas mobilis*	Sucrose	SmF	pH:4.0 Temperature: 37 °C	Jang et al. (2001b)
37	*Zymomonas mobilis* (B4286)	Sucrose	SmF	Duration: 18 h Temperature: 30 °C	Ananthalakshmy and Gunasekaran (1999b)
38	*Zymomonas Mobilis*	Sucrose	SmF	Duration: 72 h Temperature: 30 °C	Calazans et al. (2000)
39	*Bacillus subtilis*(6 strains)K,L,A,C,E,G	Sucrose	SmF	Temperature: 30 °C Volume of flask: 50 mL Duration: 24 h	Esawy et al. (2011)
40.	*Bacillus subtilis*	Sucrose	SmF	pH = 7.0; Duration: 24 h Agitation speed: 200 rpm Temperature: 30 °C	Esawy et al. (2013)
41	*Zymomonas mobilis*	Sucrose	SmF (Batch and continuous)	Incubation time: 42.3 h pH: 6.0	Silbir et al. (2014)
42	*Bacillus methylotrophicus*	Sucrose	SmF	Shaker speed: 180 rpm Temperature: 30 °C Duration: 24 h	Zhang et al. (2014)
43	*Bacillus subtilis*	Sucrose	SmF	Working volume: 450 mL	Castillo and López-Munguiá (2004)
44	*Bacillus subtilis*	Sucrose	SmF	RSM was done by taking temperature, pH and culture time as parameters	Yildirim, Tükel, Alagöz, and Alptekin (2011)
45	*Zymomonas mobilis*	Glucose, fructose, sucrose	SmF	NA	de Paula et al. (2008)
46	*Xanthophyllomyces dendrorhous*	Sucrose	SmF	Temperature: 40–70 °C. Duration: 140 h	Linde et al. (2012)
47	*Saccharomyces cerevisiae*	Sucrose	SmF	Duration: 2 days Temperature: 30 °C	Franken et al. (2013)
48	*Lactobacillus reuteri*	Sucrose	SmF	Temperature: 37 °C Duration: 48 h	Sims et al. (2011)
49	*SerratiaLevanicum*	Sucrose	SmF	Aeration: 1 vvm Temperature: 30 °C Duration: 10 h; pH: 7.0	Kikuchi et al. (2010)
50	*Bacillussp. 3B6*	Trypticase-Soy broth	SmF	Temperature: 27 °C Shaker speed: 200 rpm	Matulová, Husárová, Capek, Sancelme, and Delort (2011)
51	*Halomonas species AAD6*	Sucrose	SmF	Temperature: 37 °C pH:7; aeration: 0.1 vvm Agitation: 200 rpm	Poli et al. (2009)
52	*Zymomonas mobilis*	Sucrose	SmF	Duration: 8 days Temperature: 30 °C	Muro, Rodríguez, Abate, and Siñeriz (2000)

Table 1 (*Continued*)

S. no.	Organism	Substrate	Method (SmF/SSF)	Production conditions	Reference
53	*Zymomonas mobilis* Zsuc1	Sucrose	SmF	Working volume: 100 mL Temperature: 30 °C	Ananthalakshmy and Gunasekaran (1999a)
54	*Bacillus licheniformis*	Sucrose	SmF	Temperature: 40 °C pH:5.6; duration: 24 h	Dahech et al. (2011a)
55	*Halomonas sp.*	Sucrose	SmF	Working volume: 500 mL Temperature: 37 °C; pH:7; Aeration: 0.1 VVM; agitation: 200 rpm	Sezer, Kazak, Öner, and Akbuğa (2011)
56	*Bacillus sp. 3B6*	Sucrose	SmF	Duration: 8 days Temperature: 32 °C Agitation: 200 rpm	Matulová et al. (2011)
57	*Bacillus licheniformis*	Sucrose	SmF	pH:5.6; temperature: 40 °C Duration: 24 h	Dahech et al. (2011b)
58	*Bacillus subtilis NRC1aza*	Sucrose	SmF	Temperature: 28 to 35 °C Duration: 8–72 Agitation speed: 50–200 rpm	Abdel-Fattah, Gamal-Eldeen, Helmy, and Esawy (2012)

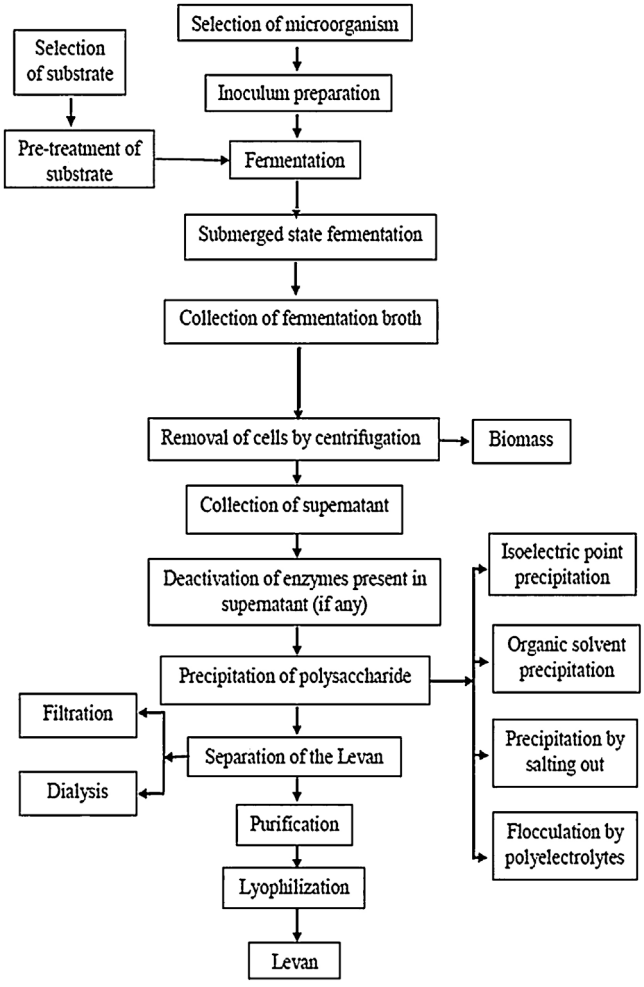

Fig. 3. Schematic representation of microbial Levan Production.

6. Characterization of levan

Post-fermentation studies require physical and chemical characterization of the obtained product, a very important step in the sample analysis. Certain biopolymers and biomolecules yield ambiguous results for the performed chemical tests. Confirming the presence of desired product in the sample using ambiguous results is not reliable. However characterization techniques determine chemical and physical properties, morphology, molecular mass, molecular structure, mechanical, thermal and few other chemical properties with utmost precision.

6.1. Determination of molecular mass

Chemical reactions involving polymerization usually yield distribution of molecular weights and shapes. The parameters used to synopsize the distribution of molecular masses are weight, average, molecular weight, number, average molecular weight, polydispersity index etc. Most widely used methods for the determination of above specified parameters include gel permeation chromatography (GPC), colligative property measurements, light scattering techniques etc.

Analysis of biopolymers usually employs gel permeation chromatography (GPC) to determine molecular weight distribution and polydispersity index. It uses Sepharose 6B columns with water or 50 mM phosphate buffer (pH 7.0) as eluents (Poli et al., 2009). Gel permeation chromatography when combined with viscometry or

Table 2
Chemical shifts of microbial Levan.

Carbon atom	Chemical shifts of Levan [^{13}C NMR (ppm)]				
	P. fluorescens (Jathore et al., 2012)	*Z. mobilis* (Jathore et al., 2012)	*L. reuteri 100-23* (Sims et al., 2011)	*B.subtilis (natto)* (Shih et al., 2005)	*B.polymyxa* (Shih et al., 2005)
C-1	60.435	60.761	61.4	60.1	60.7
C-2	104.696	104.641	105.6	104.4	104.2
C-3	76.770	77.683	77.8	76.5	77.0
C-4	75.783	75.754	76.7	75.4	75.7
C-5	80.880	80.783	81.7	80.5	80.5
C-6	63.978	63.957	64.8	63.6	63.6

low angle laser light scattering (LALLS) can efficiently determine molecular weight distribution and degree of long chain branching of polymers. Molecular weight of levan is highly dependent on microorganisms as well as the cultural parameters used during the production. Levan from *B. subtilis* (*natto*) is reported to produce both low molecular weight levan and high molecular weight levan. Low molecular weight levan is approximately 11,000 Da and high molecular weight levan is around 1800,000 Da (Shih, Chen, Wang, Wu, & Liaw, 2010). Molecular weight of *B. polymyxa* (NRRL B-18475) levan was around 2 million Da (Han, 1990). Levan from *B. subtilis natto* is composed of low molecular weight (50 kDa) and high molecular weight (568 kDa) which were 13.39% and 86.61% in composition respectively (Dos Santos et al., 2013).

6.2. Determination of molecular structure

Determination of molecular structure of the obtained sample is an important parameter in the characterization of compounds with similar molecular structures. Spectroscopic techniques such as nuclear magnetic resonance spectroscopy (NMR), Fourier transform infrared spectroscopy (FTIR), ultraviolet-visible spectroscopy; Raman spectroscopy, X-ray diffraction and mass spectroscopy are often used to determine specific chemical bonds, functional groups and stoichiometry of the unknown sample (Jathore, Bule, Tilay, & Annapure, 2012).

6.2.1. FTIR characterization

According to the principle of FTIR spectroscopy, specific chemical bonds absorb infrared energies at specific frequencies or wavelengths. This helps in the determination of the basic chemical structures of the compounds based on the spectral locations of their IR absorptions. Levan can be easily characterized using FTIR spectral locations (Jackson & Mantsch, 1995; Schmitt & Flemming, 1998). FTIR characterization of levan primarily shows hydroxyl stretching vibration C—H stretching vibration, carbonyl C═O stretching vibration, glycosidic linkage (C O C) stretching of the polysaccharide and the presence of the furanoid ring of the sugar units.

6.2.2. NMR characterization

^{13}C NMR and ^{1}H NMR spectroscopy are also widely used in the characterization of levan. Nuclear magnetic resonance spectra are highly unique, well-resolved, analytically tractable and often highly predictable for small molecules. Structural properties like Primary structure (including stereochemistry), saccharide conformation, stoichiometry of substituents, and ratio of individual saccharides in a mixture can be determined by NMR spectroscopy. It also describes about the detailed information on the presence of specific "functional groups" in the sample.^{13}C NMR spectra gives specific chemical shifts representing the carbon atoms of the structure. Comparative chemical shifts of microbial levan from different sources are given in Table 2.

6.3. Thermal properties of levan

Polymer stability and longevity is usually determined by thermal analysis of the polymer. Differential scanning calorimetry is one of the most used characterization methods in thermal analysis. Glass transition and melting transition of polymers are affected by changes in structural and compositional parameters of polymers and they in turn can be related to other performance parameters. Hence, determination of thermal properties is very important in characterizing the ability of a polymer to be used on an industrial scale. Other techniques such as dynamic mechanical spectroscopy, dielectric spectroscopy, dielectric thermal analysis, thermomechanical analysis are also used in the determination of thermal properties (Menczel & Prime, 2009). Thermal properties of levan allow it to be easily and fully thermally processed through traditional moulding and extrusion techniques. Levan also can be blended with other polymers like ethyl cellulose and chemicals like glycerol, and pressed or extruded in to films in typical polymer processing conditions to get semi transparent, cohesive and adhesive films. Levan blended films with low concentration of glycerol are too brittle to be characterized for tensile testing. But the films containing more (10–35%) glycerol have tensile module less than 0.1 GPa and glass transition temperatures (T_g) ranging from 60 °C (10 wt%) down to 0 °C (30 wt%). Since these values are too low, scope of these films usage in packaging or structural applications is less [Barone & Medynets, 2007, p. 19].

6.4. Mechanical properties of levan

Properties such as tensile strength, young's modulus of elasticity, viscosity, stress, strain etc. define the mechanical strength of the polymer. Determination of these parameters helps in estimating the potency of the polymer as well as their viability when used in an industrial scale. Several techniques are employed to study these mechanical properties and the most widely used technique is dynamic mechanical analysis. It is often used to characterize the viscoelastic behaviour of the polymers. Other techniques employed in mechanical property determination are viscometry, rheometry, and pendulum hardness etc. (Saldivar-Guerra & Vivaldo-Lima, 2013). The mechanical properties of pure levan films are very poor due to its molecular structure. High molecular weight, highly branched, compact globular structure of levan does not permit significant intermolecular entanglement. This leads to brittleness in neat levan, and low tensile modulus in glycerol-plasticized levan (Chen, Gao, & Ploehn, 2014).

7. Applications

7.1. Levan in food industry

Levan has been considered as a potential and functional biopolymer in food industry due to its high molecular weight and low viscous nature. Lactic acid bacteria like *Streptococcus salivarius,*

Streptococcus mutants, *Leuconostoc mesenteroides* NRRL B-512F, *Lactobacillus sanfranciscensis* LTH 2590 and *Lactobacillus reuteri* LB121 produce levan mostly used in food industry. Levan obtained from *Lactobacillus sanfranciscensis* LTH 2590 has shown prebiotic effects (Korakli, Pavlovic, Gänzle, & Vogel, 2003). Levan has anti-tumor (Yoo, Yoon, Cha, & Lee, 2004) effects, cholesterol-lowering effects, eco-friendly adhesive as well as bio-thickener properties as applicable in food industry (Vuyst, De Vin, Vaningelgem, & Degeest, 2001). It is naturally present in various food products and is consumed by humans in very low quantities. However, its consumption in food industry has been greatly ignored due to its limited resources (Marx, Winkler, & Hartmeier, 2000). In general, non-digestible oligosaccharides in particular fructo-oligosaccharide (FOS) are considered as prebiotics. Levan upon acid hydrolysis converts to small FOS and thus can be considered as prebiotic (Bello, Walter, Hertel, & Hammes, 2001; Huang, Lee, Ho, Lin, & Pan, 2013). Being a prebiotic, levan can significantly modulate the colonic microbiota by stimulating the growth of endogenous *Bifidobacteria*. Interestingly the *in vitro* assays also generates FOS upon acid hydrolysis (*i.e.*, use of 0.01N HCl) of levan. Ingestion of levan heptose increases the faecal counts of *Bifidiobacterium* sp. without affecting *Lactobacillus* sp. (Pharmaceutiques, 1995). Dry levan powder is non-hygroscopic and dissolves slowly in hot or cold water. It is non-toxic and non-mutagenic soluble dietary fibre which helps to improve gut function. It can beneficially affect the hosts by increasing the population of the selective lumen bacteria that result in improved host health (Kang et al., 2009). Levan is currently produced by many companies around the world and is widely used in many food products as stabilizer (Absolonne, Jossart, Coussement, & Roberfroid, 1995; Kang et al., 2009).

7.2. Levan in beverages

One efficient way to improve characteristics of dairy products is by the addition of stabilizers. Being an excellent stabilizer, emulsifier and flavour enhancer, levan is also useful in dairy products. The enzymatic or chemical hydrolytic products of levan may be used in the food industry as sweetener or dietary fibre. Levan is also widely used in the other commercial non alcoholic beverages like ultra-high-fructose syrups (UHFS) and β-(2,6)-linked fructofuranosyl oligosaccharides etc. (Bello et al., 2001).

7.3. Levan in industries

Levan finds tremendous applications in biotech and chemical industries. Levan is used to purify biological materials through a PEG/Levan two phase liquid system and selectively partitioning them into one phase. This method is used for the purification of bovine serum albumin, horse heart myoglobin and egg albumin (Chung, Kyung Kim, Song, Kim, & Rhee, 1997). Due to its excellent tensile and shear strength properties levan became a very strong competitor to many petrochemical based adhesives. Two forms of levan adhesives are commercially available by Monatana Biotech SE Inc., water based and cross linked adhesives. Water based adhesives are used for temporary bonds and indoor adhesive industries where as cross linked levan are used for water resistant purposes and for extended use (Barone & Medynets, 2007; Combie, Steel, & Sweitzer, 2004; Combie & Yavorsky, 2005)

The levan derivatives of cationic substituents have been used in home care and fabric applications, in hair care products and in oil fields (Crooks et al., 2009). Oxidized levan is also used in the synthesis of nano-structured thin films by matrix assisted pulsed laser evaporation. These films are now widely being used as coating materials in many industries to avoid the risk of poor adhesion, cracking (Sima et al., 2011). Levan, blended with exfoliated Montmorillonite clay is used in the preparation of novel nanocomposites. These nano films formed by Montmorillonite—levan blend are highly durable, transparent and flexible. The nanocomposites formed from levan are known for their improved thermal and mechanical properties which intensified their usage in many industrial and biomedical fields (Chen et al., 2014).

7.4. Levan in medicine

Levan derivatives obtained by changing the cross links between the fructo-furanoside residues are used as an inhibitor of smooth muscle proliferation and as an anti AIDS agent. This levan also can be used in healing wounds in dermatology and subcutaneous filling in dental and medical applications (Kang et al., 2009). Levan exhibits anti-clotting factor during the surgery of heart patients and also in the treatment of restenosis after angioplasty (Roberts & Garegg, 1998).

Levan exerts its anti-inflammatory properties through interferon response as skin treated with levan produced high quantities of interferon. Levan exerts an emollient effect for skin irritation caused by irritants. Inspite of its high molecular weight, levan shows good skin and cell proliferating properties (Rairakhwada et al., 2007). Along with its cell proliferating properties levan possess certain biological properties which include prevention of infection and necrosis, tumour inhibition and stimulation. It also increases cell permeability to cytotoxic agents. It has been postulated that levan showed anti-tumour activity against Sarcoma-180. *Aerobacter* levan has been used as an anti-tumour agent. The *Microbacterium* levan was used against stomach cancer and its anticancer activity increases with the complexity in branching structure of levan whereas, in certain hepatocellular carcinoma it is vice versa. Hence, the branching structure of levan prohibits the tumour growth. Increased consumption of levan lowers the caecal pH and beta glucuronidase activity and increases the number of *Bifidiobacteria* and short chain fatty acids (SCFAs) in the colon that in turn stimulates the growth of lactic acid bacteria and protects colon from carcinogens (Basit & Ibekwe, 2007; Calazans, Lopes, Lima, & De Franc, 1997; Calazans, Lima, de França, & Lopes, 2000; Henning & Hird, 1972). Levan has been shown to have immune-modulatory effect on macrophage, B and T-cells (Robertson, Papadimitriou, Walters, & Wolman, 1977) and also effectively kills small number of tumour cells and therefore can be used to reduce the tumour mass which can be followed by immunotherapy (Mathé et al., 1969). *Z. mobilis* levan is also used in cosmetic industry as it helps in moisturizing the skin.

Levan as an immunostimulant and is considered to be safe and effective against various fish diseases caused by environmental stress and other factors. Levan acts by quickly activating the non-specific defence mechanisms and protecting fish from disease causing pathogens. By using levan as an immunostimulant, an estimate of 100% survival was observed at concentration ranging from 0.1 to 1.0%. Concentrations greater than 1.0% lead to undesirable immunosuppression resulting in no protection against pathogens (Gupta et al., 2011; Rairakhwada et al., 2007).

Levan's excellent polymer properties like film forming property, viscosity and stability made it an important excipient in pharmaceutical industries. Levan is widely used as an emulsifying agent and as an encapsulating agent. Levan is also used as colour and flavour enhancer in the manufacturing of tablets and capsules (Han, 1990). Levan is used in the pharmaceutical preparations of open matrix networks. Employing levan in the carrier matrix helps the capsule to dissolve fast and produce effective pharmaceutical action in a very less time. These compositions are mainly used in the preparations of drugs which are designed to release the active pharmaceutical ingredient in the oral cavity (Kikuta, Makino, & Yamada, 1996).

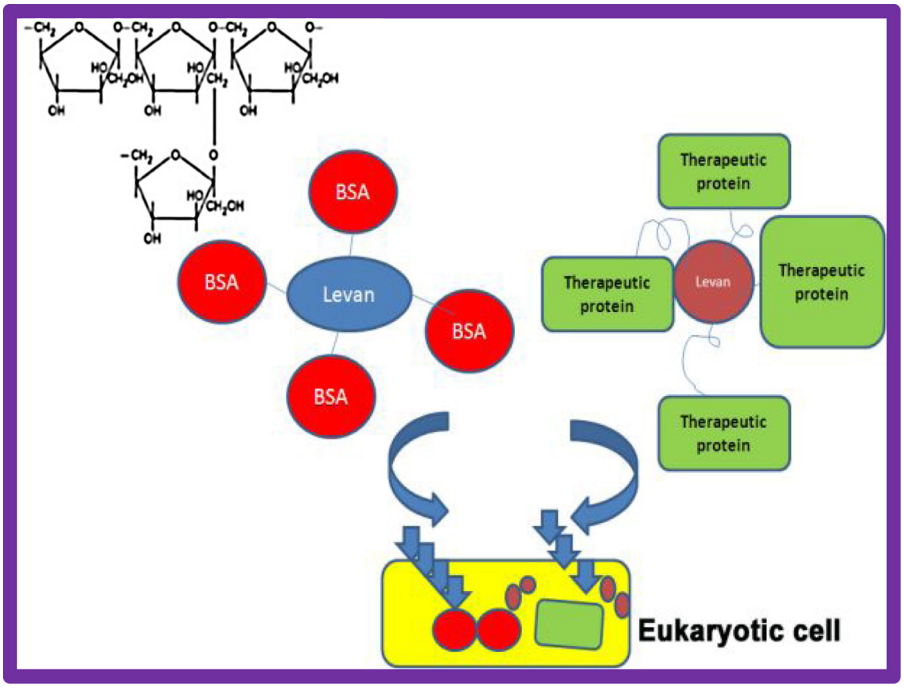

Fig. 4. The possible hypothetical figure depicting the Levan nanoparticle vehicle for delivery of therapeutics.

Levan as blood plasma volume expanders, act by replacing normal blood proteins providing osmotic pressure to pull fluid from the interstitial space into the plasma. They are used to prevent shock from haemorrhage, burns and surgery as well as reduce the risk of thrombosis and embolisms (Rehm, 2009). Administration of levan in diabetic rats through drinking water showed a significant decrease in glucose level in plasma and a significant increase in glycogen level. Levan administration also promisingly showed an increase in superoxide dismutase (SOD) and catalase in liver, kidney, pancreas and heart. It also successfully decreased the levels of thio-barbituric acid reactive substances (TBARS) in liver, kidney, pancreas and heart of diabetic rats. Furthermore, the administration of levan showed a significant decrease in hepatic and renal indices proving that levan can restore abnormal oxidative near normal levels. On the whole levan polysaccharide is proven to be efficient in inhibiting hyperglycemia, inhibition of induction of oxidative stress in diabetes and diabetic complications in adult rats (Dahech et al., 2011a).

Levan administration shows favourable positive effect in treating diabetes type 2 and hypercholesterolemia. Levan treatment positively changes plasma antioxidant enzyme activities and decreases total cholesterol, triglycerides and LDL-cholesterol which proves that levan may have potential hypolipidemic (Belghith et al., 2012b), antioxidant effects and could protect against oxidative stress linked atherosclerosis (Dahech, Ayed, Belghith, Belghith, & Mejdoub, 2013a; Dahech et al., 2013b,c).

Supplement of dietary microbial levan to common carp (*Cyprinus carpio*) juveniles showed a significant increase in total erythrocyte count and haemoglobin content. Moreover there is a notable increase in the respiratory burst activity (NBT) of blood phagocytes, lysozyme activity and the relative survival percentage of common carp (*C. carpio*) juveniles proving that levan can be used as a dietary immune-stimulant for *C. carpio* juveniles and also has an immunomodulatory effect on *C. Carpio* (Rairakhwada et al., 2007).

Derivatized levan (Acetylated levan, Phosphorylated levan and Benzylated levan) exhibits higher reducing power, scavenging activity against super oxide radical and on hydroxyl radical. They also exhibit higher anti-proliferative activity against human gastric cancer cells signifying levan's anti-oxidant and anti-tumour properties (Liu, Luo, Ye, & Zeng, 2012).

Levan is an immune-stimulatory moiety in products from the fermentation process of *B. subtilis (natto)* with soybeans as substrate and may be useful for prevention of allergic disorders with IgE production (Xu et al., 2006). Levan was also proved to be effective in inhibiting Lymphoma developed in mice. Levan showed a dose dependent effect on tumour development and inhibited the growth of tumour at the site of injection and in metastases. Levan also substantially decreased mitosis, pleomorphism and invasiveness of tumours in mice (Leibovici, Sinai, Wolman, & Davidai, 1975).

7.5. Levan in nanotechnology

Natural polymers have received more and more attention in the field of drug delivery systems. In particular, polysaccharides seem to be the most promising materials in the preparation of nanometric carriers. Due to numerous applications of nanoparticle *i.e.*, nanoreactor, nucleating reagent and drug delivery, this polysaccharide-based nanoparticles have gained rapid interest worldwide in relevance to their unique physicochemical properties, biocompatibility and self-degrading ability in human system. However, only a few polysaccharides have been reported as raw materials in preparation of nanoparticles (NPs) (Liu, Jiao, Wang, Zhou, & Zhang, 2008). Levan has gained good merits such as large number of reactive hydroxyl groups and a wide range of M_w and thus could be a promising polysaccharide for NPs preparation. To the best of our knowledge, there are very few reports on levan NPs. The self-assembled levan NPs prepared by stirring method showed a potential to deliver the selected protein BSA and these NPs synthesized by *Zymomonas mobillis* levan also exhibited high ethanol stability and substantial moisturizing effect and therefore is an active ingredient in the formulation of cosmetics (Nakapong, Pichyangkura, Ito, Iizuka, & Pongsawasdi, 2013).

Levan as a nanoparticle has gained importance of its potentiality to delivery peptides and protein drugs. Interestingly levan forms nanoparticle by self assembly in water due to its amphiphilic nature. There are very limited levan based nano-drug delivery

systems (Poli et al., 2009) such as levan obtained from *Halomonas* sp AAD6 [JCM15723, DQ131909] that is highly biocompatible and bioavailable in nature. In addition, nanostructured thin films of pure and oxidized levan polysaccharides by matrix-assisted pulse laser evaporation is reported to have high potential for cell proliferation with all coatings with certain predominance for oxidized levan (Sima et al., 2011) (Fig. 4). Interestingly, the Montmorillonite-levan nanocomposites were found to have improved thermal and mechanical properties (Chen et al., 2014). Recently, studies by our group also demonstrated the green synthesis of silver and gold nano particles and their environmental applications employing *Acetobacter xylinum* levan (Ahmed, Kalla, Uppuluri, & Anbazhagan, 2014).

8. Concluding remarks and perspectives

Significant biocompatible, biodegradable, anti-oxidant, anti-inflammatory, anti-carcinogenic, anti-AIDS and hyperglycemic inhibitor properties of microbial levan made it a competitive replacement to commercial synthetic polymers. Its wide range of application in food, medicine, pharmaceutical, cosmetic and commercial industrial sectors made it a multiuse biomaterial. Utility of microbial levan in nanotechnology in the preparation of novel nanocomposites has to be exploited for stable and eco-friendly film preparations. Their relative utilization in medical and pharmaceutical fields on a commercial scale is comparatively low and requires further investigations for effective employment on a wide range scale. Despite many industrial applications it is seldom produced and utilized due to its limited production strategies. Novel strategies for the production and purification of exopolysaccharides are being introduced to reduce the production costs and to increase the stability of biopolymers. The use of recombinant microorganisms in the production of levan on a large scale is being encouraged to increase the effective yield and reduce the production costs. Effective and economical strategies for the production and purification of such biopolymers increases the extent of their usage in industrial, medicinal and commercial cosmetic sectors which is eco-friendly and raises no environmental or health concerns on plant, animal and human life. In fact, levan has already made its way to the market as an antioxidant and antitumor, but much more has to be exploited as prophylactic medicine. The fact is that the fructose backbone makes levan a very good candidate for the formulation of nutraceuticals without any serious safety concerns. Its multiple therapeutic properties have been extensively studied, and it seems that there is a great potential for exploiting this biopolymer in various biomedical, industrial, food and pharmaceutical field that will fortify human health.

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