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Effects of abiotic factors on production of Levan by microorganisms: A review

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Abstract
Levan biopolymer is an exopolysaccharide (EPS) produced by several plant species and micro-organisms. Levan produced from the plant is of low molecular weight ranging from about 2000 to 33,000 Da and those produced from microorganisms is of high molecular weight ranging from 2 to 100 million Da. Levan produced from microorganisms is of high yield depending on the provided substrate. Therefore, levan produced from microorganisms is widely used in industrial applications. Levansucrase is the key enzyme catalyzing the production of levan from the sucrose. Many bacterial species of *Bacillus*, *Pseudomonas*, *Rhizobium* consists of Levansucrase. The synthesis of levansucrase is dependent on the culture media for the growth of microorganism. Thereby, levan production is affected by type and amount of carbon source, temperature and pH. Optimum temperature for levan production by microorganisms ranges from 37ºC to 45ºC while temperature above 47ºC decreases the levan production and optimum pH is 6-7. The levan produced by microorganisms at optimum conditions have various applications in food, cosmetic, pharmaceutical industries.

Keywords: biopolymer, exopolysaccharide, fructans, levan, levansucrase

1. Introduction
Fructans are fructose polymers produced by several plants and microorganisms, differentiated on the basis of fructose linkage position [1]. Levan is one of the fructooligosaccharide consisting of D-fructose linked by beta-2, 6 glycosidic bond synthesized as non-structural storage carbohydrate [2]. It is an extracellular biopolymer produced mainly by biennial and perennial plants having low molecular weight of approximately 2000 to33000Da [3]. High molecular weight levan is majorly synthesized by many microorganisms under both oxic and anoxic conditions such as *B.subtilis*, *B.lentus*, *Z.mobilis*, *Lactobacillus sanfranciscensis*, *P. brassicacearum*, *Microbacterium laevaniformans* as their major metabolic product [1, 4–7]. Apart from these some soil bacteria's also contribute in levan production for instance *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium* [8]. Table 1 depicts the different microorganisms that produce the levan.

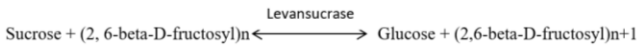
Table 1: List of different microorganism that produce levan

Microorganism	Substrate	Production(g l^{-1}) (Dry weight)
Saccharomyces Cerevisiae	Fructose, Sucrose, Glucose media.	Detectable amount
Bacillus licheniformis	Sucrose as carbon source	50.25
Halomonas smyrnensis	Sucrose from sugar beet, rock salt samples	18.06
Bacillus atrophaeus	Sucrose	3.5
Acinetobacter nectaris	Sucrose as substrate	1.5-3
Bacillus polymyxa	Sugarcane juice, beet molasses.	46
Bacillus lentusV8	Sucrose, black strap, cane molasses	57.97
Pseudomonas spp.	Sucrose, Fructose	7.77
	N-source casein.	8.52
Microbacterium laevaniformans	Date syrup	10.48
	Sucrose medium.	48.9
Lactobacillus sanfranciscensis	Sucrose medium	25.7
Leuconostoc citreum strain BD1707	Tomato juice supplemented with sucrose.	28
Zymomonas mobilis	Sucrose	5.7-12.6

Levan is having unique and particular characteristic feature of being odorless, non-toxic, and tasteless. It is easily fermented by the bacteria that live in the intestines after consumption [9]. Therefore, levan possessed wide range of applications in food industry as bio-adhesive, bio-surfactant, sweeteners and thickeners because of the potential to be easily degraded by the probiotic bacteria [10]. It is also considered as a stored carbohydrates and widely used as thickeners, edible films, sweetener, encapsulating agent and a texture forming compound [11]. It also possessed the potential activity applications in drug prescription, printing, cosmetics, anticancer, antitumor agents whose activities depend on chain length and the degree of branching of levan [12, 13]. Levan produced from *Zymomonas* act as a potential

antitumor agent [14]. Levan showed potential as antiviral agents against avian influenza HPAI, H5N1 and adenovirus type 40 [15].

Production of microbial levan as exopolysaccharides is dependent on the nutrient composition in culture medium and the environmental parameters. The chemical structure and physical properties of levan have been extensively characterized, in terms of molecular weight, linkage type, sugar components, and viscosity [5, 16]. All these characteristics highly influence the rheological functions of levan polysaccharides and affect the quality of food [17]. Levan is synthesized from sucrose catalyzed by the action of levansucrase, which is an extracellular enzyme [18].



The Levansucrase enzyme catabolizes the sucrose and converts fructose into levan. This reaction is known as "transfructosylation". Transfructosylation process has been observed as an applicable synthetic path for the synthesis of novel (2, 6)-FOSs and levan [19]. The major drawback with the levan production is the low availability of levansucrase. *Bacillus amyloliquefaciens*, is one of the dominant bacterial cultures with excessive ability for enzyme production [20]. *B. Amyloliquefaciens* cultivated on media containing sucrose and xylose has been suggested to provide extracellular Levansucrase [21]. A few records [2, 20] had found catalytic activity of both intra and extracellular Levansucrase from *B. amyloliquefaciens*.

2. Production of Levan

Sources

The principal source of carbon used for production of levan is sucrose. Concentration of sucrose was dependent on the cultured microorganism incubated at 30^0 to 40^0C with pH varied from 7-8. Levan can be produced from various microorganisms grown in sucrose containing medium. It can be produced by both gram-positive and gram-negative bacteria, including *Bacillus subtilis* [22], *Z. mobilis* [23], *Gluconacetobacter diazotrophicus* [24], *Pseudomonas syringae pv. Phaseolicola* [25], *Rahnella aquatilis* [18] and *Leuconostoc mesenteroides* [26]. Composition of culture medium depends on the species of organism cultured for growth and production of levan. Levan produced from *rhizobia* species required the YEMA medium consisting of mannitol, K2HPO4, MgSO4.7H2O, MnSO4 and NaCl [6, 8]. *Rhizobia* species can be grown on another medium Rhizobium Defined Medium (RDM) [8].

For ordinary rhizobia culture, a stock is maintained on YMA medium (0.4 g l^{-1} yeast extract, 10 g l^{-1} mannitol, 0.5 g l^{-1} K2HPO4, 0.2 g l^{-1} MgSO4, and 0.1 g l^{-1} NaCl, 9.0 g l^{-1} agar, pH 7.0) and sub cultured periodically. To verify the purity of every strain culture, the YMA medium became supplemented with Congo Red (25 µg ml^{-1}). The cultures had been incubated at 30 °C for 24 h [27]. For comparative analyses of EPS, production obtained from the rhizobial culture, the monosaccharide compositions, and the FTIR and NMR analyses of the EPS of the strains, pre-inoculum and batch experiments have been done using Rhizobium described medium (RDM) (0.23 g l^{-1} K2HPO4, 0.1 g l^{-1} MgSO4, and 1.1 g l^{-1} C5H8NaO4.H2O, 4 ml l^{-1} glycerol, pH 6.8) [27, 28].

Levan is also produced from the species of *Bacillus*. The culture medium used or the growth of it is nutrient media supplemented with $MgSO_4$, 7H20, K2HPO$_4$ [6]. LB Media and PYD are also used for the growth of *bacillus spp.* [21].

Production Methods

Microorganism is isolated from the sample and cultures in medium using spread plating or streaking approach [29]. After that, inoculum is prepared by adding a loop complete of subculture into the media containing sucrose, yeast extract, $MgSO_4$, $7H_2O$, $(NH_4)_2SO_4$, KH_2PO_4, $MnSO_4$ and incubated in orbital shaker at preferred temperature till most fulfilling increase is accomplished [19]. The inoculum is further plated onto the agar plates with appropriate concentration of sucrose and plates are incubated at 37°C. Once most growth is completed, culture is centrifuged at 5000 rpm for10 minutes and the enzyme (Levansucrase) produces by micro-organisms is break free the pellet. The pellet is then re-suspended into the phosphate buffer and cellular disruption is done by sonication [21].

Fermentation Technique

There are mainly two methods for the production of levan from levansucrase on a commercial scale. These are: 1) Batch Fermentation [30] and 2) Continuous fermentation technique [30].

Batch fermentation is a closed technique in which medium is added once within the fermenter and then the culture is inoculated and obtained product is eliminated in the end. In batch fermentation, media used is consisting of yeast extract, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, K2HPO4, MnSO4, distilled water and sucrose as main carbon source. Culture medium is sterilized at 15psi for 15-20mins at 121oC. Sterilized medium is delivered into the Erlenmeyer flask and inoculated with the precise amount of inoculum and incubated in rotary or orbital shaker at 200-500 rpm at the temperature 37°C for 24 h. [6]. The pH and temperature may vary according to the species of microorganism.

Continuous levan production from *Z. mobilis* was carried out using a jacketed Pyrex column packed with 2.0–2.4 mm diameter Ca-alginate beads containing entrapped bacterial cells [30]. After the bioreactor packed with the Ca-alginate beads, the production medium was fed from the bottom of the column continuously. Effluent liquid overflowed from the outlet port at the top of the column. The optimum pH and initial substrate concentration were used at the temperature of 28ºC of the bioreactor. The dilution rates were 0.14, 0.22, 0.29 and 0.4 h^{-1} throughout the packed-bed bioreactor experiments. Levan production was monitored by measuring the concentration of the polymer in the effluent liquid overflowing from the column [30]

Levan Purification and Extraction

At the end of fermentation, cell sample was centrifuged at 10,000 rpm to lyse the bacterial cell which is further washed twice using distilled water and dried at 70ºC to 90ºC. Cell free supernatant was used to precipitate the levan polymer using ice cold ethanol or acetone. Then levan precipitate was collected by centrifugation at 10000 rpm for 10 minutes. The precipitated pellet was washed with distilled water, freeze dried and stored at -20ºC for further analysis and characterization [5].

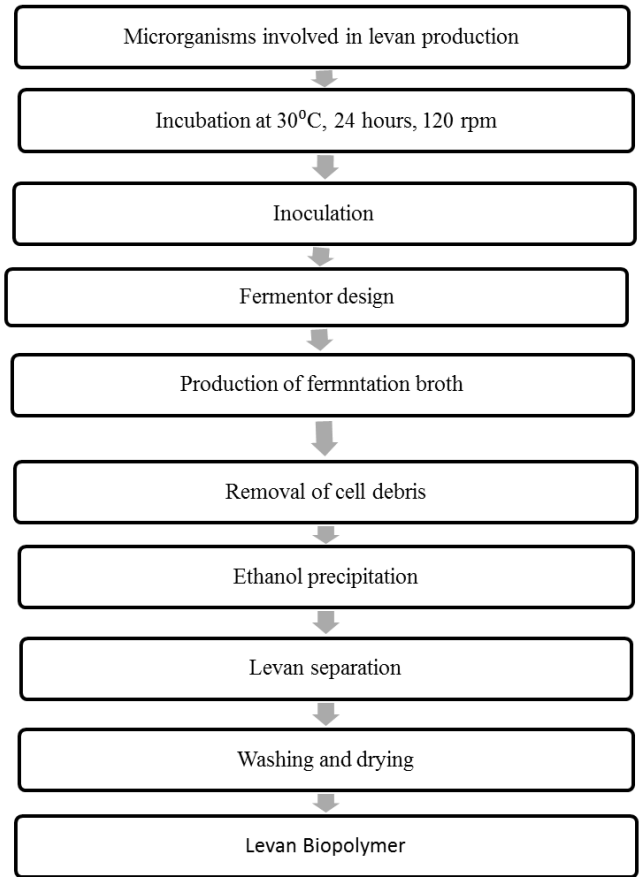


Fig 1: Flow chart for levan production

Characterization of Levan

TLC

0.01 g weight of levan was dissolved in 400µl of 1N HCl and incubated at temperature 70°C for 3hrs for TLC analysis [31]. Then, a 10 µl of levan and other sugar suspensions of fructose, glucose and sucrose were spotted at equivalent distance by capillary tube at 2cm above from the bottom edge of the silica plate. Then, TLC plate was placed in a jar containing the separation solution which is composed of propanol : n-butanol : acetic acid : distilled water in the ratios of 5:7:2:4. Levan and other sugar suspensions were diffused through the silica gel plate. Further, the plates were dried at 37^0C [32] and sprayed by the mixture of ethanol and H_2SO_4 with the ratio of 9:1 and placed in an oven at 90°C for 5-10min [33]. Levan components were appeared as a dark colored spot.

FTIR spectroscopic analysis

Fourier transform infrared (FTIR) spectra analyze the functional groups of purified EPSs. Combination of 1 mg of EPSs and 100mg of dry potassium bromide was pelleted for FTIR evaluation. The FTIR spectra were obtained on a Paragon 1000, Perkin-Elmer spectrometer between 400-4000 wave lengths (cm^{-1}) [34–36]. Levan is a biopolymer consisting of five-membered ring fructose and resulted in obtaining the characteristic peak of carbohydrates with FTIR analysis [7].

Process Parameters

There are many factors that influence the production of levan such as temperature, carbon source, pH and many more factors. The optimum conditions for the production process vary depending on the microbial source.

***Temperature*:** The optimum temperature required for the levan production ranges from 25ºC to 37ºC. Temperature above the range may decrease in levan production and production inhibits at temperature above 47ºC. There is exception in temperature range depending on the type of microorganism used for levan production. The microbial species which grows at high temperature are able to produce levan at equivalent temperature with high yield [4]. Temperature for levan production also varies from one organism to another. Researchers in their study used *Zymomonas mobilis* to study the amount of levan production at different temperature and found the maximum concentration of levan (27.2 g l^{-1}) at 25ºC. And negligible amount of levan production was observed at temperature ranging between 35ºC to 40ºC [37]. Few authors [5, 38] studied the effect of temperature on levan production in *Bacillus lentus V8 Strain* using the subculture media consisting of sucrose or black strap sugar cane molasses. The culture was incubated at different temperature ranging from 25ºCto 35ºC. The maximum levan production of 44 g l^{-1} was observed at 30ºC and above this temperature sudden decline in production was observed [39].

pH: Microbial growth depends on the pH of media. The difference in the pH will alter the growth of microorganism and thereby effect the levan production. The amount of levan decreased when pH maintained above 7 and inhibited below pH 4. The maximum amount is observed at pH 6-7, therefore it is considered as optimum pH for the levan production [40]. In order to find the pH effect on the production of levan, scientists studied on microbial specie *Zymomonas mobilis* which grows at pH 4 but the optimum pH for levan production is ranging from 5-6.5. Therefore, *Z. mobilis* was grown on different culture medium with pH 4 and 5 respectively consisting of sucrose as carbon source. The cultured medium was incubated at 30^0C for 24, 48, 72 and 96 hours and the result observed that at pH 4 the production of levan was less as compared to levan produced by *Z. mobilis* in cultured medium of pH 5 [41].

Substrate

***Carbon Source*:** Microorganisms utilize carbon source for their growth and in the production of levan. Carbon source is one of the important factors that impact the quality and quantity of levan production [42–44]. There are many carbon sources which are used for levan production such as sucrose, glucose, fructose, galactose, maltose, mannose and mannitol. However, sucrose is widely used for levan production because it is easily metabolized by micro-organisms and gives the high yield of levan as compared to other carbon sources. It has been observed from the numerous studies that increase concentration of sucrose results in high microbial growth and hence increase production of the levan. Scientists [45], in their studies used date syrup for levan production and observed that *Paenibacillus polymyxa* utilized date syrup extensively as a substrate for the production of levan. The most suitable condition for levan production utilizing 20% date syrup as substrate was 37ºC and pH 7 at incubation time of 48 hours. They found that levan yield was 28 g /l using date syrup as substrate while the yield increased to 48 g l^{-1} by using sucrose as a substrate. The difference in the levan yield was due to ease metabolization of sucrose by *Paenibacillus polymyxa* and resulted in the high amount of levan production [7, 46]. Date syrup consists of the combination of sugars which

cannot be easily metabolized by the micro-organisms and leads to the decrease in the production of levan [47]. In other study, combination of sugars was, used for levan production from *Bacillus subtilis.* It was observed that 2% sucrose, maltose and fructose produced 2.66 g l^{-1}, 1.42 g l^{-1} and 0.96 g l^{-1} of levan respectively. Glucose and lactose were also able to utilize by microorganisms to yield maximum of about 1.8 g l^{-1} of levan concentration. The amount of levan production depends on the substrate used as a carbon source by microorganisms [48]. The following Table 2 shows the variation in levan production using different carbon source

Table 2: Table for distinctive carbon supply used for Levan production [48]

Different Carbon Sources	Levan Production (g l^{-1})
Xylose	5.63
Galactose	3.58
Glycerol	6.08
Glucose	6.37
Fructose	4.96
Maltose	2.37
Sucrose	8.32
Lactose	4.12
Mannose	2.51
Starch	2.07
Dextrin	3.15

Nitrogen supply and Salt: Levan production by microorganism also requires the nitrogen source. Various nitrogen substrates like peptone, NH_4Cl, $(NH_4)_2SO_4$, CH_4N_2O are used as primary nitrogen source for levan production. Apart from nitrogen, elements such as phosphorous salt and magnesium ions increase the levan production efficiently. Khani *et al*., 2016 started studying to evaluate the impact of nitrogen supply on extracellular polymeric substances (EPS) production by *Chryseobacterium indologenes* MUT.2. They tested eight different nitrogen sources (glycine, glutamic acid, aspartic acid, proline, ammonium sulfate, sodium nitrate, yeast extract, beef extract and peptone) for EPS production. These N-sources were added on the basis of an equivalent N-content in liquid culture medium of LB for 96 h at 30ºC to evaluate the effect of nitrogen source in EPS production. The study resulted in increased production of EPS (5g l^{-1}) utilizing glutamic acid as nitrogen source [49].

Effect of Metal ion: Metal ions behave as a co-factor and interact with enzyme which influences the field of activation, inhibition and stabilization in enzyme-substrate reactions [50]. The studies were done using 0.2 to 0.8 M concentration of each potassium, sodium and zinc monovalent ions to evaluate their effect on the enzyme activity after interaction. Levansucrase activity increased after the interaction with sodium, potassium and zinc ions in *B. subtilis natto* CCT 7712 strain [4].

Levansucrase activity was partially inhibited after interacting with divalent cations (Ba^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+} and Mn^{2+}) of 0.2 to 0.8 M concentration. Levansucrase interaction with Zn^{2+} responded differently, activity of the enzyme was inhibited at 0.2 M concentration and further activity was increased with the concentration. However, levansucrase from Bacillus spp. was inactivated by Zn^{2+} and activated by Fe^{2+} [51]. And furthermore, Fe^{2+} weakly inhibited the activity of levansucrase isolated from *Acetobacter diazotrophicus* [32, 52]. Therefore, the result showed that activation or inhibition of the enzyme activity is not only related with cation but also with difference in their concentration and synergic effect of cation and anion in their salt form. The study was done to analyze the effect of trivalent ions (Fe^{3+} and Al^{3+}) on catalytic activity of levansucrase. It was observed that both Fe^{3+} and Al^{3+} inhibited the activity of levansucrase by 12% and 18% respectively.

3. Conclusion

Excellent biocompatiblity, biodegradablity, anti-cancer, anti-oxidant, anti-AIDS, anti-inflammatory and hyperglycemic inhibitor traits of levan offered it an affordable alternative to commercialized artificial polymers. It has the broad range of applications in nourishment, drugs, pharmaceutic, cosmetic and professional market sectors offered it as a multiuse biomaterial. Irrespective of multiple commercial uses, it is rarely used and manufactured because of its limited production approaches. Unique approaches intended for the formulation and so refinement of biopolymers are increasingly being contributed to decrease the formulation expenses and also to improve the reliability of biopolymers. The utilization of microbes for the formulation of levan biopolymer on a sizable level is definitely being motivated to boost the profitable production and so decrease the formulation expenses. Competent and cost-effective approaches intended for the formulation and refinement of similar biopolymers raises the amount of their utilization in commercial, therapeutic and industrial aesthetic industries which is definitely eco-friendly and accelerates no environmental or health issues foe animals and plants. This review proposed a summary analysis of levan biosynthesis, properties and potential applications in various industrial sectors.

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