LWT - Food Science and Technology 138 (2021) 110619

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Optimization and extraction of edible microbial polysaccharide from fresh coconut inflorescence sap: An alternative substrate

Gopinath Mummaleti, Chayanika Sarma, Sureshkumar Kalakandan [**], Vignesh Sivanandham [*], Ashish Rawson, Arunkumar Anandharaj

Department of Food Safety and Quality Testing, Indian Institute of Food Processing Technology, Thanjavur, 613 005, Tamil Nadu, India

ARTICLE INFO

Keywords:
Levan
Fresh Coconut Inflorescence Sap
Fed-Batch Fermentation
Bacillus subtilis, Downstream Process

ABSTRACT

The objective of the study is to evaluate the potential usage of fresh coconut inflorescence sap (FCIS) as a fermentation medium for levan production using *Bacillus subtilis*. The fermentation conditions such as temperature, pH and agitation speed were optimized using Response Surface Methodology (RSM). The levan yield was enhanced by optimizing the downstream process by altering the pH (5–12) with different solvents (Ethanol, Methanol and Isopropanol) at different ratios. The yield was enhanced further by fed batch fermentation process by feeding with sucrose alone or sucrose and yeast extract. The maximum levan yield was observed as 51.84 g/L at the optimized conditions (Temperature - 35 °C, pH 6.5, and Agitation speed - 150 rpm) using Response Surface Methodology. Whereas, in fed-batch fermentation process the levan yield was increased to 62.1 g/L. The maximum levan was obtained at pH 10 with ethanol at 1:5 ratio. The obtained levan was characterised and confirmed by TLC, FTIR, NMR and GPC analysis. To best of our knowledge, this is the first study conducted to produce levan from FCIS and results showed that FCIS can be a natural low-cost substrate for levan production.

1. Introduction

Microbial polysaccharides are natural water-soluble substances produced by microorganisms in larger quantities with lesser time. These polysaccharides such as xanthan, gellan, curdlan, pullulan, etc., have potential application in pharmaceutical, food and cosmetic industries due to their diverse properties as thickeners, gelling agents, stabilizers, film forming, anti-tumor and anti-microbial activities (Bondarenko et al., 2015; Domżał-Kędzia et al., 2019; Giavasis, 2013; Gomes et al., 2018). Levan is one such polysaccharide produced by a wide range of microorganisms such as *Lactobacillus johnsonii*, *Lactobacillus gasserii*, *Bacillus subtilis*, *Aerobacter levanicum*, *Zymomonas mobilis*, *Bacillus polymyxa* and *Corynebacterium laevaniformans* (Han, 1990) and certain plant species such as *Dactylis glomerata, Poa secunda and Agropyron cristatum, Triticum aestivum* and *Hordeum vulgare* (Gupta et al., 2011).

Levan is a fructose polysaccharide produced through transfructosylation reaction from sucrose-based substrate catalysed by levansucrase (beta-2, 6 fructan: glucose-fructosyl transferase, EC 2.4.1.10) enzyme. Levansucrase catalyse sucrose hydrolysis, followed by fructose polymerization reactions. Levan structurally composed of β-2, 6 and β-2, 1 linked D-fructofuranosyl residues in main chains and side chains were linked with D-glucosyl terminal residue (Ragab et al., 2019). Levan has useful properties like low viscosity, solubility in water and oil, rheological properties, compatibility with salts and surfactants, stable to heat, acid and alkali, film-forming ability and water holding capacity (Sezer et al., 2011). With all these properties, levan can be used as an emulsifier, stabilizer, thickener, encapsulating agent and biodegradable films (Küçükaşik et al., 2011). Several factors such as temperature, pH, incubation time, sucrose concentration, medium composition and mineral concentration in the medium affected the levan production (Rütering et al., 2016).

As levan is a polymer of fructose, it can be used as a natural sweetener. The microbial levan is gaining importance in food applications because of its wide range of properties and health benefits. *In vitro* studies showed that levan anti-tumor activity against eight different tumor cell lines (Calazans et al., 2000; Queiroz et al., 2017). It is capable of mitigating the adverse effects of heavy metal contamination in the environment (Lončarević et al., 2019). The sulphates, phosphates and acetates of levan polymers act as anti-AIDS agents (Liu et al., 2012), prebiotic substances (Gupta et al., 2011) and hypocholesterolemic

---

[*] Corresponding author.

[**] Corresponding author.

E-mail addresses: k.sureshkumar@iifpt.edu.in (S. Kalakandan), vignesh@iifpt.edu.in (V. Sivanandham).

https://doi.org/10.1016/j.lwt.2020.110619
Received 19 August 2020; Received in revised form 10 November 2020; Accepted 21 November 2020

effects (Belghith, Dahech, Belghith, & Mejdoub, 2012). Levan protects liver, pancreas, heart and kidney tissues from the damage caused by superoxide (O_2^-), hydroxyl (OH^-) and hydrogen peroxide (H_2O_2) free radicals (Dahech et al., 2011). The high and low molecular weight levan were produced simultaneously in fermentation process (Tanaka et al., 1980). High molecular weight levan is capable of lowering blood cholesterol levels and anti-tumor activity (Calazans et al., 2000).

Low molecular weight levan has been proved to have better encapsulation efficiency and sustained release of the drugs (Bondarenko et al., 2015). Especially in pharmaceutical industry, the addition of levan enhances the colour coating on tablets and capsules (Han & Watson, 1992). The functional molecule upon encapsulation with the levan matrix exhibited faster dissolution resulting in a quicker therapeutic effect, further oral drugs available in the market are coated with levan to mask the bitterness while ingestion (Domżał-Kędzia et al., 2019). The *in vitro* studies showed that levan was found to be a potential immunomodulator by stimulating the proliferation of spleen cells and inducing the expression of TNF-α thereby suppressing inflammation (Xu et al., 2016). Moreover, the edible films made of cassava starch used as antimicrobial agent and packing material which incorporated with levan polysaccharide showed higher tensile strength, solubility and elongation and low water vapour permeability (Mantovan et al., 2018).

The levan produced in previous studies (Bae et al., 2008; Shih & Yu, 2005; Szwengiel & Wiesner, 2019) by using of synthetic medium which is very costly as the chemical ingredients account for higher production costs. A low-cost alternative high yielding substrate is required for levan production. *Cocos nucifera* existed from millions of years and grow mostly in tropical areas. The sweet sap produced from unblossomed mature spadix which is used as a natural drink. The fresh coconut inflorescence sap (FCIS) is highly nutritious with all the vital nutrients (Sudha et al., 2019). The sap is not commercialized successfully due to its tendency of auto-fermentation by naturally occurring environmental microorganisms. The traditional tapping process of FCIS is mostly unhygienic and also responsible for natural fermentation in short span. The fermented sap contains 5–8% of alcohol and is called sweet toddy whereas unfermented sap without alcohol is called neera (Misra, 2016).

The fresh sap contains sucrose, glucose, fructose (as carbon sources for fermentation), amino acids and micronutrients (Naik et al., 2020., Ameetha et al., 2018). The FCIS though it is rich in nutritive value it cannot be consumed due to its fermentation nature. The rich sucrose, amino acids and micronutrients containing FCIS could be an alternate low-cost substrate for levan production. Till now to the best of our knowledge, no report was found to use FCIS as a fermentation medium for levan production. This study was conducted to optimize the fermentation conditions for production of levan in large scale using a cheap substrate.

In this study, the main objectives are a) to standardize the test culture for fermentation process; b) to optimize the fed-batch fermentation process with low cost medium (FCIS alone, FCIS + sucrose, FCIS + sucrose + yeast extract); c) to enhance the recovery of levan (downstream process) by using of three different kinds of solvents separately and each solvent is used at four different ratios; d) To compare the levan yield at different time intervals of fermentation process.

2. Materials and methods

2.1. Materials

The media such as nutrient agar, yeast extract and agar were purchased from HiMedia Pvt. Ltd, Mumbai, India. The chemicals (AR grade) such as isopropanol, methanol, ethanol, HCl, sodium hydroxide and others were purchased from Sigma-Aldrich, Mumbai, India. The sterile Millipore water was used in this entire study. The pH adjustment in all the experiments were done with 1 N HCl and 1 N NaOH.

Table 1

Coded independent variables and levels of the experimental design.

Factors	Symbols	Actual levels of coded factor		
		−1	0	1
Temperature	A	30	35	40
pH	B	5	6.5	8
Agitation speed	C	120	150	180

2.2. Microorganism

The freeze-dried culture of *Bacillus subtilis* (NCIM 5021) was procured from NCIM, Pune India. The microorganism was revived and stored at 4 °C on nutrient agar slants as per the NCIM instructions and subcultured freshly every time before the experiments.

2.3. Optimization of Bacillus subtilis culture at different temperatures

The 100 μL of standardised (based on 0.5 McFarland standards, the microbial culture was adjusted to approximately 10^8 CFU/mL) *Bacillus subtilis* culture was inoculated in 10 mL of nutrient broth containing test tubes and incubated at different temperatures of 25, 30, 35 and 40 °C to find the suitable growth temperature. After 24 h incubations, the growth of the organism was estimated by measuring the OD at 600 nm in UV visible spectrophotometer.

2.4. Preparation of fermentation medium

The sugar profile, protein and mineral content of the FCIS were measured according to the AOAC (21st edition 2019) methods. The sugar profile was measured using AOAC 982.14, protein using Lowry method and mineral composition using AOAC 2011.14 methods. The FCIS was collected every day morning, pH (5–8) was adjusted according to the experimental design, autoclaved and used as fermentation medium.

2.5. Fermentation

About 1 mL of standardised subculture was transferred to the fresh autoclaved (FCIS) fermentation medium and incubated for 4 h till the culture enters into the lag/log phase with the absorbance 0.50 and used as inoculum for fermentation. About 1 mL of seed culture was added to the substrate medium in 250 mL Erlenmeyer flask containing 100 mL FCIS and incubated for 24 h in shaking incubator.

2.6. Optimization of fermentation conditions using response surface methodology (RSM)

The central composite design was used for the optimization of levan production by using Design Expert software V11.1.0.1. The response surface methodology (Jiang, 2010) is suitable for fitting the data in a quadratic model and optimizing the response. The interaction between the parameters and effect of independent variables on responses can be statistically explored using this method. Temperature, initial pH and agitation speed were chosen as independent variables at three levels for each factor and yield of levan as the dependent variable to find the optimal conditions for the production of levan in the batch process. Table 1 shows the range of variables of temperature, initial pH and agitation speed for RSM.

2.7. Isolation and purification of levan

The fermented medium was centrifuged at 14083g for 10 min (Abou-taleb et al., 2015). Further, the supernatant was separated and added 2 vol of 100% ice-cold ethanol at 4 °C then stored for 24 h at 4 °C to precipitate out the exopolysaccharide. The precipitate was separated

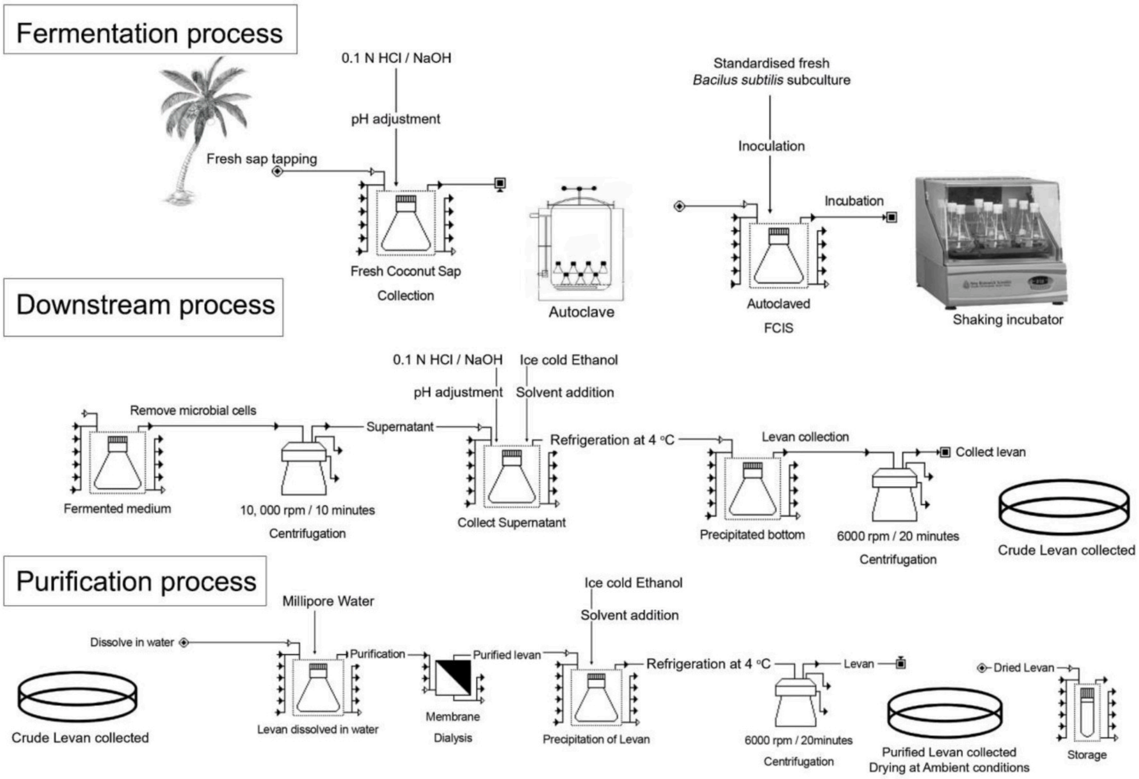

Fig. 1. Pictorial representation of fermentation and downstream process.

by centrifugation at 5070g at 4 °C for 20 min and the pellet was collected. The pellet was dissolved in deionized water and the solution was dialyzed against the demineralized water to remove the unfermented sugars and small molecules. The polysaccharide was again precipitated using two volumes of 100% ethanol and the precipitate was air-dried at room temperature for 24 h and weighed (Abou-taleb et al., 2015; Chidambaram et al., 2019).

2.8. *Optimization of downstream process*

After fermentation, the downstream process was optimized to increase the recovery of levan. The effect of pH, type of solvents and volume of solvent were studied in the optimization of downstream process. The fer mented medium (FCIS) was collected and centrifuged at 14083g at 4 °C for 20 min. The supernatant was collected and the initial pH was optimized. The pH of supernatant was adjusted to 5–12 and precipitated using 1:2 vol of ethanol (Chidambaram et al., 2019). At the optimized pH (pH 10), the effect of three solvents ethanol, methanol and isopropanol on precipitation were studied. Each solvent was studied at four different ratios 1:2, 1:3, 1:4 and 1:5 (Fig. 1).

2.9. *Estimation of time of maximum levan production*

The *B. subtilis* culture was inoculated in the FCIS medium and kept for fermentation at 35 °C, pH 6.5 and 150 rpm agitation speed. The fermentation process was stopped at different time intervals (10, 15, 20, 24 h) and the downstream process was carried out to find the maximum levan production.

2.10. *Optimization of the fermentation process by fed-batch method*

The fed-batch fermentation method was carried to improve the yield of levan. Two different supplements such as sucrose and yeast extract (nitrogen source) were used in the combination with FCIS medium. About 50 mL FCIS medium was inoculated with 1000 μL of standardised *B. subtilis* culture in different sterile conical flasks for the fermentation process. 2.0 g sucrose was supplemented with FCIS medium at an interval of 8, 11, 14 and 17 h, similarly 1.5 g of sucrose +0.5 g of yeast extract was supplemented with FCIS medium at an interval of 11, 14, 17 and 20 h, respectively.

2.11. *Effect of natural fermentation of FCIS on levan production*

The effect of natural fermentation of FCIS due to natural microflora present in the neera was studied by allowing the FCIS to ferment for 2, 4, 6, 8, 10 and 12 h. Then, the fermented FCIS was autoclaved and inoculated with the *Bacillus subtilis* culture. After fermentation (20 h), the levan yield was measured. One experiment was conducted by inoculating the *Bacillus subtilis* without autoclaving the neera.

2.12. *Confirmation and characterization of levan polysaccharide*

2.12.1. *Thin-layer chromatography*

Thin-layer chromatography method was carried out to identify the composition of the levan using silica gel coated TLC plate. 0.01 g was dissolved in 1 N HCl and incubated at 70 °C for 3 h. About 10 μL of the sample was spotted on the TLC plate along with the sucrose, glucose and fructose solutions. The mobile phase is a mixture of *n*-butanol: 2-propanol: distilled water: acetic acid (ratio of 7:5:4:2) and allowed to diffuse through the TLC plate in a closed jar. After 1 h (till the mobile phase reaches 1 cm below the upper end), the plate was dried and sprayed with ethanol + H_2SO_4 mixture (ratio of 9:1) (Kojima et al., 1993) and placed in the oven at 90 °C for 5 min to complete the reaction and relative flow was measured.

2.12.2. *FT-IR analysis*

The functional groups of dialyzed polysaccharide was identified by FT-IR analysis (Košťálová & Hromádková, 2019). FT-IR spectrum was

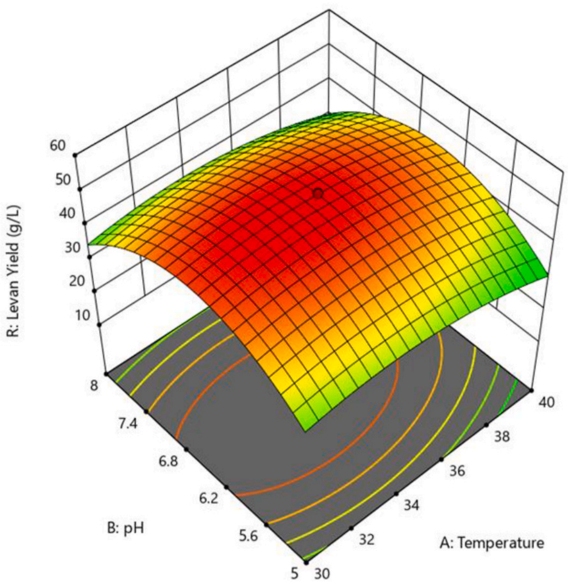

Fig. 2. Effect of temperature and pH on levan production.

obtained by the instrument Nicolet iS50 FT-IR spectrophotometer with a DTGS KBr detector controlled by Omnic 9.9.549 software. The spectrum was collected at a resolution of 4 cm^{-1} from 4000 to 400 cm^{-1}.

2.12.3. NMR analysis

^{1}H NMR analysis and ^{13}C NMR analysis were carried out in Bruker 400 MHz instrument. The sample was prepared in D_2O (Li et al., 2017). The chemical shifts were obtained in ppm.

2.12.4. Molecular weight by gel permeation chromatography

The molecular weight of the levan was measured by Agilent 1260 Multidetector system with PL aquagel – OH 40 column. The 100 μL of sample (concentration 50 mg/mL) was injected using eluent as buffer prepared by mixing 0.2 M $NaNO_3$ and 0.01 M Sodium phosphate at flow rate 1 mL/min (Singh et al., 2018). The oven temperature was maintained at 40 °C. The number average molecular weight (M_n), Weight average molecular weight (M_w), Higher average molecular weights (M_Z) (M_{Z+1}) and Polydispersity index were measured against the dextran standards using refractive index detector.

2.13. Statistical analysis

The experimental design, plotting response surface plots, regression and ANOVA for RSM were carried out using Design Expert version 11.1.0.1 (Stat-Ease, Inc., Minneapolis, USA). The fed batch fermentation and downstream process experiments were conducted in triplicates and statistical analysis (ANOVA and Duncan's multiple range test) (Nageswara Rao, 2007) were carried by using SPSS 25.0 software.

3. Results and discussion

3.1. Fermentation medium

The *Bacillus subtilis* showed the maximum growth at 35 °C followed by 30 °C, 40 °C whereas minimum growth was observed at 25 °C. The FCIS was composed of (g/L) carbohydrate 191.26, sucrose 172.31, glucose 16.21, fructose 6.23, protein 2.47, potassium 0.91, sodium 0.15, phosphorous 0.064, magnesium 0.027, copper 0.0043 and manganese 0.00099. The FCIS was used directly as fermentation medium without the addition of any nutrients.

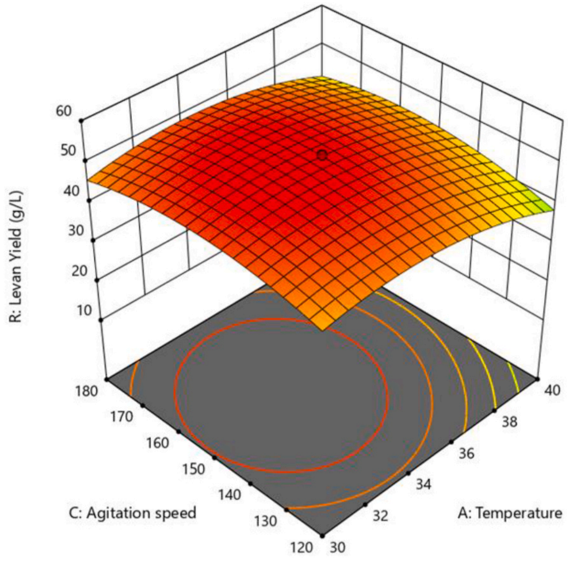

Fig. 3. Effect of temperature and agitation speed on levan production.

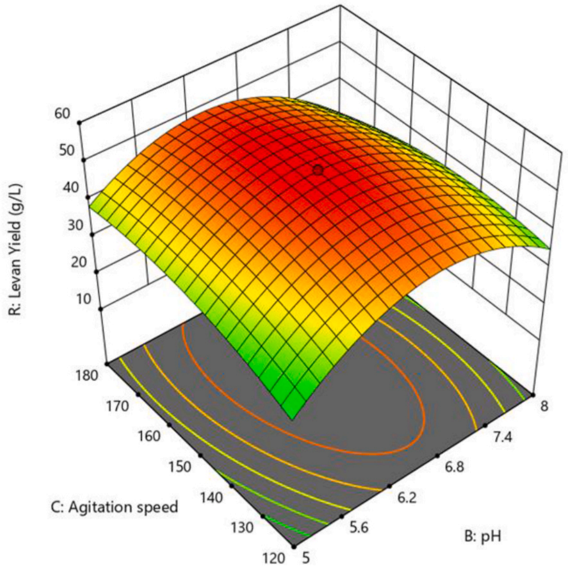

Fig. 4. Effect of pH and agitation speed on levan production.

3.2. Optimization of fermentation conditions using RSM

The levan yield was optimized by conducting 20 experiments with different combinations of temperature (30–40 °C), initial pH (5–8) and agitation speed (120–180 rpm) (Figs. 2–4).

3.2.1. Effect of temperature on levan yield

The increased levan production was observed (Fig. 2) with the increase in temperature and reached the maximum at 35 °C ($P < 0.0001$). The study clearly indicated that the maximum growth of the *Bacillus subtilis* reached at 35 °C, on extrapolation the levan yield decreased considerably. This may be due to a decrease in the bacterial growth (Ni et al., 2018; Wu et al., 2013) and it confirms that the temperature plays a significant role on the levan production.

3.2.2. Effect of initial pH on levan yield

Similarly, the pH plays an important role on the bacterial growth and levan production. The maximum levan production was observed at the initial pH 6.5 (Fig. 4), which is almost near to the natural pH of FCIS 6.8

Table 2
Central composite design and experimental results.

Run	Factor 1	Factor 2	Factor 3	Response
	A: Temperature	B: Initial pH	C: Agitation speed	R: Levan yield
	oC	pH	RPM	g/L
1	40	8	180	25.22
2	30	5	180	36.98
3	30	8	120	34.25
4	35	6.5	200	51.68
5	40	5	180	31.26
6	35	6.5	200	41.86
7	35	4	150	12.92
8	40	8	120	27.21
9	40	5	120	22.09
10	30	8	180	27.42
11	30	5	120	28.86
12	35	6.5	100	38.21
13	43	6.5	150	34.25
14	35	6.5	150	51.89
15	35	6.5	150	51.89
16	35	6.5	150	51.26
17	35	6.5	150	52.17
18	35	6.5	150	51.28
19	27	6.5	150	42.59
20	35	9	150	11.85

± 0.32 which was measured immediately after the collection. The initial pH is an important deciding factor for the production of levansucrase enzyme which polymerises the sugar molecules. Belghith and his co-workers (2012) demonstrated that pH 6.5 is optimum for maximum levansucrase enzyme synthesis that promotes polymerization of sugars. The maximum levan yield of levan was observed as 56.0 g/L at 17% sucrose concentration which is naturally present in the FCIS. Additionally, Laddha and Chitanand (2017) also observed that artificial supplementation of 10% sucrose in fermentation media yielded 30.6 g/L of levan by using *Bacillus subtilis*. In a similar study reported by Ni et al. (2018) with *Lactobacillus reuteri* using artificially prepared fermentation medium containing 500 g/L of sucrose and 13 U/g of levansucrase enzyme dosage, showed maximum levan yield of 183 g/L at pH 6.0 and 35 °C temperature.

3.2.3. *Effect of agitation speed on levan yield*

The agitation of fermentation plays a significant role in the culture growth by providing necessary aeration. The results of the present study showed that agitation speed had significant effect ($P < 0.0001$) on levan production and maximum production was observed at 150 rpm. The levan production by *Bacillus polymyxa* showed three times increase in levan production upon agitation as compared to non-agitated media, further vigorous agitation led to decrease in levan production (Han, 1989). Boa and LeDuy (1987) explained that increase in agitation speed causes better mixing and aeration in fermentation media. However, a higher agitation speed may cause high shear rate and autolysis of cells leading to the lower production of levan polysaccharide (Cheng et al., 2011; Melo et al., 2007).

3.2.4. *Effect of combination of the treatment factors on levan yield*

The combined effect of temperature and pH, agitation speed and temperature and agitation speed and pH were significant at $P < 0.01$, $P < 0.05$ and $P < 0.0001$ respectively. The combined parameters were subsequently used for optimization of conditions in RSM. Alteration in these parameters is presumed to significantly affect the microbial growth and the levan yield (Figs. 2–4).

3.2.5. *Optimization*

The levan yield was observed to be mostly influenced by parameters such as temperature, pH and agitation speed and levels of parameters were determined according to preliminary observations and previous studies (Abou-taleb et al., 2015; Wu et al., 2013). The response surface methodology method was suitable for optimizing the parameters with minimum number of experiments and interaction between the parameters can be analysed. The design of the study and results are shown in Table 2. The results of levan yield were fitted in second-order polynomial equation. The regression coefficients were calculated and levan production modelled by the quadratic equation indicating yield (Y), temperature (A), initial pH (B) and agitation speed (C) given below.

$$Y = 51.6892–2.61818 \text{ x } A - 0.504473 \text{ x } B + 1.06969 \text{ x } C + 0.40625 \text{ x } AB + 0.73625 \text{ x } AC -3.26375 \text{ x } BC - 4.65538 \text{ x } A^2 - 13.8601 \text{ x } B^2 - 4.08439 \text{ x } C^2$$

The mathematical model predicted the experimental values adequately and significant effect of independent factors (temperature, initial pH and agitation speed) on response (levan yield) was observed. The *F*-Test and analysis of variance were done for response surface quadratic model to measure the statistical significance of the model. The ANOVA for the response surface quadratic model and responses for levan production were shown in Table 3. The computed *F* value was 1157.13 and P value was <0.0001 which indicated that the model was highly significant. The multiple correlation coefficient (R^2) was 0.9931 and was considered to have a good correlation between predicted and experimental values. It also depicted that 99.31% of the variability of response can be explained. The lack of fit found to be insignificant (*P* value = 0.0907) and it indicated that the model was fit. The smaller *P* value indicated more significance to corresponding coefficient (Table 3).

The optimum condition of the fermentation process obtained by differentiation of the quadratic model for achieving maximum levan production (A = 35 °C, B = 6.5 and C = 150). According to the RSM model, the optimal levan production at these conditions was predicted as 51.89 g with 1 L of FCIS as fermentation medium. The experiments were conducted thrice to confirm the goodness and the yield of levan was observed as 51.84 g/L at the optimized conditions.

Table 3
ANOVA for Quadratic model.

Source	Sum of Squares	df	Mean Square	*F*-value	*P*-value	
Model	3213.92	9	357.10	1157.13	<0.0001	Significant
A-Temperature	93.62	1	93.62	303.35	<0.0001	
B-pH	3.48	1	3.48	11.26	0.0073	
C-Agitation speed	15.63	1	15.63	50.64	<0.0001	
AB	1.32	1	1.32	4.28	0.0655	
AC	4.34	1	4.34	14.05	0.0038	
BC	85.22	1	85.22	276.13	<0.0001	
A^2	312.33	1	312.33	1012.05	<0.0001	
B^2	2768.46	1	2768.46	8970.72	<0.0001	
C^2	240.41	1	240.41	779.02	<0.0001	
Residual	3.09	10	0.3086			
Lack of Fit	2.42	5	0.4846	3.65	0.0907	Not significant
Pure Error	0.6634	5	0.1327			
Cor Total	3217.01	19				

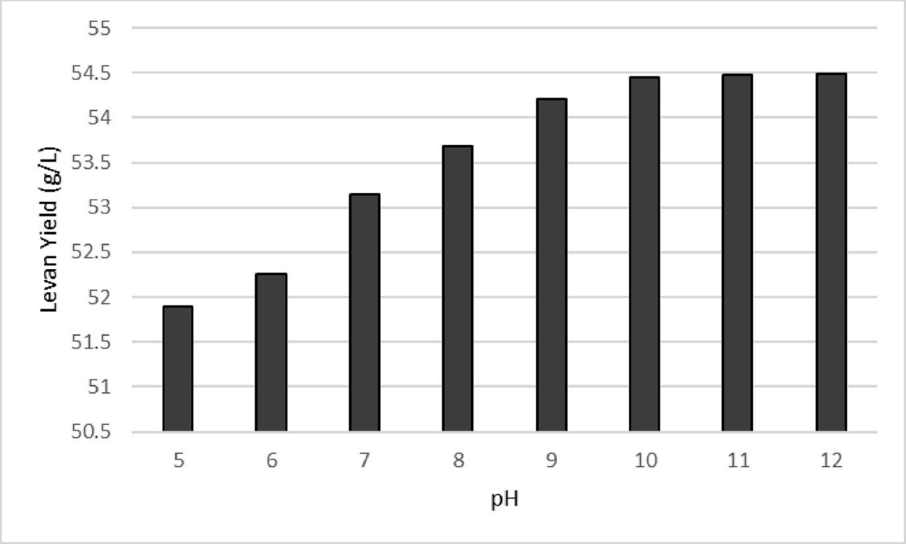

Fig. 5. Effect of pH in downstream process on levan yield.

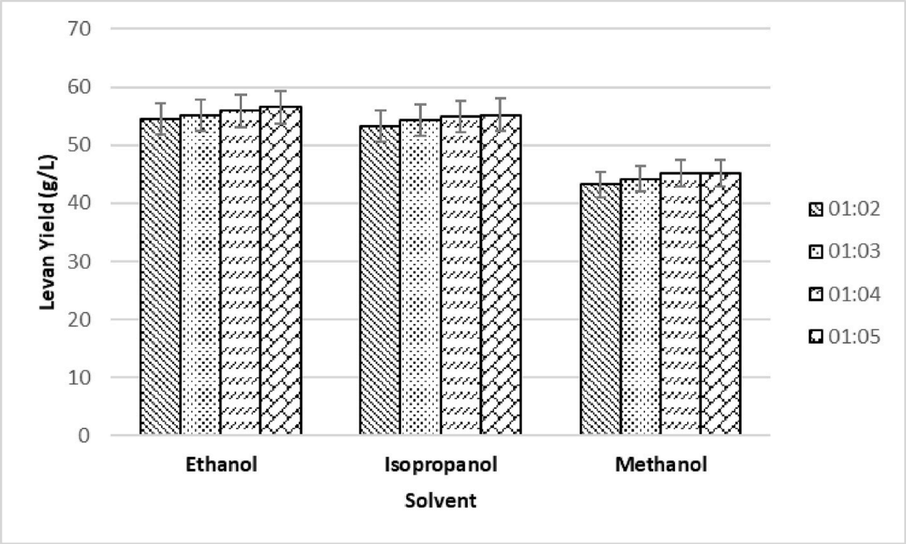

Fig. 6. Effect of different solvents each at different ratios on levan yield.

3.3. *Optimization of downstream process*

The effect of pH on levan precipitation was studied at optimized fermentation conditions. The fermented medium was centrifuged at 14083g for 10 min and the supernatant was collected. The pH of supernatant was adjusted from 5 to 12 and precipitated with ethanol (ratio 1:2 vol of supernatant and ethanol) (Fig. 5). The results showed there was a significant increase in the levan yield with an increase in the pH. There was no significant difference in the yield beyond the pH 10. The levan was highly stable and withstand a wide range of pH. The influence of physicochemical factors on levan were studied and results showed that levan structure was stable at a pH 4–11 and high temperatures up to 70 °C (Vina et al., 1998). The supernatant was maintained at pH 10 and by use of three different kinds of solvents separately and each solvent was used at four different ratios were added to the supernatant and yield was measured. Fig. 6 represents the results of yield by using of three different kinds of solvents separately and each solvent was used at four different ratios. The significant increase of the precipitation was observed for an increase in the volume of solvent up to the 1:5 ratio. The maximum precipitation of levan (56.49 g/L) was found with ethanol at the ratio of 1:5. Methanol showed the least precipitation when compared to the other two solvents. The maximum yield of levan (0.327 g/g) was obtained at pH 10 and a 1:5 ratio of ethanol (when used only FCIS as fermentation medium without addition of other nutrients). Chidambaram and his group (2019) explained that maximum precipitation was observed at pH 11 and 1:5 isopropanol when formulated fermentation medium used.

3.4. *Estimation of time of maximum levan production*

The fermentation was carried out at different time interval such as 10, 15, 20 and 24 h. The maximum levan production of 57.2 g/L was observed at 20 h and a slight decrease in the levan was observed at 24 h; this may be due to the utilization of levan as a carbon source for the microbial growth. Han (1989) stated that 10 days were required for maximum levan production, using *Bacillus polymyxa* whereas maximum levan yield was observed with 21 h when *Bacillus subtilis* was used as prime strain (Wu et al., 2013).

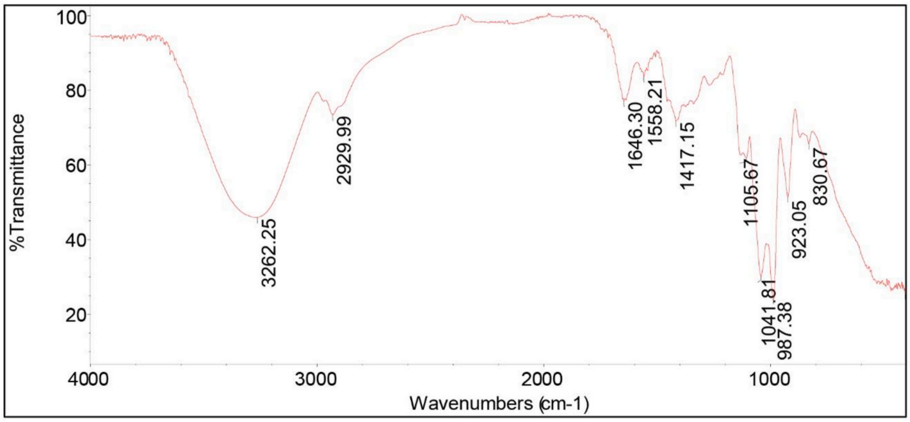

Fig. 7. FTIR spectrum of Levan.

3.5. Optimization of fed-batch fermentation

The different combinations of media were used in the fed-batch fermentation process and the medium formulation play an important role in levan production. The FCIS in combination with sucrose (carbon source) and yeast extract (nitrogen source) were used to improve the levan production. The highest levan yield (62.1 g/L) was achieved with the FCIS medium + 1.5 g sucrose + 0.5 g yeast extract at 17 h. The results indicate that the levan yield depends on both the sucrose and nitrogen source. Our results is in line with the previous reported studies which showed that the levansucrase enzyme production was effected by both sucrose and nitrogen source concentration (Belghith, Dahech, Belghith, & Mejdoub, 2012; Silbir et al., 2014). The maximum levansucrase enzyme production was observed when yeast extract was used as a nitrogen source than other nitrogen sources such as corn steep liquor, peptone, tryptone, urea, sodium nitrate and ammonium nitrate (Belghith, Dahech, Belghith, & Mejdoub, 2012; Silbir et al., 2014).

3.6. Effect of natural fermentation of FCIS on levan production

The natural microflora present in the FCIS was responsible for ethanol production which may affect the levan production, if the FCIS was not autoclaved immediately after tapping. The effect of ethanol produced by natural fermentation due to delay in autoclave on levan production was also studied. The results show that there was no significant change in the levan yield till 4 h delay in autoclaving the FCIS. The significant decrease in the levan yield was observed beyond 4 h and complete absence was observed when autoclave was delayed for 12 h. The decrease in levan yield may be due to the decrease in the carbon sources and ethanol production (Xia, 2011) by other microorganisms, which also declined the growth of *Bacillus subtilis*. The levan was not obtained when fermentation experiment was conducted without autoclaving the FCIS and this may be due to the growth of other natural microflora and ethanol production.

3.7. Confirmation and characterization of levan polysaccharide

The Levan obtained from the media is subjected to dialysis, the final yield before and after dialysis is provided in Supplementary Table 1. The dialyzed levan was analysed by TLC to determine the monosaccharide composition. The R_f values of the levan, fructose, sucrose and glucose were obtained as 0.48, 0.48, 0.41 and 0.44, respectively. The R_f value of levan and fructose was similar and confirmed that the levan was composed solely of fructose. The earlier reports (Kojima et al., 1993; Laddha & Chitanand, 2017) stated that the TLC method easily identified the levan composition (only fructose). The molecular structure of compounds can be determined by FT-IR and NMR spectroscopy methods.

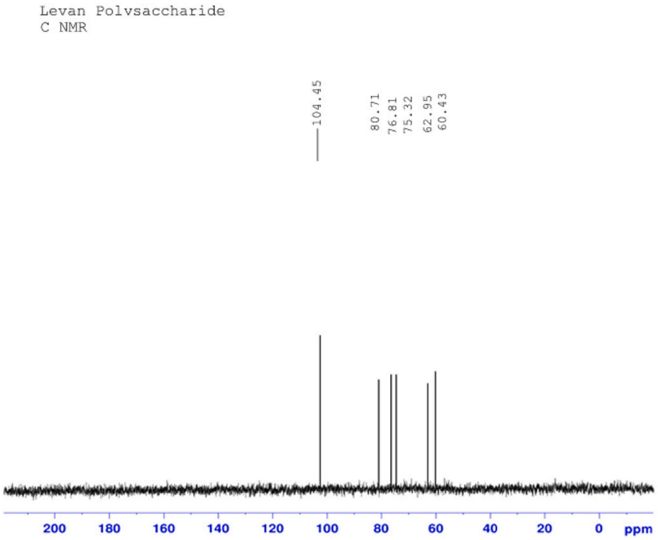

Fig. 8. C NMR spectrum of levan.

FT-IR spectrum (Fig. 7) was used to identify the functional groups of levan. The hydroxyl (OH) vibrations of polysaccharide exhibited by a strong band at 3262.25 cm^{-1} and band 2929.99 cm^{-1} was carbon-hydrogen stretching; the fingerprint region confirms the

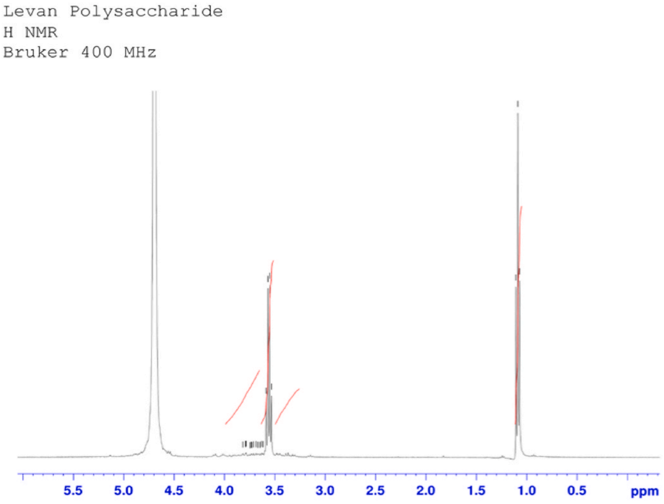

Fig. 9. H NMR spectrum of levan.

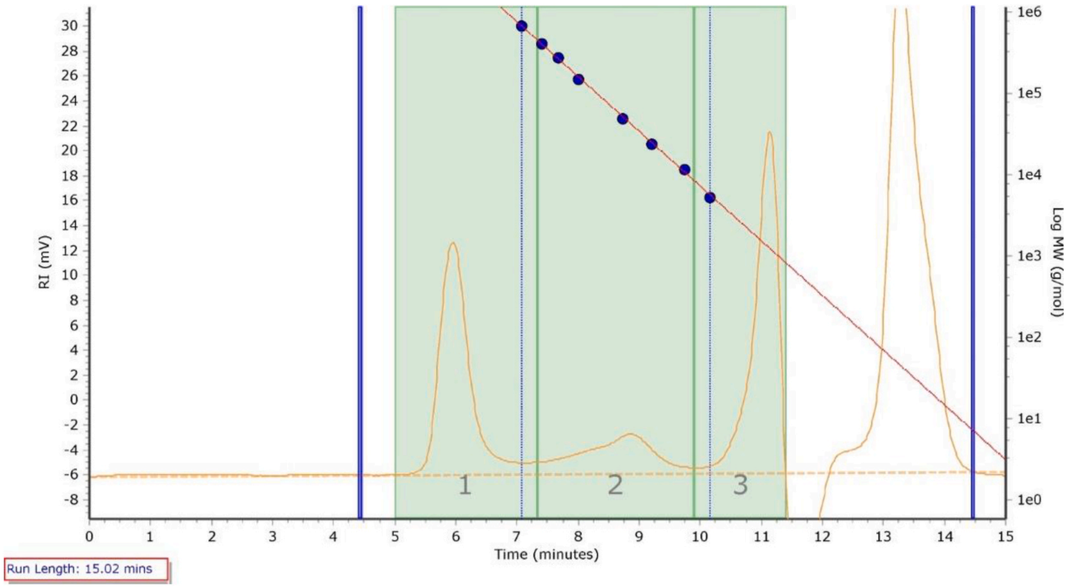

Fig. 10. Gel permeation chromatogram of Levan.

Table 4
Molecular weight characteristics of Levan.

Peak	Mn (g/mol)	Mw (g/mol)	Mz (g/mol)	Mv (g/mol)	PD
Peak I	2727254	3614672	4212891	4136104	1.325
Peak 2	43919	95791	185308	172540	2.181
Peak 3	1409	1623	2053	1962	1.152

presence of fructose (Barone & Medynets, 2007). The band 1646.30 cm^{-1} specify the bound water, 1417.15 cm^{-1} assigned the C–H stretch and spectrum around 1105.67 cm^{-1} and 1041.81 cm^{-1} corresponds to glycosidic linkage. The peaks between 1200 cm^{-1} to 900 cm^{-1} considered as fingerprint regions for polysaccharides (Fellah et al., 2009). The peaks between 923.05 cm^{-1} and 830.67 cm^{-1} correspond to the pyran ring of levan (Dahech et al., 2013). The hydroxyl C–H stretching, glycosidic linkages and fructose were major bands indicating the compound was levan.

Nuclear Magnetic Resonance spectroscopy can be used to determine the detailed structure of levan. The ^{13}C NMR spectrum (Fig. 8) showed six resonances 60.43 (C1), 104.45 (C2), 76.81 (C3), 75.32 (C4), 80.71 (C5) and 62.95 (C6). The chemical shift 104.45 carbon corresponds to the β configuration at C2 of fructose. The C6 signal at 62.95 ppm corresponds to the unique β (2–6) fructofuranoside (Han, 1989). The ^{1}H NMR (Fig. 9) spectrum showed seven chemical shifts 3.74 (H-1a), 3.68 (H-1b), 4.12 (H3), 4.07 (H4), 3.89 (H5), 3.86 (H-6a) and 3.55 (H-6b) ppm. The strong C3, C4 and C5 shifts were closely grouped, which is the unique character of levan (Han & Clarke, 1990). The FT-IR and NMR spectrum were compared with the Sigma Aldrich standard and spectrum of previous study (Shih et al., 2005). The spectrum obtained was similar to the standard and spectrum of levan of previous studies (Mamay et al., 2015; Rütering et al., 2016; Shih et al., 2005).

The levan collected after dialysis through the membrane with molecular weight cut-off (14 kDa) was analysed for molecular weight using Gel permeation chromatography. The results showed three peaks (Fig. 10), both low and high molecular weight levan were observed. The amount of low molecular weight levan was higher than high molecular weight levan (Table 4).

4. Conclusion

The work was done to produce levan from an alternative low-cost substrate coconut inflorescence sap. Central composite design can be successfully used to optimize the production process and study the combined effect of temperature, initial pH and agitation speed on levan production. The study showed that both sucrose and nitrogen source yeast extract have effect on levan yield. The levan was composed solely of fructose and structural confirmation was done by FT-IR and NMR spectroscopy. The molecular weight was analysed and observed that both low and high molecular weight levan was formed. Overall, the levan yield was very high at fresh sap sucrose concentration when compared to other studies. Coconut inflorescence sap can be the best alternative and cheap source for levan production. The levan production will be a costly process as the synthetic medium requires pure chemicals which are costlier. The results of the study showed that the production of levan at low cost is feasible with coconut inflorescence sap in less time with *Bacillus subtilis*.

CRediT authorship contribution statement

Gopinath Mummaleti: Performed all the experiments. **Chayanika Sarma:** Performed a part of the experiments in the study. Suresh Kumar Kalakandan, Supervision. **Vignesh Sivanandham:** Conceptualization, Formulation of the objective, concept of the study and designed the study. **Ashish Rawson:** Formal analysis, and verification of data. **Arunkumar Anandharaj:** Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare no potential conflict of interest.

Acknowledgement

We thank Dr. C. Anandharamakrishnan, Director, Indian Institute of Food Processing Technology, Thanjavur for providing the facility to carry out the research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.110619.

References

Abou-taleb, K., Abdel-Monem, M., Yassin, M., & Draz, A. (2015). Production, purification and characterization of levan polymer from Bacillus lentus V8 strain. *British Microbiology Research Journal, 5*(1), 22–32. https://doi.org/10.9734/bmrj/2015/12448

Ameetha, G., Madhavan, M., & Rajamohan, T. (2018). Exploring the potential of coconut based beverages as functional foods. *11*(7), 1066–1072.

Bae, I. Y., Oh, I. K., Lee, S., Yoo, S. H., & Lee, H. G. (2008). Rheological characterization of levan polysaccharides from Microbacterium laevaniformans. *International Journal of Biological Macromolecules, 42*(1), 10–13. https://doi.org/10.1016/j.ijbiomac.2007.08.006

Barone, J. R., & Medynets, M. (2007). Thermally processed levan polymers. *Carbohydrate Polymers, 69*(3), 554–561. https://doi.org/10.1016/j.carbpol.2007.01.017

Belghith, K. S., Dahech, I., Belghith, H., & Mejdoub, H. (2012). Microbial production of levansucrase for synthesis of fructooligosaccharides and levan. *International Journal of Biological Macromolecules, 50*(2), 451–458. https://doi.org/10.1016/j.ijbiomac.2011.12.033

Belghith, K. S., Dahech, I., Hamden, K., Feki, A., Mejdoub, H., & Belghith, H. (2012). Hypolipidemic effect of diet supplementation with bacterial levan in cholesterol-fed rats. *International Journal of Biological Macromolecules, 50*(4), 1070–1074. https://doi.org/10.1016/j.ijbiomac.2012.02.024

Boa, J. M., & LeDuy, A. (1987). Pullulan from peat hydrolyzate fermentation kinetics. *Biotechnology and Bioengineering, 30*(4), 463–470. https://doi.org/10.1002/bit.260300402

Bondarenko, O. M., Ivask, A., Kahru, A., Vija, H., Titma, T., Visnapuu, M., Joost, U., Pudova, K., Adamberg, S., Visnapuu, T., & Alamäe, T. (2015). Bacterial polysaccharide levan as stabilizing, non-toxic and functional coating material for microelement-nanoparticles. *Carbohydrate Polymers, 136*, 710–720. https://doi.org/10.1016/j.carbpol.2015.09.093

Calazans, G. M. T., Lima, R. C., De França, F. P., & Lopes, C. E. (2000). Molecular weight and antitumour activity of Zymomonas mobilis levans. *International Journal of Biological Macromolecules, 27*(4), 245–247. https://doi.org/10.1016/S0141-8130(00)00125-2

Cheng, K. C., Demirci, A., & Catchmark, J. M. (2011). Evaluation of medium composition and fermentation parameters on pullulan production by Aureobasidium pullulans. *Food Science and Technology International, 17*(2), 99–109. https://doi.org/10.1177/1082013210368719

Chidambaram, J. S. C. A., Veerapandian, B., Sarwareddy, K. K., Mani, K. P., Shanmugam, S. R., & Venkatachalam, P. (2019). Studies on solvent precipitation of levan synthesized using Bacillus subtilis MTCC 441. *Heliyon, 5*(9), Article e02414. https://doi.org/10.1016/j.heliyon.2019.e02414

Dahech, I., Belghith, K. S., Hamden, K., Feki, A., Belghith, H., & Mejdoub, H. (2011). Antidiabetic activity of levan polysaccharide in alloxan-induced diabetic rats. *International Journal of Biological Macromolecules, 49*(4), 742–746. https://doi.org/10.1016/j.ijbiomac.2011.07.007

Dahech, I., Fakhfakh, J., Damak, M., Belghith, H., Mejdoub, H., & Belghith, K. S. (2013). Structural determination and NMR characterization of a bacterial exopolysaccharide. *International Journal of Biological Macromolecules, 59*, 417–422. https://doi.org/10.1016/j.ijbiomac.2013.04.036

Domżał-Kędzia, M., Lewińska, A., Jaromin, A., Weselski, M., Pluskota, R., & Łukaszewicz, M. (2019). Fermentation parameters and conditions affecting levan production and its potential applications in cosmetics. *Bioorganic Chemistry, 93* (December 2018), 1–8. https://doi.org/10.1016/j.bioorg.2019.02.012

Fellah, A., Anjukandi, P., Waterland, M. R., & Williams, M. A. K. (2009). Determining the degree of methylesterification of pectin by ATR/FT-IR: Methodology optimisation and comparison with theoretical calculations. *Carbohydrate Polymers, 78*(4), 847–853. https://doi.org/10.1016/j.carbpol.2009.07.003

Giavasis, I. (2013). Production of microbial polysaccharides for use in food. In *Microbial production of food ingredients, enzymes and nutraceuticals*. Woodhead Publishing Limited. https://doi.org/10.1533/9780857093547.2.413.

Gomes, T. D., Caridade, S. G., Sousa, M. P., Azevedo, S., Kandur, M. Y., Öner, E. T., Alves, N. M., & Mano, J. F. (2018). Adhesive free-standing multilayer films containing sulfated levan for biomedical applications. *Acta Biomaterialia, 69*, 183–195. https://doi.org/10.1016/j.actbio.2018.01.027

Gupta, S., Das, P., Singh, S., Akhtar, M., Meena, D., & Mandal, S. (2011). Microbial levari, an ideal prebiotic and immunonutrient in aquaculture. *World Aquaculture, 42*(1), 61.

Han, Y. W. (1989). Levan production by Bacillus polymyxa. *Journal of Industrial Microbiology, 4*(6), 447–451. https://doi.org/10.1007/BF01569641

Han, Y. W. (1990). Microbial levan. *Advances in Applied Microbiology, 35*(C), 171–194. https://doi.org/10.1016/S0065-2164(08)70244-2

Han, Y. W., & Clarke, M. A. (1990). Production and characterization of microbial levan. *Journal of Agricultural and Food Chemistry, 38*(2), 393–396. https://doi.org/10.1021/jf00092a011

Han, Y. W., & Watson, M. A. (1992). Production of microbial levan from sucrose, sugarcane juice and beet molasses. *Journal of Industrial Microbiology, 9*(3–4), 257–260. https://doi.org/10.1007/BF01569633

Jiang, L. (2010). Optimization of fermentation conditions for pullulan production by Aureobasidium pullulan using response surface methodology. *Carbohydrate Polymers, 79*(2), 414–417. https://doi.org/10.1016/j.carbpol.2009.08.027

Kojima, I., Saito, T., Iizuka, M., Minamiura, N., & Ono, S. (1993). Characterization of levan produced by Serratia sp. *Journal of Fermentation and Bioengineering, 75*(1), 9–12. https://doi.org/10.1016/0922-338X(93)90169-9

Košťálová, Z., & Hromádková, Z. (2019). Structural characterisation of polysaccharides from roasted hazelnut skins. *Food Chemistry, 286*(February), 179–184. https://doi.org/10.1016/j.foodchem.2019.01.203

Küçükaşik, F., Kazak, H., Güney, D., Finore, I., Poli, A., Yenigün, O., Nicolaus, B., & Öner, E. T. (2011). Molasses as fermentation substrate for levan production by Halomonas sp. *Applied Microbiology and Biotechnology, 89*(6), 1729–1740. https://doi.org/10.1007/s00253-010-3055-8

Laddha, N. B., & Chitanand, M. P. (2017). Screening of levan producing bacteria from soil collected from jaggery field. *International Journal of Pharmaceutical, Chemical and Biological Sciences, 7*(3), 202–210.

Liu, J., Luo, J., Ye, H., & Zeng, X. (2012). Preparation, antioxidant and antitumor activities in vitro of different derivatives of levan from endophytic bacterium Paenibacillus polymyxa EJS-3. *Food and Chemical Toxicology, 50*(3–4), 767–772. https://doi.org/10.1016/j.fct.2011.11.016

Li, Q., Wang, W., Jia, H., & Zhang, Y. (2017). Molecular structural properties of a polysaccharide isolated and purified from Sophora japonica pods and its relationship to their rheology. *International Journal of Food Properties, 20*(11), 2844–2854. https://doi.org/10.1080/10942912.2016.1255897

Lončarević, B., Lješević, M., Marković, M., Anđelković, I., Gojgić-Cvijović, G., Jakovljević, D., & Beškoski, V. (2019). Microbial levan and pullulan as potential protective agents for reducing adverse effects of copper on Daphnia magna and Vibrio fischeri. *Ecotoxicology and Environmental Safety, 181*(May), 187–193. https://doi.org/10.1016/j.ecoenv.2019.06.002

Mamay Wahyuningrum, D., & Hertadi, R. (2015). Isolation and characterization of levan from moderate halophilic bacteria Bacillus licheniformis BK AG21. *Procedia Chemistry, 16*, 292–298. https://doi.org/10.1016/j.proche.2015.12.055

Mantovan, J., Bersaneti, G. T., Faria-Tischer, P. C. S., Celligoi, M. A. P. C., & Mali, S. (2018). Use of microbial levan in edible films based on cassava starch. *Food Packaging and Shelf Life, 18*, 31–36. https://doi.org/10.1016/j.fpsl.2018.08.003. February.

Melo, I. R., Pimentel, M. F., Lopes, C. E., & Calazans, G. M. T. (2007). Application of fractional factorial design to levan production by Zymomonas mobilis. *Brazilian Journal of Microbiology, 38*(1), 45–51. https://doi.org/10.1590/S1517-83822007000100010

Misra, B. (2016). Neera: The coconut sap: A review. *International Journal of Food Sciences & Nutrition, 1*(4), 35–38.

Nageswara Rao, G. (2007). Statistics for agricultural sciences. *Journal of Chemical Information and Modeling, 53*(Issue 9). https://doi.org/10.1017/CBO9781107415324.004

Ni, D., Xu, W., Bai, Y., Zhang, W., Zhang, T., & Mu, W. (2018). Biosynthesis of levan from sucrose using a thermostable levansucrase from Lactobacillus reuteri LTH5448. *International Journal of Biological Macromolecules, 113*, 29–37. https://doi.org/10.1016/j.ijbiomac.2018.01.187

Queiroz, E. A. I. F., Fortes, Z. B., da Cunha, M. A. A., Sarilmiser, H. K., Barbosa Dekker, A. M., Öner, E. T., Dekker, R. F. H., & Khaper, N. (2017). Levan promotes antiproliferative and pro-apoptotic effects in MCF-7 breast cancer cells mediated by oxidative stress. *International Journal of Biological Macromolecules, 102*, 565–570. https://doi.org/10.1016/j.ijbiomac.2017.04.035

Ragab, T. I. M., Malek, R. A., Elsehemy, I. A., Farag, M. M. S., Salama, B. M., Abd EL-Baseer, M. A., Gamal-Eldeen, A. M., El Enshasy, H. A., & Esawy, M. A. (2019). Scaling up of levan yield in Bacillus subtilis M and cytotoxicity study on levan and its derivatives. *Journal of Bioscience and Bioengineering, 127*(6), 655–662. https://doi.org/10.1016/j.jbiosc.2018.09.008

Rütering, M., Schmid, J., Rühmann, B., Schilling, M., & Sieber, V. (2016). Controlled production of polysaccharides-exploiting nutrient supply for levan and heteropolysaccharide formation in Paenibacillus sp. *Carbohydrate Polymers, 148*, 326–334. https://doi.org/10.1016/j.carbpol.2016.04.074

Sezer, A. D., Kazak, H., Öner, E. T., & Akbua, J. (2011). Levan-based nanocarrier system for peptide and protein drug delivery: Optimization and influence of experimental parameters on the nanoparticle characteristics. *Carbohydrate Polymers, 84*(1), 358–363. https://doi.org/10.1016/j.carbpol.2010.11.046

Shih, I. L., & Yu, Y. T. (2005). Simultaneous and selective production of levan and poly (γ-glutamic acid) by Bacillus subtilis. *Biotechnology Letters, 27*(2), 103–106. https://doi.org/10.1007/s10529-004-6936-z

Shih, I. L., Yu, Y. T., Shieh, C. J., & Hsieh, C. Y. (2005). Selective production and characterization of levan by Bacillus subtilis (Natto) Takahashi. *Journal of Agricultural and Food Chemistry, 53*(21), 8211–8215. https://doi.org/10.1021/jf058084o

Silbir, S., Dagbagli, S., Yegin, S., Baysal, T., & Goksungur, Y. (2014). Levan production by Zymomonas mobilis in batch and continuous fermentation systems. *Carbohydrate Polymers, 99*, 454–461. https://doi.org/10.1016/j.carbpol.2013.08.031

Singh, R. S., Chauhan, K., Pandey, A., Larroche, C., & Kennedy, J. F. (2018). Purification and characterization of two isoforms of exoinulinase from Penicillium oxalicum BGPUP-4 for the preparation of high fructose syrup from inulin. *International Journal of Biological Macromolecules, 118*. https://doi.org/10.1016/j.ijbiomac.2018.07.040 (July), 1974–1983.

Sudha, R., Niral, V., Hebbar, K. B., & Samsudeen, K. (2019). *Coconut inflorescence sap. Current science*. https://doi.org/10.18520/cs/v116/i11/1809-1817. June.

Szwengiel, A., & Wiesner, M. (2019). Effect of metal ions on levan synthesis efficiency and its parameters by levansucrase from Bacillus subtilis. *International Journal of Biological Macromolecules, 128*, 237–243. https://doi.org/10.1016/j.ijbiomac.2019.01.155

Tanaka, T., Oi, S., & Yamamoto, T. (1980). The molecular structure of low and high molecular weight levans synthesized by levansucrase. *Journal of Biochemistry, 87*(1), 297–303. https://doi.org/10.1093/oxfordjournals.jbchem.a132737

Vina, I., Karsakevich, A., Gonta, S., Linde, R., & Bekers, M. (1998). Influence of some physicochemical factors on the viscosity of aqueous levan solutions of Zymomonas mobilis. *Acta Biotechnologica, 18*(2), 167–174. https://doi.org/10.1002/abio.370180214

Wu, F. C., Chou, S. Z., & Shih, I. L. (2013). Factors affecting the production and molecular weight of levan of Bacillus subtilis natto in batch and fed-batch culture in fermenter. *Journal of the Taiwan Institute of Chemical Engineers, 44*(6), 846–853. https://doi.org/10.1016/j.jtice.2013.03.009

Xia. (2011). Chemical composition changes of post-harvest coconut inflorescence sap during natural fermentation. *African Journal of Biotechnology, 10*(66), 14999–15005. https://doi.org/10.5897/ajb10.2602

Xu, X., Gao, C., Liu, Z., Wu, J., Han, J., Yan, M., & Wu, Z. (2016). Characterization of the levan produced by Paenibacillus bovis sp. nov BD3526 and its immunological activity. *Carbohydrate Polymers, 144*, 178–186. https://doi.org/10.1016/j.carbpol.2016.02.049