Characterization of levan produced from coconut inflorescence sap using *Bacillus subtilis* and screening of Levan producing bacteria

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ABSTRACT

Levan is a biopolymer formed by β-(2,6) linkage having β-(1,2) linkages on its branch points and contains long chain of fructose units in its chemical structure . Levan is an extracellular polysaccharide composed predominantly of D-fructose residues joined by glycosidic bonds.The Thermal and functional properties of the levan produced by Bacillus subtilis from coconut inflorescence sap. Levan is thermally stable even at about 300C . Screening the levan production by using emulsifying, water and oil holding capacity, interestingly levan Exhibited anti-inflammatory, anti-oxidant activity . The potential of levan producing from sucrose-rich substrate sugarcane, molasses and pineapple extract where explored individual and in combination with coconut inflorescence sap.

KEYWORDS : Levan, coconut inflorescence sap, anti-oxidant , anti-inflammatory, polysaccharide, antimicrobial

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INTRODUCTION :

Levan is a biopolymer formed by β-(2,6) linkage having β-(1,2) linkages on its branch points and contains long chain of fructose units in its chemical structure . It develops in the course of a trans-glycosylation reaction with the participation of the levansucrase. Levansucrase is an enzyme produced by gram-positive bacteria such as Bacillus subtilis, Lactobacillus reuteri, gram-negative bacteria, such as Zymomonas mobilis, Halomonas sp. , Erwiniaamyl ovora , Pseudomonas syringaee P. chlororaphis and fungi, such as Streptococcus mutans and Actinomyces viscosus . B. subtilis is considered as a model to study gram-positive bacteria due to their genetic and physiological characteristics and for not pathogenic potential, non-colonizing tissues and good ability to form spores. Furthermore, it is naturally found in soil and for these reasons it has been the microorganism of choice for obtaining levansucrase

It can be found in plants and many microbial strains, microbial levans are much larger than those produced by plants with multiple branches with molecular weights ranging from 2 to 100 million Da. and more beneficial in: economical, industrially, food, medicine, pharmaceutical, cosmetic and commercial industrial sectors, while plant levans generally have molecular weights ranging from 2000 to 33,000 Da. In addition to its other uses as

stabilizers, emulsifiers, thickeners, fillers, low-calorie natural sweetener, encapsulating flavors andperfumes, and its ability to form edible wrappers, its highability to break down some free radicals and preventoxidation. Levan also has healthy properties as it is anti-cancer and antidiabetic, as well as anti-oxidant and anti-inflammatory activities, In cosmetic processes and other therapeutic materials reported that levan could be used as a blood plasma substitute and lower cholesterol in the body. In food, it used as a stabilizer, thickener, fat substitute and color and flavor mentioned that levan used as industrial gums (gum Arabic) and fillers ,fat substitues ,sweeteners

The production of levan is influenced not only by the carbon source, but also by other fermentation conditions such as temperature, pH, type of fermentation, cell growth rate and oxygen content in the fermentation medium.Bacillus is one of the most important bacteria used in the production of microbial levan. 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

The fermentation conditions for levan production from coconut inflorescence sap using *B. subtilis* were optimized and confirmed levan through structural elucidation. The present study was aimed to study the thermal and functional properties of levan produced by *B. subtilis* from coconut inflorescence sap. The sensory acceptability of the cake coated with cream made by replacing icing sugar. The potential combination of low-cost substrates such as sugar cane molasses, coconut inflorescence sap, and pineapple extract for levan production was explored

In medicine, it can be used as a plasma substitute, drug activity prolonger, antiobesity and lipid-lowering agent, osmoregulator, cryoprotector, or antitumor agent. The application potential of this biopolymer in the entire sectors is stimulating an intense and constant search aiming for a better new options so that we can get better alternative. In this context the present work was aimed to screen levan producers , which is the rich source of various bacterial species.

MATERIALS AND METHODS

Materials

The fresh coconut inflorescence sap was tapped freshly every time on the day of experiment. The coconut inflorescence sap 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, mag-nesium 0.027, copper 0.0043, and manganese 0.00099 was used as a fermentation medium . The freeze-dried B. subtilis culture used in this study was procured from National Chemical Laboratory, Pune. The culture was revived and stored at 4 ◦C in agar slants and sub-cultured every time before the experiments. The micro-biological culture media and chemicals of AR grade were used in this study purchased from Himedia Pvt. Ltd and Sigma-Aldrich, Mumbai, India. The sterile Millipore water was used in the entire study. The pH adjustment in all the experiments was made with 1.0 N HCl and 1.0 N NaOH. The heavy whipping cream, vanilla essence, and sugar were purchased from the local market of Thanjavur, India.

FERMENTIVE PRODUCTION OF LEVAN FROM COCONUT INFLORESCENCE SAP

The levan used in this study was produced at optimized fermentation and downstream conditions from our previous research (Mummaleti et al., 2021). About 1 mL of freshly standardized subculture was added to fresh autoclaved coconut inflorescence sap with initial pH of 6.5 and incubated in the Excella E24R Bench top shaking incubator (New Brunswick Scientific, USA) at a temperature of 35 ◦C and 150 rpm agitation speed for 20 h. The fermented medium was centrifuged (Remi C23 plus, Remi Elektrotechnik LTD, Vasai, India) at 14083 g for 10 min (Abou-taleb et al., 2015), and the supernatant was collected. The levan was precipitated by adding five volumes of 100% ice-cold ethanol and storing it at 4 ◦C for 24 h. The precipitated levan was collected by centrifugation at 5070g at 4 ◦C for 20 min (Chidambaram et al., 2019). The pellet of crude levan obtained was dissolved in deionized water and

dialyzed against the demineralized water to remove the small molecules and unfermented sugars. The purified levan was precipitated again by adding 100% ethanol. The precipitated levan was air-dried at room temperature for 24 h and used for further study.

SCREENING OF LEVAN PRODUCING BACTERIA

Ten fold serial dilutions of the tube showing turbidity were prepared. 1ml of diluted sample was spread inoculated on 5% and 10% sucrose agar plates. Plates were incubated at 300C for 48hrs. Organisms producing mucoid colonies were selected as levan producer. Cultural and morphological characters of colonies were studied and colonies were sub-cultured on nutrient agar slants and preserved for further studies.

CHARACTERIZATION OF LEVAN POLYSACCHARIDE

Thermal properties

The Differential scanning calorimetry (DSC) (Mettler Toledo, USA) was conducted to study the thermal properties of levan polysaccharides (Taylan et al., 2019). The 5 mg of levan sample was placed in an aluminum pan sealed, and properties were studied during heating and cooling. The heating and cooling rate was 10 ◦C/min from 10 to 500 ◦C and then 500 to 10 ◦C.

Thermal Stability

Thermal stability of levansucrase was examinatedin two steps. First,the enzyme was incubating at different temperatures (30, 50, 70 and 90oC) and pH 5.0 for 16 h and in second step the enzyme was incubated at 30 and 50°C and pH 5.0 for 10 days. Experiments were performed in triplicate, levansucrase activity were determined and the results expressed as a percentage of residual activity compared to control

Effect of salts

Fermentations were carried out to evaluate the influence of NaCl, KCl, and Na2SO4 (0.2 to 0.8 M)using pre-defined optimal conditions of levansucrase production.

Levan Determination

Levanwas precipitated from CFE with 3 volof absolute ethanol at 4°C for 12 h, centrifuged at 18.060xg at 4°C for 20 min and hydrolyzed with 0.1 M HCl at 100°C for 1 h [35], neutralized with NaOH 2M and determinated by reducing sugars according to Somogyi and Nelson [36] with fructose as standard.

Oil holding capacity

The oil holding capacity is defined as the amount oil absorbed by a unit weight material which refers to adsorption of organic compounds on substrate surface (Haddar et al., 2021). The oil holding capacity of levan was measured according to Haddar et al. (2021). About 500 mg of levan was mixed with 10 mL of sunflower oil and kept for 60 min at ambient temperature. The sample was then centrifuged at 5070 g for 30 min. The oil holding capacity was determined as the weight of contents in the tube after draining divided by the weight of dried levan and expressed as % weight of dried levan.

Determination the optimal conditions for levan production Different conditions were used to determine the optimum one for increasing the production of levan, these conditions include; Effect of different:

1. Carbon sources

Mineral salt broth containing 20% of different carbon sources (lactose, glucose) were prepared and inoculated with 1% of activated bacterial culture broth (optical density, 0.1), incubated at 37°C for 48h. levan was extracted and dry weight was weighted after incubation periods, and compared with the dry weight of levan that extracted from mineral salt broth containing 20% of sucrose previously prepared and inoculated with 1% of activated bacterial culture.

2. Nitrogen sources

Mineral salt broth media with optimum carbon source were prepared with additional of two nitrogen sources (yeast extract and peptone) at concentration (1%), and inoculated with 1% of activated bacterial culture broth, incubated at 370C for 48h. after incubation periods, levan was extracted and dry weight was weighted.

3. pHs value

Mineral salt broth media with 20 % of optimum carbon source were prepared with different pHs value (5, 6, 7, 8, 9 and 10), and inoculated with 1% of activated bacterial culture broth, incubated at 370C for 48h. levan was extracted after incubation periods and dry weight was weighted.

4. Temperature

Mineral salts broth media with optimum source of (carbon, nitrogen) and optimum pH value were prepared and inoculated with 1% of bacterial culture broth, incubated at different temperatures (370C, 450C and 500C) for 48h, after incubation, levan was extracted, and dry weight was weighted.

ANTIOXIDANT ACTIVITY

DPPH assay. According to the procedure suggested by Srikanth et al. (2015), the antioxidant activity of the levan was evaluated via in-vitro 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity. DPPH with 0.1 mM concentration was prepared in ethanol. The 1 mL of aqueous levan solution of different concentrations (0, 50, 100, 200, 300, 400, 500, 1000 and 1200 µg/mL) mixed with 300 µL of freshly prepared DPPH reagent and incubated in the dark for 30 min at room temperature. After incubation, the absorbance was measured at 517 nm using a UV visible spectrophotometer (UV – 1800 Shimadzu, Japan). The mixture of aqueous levan and ethanol was taken as blank, and a mixture of DPPH and ethanol was taken as a control. The antioxidant activity was measured as % inhibition of free radical formation by levan and calculated using the formula.

% Inhibition = (Absorbance of controltAbsorbance of sample)/Absorbance of control x100

LEVANSUCRASE ASSAY

Levansucrase assays were performed according to themethod of Yanase et al. (13). Culture filtrate (0.5 mL) was incubated with. 1 mL 20%sucrose (Sigma) and 0.5 mL 0.1 M acetate buffer at pH 5.2, and incubated at 30 C for15 min. The decreasing amounts of sugars produced were measured by glucoseoxidase kits.Polymer

ANTIMICROBIAL ACTIVITY

The in-vitro antibacterial activity of levan was determined by the standard agar disc diffusion method against the pathogenic microorganisms (Carson & Riley, 1995). The standard bacterial cultures Listeria monocytogenes,

Salmonella abony, Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa were purchased from the National chemical laboratory, Pune, and used in this study. The sterile Muller Hinton agar plates were prepared and inoculated with bacterial cultures using a sterile swab. The sterile Himedia disc (0.7 cm) was saturated with 100 µL of levan sample (50, 100, 200 µg concentration) and placed on the agar plates. The plates were then incubated at 37 ◦C for 24 h, and antimicrobial activity was determined by measuring the Zone of Inhibition.

Effect of incubation temperature on levan production

Different temperature degrees (25, 32, 37, 45 and 50)°C were examined for the substitute media of levan production by local isolate, initial pH of media was 6.8, other optimum conditions were used as the conditions of the levan production.

Effect of initial pH on levan production

To evaluate optimum pH for levan production from substitute media by local isolate, pH of production media was adjusted to 4, 4.5, 5, 5.5, 6, 6.5, 6.8, 7.5 and 8 by using HCl (0.2 M) or NaOH (0.1 M). The optimal conditions were obtained from previous steps were used.

ANTI-INFLAMMATORY ACTIVITY

The protein denaturation method was used to study the anti-inflammatory activity of levan according to the procedure given by Srikanth et al. (2015). The 0.05 mL aqueous levan solution of different concentrations (0, 50, 100, 150, 200, 250, 300, 350, 400 and 500 µg/mL) were mixed with 0.45 mL of Bovine serum albumin (BSA) (5% w/v). The sample’s pH was adjusted to pH 6.3 with 1N HCl and incu-bated at 37 ◦C for 30 min and 57 ◦C for 3 min. Later, samples were cooled, and 2.5 mL of phosphate buffer saline was added, and absor-bance was measured at 416 nm. The Diclofenac sodium was taken as a standard for comparison, and % inhibition was calculated using the formula. Where Test control is 0.45 mL BSA +0.05 mL distilled water; product control is 0.45 mL distilled water +0.05 mL of levan.

% Inhibition = 100 − (Absorbance of the test solution − Absorbance of the product contol)/Absorbance of the test control x100

TLC OF LEVAN

Hydrolyzed samples were applied to Silica Gel plates saturated with solvent 1 Butanol: 2 propanol: water: acetic acid [7:5:4:2]. Standards of glucose [Hi media Pvt. Ltd.] and fructose were run and plates were sprayed with 5% sulfuric acid in methanol, air dried and then heated at 1100 C until spots appeared.

CONCLUSION

The present work was conducted to study the thermal and functional Effect of levan. The study revealed that the levan polysaccharide is highly thermally fixed. The application of levan in thermally dehydrated foods and packaging film synthesis through blow molding and extrusion can be explored further. Antioxidant, anti-inflammatory, and antimicrobial activity against pathogenic microorganisms indicates the strong biological activity of levan, and its application in various pharmaceutical and biomedical industries can be studied. The levan polysaccharide can be used as a bio-emulsifier, an excellent alternative for synthetic chemical emulsifiers. The levan

polysaccharide was produced using sugar cane molasses, coconut inflorescence sap, and pineapple extract in various combinations and had a maximum levan of 69.40 g/L. The coconut inflorescence sap is observed to be a potential accessory substrate for total levan production.

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