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Antioxidant and anti-inflammatory levan produced from *Acetobacter xylinum* NCIM2526 and its statistical optimization

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ABSTRACT

Levan is a homopolymer of fructose naturally obtained from both the plants and microorganisms. Along with the general properties of a biopolymer 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 and hyperglycaemic inhibitor. In this study, we have demonstrated the microbial production of therapeutically potential levan by batch fermentation process in sucrose rich medium using *Acetobacter xylinum* NCIM 2526. The produced Levan was characterized using various physicochemical techniques such as FTIR, ^{1}H NMR, ^{13}C NMR spectroscopy, TGA and HPLC. The biomedical potential of the isolated *A. xylinum* levan for its anti-oxidant and anti-inflammatory activities was exploited *in vitro*. Further the present study also focused on the optimization of levan production using one factor at a time approach followed by a statistical method, central composite design (CCD) with selected variables. The yield of levan was increased significantly from 0.54 to 13.25 g/L with the optimized variables.

1. Introduction

Levan is a branched exopolysaccharide of D-fructo-furanosyl residues joined together by β-(2,6) linkages in the core chain and β-(2,1) linkages at branching points (Arvidson, Rinehart & Gadala-Maria, 2006). It is strictly non-reducing and resistant to action of amylases and invertases secreted by yeast. Levan exhibits some of the most important biomedical and functional food properties owing to properties like biodegradability, biocompatibility and also the ability to form nanoparticles as well as films. Levan was shown to have prominent prebiotic effects and hypocholesterolaemic effects (Yamamoto et al., 1999) as it produces di-fructo-furanose (Song, Bae, Lee, Lee, & Rhee, 2000), fructose and fructo-oligosaccharides (Jung Kang, Lee, Lee, & Lee, 1999) upon acid hydrolysis in the stomach. Levan shows some promising pharmacological activities like antioxidant, anti-inflammatory, anti-carcinogenic and anti-tumour (Calazans, Lopes, Lima, & De Franc, 1997). In food industry, levan is widely used as an emulsifying and encapsulating agent, colouring and flavouring vehicle (Han, 1990) and as fat substitute (Vijn & Smeekens, 1999). Dietary supplementation of levan can improve the growth performance, digestibility and beneficial effect on the immune response during inflammatory challenge**.** Levan can be used as a new additive for colon specific films and also as a coating for solid oral drugs for specific release (Dos-Santos, Gómez-Pineda, Colabone-Celligoi, & Cavalcanti, 2013). These outstanding properties of levan made it a versatile polymer that is currently being used in many commercial sectors including food, medicine, pharmaceutical, cosmetic and industrial sectors (Ahmed, Kalla, Uppuluri, & Anbazhagan, 2014; Gupta et al., 2010; Kang et al., 2009; Park et al., 2003; Rairakhwada et al., 2007).

Levan is naturally produced from a wide range of microorganisms including bacteria, fungi, and algae and is also produced from a few plant species (Laws, Gu, & Marshall, 2001). Microbial levan are produced from sucrose-based substrate by trans-fructosylation reaction of levansucrase (beta-2,6 fructose; D-glucose-fructosyl transferase EC2.4.1.10) secreted by a variety of microorganisms (Corrigan & Robyt, 1979; Dedonder, 1966) including *Zymomonas, Halomonas, Xanthomonas, Sachharomyces, Acetobacter, Bacillus, Streptococcus, Pseudomonas* (de Oliveira, da Silva, Buzato, & Celligoi, 2007; Dos-Santos et al., 2013; Küçükaşik et al., 2011; Shih, Chen, & Wu, 2010; Silbir, Dagbagli, Yegin, Baysal & Goksungur, 2014; Yoshida, Suzuki & Yagi, 1990).

The fermentative levan production of microbial usage usually requires a high carbon source. Hence sucrose is mainly employed

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in most fermentative productions to achieve high molecular weight levan. Interestingly, sucrose has low inhibitory effects against levansucrase, the enzyme responsible for the synthesis of levan, and thereby increasing the possibility of achieving high polysaccharide yields (Thompson & He, 2006). The current study mainly focuses on the enhanced production of therapeutically important levan using sucrose containing medium from *Acetobacter xylinum* NCIM 2526 with a special emphasis on the statistical optimization through central composite design (CCD).

2. Materials and methods

2.1. Chemicals and media components

Bovine serum albumin (BSA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ascorbic acid, Diclofenac sodium were procured from Sigma, India. Nutrient agar, peptone, malt extract were obtained from Himedia, India. All the other chemicals used in this study were procured from Merck Laboratories Pvt., Ltd. (Mumbai, India).

2.2. Microorganism and its maintenance

A. xylinum NCIM 2526 was procured from National Centre for Industrial Microorganism, NCL (Pune, India). Glycerol stocks were maintained at −20 °C and working stock cultures were maintained at 4 °C. *A. xylinum* NCIM 2526 was cultured in Luria-broth liquid medium (6% sucrose, 1.5% peptone, 0.2% ammonium sulphate, 0.1% potassium dihydrogen phosphate, 0.1% magnesium sulphate heptahydrate, pH 6.8) at 28 °C. Twenty four hour well grown culture was used as an inoculum for the levan production throughout the study.

2.3. Production and isolation of levan

The levan production medium contains sucrose 40.0 (g/L), Bacteriological peptone 10.0 (g/L), Ammonium sulphate ($(NH_4)_2SO_4$) 1.0 (g/L), Potassium dihydrogen phosphate (KH_2PO_4) 1.0 (g/L) and Magnesium sulphate ($MgSO_4{\cdot}7H_2O$) 1.0 (g/L) (Meng & Fütterer, 2003). After 60 h incubation, the exopolysaccharide, levan was harvested from the fermented broth. To remove the dissolved protein, the broth was boiled at 100 °C for 30 min. After cooling, the broth was centrifuged for 30 min at 8000 rpm. The resultant supernatant was further boiled for 5 min to deactivate any residual extracellular enzymes. After cooling down to room temperature, the pH of the supernatant was adjusted to 10 by using 1 M KOH. To this, two volumes of cold 80% ethanol were added to precipitate the levan. Further, the addition of 1% Calcium Chloride under rigorous stirring for 20 min supported the precipitation. The precipitate was collected by centrifugation for 15 min at 13,000 rpm. The resultant pellet was washed with 1.5 volumes of cold 80% ethanol, freeze dried and weighed (Jathore, Bule, Tilay & Annapure, 2012).

2.4. Identification and structural characterization of levan

The presence of fructose in levan isolated from *A. xylinum* was identified by Potassium ferricyanide (PF) sugar test, briefly; 1 mL PF (10 mg/mL) in sodium hydroxide (20%) aqueous solution was added to 1 mL hydrolyzed levan (1 mg/mL). The content was gently shaken and colour change was closely monitored. Fructose and glucose were served as control.

The functional group present in the isolated polysaccharide was identified by FTIR spectroscopy. FTIR spectroscopic measurements were carried out using Perkin Elmer spectrum-one instrument in the diffuse reflectance mode at a resolution of 1 cm^{-1} in KBr pellet. ^{13}CNMR and ^{1}H NMR was measured in a Bruker 300 MHz instrument. (Ahmed et al., 2014).

Further the isolated levan was hydrolyzed by 2 M sulphuric acid at 105 °C for 4 h and then analysed by HPLC. Chromatographic separation was performed with PEAK high performance liquid chromatography having LC-P7000 isocratic pump, equipped with PEAK LC-UV7000 variable wavelength detector. UV detection was made at 195 nm with column temperature of 30 °C. The flow rate was set to 0.6 mL/min and injections of 20 μL were made. Acetonitrile and deionized water (80% and 20%) used as mobile phase (Rahman, Hasan, Hussain & Jahim, 2008). Glucose, fructose and fructo oligosaccharides were used as standards. Thermal stability of the purified levan was determined by measuring the thermogram under nitrogen atmosphere (TA Instruments, SDT Q 600, USA).

2.5. Optimization of levan production using one factor at a time method

Medium development is an essential prerequisite to get higher productivity using any microbial strain. The strain production potential not only depends on the genetic nature, but also on nutrients supply and cultural conditions. So it is important to know the suitable nutrients and cultural conditions required to achieve higher productivity. Fermentative factors like fermentation time, carbon and nitrogen source, inoculum level and initial pH, effecting the secretion of levan by *A. xylinum* were optimized. The strategy adopted for standardization of fermentation parameters was to evaluate the effect of an individual parameter and incorporate it at standard level before standardizing the next parameter. Unless otherwise mentioned all the measurements recorded in the study were performed in triplicates and the mean values with SE were reported.

2.5.1. Effect of fermentation time on levan production

In order to determine the optimum fermentation time, the inoculated culture flasks were incubated in orbital shaking incubator at 30 °C with 150 rpm agitation speed. Samples were drawn aseptically at 6 h intervals and estimated for biomass formation and the levan production.

2.5.2. Effect of different nitrogen sources on levan production

Six different nitrogen sources (Meat peptone, yeast extract, meat extract, bacteriological peptone, ammonium chloride, ammonium hydrogen carbonate) were employed in order to study the effect of nitrogen source on levan production. Further the concentration of the best nitrogen source was optimized.

2.5.3. Effect of pH on levan production

To determine the effect of pH on the production of levan, the production medium was adjusted to various pH ranges from 5.0 to 8.0 and dispersed in 250 mL flasks and each flask was sterilized, inoculated and incubated.

2.5.4. Effect of sucrose concentration on levan production

To study the effect of sucrose concentration on levan production different concentrations of sucrose (20, 30, 40, 50, 60, 70 g/L) were supplemented to levan production medium and dispersed in 250 mL flasks and each flask was sterilized, inoculated and incubated.

2.5.5. Effect of initial levan concentration on levan production

The addition of preformed levan to the production medium accelerates the levan polymerization and offers more yields of levan with higher molecular weight (Han, 1990). So, to study the effect of initial concentrations of levan, levan was added to production media at different concentrations (0.1 g/L, 0.2 g/L, 0.3 g/L, 0.4 g/L, 0.5 g/L and 0.6 g/L).