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Screening and Characterization of Levan Secreted by Halophilic Bacterium of *Halomonas* and *Chromohalobacter* Genuses Originated from Bledug Kuwu Mud Crater

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Abstract

Five bacterial isolates from the genus of *Halomonas* and *Chromohalobacter* isolated from Bledug Kuwu mud crater, located in mainland Purwodadi-Grobogan, Central Java, Indonesia, have been assayed for levan production. Initial screening was conducted on modified Belgith medium using sucrose as the major carbon source, in which the colonies of levan-producing bacteria will have a slimy mucoid appearance when grown on this medium. The screening results showed only one positive bacterial species, which was *Chromohalobacter japonicus* BK AB18 that identified as potential levan producer. Thermal stability of the isolated levan has been charachterized by TGA, which gave approximate decomposition temperature about 211 ^{o}C. The structure of the levan has been elucidated by FTIR and NMR spectroscopies. FTIR spectrum of the isolated levan displayed high similarity to that of levan isolated from *Bacillus methylotrophicus*. The chemical shifts of carbon and proton NMR spectra of the isolated levan also exhibited high similiarity to those of levan isolated from *Pseudomonas fluorescens* and *Zymonas mobils*.

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Keywords: levan, *Halomonas* and *Chromohalobacter japonicus* BK-AB18

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Nomenclature

TGA	Thermal gravimetric analysis
FTIR	Fourier transform-infrared
NMR	Nuclear magnetic resonance

1. Introduction

Levan is biopolymer composed of repeating units residue of β-(2→6)-D-fructofuranosyl linkages, which is naturally the main carbohydrate component contained in the grass[1]. Levan consistent structure composed of short straight chain of β-(2→6)-D-fructofuranosyl linkages (5.5 to 8.7 kDa) with a variation of the branch through the β-(2→1)-glycoside bonds. However, levan generated by a variety of bacteria typically have a consistent straight-through bonding chain β-(2→6)-glycoside bonds with a molecular weight of approximately 1693 to 1851 kDa[2].

Prebiotics are a functional food ingredient that selectively fermented by certain microbes, thus allowing changes in the composition and activity of microflora in the digestive tract, which in turn provide health benefits to its host. There are three criteria for prebiotic ingredients, namely resistance to hydrolysis activity of mammalian enzymes, fermented by intestinal microflora, and selectively stimulate the growth and activity of intestinal bacteria[3]. The levan-type-FOSs have been found to satisfy all 3 prebiotic criteria. Levan can be selectively fermented by certain bacteria resulting in increased growth and activity of bacteria in the colon. *Bifidobacteria* and *Lactobacilli* belong to the type of probiotics which utilizes levan as food. The bacterial can produce the enzyme β-fructofuranosidase capable of hydrolyzing levan. Fermentation products will selectively stimulate the growth of lactic acid bacteria cause a significant increase in the number of good bacteria in the digestive tract so that eventually will inhibit the growth of pathogenic bacteria that cause disease[3].

In addition, it is known that levan produced by the bacterium *Bacillus polymyxa* has the ability to reduce mineral impurities in the ore bauxite[4]. The bacteria secrete extracellular polysaccharides (ECP), protein, and some organic acids, such as acetic acid, oxaloacetic acid, and formic acid. The addition of sucrose in the experiment will secrete levansucrase enzyme by the bacteria. This enzyme catalyzes the formation of levan with the presence of sucrose. Levan presence around the bacterial cell wall will facilitate the binding of mineral impurities from bauxite ore matrix. The binding of metal ions are useful for the growth and metabolism of the bacterial cell. The presence of metal ions will increase the secretion of levan in excess, which can act as a diffusion barrier to prevent the entry of unwanted metal ions into the cell[4].

Indonesia has a wealth of high biodiversity, because it has many unique natural resources as a habitat for the growth of bacteria owing high potential in producing high market value bioproducts, such as levan. One of the unique bacterial habitats was found in the region of Central Java Purwodadi, Grobogan, Bledug Kuwu, where at this location there is a mud volcanic crater that periodically spit brine to the surface. The uniqueness of this habitat has encouraged us to explore the potential of bacteria in producing levan.

In current study, screening of halophilic bacterium having ability to secrete levan from *Halomonas* and *Chromohalobacter g*enuses originated from Bledug Kuwu Mud Crater and the characterization of the levan produced by selected bacterium is reported.

2. Materials and methods

2.1. Materials

Microorganisms used for this study is five isolates halophilic genus of *Halomonas* and *Chromohalobacter* i.e, *Halomonas elongata* BK-AB8, *Halomonas elongata* BK-AG18, *Halomonas eurihalina* BK-AB15, *Halomonas meridiana* BK-AB4 and *Chromohalobacter japonicus* BK-AB18 available in the Biochemistry Laboratory, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology. These five bacterial isolates was isolated from the mud crater of Bledug Kuwu located in the mainland Purwodadi-Grobogan, Central Java, Indonesia.

2.2. Screening mediums and methods

The medium used for screening the bacteria potentially produced levan contained 1% tryptone, 0.5% yeast extract, 1.5% bacto agar, 0.25% K_2HPO_4, 10% NaCl, and 20% sucrose. The plates were incubated at 37 ^oC in constant temperature incubator for 24 hours. These bacteria were identified as a levan producer as it had a slimy mucoid appearance, which indicated the production of levan polymer from sucrose.

2.3. The production of Levan

The production of levan polymer was performed using bacterial isolates having the best potentially produced levan in the optimum medium for levan production contained 1% tryptone, 0.5% yeast extract, 0.25% K_2HPO_4, 7.5% NaCl, and 7.5% sucrose. The media was autoclaved at 121 ^oC for 15 min and then cooled to room temperature before inoculation. The incubation was carried out in a rotary shaker (150 rpm) at 37 ^oC for 24 hours. The supernatant were obtained after centrifugation at 9820 × g for 15 minutes at 4 ^oC. The levan product was then precipitated by adding 2 volumes of absolute ethanol and harvested by centrifugation at 9820 × g for 25 minutes at 4 ^oC. The levan product was dried using freeze drier for 8 hours.

2.4. Levan characterization and identification methods

2.4.1. The Thermal Analysis Gravimetric (TGA) measurements

Thermal analysis gravimetric is a technique to measure the change weight of a compound as a function of temperature or time. The results were plotted with temperature on the X-axis and mass loss on the Y-axis. Thermal decomposition of levan polymer was measured using TGA-LINSEIS STA Platinum Series 1500, at a heating rate of 10 °C/ min from 40 to 450 °C, at Chemical Engineering Laboratory, Faculty of Industrial Technic, Bandung Institute of Technology.

2.4.2. The Fourier Transform-Infrared (FTIR) spectra measurements

The functional groups within the structure of levan isolated from the bacteria studied were determined using fourier transform infrared (FTIR) spectrophotometer. The sample was mixed with KBr in a ratio of 1:10, ground thoroughly and then pressed into a 1 mm pellet. The spectra were recorded in the absorbance from the wavenumber of 4000 to 500 cm^{-1} on an Alpha Bruker FTIR (Thermo Fisher Scientific, USA) at Inorganic Laboratory, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology.

2.4.3. The ^1H and ^{13}C NMR spectra measurements

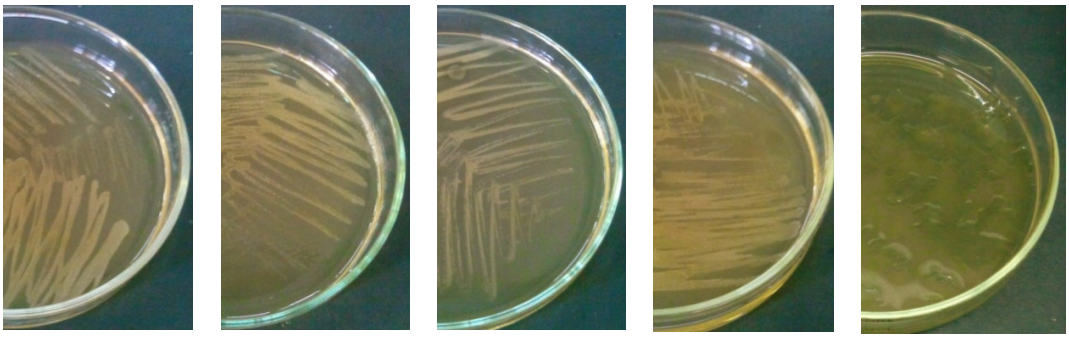
Nuclear magnetic resonance (NMR) spectroscopy has been used to analyze the microscopic physical and chemical structures of molecules. NMR has become the finest technique for looking closely at the composition of organic compounds. In this research, the sample of the isolated levan polymer was dissolved in D_2O. The NMR spectra measurements of the isolated levan were performed on a DELTA2_NMR-500.16 MHz (^1H NMR) and 125.77 MHz (^{13}C NMR) at Chemistry Research Center, Indonesian Institutes of Sciences (LIPI), Serpong.

3. Results and Discussion

3.1. The screening of the potential bacteria in levan production

The screening of bacteria was performed on a modified medium of Belgith (2012) in order to obtain potential levan-producing bacteria. In this work, we used halophilic bacteria for the screening, as a result the addition of NaCl on the screening medium was crucial to the growth of bacteria. The addition of NaCl on the screening medium also provide benefits to prevent contamination from other type of bacteria. Five isolates bacteria of the genus of *Halomonas* and *Chormohalobacter* i.e, *Halomonas meridiana* BK-AB4, *Halomonas elongata* BK-AB8, *Halomonas eurihalina* BK-AB15, *Halomonas elongata* BK-AG18, and *Chromohalobacter japonicus* BK-AB18 were screened for its potential in producing levan. The presence of sucrose as the substrate in the screening medium induced the

bacteria to secrete levansucrase. The early stages of levansucrase catalytic process was the formation of fructosyl-enzyme intermediate. After that, fructosyl units was transferred to sucrose acceptors. Then it was subsequently forwarded to the polymerization reaction through the transferred fructosyl units on various fructo-oligosaccharides acceptors forming the levan polymer. The appearance of slimy mucoid on screening medium indicated the production of levan polymer from sucrose[5] (Fig. 1).



(a) (b) (c) (d) (e)

Fig. 1. The screening results of five halophilic bacteria from the genus of *Halomonas* and *Chromohalobacter*. (a) *Halomonas meridiana* BK-AB4, (b) *Halomonas elongata* BK-AB8, (c) *Halomonas eurihalina* BK-AB15, (d) *Halomonas elongata* BK-AG18, and (e) *Chromohalobacter japonicus* BK-AB18.

The five bacteria grew well on the screening medium. The appearance of colonies on each of the bacteria was observed (Fig. 1). However, among five of the screened bacteria only *Chromohalobacter japonicus* BK-AB18 that produced slimy mucoid on the screening medium indicating that it was a potential levan producer (Fig. 1.e). Levan was known to have been produced by various genuses of bacteria e.i. *Halomonas*[6], *Bacillus*[1,5,7-9], *Zymomonas*[10-11], *Pseudomonas*[12-14], and *Gluconacetobacter*[15]. But among the bacteria that have been reported, there is no information about the potential of halophilic bacteria from the genus of *Choromohalobacter* in producing levan. Therefore, our finding is the first report for the levan producing bacteria from the genus of *Chromohalobacter*.

3.2. Thermal Analysis Gravimetric (TGA) of the isolated levan

The stability of levan produced by *Choromohalobacter japonicus* BK-AB18 was evaluated by measuring its decomposition temperature by thermal gravimetric analysis (TGA). In principle, TGA measures mass reduction due to material decomposition in the course of heating from room to high temperature. The decomposition temperature is defined as the temperature at the highest/lowest peak appear at the derivative curve of the resulted thermogram. TGA analysis on levan produced by *Choromohalobacter japonicus* BK-AB18 found its decomposition temperature at 211^{o}C (Fig. 2). As comparison, levan produced by *Halomonas smyrnenis* AAD6 have the decomposition temperature about 253 ^{o}C[6]. This difference was likely correlated to the average chain length of the produced levan. The longer of a levan the higher its decomposition temperature.

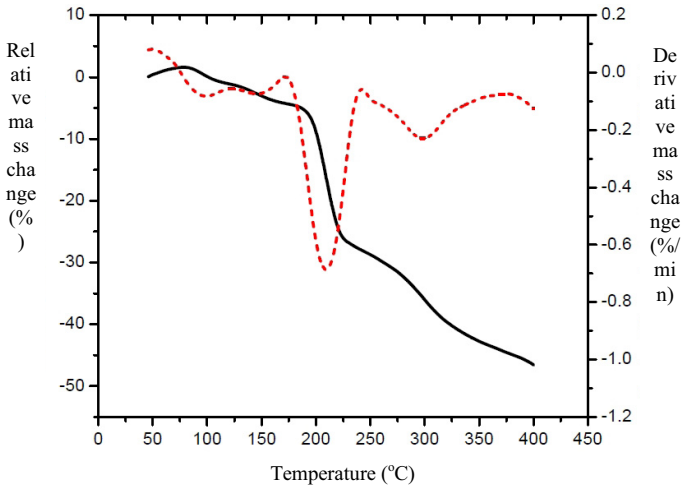


Fig. 2. The thermogram of levan produced by *Chromohalobacter japonicus* BK-AB18

3.3. The Fourier Transform-Infrared (FTIR) spectroscopy analysis of levan

The FTIR analysis was conducted to confirm the similarity between the FTIR spectrum of levan produced by *Choromohalobacter japonicus* BK-AB 18 (Fig. 3.a) and that of levan produced by *Bacillus methylotrophicus*[9] (Fig. 3.b). Both spectra displayed high similarity in the pattern of peak transmittance as follows: (A) the O-H stretching at the wavenumber range of 3600-3200 cm^{-1}, (B) the C-H stretching at the wavenumber range of 3000-2800 cm^{-1}, (C) the vibration of C=O at the wavenumber of 1641.16 cm^{-1}, (D) the fingerprint of β-glycoside bond was found at 2090 cm^{-1}, (E) the region of typical carbohydrate at the finger prints wavenumber range of 1000-800 cm^{-1}. Based on this comparison, it can be concluded that the isolated levan produced in this research having the same structure with levan standard produced from *Bacillus methylotrophicus*[9].

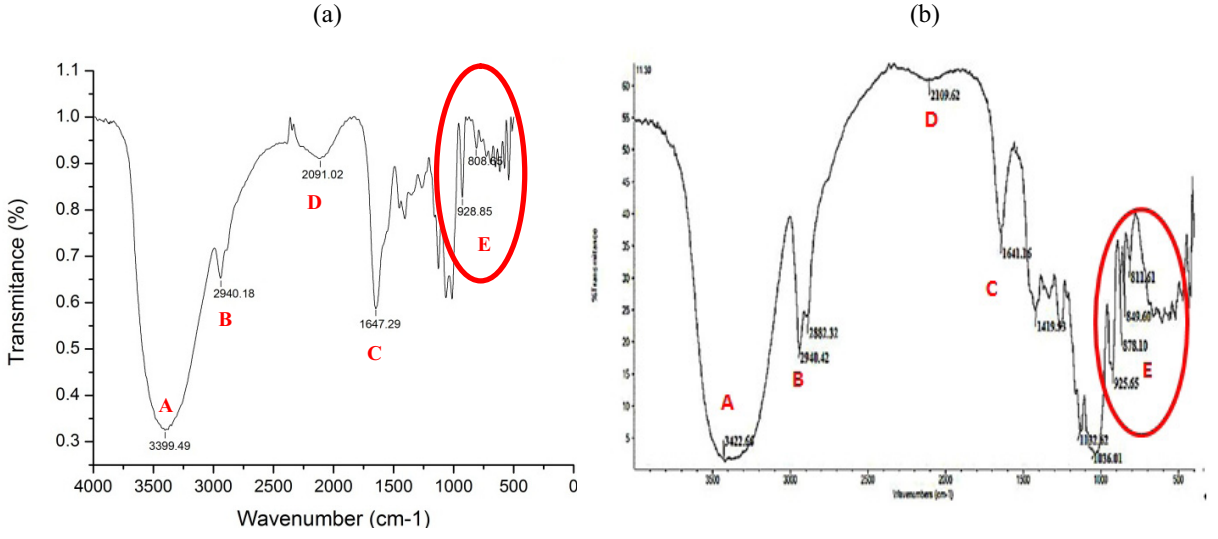


Fig. 3. The FTIR spectra of: (a) Levan produced by *C. japonicus* BK-AB 18; and (b) levan produced by *Bacillus methylotrophicus*[9]

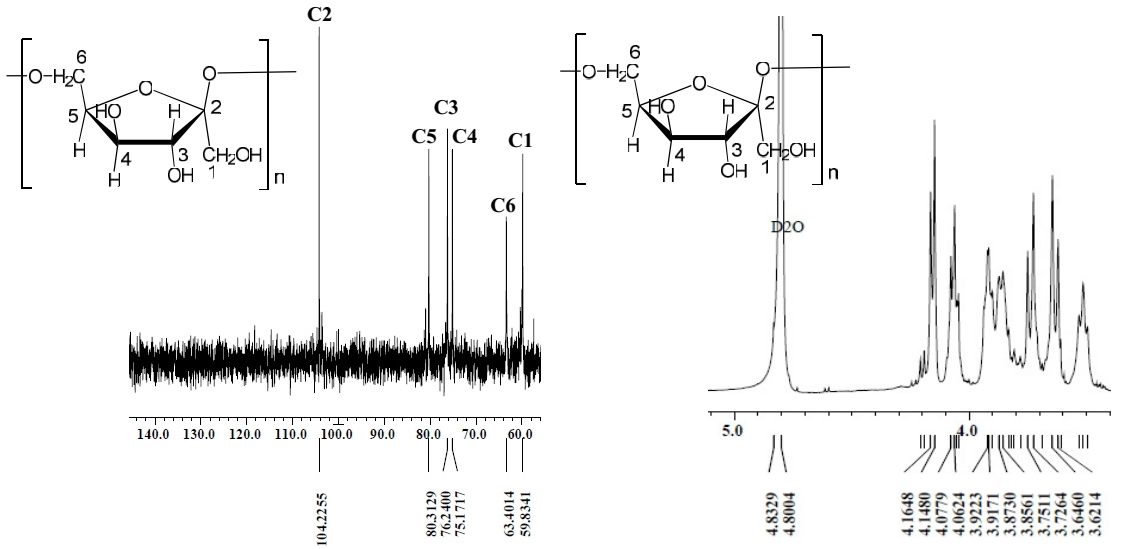
3.4. The NMR analysis of levan

The ^{13}C NMR spectra of levan produced by *Choromohalobacter japonicus* BK-AB18 showed 6 major resonances for carbon signals (Fig. 4.a). Comparing the chemical shift of ^{13}C NMR spectrum between our levan sample and that produced by the other bacteria previously reported[11,14] was presented in Table 1. The carbon signals from all levan ^{13}C NMR spectra showed the similarity in terms of their chemical shifts. The chemical shifts of levan's carbons were attributed to β-configurated fructofuranose units as a result of the comparison with that of the standard methyl glycoside[16]. The ^{1}H NMR spectrum also showed the proton chemical shift signals related to the fructose as monomer of levan (Fig. 4.b).

Table 1. The comparison of carbon chemical shift signals of the isolated levan produced from various bacteria

Bacteria producing levan	The carbon chemical shifts of isolated levan (ppm)					
	C1	C2	C3	C4	C5	C6
Zymonas mobils[*)]	60.767	104.641	77.683	75.754	80.783	63.957
Pseudomonas fluorescens[**)]	60.435	104.696	76.770	75.783	80.880	63.978
Chromohalobacter japonicus BK-AB18	59.834	104.226	76.240	75.172	80.313	63.401

[*)]*As reported by Han and Clarke*[11] ; [**)]*as reported by Jathore et al*[14].



(a) (b)

Fig. 4. (a) The ^{13}C NMR, and (b) The ^{1}H NMR spectrum of the isolated levan produced by *Chromohalobacter japonicus* BK-AB18

4. Conclusions

The screening results of five halophilic bacteria from the genus of *Halomonas* and *Choromohalobacter* found *Chromohalobacter japonicus* BK-AB18 as the most potential source for levan production. TGA, FTIR, and NMR analysis displayed high similarity of levan produced by *Choromohalobacter japonicus* BK-AB18 with levan produced by the other bacteria.

Acknowledgements

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