

Screening, Purification and Characterization of β -Glucan from a Novel Strain *Bacillus cereus* LVK13 (KC 898956)

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Abstract: Exopolysaccharides are non-toxic, biodegradable and environmental friendly microbial metabolites that play a pivotal role in food and pharmaceutical sectors. Biotechnological means of β -Glucan production was found to be most economical and realistic method. Present study investigates one such possibility to explore the roduction of β -Glucan, an exopolysaccharide from a novel strain isolated from the field soil of Sathyamangalam region, Tamil Nadu, India. Taxonomical investigation of the isolate was carried out according to Bergey's manual of determinative bacteriology and further validated by molecular analysis. The isolate represents a new member of *Bacillus cereus* and hence was designated as *Bacillus cereus* LVK13. Nucleotide sequence of this novel bacterium has been deposited with NCBI and GenBank accession number (KC 898956) was obtained. β -Glucan producing capability of this novel strain was monitored periodically and found that the maximum productivity was obtained during 68-76 hrs of fermentation. Thin Layer Chromatography and Fourier Transform Infrared spectroscopy were employed to characterize the exopolysaccharide and identified as β -1,3-Glucan.

Keywords: β -Glucan, Exopolysaccharides, *Bacillus cereus* LVK 13.

Introduction

Microbial exopolysaccharides (EPS) are soluble or insoluble polymers secreted by microorganisms¹. They are widely used in food industries as stabilizing and gelling agent based on their rheological property². Wang et al (2008) discusses the emerging applications of microbial polysaccharides as flocculants, absorbents, heavy metal removal and drug delivery agents³. The production of β -glucan has drawn considerable interest because of its unique rheological and thermal gelling properties. One of the unique features is that its aqueous suspensions do not return to native liquid state once being heated⁴. Currently, majority of the food products such as jelly, noodles, edible fibers and new calorie-reduced products utilize Curdlan (β -1,3-Glucan) for its gelling property^{5,6}. *Bacillus* sp. and strains produce a variety of EPS such as levan⁷, β -1,3-Glucan⁸, and heteropolymers mainly composed of neutral sugar⁹ and sugar-protein conjugate¹⁰. Some EPS from *Bacillus* have shown excellent emulsifying, flocculating, heavy metal removal capacity and pharmaceutical activity¹¹. In the present study, methods for isolating β -1,3-glucan are efficient and low cost when compared with other extraction methods, providing high quality products from *Bacillus cereus* LVK13, which may be useful in commercial applications.

Materials and Methods

Isolation of glucan producing microorganisms

The extract of agricultural field soil collected from different regions of sathyamangalam, Tamil Nadu was serially diluted and plated on aniline blue agar (ABA) medium containing the following ingredients (per L); 20 g sucrose, 5 g yeast extract, 0.05 g aniline blue, 3 g CaCO₃ and 20 g agar¹². The β-glucan producers that form intense blue colour colonies will be isolated from ABA plates after incubation at 30°C for 48 hours.

Taxonomical investigation of β-glucan producing isolate

Phenotypic and biochemical characterisation

The promising strain that produced blue colour colonies on aniline blue agar was subjected to taxonomic investigation using classical morphological, physiological and biochemical tests according to the Bergey's manual of determinative bacteriology. Gram staining, endospore staining, motility determination, starch hydrolysis, oxidase test, catalase test, IMViC test (Indole, Methyl red-Voges proskauer, Citrate utilization) and triple sugar iron agar test were carried out to predict the genus and species.

Molecular approach: 16s rDNA sequencing

Genomic DNA was isolated from the culture using Chromous Genomic DNA isolation kit. PCR was performed in order to amplify the 16S ribosomal DNA of the test strain. The following universal primers, forward: 5'-AGAGTRTGATCMTYGCTWAC-3' and reverse: 5'-CGYTAMCTTWTACGRCT-3' were used and the PCR program was set to initial denaturation at 96°C for 1 min, denaturation at 96°C for 10 sec, hybridization at 50°C for 5 sec and elongation at 60°C for 4 min for a total of 25 cycles. The PCR products were excised from agarose gel purified with Chromous kit and sequenced using DNA sequencer ABI 3500 XL. Sequence similarity search was made for the 16S rDNA sequence using BLAST. Multiple sequence alignment was done using CLUSTAL W and the phylogenetic tree was constructed using neighbour-joining method^{13,14}.

Production of β-glucan

10 ml of seed culture (*Bacillus cereus* LVK13) cultivated at 30°C for 12 hrs using nutrient broth (HiMedia) was inoculated into 1 litre of mineral salt medium containing the following ingredients (g/L); KH₂PO₄ - 1.74, CaCl₂.2H₂O - 0.015, K₂HPO₄ - 0.49, MnCl₂.4H₂O - 0.01, Na₂SO₄.10H₂O - 3.7, Citrate - 0.21, MgCl₂.6H₂O - 0.25, NH₄Cl - 1.5, FeCl₃.6H₂O - 0.024 supplemented with 10% sucrose as a carbon source to induce glucan production and incubated in a rotary shaker at 30°C for 4 days¹². The product was analysed periodically upto 96 hrs.

Purification of β-glucan

The fermentation broth was centrifuged at 5000 rpm for 15 min and the resultant cell free supernatant was precipitated for β-glucan using 3 volumes of ice cold ethanol followed by incubation at 4°C for 12 hours. After incubation, the pellets were recovered, washed with distilled water and the crude glucan was obtained by lyophilization. The crude sample was then dissolved in 0.1 M NaCl and size exclusion chromatography was performed using Sephadex G-100 as stationary phase. The fractions were collected at a rate of 0.1 mL/min and glucan content in each fraction was determined by measuring the total carbohydrate content using phenol sulphuric acid method. The high carbohydrate containing glucan fractions were pooled, lyophilized and subjected to product characterization¹².

Characterization of β-Glucan

The purified glucan hydrolysate was prepared for thin layer chromatographic (TLC) analysis by dissolving 50 mg of glucan in 5 mL of 2 N sulphuric acid, hydrolyzed at 100°C for 5 h and loaded onto a TLC plate. The developing solvent system used was acetone-butanol-water (4:5:3) and the chromatograms were observed after α-naphthol (0.5% (w/v) α-naphthol and 5% (v/v) sulfuric acid in ethanol) treatment¹². Chemical characterization of the produced glucan was analysed by Fourier-transform infrared (FT-IR) spectrometry. The FT-IR spectra were recorded between 500 to 4000 cm⁻¹ using the ABB MB 3000 FT-IR analyzer.

Results and Discussion

Isolation and Identification of β -glucan producing isolate

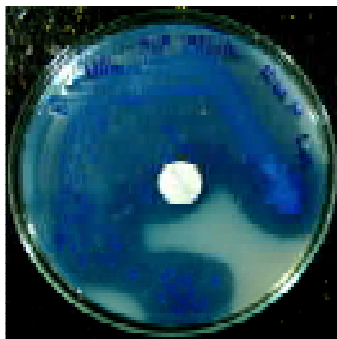


Fig. 1: Intense blue colour colonies of β -Glucan producing isolate on aniline blue agar

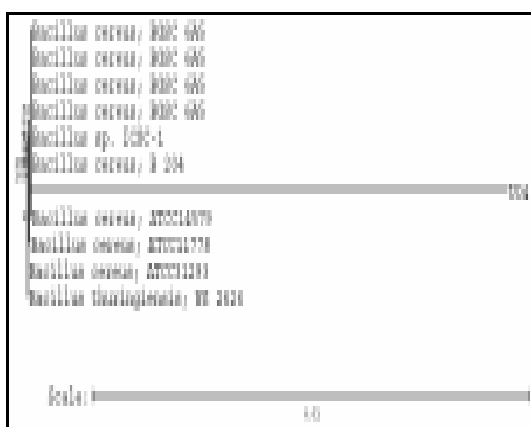


Fig. 2: Phylogenetic relationship of *Bacillus cereus* LVK13 (UO4)

The microorganisms were isolated by standard microbiological procedures. Among 4 strains isolated from aniline blue agar, only *Bacillus cereus* LVK13 strain was picked based on the intensity of the blue colour colonies as reported by Naganishi et al (1974)¹⁵. (Fig.1) The isolate was morphologically and biochemically confirmed as *Bacillus sp.*, and the results were detailed in Table 1 and 2. The evolutionary history was inferred using the Neighbour-Joining method. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analysis was conducted using MEGA 4.0 software^{13,14}. (Fig.2) Nucleotide sequence of this novel bacterium has been deposited at NCBI, USA gene database (Accession No.KC 898956).

Table - 1: Phenotypic Characterization of glucan producing isolate

S. No.	Name of the technique	Result
1	Gram staining	Gram positive; rod shaped









2	Endospore staining	Vegetative cells (Pink) & Endospores (Green)	
3	Motility determination (Hanging drop method)	Motile	

Table - 2: Biochemical Characterization of glucan producing isolate

S. No.	Name of the test / procedure	Result	
1	Indole	Negative	
2	Methyl red	Negative	
3	Voges-Proskauer	Positive	
4	Citrate utilization	Positive	
5	Catalase	Positive	
6	Oxidase	Positive	
7	Urease	Negative	
8	Lactose fermentation	Negative	
9	Sucrose fermentation	Positive	
10	Glucose fermentation	Positive	
11	Gas production	Negative	
12	H ₂ S production	Negative	

Screening for β -glucan

The production of β -Glucan was plotted against time in hours. (Fig.3) The exopolysaccharide concentration was determined using Phenol-sulphuric acid method of analysing total carbohydrate content in the sample. Each sample was assayed in triplicates. The productivity of the glucan was tremendously increased

between 68 - 76 hrs of fermentation. Kumar et al (2005) reported that the maximum production of glucan was obtained in 70-84 hours of fermentation¹. The present study suggests the increased production of glucan with reduced fermentation time. Even though, the yield of glucan is poor; it can be improved by optimizing the physiological conditions and strain itself. Immune modulatory effects of the exopolysaccharide produced by *B. cereus* LVK 13 may have different property to be used as animal feed additive from that of *P. polymyxa* JB115 as reported by Hee et al (2007)¹².

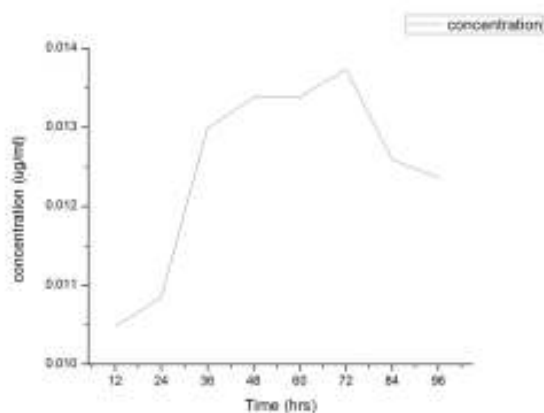


Fig. 3: Graphical representation of β -Glucan production by *Bacillus cereus* LVK13 in mineral salt medium

Characterization of β -Glucan

The cells were harvested by centrifugation and removed. The cell free broth was purified for β -Glucan and the resulting product was analyzed by Thin Layer Chromatography and FTIR. The thin layer chromatography was carried out using the standard procedure as described by Hee et al (2007) and obtained the R_f value similar to glucose which is taken as reference¹². (Fig.4) The peaks obtained from FTIR suggests that there is more C-OH; C-H and aldehyde bonds are present in the sample. This ascertains the possibility of β Glucan presence. Broad band at 3375 cm^{-1} corresponds to the stretching vibration modes of -OH groups, the peaks at 900 and 1160 reveals the presence of β 1,3-glycosidic linkage. It may validate the presence of β -1,3-Glucan (Curdlan). Also other significant peaks at 1165, 1234 and 1373 shows the presence of $C_1 - O - C_3$, C - O and CH ; CH_2 respectively¹⁵. (Fig.5)

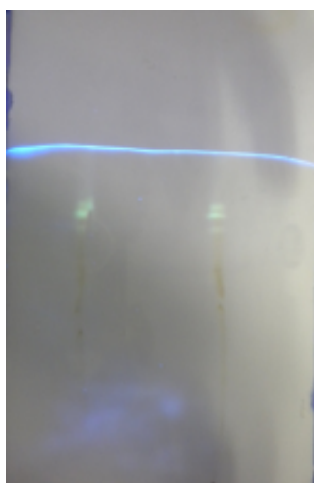


Fig. 4: Characterization of β -Glucan by Thin Layer Chromatography

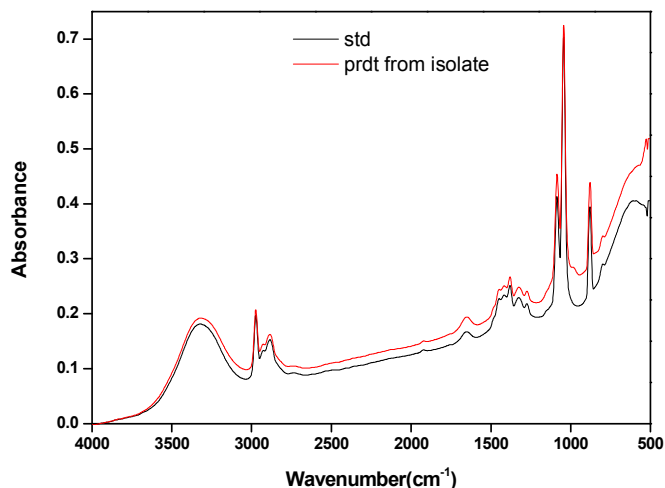


Fig. 5: Characterization of β -Glucan by Fourier Transform Infrared Spectroscopy

Conclusion

In this study, *Bacillus cereus* LVK13 was isolated and identified. The sequence was deposited in NCBI gene repository with accession number KC 898956. Morphological and biochemical studies were carried out to confirm the bacterial genus. The microorganism is able to consummate the production of β -Glucan which is characterized using Thin Layer Chromatography and Fourier Transform Infrared Spectroscopy studies. Further, enhancement of the species will be done by optimizing the growth and process condition to meet the market potential of the exopolysaccharides. In future, SEM and XRD analysis will be carried out to validate the findings of the presented work. The strain may be of commercial interest due to its ability to produce the exopolysaccharide at pH 7.0 and 37°C.

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