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# Biosynthesis of Applicable Levan by a New Levan Producing Moderately Halophilic Strain *Chromohalobacter salexigens* and its Biological Activities

**[1]Sherif M. Husseiny, [2]Fatma A. Sheref, [3,4] Hassan Amer and [2]Tamer A. Elsakhawy**

*[1]Department of Botany, Faculty of Women for Art, Science & Education , Ain shams University, Cairo, Egypt.*
*[2]Department of Microbiology, Soils, Water and Environment Res. Inst., Agric. Res. Center. Giza, Egypt.*
*[3]Department of Chemistry of Natural and Microbial Products, National Research Centre, Dokki,Giza, Egypt.*
*[4]Department für Chemie UFT-Haus Universitäts- und for schungszentrumTulln (UFT) Konrad Lorenz Straße 24 3430 Tullnan der Donau, Austria.*

## ABSTRACT

A newly isolated moderately halophilic bacterial strain from Egyptian hyper saline source was identified as *Chromohalobacter salexigens* and was selected for its ability to produce extracellular levan. Different environmental and nutritional factors were optimized. These factors including initial pH, temperature, nitrogen source, sodium chloride concentration, phosphorous source in addition to the sequence incubation at optimum and cold temperature. The resulted polymer was identified by paper chromatography, NMR and FT-IR spectroscopy. Also chemical modifications (carboxymethylation and sulphation) were proceeded and the biological activity (fibrinolytic, prebiotic activity) of resulted levan and its derivatives were also determined. The highest quantity of levan (11.9 g $l^{-1}$) was produced at pH 8, sodium chloride concentration 15%, ammonium sulphate as nitrogen source and sequence incubation at optimum followed by cold temperature. Chemically modified levan recorded high fibrinolytic and prebiotic activity compared with the original one. Hence with this work, *Chromohalobacter salexigens* has been described as a levan producer microorganism for the first time according to our knowledge.

***Key words:*** levan, Chromohalobacter, sulphation, carboxymethylation

## Introduction

Exopolysaccharides (EPS) are high molecular weight carbohydrate polymers that make up a substantial component of the extracellular polymers surrounding most microbial cells in the marine environment. They assist the microbial communities to endure extremes of temperature, salinity, and nutrient availability. It is to be expected that their biopolymers will also have some unique properties to adapt to such extreme conditions. Hence, most research is focused on the identification of EPS producing extremophiles with the idea that these microorganisms survive environmental extremes of desiccation, temperature, pressure, salinity and acidity (Poli *et al.,* 2009; Satpute *et al.,* 2010 and Esawy *et al.,* 2011).

Levan is one of EPS mainly highly branched fructose homopolysaccharide, in which the main interglycoside linkage is β-(26) glycosidic bonds. Levansucrase is responsible for synthesizing levans by transferring fructose moiety of sucrose to pre-existing acceptor molecules (Dahech *et al.,* 2011).

Applications of levan have been suggested in various aspects, particularly in food (Han and Watson, 1992; Yamamoto *et al.,* 1999 and Esawy *et al.,* 2013). Levan acted also as a prebiotic changing the intestinal microflora and this is offering beneficial effects when present in the human diet. Levan and its partially hydrolyzed products were fermented by intestinal bacteria including *Bifidobacteria* and *Lactobacillus* species (Marx *et al.,* 2000; Duboc and Mollet, 2001).

Levans are primarily produced from various microorganisms such as *Pseudomonas* (Jathore et al., 2012), *Zymomonas* (Silbir *et al.,* 2014) and *Bacillus* (Esawy *et al.,* 2013). Hypersaline environments are known to harbor a variety of EPS producing marine bacterial strains such as moderately halophilic bacteria of the genus *Aphanocapsa halophytica* (Matsunaga *et al.,* 1996), *Halomonas* (Bouchotroch *et al.,* 2000), *Halomonas maura* (Arias *et al.,* 2003), *Halomonas alkaliphila,* strain CRSS (Poli et al., 2004), *Halomonas ventosae* and *Halomonas anticariensis* (Mata *et al.,* 2006), *Halomona scerina* (Gonzalez-Domenech *et al.,* 2008).

In this study, a new levan-producing moderately halophilic bacterial strain *C. salexigens* was isolated and characterized. The structure of the biosynthetic product was investigated by different techniques and the results indicated to levan polymer. Nutritional and environmental factors for biomass and EPS production, like carbon,

**Corresponding Author:** Tamer A. Elsakhawy, Department of Microbiology, Soils, Water and Environment Res. Inst., Agric. Res. Center. Giza, Egypt.
E-mail: drelsakhawy@yahoo.com

nitrogen sources, different sucrose concentrations, sodium chloride concentrations, initial pH values, incubation temperature, and incubation time were investigated. At the end, the most favorable conditions for levan production were developed.

## Materials and Methods

*Microorganism and growth conditions.*

Water samples were collected from a shallow small pond of approximately 50 cm depth which supplied by the sea water percolating from the Suez Canal in El-kantara Gharb behind wastewater treatment plant of Elkantara Gharb, Ismailia, Egypt in July 2011. It was stored into sterile bottles and transferred in ice box to the laboratory. Gibbons complex medium (SGCM) (Sehgal and Gibbons, 1960) contained g/l: (7.5 casamino acids, 10 yeast extract, 2.0 KCl, 3.0 sodium citrate, 20.0 $MgSO_4.7H_2O$, 200 NaCl, 0.01, $FeCl_2.7H_2O$ and 20 agar. The medium was adjusted to pH 7.0 by 0.5 M NaOH before autoclaving at 121$^oC$ for 15 minutes. It was used for isolation and purification. Twenty five ml of sterile SGCM broth were inoculated by 1ml of water sample and incubated at 30$^oC$ on a rotary shaker at 150 rpm for seven days. Isolation and purification were carried out by pour plate and streaking techniques. Isolates were screened for levan production on the modified SGC agar and broth medium (supplemented with 50 g/l sucrose). Isolation of EPS producer was firstly selected on the basis of slimy mucoid appearance of colonies. Pure microbial cultures were stored at 4$^oC$ on the isolation medium.

*Characterization of the selected isolate*

The selected isolate was firstly characterized by Gram stain, motility test and some biochemical reactions like catalase production, oxidase test, hydrogen sulphide production, indole production and nitrate reduction.

*Molecular identification on the basis of 16S rRNA.*

The DNA from bacterial cultures was extracted using a protocol of GeneJet genomic DNA purification Kit (Fermentas). The 16S rRNA gene of the isolate strain was amplified by adding the following components to a thermocyclermicrotube :- Maxima® Hot Start PCR Master Mix (2X) 25$\mu$L, 16SRNA forward primer 1$\mu$L, 16SRNA reverse primer(of each 8 primer) 1$\mu$L, Template DNA 5$\mu$L and water, nuclease free 18$\mu$L. Universal primers Forward and Reverse (F, 5-AGA GTT TGA TCC TGG CTC AG-3 and R, 5- GGT TAC CTT GTT ACG ACT T -3) were used to obtain a PCR product of ~1.5 kb. The sample was placed in a hybridthermal reactor thermocycler (Maxima Hot Start PCR Master Mix (Fermentas), initially denatured (enzyme activation) for 10minute at 95◦C for 1 cycle and denaturated for 30s at 95◦C, annealing for 1min at 65◦C then extention for 1min at 72◦C. This was followed by a final elongation step for 10 minutes at 72◦C. The PCR products were analyzed on 1% (w/v) agarose gels and sent to GATC (Germany) for sequencing using ABI 3730xl DNA sequincer. Sequence data were imported into the BioEdit version 5.0.9 sequence editor; base-calling was examined, and a contiguous sequence was obtained. The full sequence was aligned using the RDP Sequence Aligner program. Sequences used in the phylogenetic analysis were obtained from the RDP and GenBank databases.A dendrogram was constructed using the neighbour-joining method. Confidence in tree topology was determined.

*Optimization of EPS production*

To establish which conditions lead to optimum levan production the following environmental and nutritional parameters studied : incubation under different pH (6-10), incubation temperature (10,25,30,40,45 ◦C), sodium chloride concentration (0, 5,10,15,20 and 25%, w/v), nitrogen source, where the amount of yeast extract and proteose peptone (10 g/l, 7.5 g/l) respectively were decreased to(1 g/l, 0.75 g/l) and replaced with other inorganic nitrogen sources equivalent to 0.2% ammonium chloride. Also sequence incubation at optimum temperature followed by incubation at 4◦C for 24 h was studied. Levan production were monitored in batch cultures in 100-ml Erlenmeyer flasks containing 20 ml medium (3 replicate flasks per experiment). Bacterial growth was determined by measuring optical density at 550 nm. All experiments were processed under the optimum condition resulting from each experiment.

*EPS production and purification*

The most EPS producing organism was cultivated as described above. After 96 hours of incubation under optimum conditions, the culture was centrifuged at 5000 rpm for 10 minutes to remove cells. The culture filtrate was dialyzed against deionized water for 48 h with dialysis membrane (Mr Cut off 104-12.103, diameter 60 mm) to remove the unfermented sucrose, and any fermentation products with low molecular weight. The dialysate was frozen with liquid nitrogen and freeze dried to levan without precipitation with ethanol (Esawy *et al.,* 2013)

*EPS identification*
*Chromatographic analysis*

Acid hydrolysis was done using 0.1 N HCl in boiling water bath for 1 h. Hydrolysate was analyzed by descending spot test on paper-chromatography using Whatman No.1 and solvent system n-butanol:acetone:water (4:5:1, v/v/v) (Tanaka *et al.,* 1978) and sprayed with aniline phthalate for identifying sugars in EPS samples (Block *et al.,* 1995).

*Total carbohydrates determination*

One ml 5% phenol solution was added to $10^{-2}$ diluted solution of fermented broth and 5 ml of concentrated $H_2SO_4$ were added rapidly to mixture shake and set aside for 10 min at room temperature, then at 20-30°C for 20 min. Thereafter, the color density was measured at 490 nm using glucose as standards (Dubois *et al.,* 1956).

*Attenuated Fourier Transform Infra-Red (FT-IR) Spectroscopy*

ATR-FTIR spectra from three samples (32 scans per sample, spectral resolution, 4 $cm^{-1}$; wave number range, 4000- 650 $cm^{-1}$ using a single reflection attenuated total reflectance (ATR) device (MIRacle, Pike Technologies, www.piketech.com) and a DLATGS detector) were recorded with a Bruker FT-IR spectrometer (Vertex 70). All samples used for infrared measurements were stored in a drying oven for three days at 50°C. Thereafter, they were stored in a desiccator overnight over silica gel before measurement.

*H NMR and 13C NMR spectroscopy*

NMR spectra were recorded at 27°C unless otherwise stated with a Bruker Avance III 600 instrument employing standard Bruker NMR software. $^{1}H$ spectra in $D_2O$ were referenced to DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) in $D_2O$ as external standard. $^{13}C$ NMR spectra were referenced to 1,4 dioxane in $D_2O$ (δ 67.4 ) as external standard. Coupling constants were reported in Hz and chemical shifts (δ) in ppm.

*Modification of bacterial levan:*
*Carboxymethylation:*

The carboxymethylation reaction was adopted according to the literature (Rahul *et al.,* 2014) as follows: Levan-like polysaccharide (1.0 g, 6.17 mmol) was suspended in 20 mL isopropanol in a round bottom flask with constant stirring at room temperature. The resulting mixture was heated to 50 °C and purged with nitrogen for 1 h. Required amount of aqueous solution of sodium hydroxide was added to the above mixture to keep pH at 11 constant and was stirred for 30 min. Afterwards, the sodium chloroacetate (2.6 g, 22.3 mmol) was added in small batches under constant stirring and pH 11 and the reaction was continued for 4 h. The reaction mixture was cooled gradually and the excess alkali neutralized with dilute. HCl bringing the pH to 7. The solution was dialyzed against deionized water for 48 h with dialysis cellulose acetate membrane ($M_r$ cut off $10^4$-$13x10^3$, diameter 60 mm) to remove the salts. Dialysate was frozen with liquid nitrogen and freeze-dried to afford carboxymethylated levan "CM-Levan".

*Sulphation*

Levan-like polysaccharide was sulfated in dimethylformamide (DMF) according to literature (Zhang *et al.,* 2009). In brief, 1.0 g (6.17 mmol) of biopolymer was suspended in 20 ml anhydrous DMF at room temperature for 2 hrs followed by addition of 3.5 g (22 mmol) $SO_3$-pyridine complex in 10 ml DMF. The reaction mixture was maintained at 0 °C for 4 hrs. After the reaction, the mixture was adjusted to pH 7 by 1 M NaOH solution. The mixture was dialyzed against deionized water for 48 h with dialysis cellulose acetate membrane ($M_r$ cut off $10^4$-$13x10^3$, diameter 60 mm). The precipitate was frozen with liquid nitrogen and freeze-dried to afford sulphated levan "SA-Levan".

*Biological activity of original and modified polymer.*
*Evaluation of fibrinolytic activity*

Fibrinolytic activity of original levan and sulphated levan was performed as described in (Helmy *et al.,* 2007). Sets of three-hard glass test tubes (31 mm× l00 mm) were cleaned by immersions overnight in chromic acid. To each tube 0.8 ml of 0.9% saline solution, 1 ml plasma and 0.2 ml of 2% calcium chloride solution were added. After mixing, the tubes were placed in water bath at 37 ◦C and when clotting was complete, 2000 Ug of heamoclar or tested samples was added individually. After 30 minute of incubation at 37 ◦C. Lyses percentage of plasma clots was recorded by using measuring cylinder.

*Determination of prebiotic activity*

Prebiotic activity assay was preceded according to Anprung and Sangthawan, (2012). *Lactobacillus acidophilus* and Escherichia *coli* were used in this study. *L. acidophilus* was prepared by streaking onto DeMan,

Rogosa, and Sharpe (MRS) agar medium and for *E. coli* onto Tryptic soy agar (TSA). Then, they incubated at 37ºC for 24-48 h under aerobic conditions. After that, one colony from each plate was transferred into 10 ml of MRS broth for *L. acidophilus* and Tryptic soy broth (TSB) for *E. coli* and incubated overnight. For *L. acidophilus*, an additional transfer of 1% (v/v) was transferred to (MRS) broth with 1% w/v glucose or 1% w/v levan For *E. coli*, an additional transfer of 1% (v/v) was transferred to minimal medium broth with 1% w/v glucose or 1% w/v samples and incubated overnight.

The prebiotic activity score was determined using the following equation; prebiotic activity score equal

$$\left[\frac{\left(\text{prebiotic log}\frac{cfu}{ml}\text{ on the prebiotic at 24 hr} - \text{probiotic log}\frac{cfu}{ml}\text{ on the prebiotic at 0 hr}\right)}{\left(\text{probiotic log}\frac{cfu}{ml}\text{ on glucose at 24 hr} - \text{probiotic log}\frac{cfu}{ml}\text{ on glucose at 0 hr}\right)}\right] - \left[\frac{\left(\text{enteric log}\frac{\text{cfu}}{\text{ml}}\text{ on the prebiotic at 24 hr} - \text{enteric log}\frac{\text{cfu}}{\text{ml}}\text{ on the prebiotic at 0 hr}\right)}{\left(\text{enteric log}\frac{\text{cfu}}{\text{ml}}\text{ on glucose at 24 hr} - \text{enteric log}\frac{\text{cfu}}{\text{ml}}\text{ on glucose at 0 hr}\right)}\right]$$

Where CFU is colony forming unit.

## Results

*Isolation, selection and identification of the most potent levan producer*

Six of Twenty three isolates showed slimy, mucoid, smooth appearance on solid media. isolate 1 was the most potent EPS producer, (2.3mg/ml). It was strictly aerobic microorganism ,Gram negative, non-spore forming, rod shaped cells. Catalase, casein hydrolysis, nitrate reduction were positive and optimum sodium chloride was 10 %. Result by the BLASTN analysis showed that it is more close to *Chromohalobacter salexigens* DSM 3043 with the maximum identity of just 99% Fig. (1)

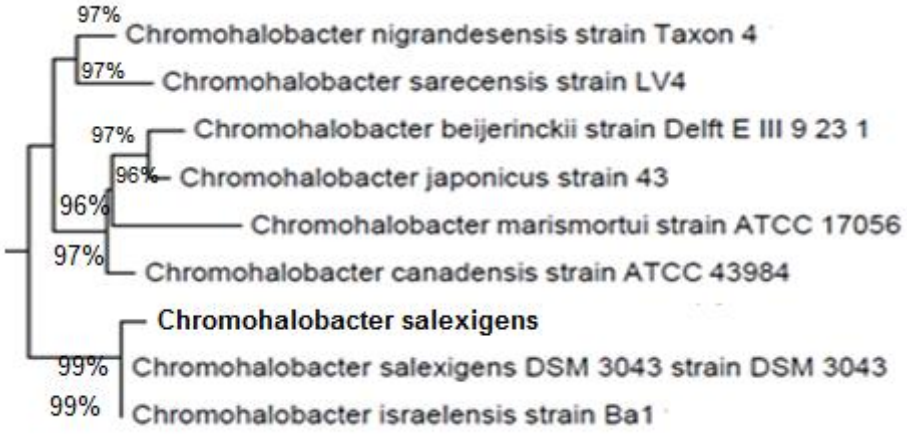



**Fig 1**. Phylogenetic tree constructed based on the 16S rDNA gene sequences.

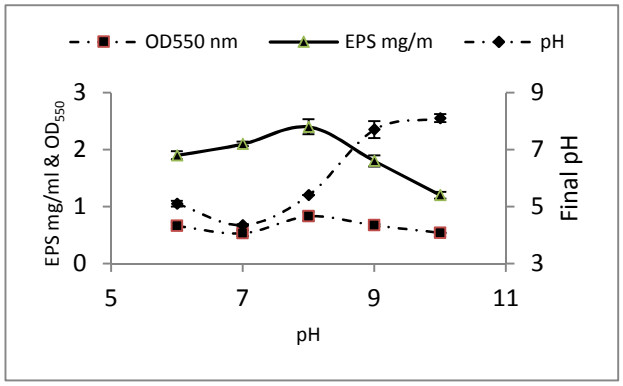
*Parameters controlling levan Production*

It was found that the maximum EPS (2.4 mg/ml) and biomass concentration were obtained at an initial pH of 8.0 Fig (2A). The optimum temperature for biomass and EPS production (2.4 mg/ml) was 30ºC (Fig 2B). The effect of sodium chloride concentrations on the EPS and biomass production is shown in Fig (2C). Maximum EPS concentration (2.56 mg/ml) was obtained in cultures grown at 15% sodium chloride.

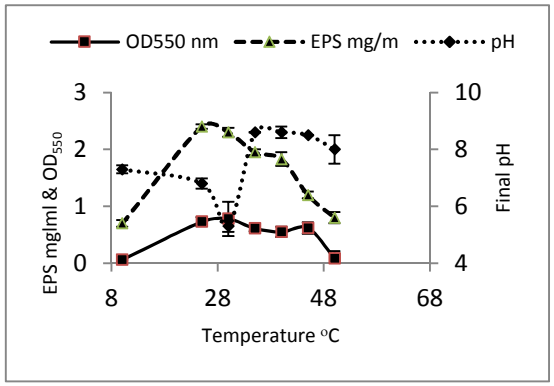
Results recorded in (Fig 2D), indicated that, ammonium sulphate was the best nitrogen source for the production of EPS (3.3 mg/ml). The final pH affected greatly with different nitrogen sources, where the final pH decreased greatly with most nitrogen sources except nitrate and urea where the final pH increased lightly and the EPS production decreased sharply. All phosphorus sources showed sharp increase in biomass compared with control. In the other hand, $KH_2PO_4$ gave the highest EPS production (5.7 mg/ml) (Fig 2E).

The step of incubation at optimum temperature for 72 h followed by incubation at 4ºC for 24 h (Table 1) showed a sharp increase in polymer production where the production reached 11.9 mg/ml.

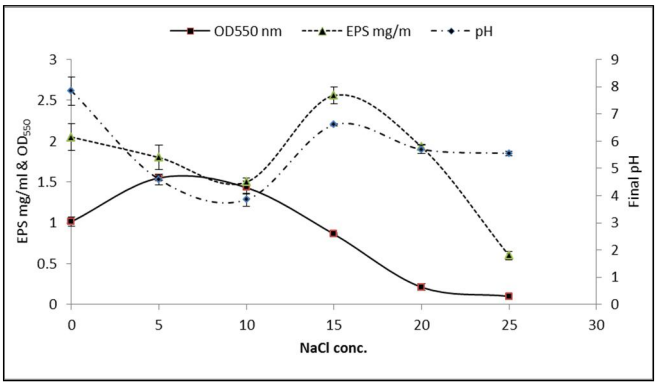
A



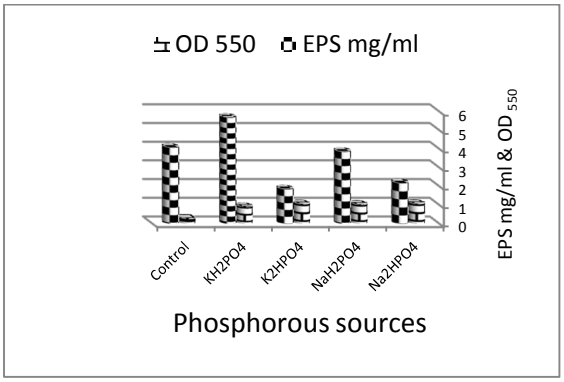

B




C




E




D

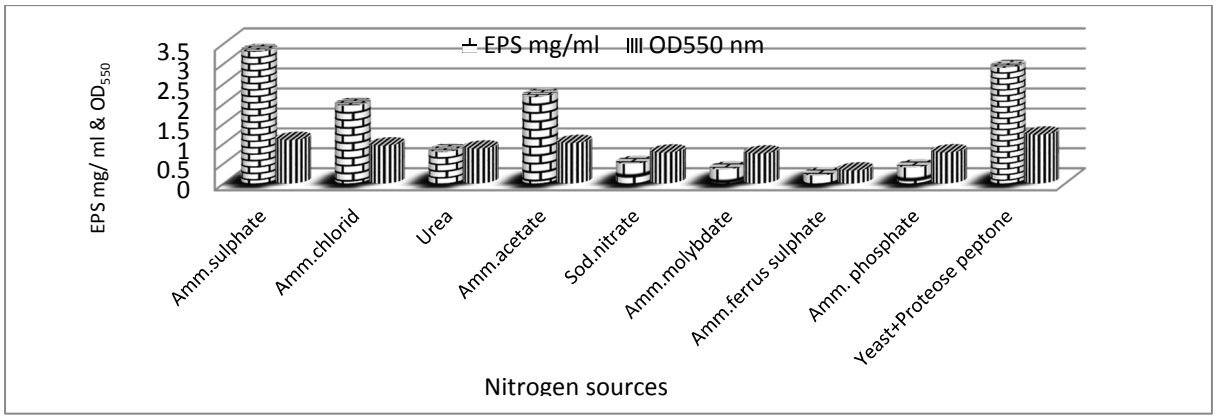



**Fig 2.** Effect of nutritional and environmental conditions on the production of levan by *Chromohalobacter salexigens*, **A**. Effect of pH, **B.** Effect of temperature, **C.** Effect of concentration of NaCl, **D.** Effect of nitrogen source, **E.** Effect of phosphorous source.

**Table 1**: Effect of different incubation temperatures and sequence incubation at 4°C for 48 hours.

| Incubation period (h) at 30°C and then left at 4°C for 48 h | *C. salexigens* | |
|---|---|---|
| | OD $_{550nm}$ | EPS mg/ml |
| 24 | 0.185±0.03 | 4.3±0.2 |
| 48 | 0.963±0.07 | 6.8±0.32 |
| 72 | 1.126±0.12 | 11.9±0.10 |
| 96 | 1.277±0.05 | 10.1±0.31 |
| 120 | 1.236±0.11 | 7.1±0.22 |

*Chemical characterization of exopolysaccharide:*
*FTIR spectrum of levan*

As shown in the Figure (3A), the FTIR spectrum showed a strong band at 3300 $cm^{-1}$, which was attributed to the hydroxyl stretching vibration of the polysaccharide. The bands at 2950 and 1910 $cm^{-1}$ were due to C-H stretching vibration of $CH_2$ and CH groups, respectively. The strong absorption at 1020 $cm^{-1}$ was dominated by the glycosidic linkage (C-O-C) stretching vibration contribution. Characteristic absorption at 930 $cm^{-1}$ and 820 $cm^{-1}$ was also observed, indicating the presence of the furanoid ring of the sugar units.

*$^{13}C$ and $^{1}H$ NMR spectroscopy of levan*

The $^{1}H$ NMR spectrum of exopolysaccharide showed the presence of intense signals relating to the fructose moieties (Fig.3B). These signals were observed at 3.48 (t, 9.5 Hz, H-61b); 3.64 (d, 12.3 Hz, H-1b); 3.69 (d, 12.3 Hz, H-1a); 3.80-3.95 (m, H-5, H-6a); 4.02 (t, 8.0 Hz, H-4) and 4.11 (d, 8.4 Hz H-3). All of these assignments have been performed by the analysis of HCOSY (Fig. 3C), HSQC (Fig.3D), HMBC (Fig. 3E) and APT (Fig. 3F).

The HCOSY spectrum showed cross peaks between H-3/H-4; H-4/H-5 and H-5/H-6. The concomitant attribution of the chemical shifts for all of the carbons of the fructofuranosyl moieties has been obtained from HSQC and HMBC spectra.

The HSQC spectrum (Fig.3D) showed cross peaks between H6a and H6b/C6, H1a and H1b/C1, H5/C5, H4/C4, H3/C3 and no cross peak between C-2 and any other confirmed its quaternary anomeric carbon character.

The HMBC spectrum (Fig. 3E) showed cross peaks between H3/C4 and especially H1/C2. This latter proved the beta-(2-1) linkage between two fructofuranosyl moieties. However the beta-(2-6) linkages have been confirmed only by the presence of a down field shifted signal at 64.18 ppm (C-6) in the $^{13}C$ NMR spectrum.

As shown in (Fig. 3F) six main resonance shifts at 60.7(C-1); 64.2(C-6); 76(C-4); 77.1(C-3); 81.1(C-5) and 105 ppm (C-2) relating to a quaternary anomeric carbons were detected in the $^{13}C$ NMR spectrum, corresponding to levan-like polysaccharide.

*Modification of bacterial levan:*
*Preparation and characterization of carboxymethylated levan*

Carboxymethylated levan (CM-levan) have been synthesized by derivation of bacterial exopolysaccharides using carboxymethylation. In order to reduce the percentage of by product formed, the reaction was carried out in an oxygen-free inert atmosphere. The reaction follows Williamson's etherification protocol and in the first step, in the presence of a base, alcoholic functionality converted to an alkoxide. The base performs the dual role of a reactant as well as a catalyst and was taken in slight excess. The generated adduct, undergoes nucleophilic substitution with sodium monochloroacetic acid to give the carboxymethyl derivative.

The reaction temperature was maintained at 50$^{o}$C. Although at relatively high temperature the reaction was faster, the chances of the formation of the by product (hydroxyl acid) increased CM-levan from isolated polysaccharides. As evident from (Fig. 4A), spectrum of CM-levan has a broad peak centered at 3300 $cm^{-1}$ from the O-H stretching of associated glucose and fructose units in the polysaccharide backbone. Two bands around 2950 $cm^{-1}$/2910 $cm^{-1}$ corresponded to C-H stretch and peak at 1020 $cm^{-1}$ could be assigned to glycosidic bond. The bands at 930 $cm^{-1}$ and 820 $cm^{-1}$ corresponded to furanose ring. Apart from these, there are two additional characteristic peaks at 1590 and 1420 corresponded to antisymmetric and symmetric stretching for carboxylate group, respectively.

*Preparation and characterization of sulphated levan*

Chemical sulphation of polysaccharides is one of the most utilized procedures to produce heparin-like polymers. This type of chemical modification is reproducible and allows the employing of high amounts of sulphated polysaccharides. The FT-IR spectrum of levan showed a broad stretching intense characteristic peak at around 3300 $cm^{-1}$ for OH and a narrow stretching characteristic peak at around 2950 $cm^{-1}$ for C-H (Fig. 4B). The peaks of 1134 and 1031 $cm^{-1}$ were referred to C-O stretching vibration of ring ether C-O-C and O-H variable angle vibration of C-O-H, respectively. Moreover, the absorption peak at 2933 $cm^{-1}$ of CH-stretching vibration became weaker and moved to 2967 $cm^{-1}$, suggested the sulfate substitution may occur on the position of C-6.

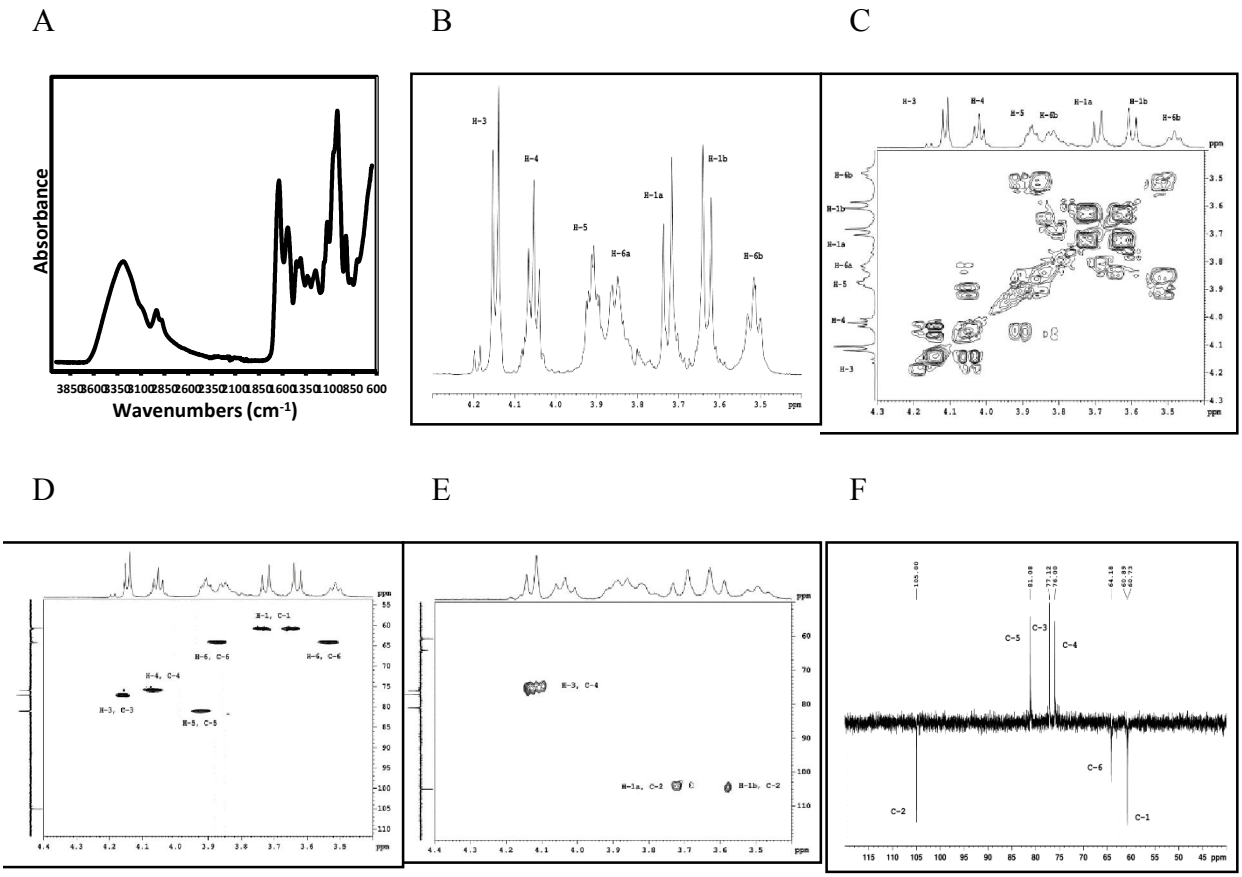



**Fig 3:** Structure identification of levan; **A.** Fourier transform Infra-redi spectroscopy spectrum of levan , **B.** 600-MHz 1D $^{1}H$ NMR spectrum of levan, **C.** 2D-COSY spectrum of levan recorded in $D_2O$, **D.** 2D-HSQC spectrum of levan recorded in $D_2O$, **E.** 2D-HMBC spectrum of levan recorded in $D_2O$**, F.** 1D-APT spectrum of levan recorded in $D_2O$.

*Fibrinolytic activity analysis of levan and sulphated levan.*

In vitro fibrinolytic activity analysis of levan and its sulphated derivative was done and compared with standard fibrinolytic compound heamoclar (pentosan sulfuric polyester, product of ClinMidy, Paris). The results showed that the sulphated levan exhibited 62% fibrinolytic activity while standard heamoclar "pentosan sulfuric polyester recorded (57%) by the same amount.

*Prebiotic activity score*

*Lactobacilli* strain was chosen to test the prebiotic activity score because it is used in dairy foods and has good potential probiotic properties. From the determination of prebiotic activity of levan (sample without modifications) compared with its carboxymethylated derivative. The amount of bacterial cell grown in media with levan and glucose for 24 h was recorded (shown in Table 2). The genus *L. acidophilus* grown with carboxymethylated levan was higher than ones grown on media with original levan and media with glucose. On the other hand, *E.coli* ATCC 25922 (representative of Enteric bacteria) grown on glucose (no prebiotic) was higher than other with prebiotic. It was found that the highest prebiotic activity scores as $\log_{10}$ (CFU/ml) when *L. acidophilus* grown on media with carboxymethylated levan was 2.43**.**

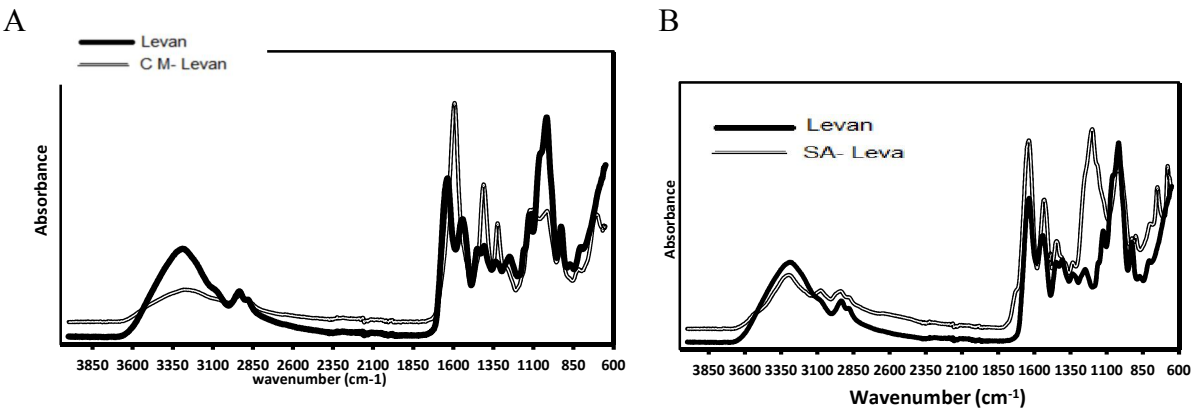



**Fig 3. A**. FT-IR spectrum of CM-levan**, B.** FT-IR spectrum of SA-levan**.**

**Table 2**: Prebiotic activity of levan produced by *Chromohalobacter salexigens* and its carboxymethylated derivatives of levan

| Bacterial culture | Cell density [$\log_{10}$(CFU/ml)] | | |
|---|---|---|---|
| | levan | CM levan | Glucose |
| *L. acidophilus* | 2.3 ± 0.03 | 2.43 ±0.00 | 2.2 ±0.04 |
| *E. coli* | 1.6 ±0.024 | 1.71 ±0.02 | 2.33 ±0.01 |

** CM= carboxymethylated derivative of levan*

## Discussion

Halophilic bacteria were targeted by isolation. Localities contained high sodium chloride concentration in addition to other salts selected as sources of halophilic bacteria. The effect of solar energy on the small ponds formed from the water percolating from Swiss canal in Elkantara gharb region, Ismailia, Egypt made it ideal location for isolation of such organisms.

After several dilutions and repeated sub culturing process, isolation of halophilic bacteria was carried out by using the SGC medium. Luxuriant growth was observed at pH 7 and temperature of 37$^{o}$C for 24 hours.

Carbon source plays a very important role in growth and polysaccharide production, so the SGC medium was supplemented with 50 g per liter sucrose for levan production in the beginning of optimization process. Transfructosylating activity, act on sucrose by cleaving the β (1, 2) linkage and transferring the fructosyl group to an acceptor molecule such as sucrose, releasing glucose. This reaction yields fructooligosaccharides (FOS),i.e. fructose oligomers mainly composed of 1-kestose (GF2) and 1F-fructosyl nystose (GF4) in which fructosyl units are bound at the β (2, 1) position of sucrose. Sucrose supported maximum EPS production which was in complete accordance with (Belghith *et al.,* 2012; Ko *et al.,* 2000; lee *et al.,* 2001) where sucrose was the best carbon source for the production of EPS from *Hahella chejuensis*.

Moreover, it is known that levan is generally formed by a repeated unit of beta-fructose residues linked by beta-(2-6)-gylcosidic bonds with some beta-(2-1) branches. The value of the $^{13}$C chemical shift of the anomeric carbon of the fructofuranosyl moieties (C-2 =105 ppm) confirms its beta-configuration (Shih *et al.,* 2010).

With respect to the nitrogen sources, in general it was observed that the culture showed a preference for reduced form of nitrogen sources to support both bacterial growth and maximal EPS production. (Silbir *et al.,* 2014) investigated levan production in batch and continuous fermentation systems by *Zymomonas mobilis* 38 B-14023 using ammonium sulphate as nitrogen source. Also, ammonium sulphate was the nitrogen source used by (Küçükaşik *et al.,* 2011) for production of levan from *Halomonas* sp. AAD6 (JCM15723, DSM 21644.

Levansucrase is an extracellular enzyme whose function is to catalyze levan synthesis from sucrose by transfructosylation. The enzyme accumulates in the periplasm before being excreted and appears to adopt its final conformation in the periplasm. The optimal temperature for levansucrase synthesis depends on the bacterial strain: for *Zymomonas mobilis*, it is 0 $^{o}$C whereas for *B. subtilis*, it is over 10 $^{o}$C. (Donot *et al.,* 2012)

It found that the high production of levan (11.9 mg/ml) by *C. salexigens* at 4$^{o}$C for 24 hours after incubation at 30$^{o}$C for 48 hours.

The studies with regard to the mechanism of sulphation reaction using pyridine.$SO_3$ complex had showed that the controlling transition state has only weak N–S and O–S bonds and is symmetrical. Sulfate group transfer between nitrogen of pyridine.$SO_3$ complex and oxygen is consistent with a concerted ‘in line’ sulfate group transfer or an open ‘exploded’ transition state (Hopkins *et al.,* 1983). Thus the strong nucleophilicity of the

oxygen atoms in the polysaccharides was necessary for high degree of sulphation. The FT-IR spectrum of levan and its sulphated derivative are in agreement with other levans and sulphated polysaccharides (Sun *et al.,* 2009; Wei *et al.,* 2012; Yang Du and Huang, 2002). The variable angle vibration absorption peak of 1089 $cm^{-1}$ decreased significantly, which could be the result of hydroxyl group replaced partly by sulfate groups. (Sudipta *et al.,* 2012).

Characterization of produced polysaccharide depends on IR, 1H-NMR and 13C NMR. All the previous tests confirmed each other and indicated that fractions were mainly fructose. Also, comparison of levan fractions and fructose spectra proved that fructose was the dominating component. The broad band at 1650 $cm^{-1}$ IR spectrum was due to the bound water (Park, 1971). Spectral data was in agreement with Yoo *et al.,* (2004); Tanaka *et al.,* (1980); Tomasic *et al.,* (1978).

NMR analysis showed that the bacterial polysaccharide (levan) was composed only of fructose units, as shown by Barrow et al. (1984); Yoshida and Suzuki, (1990) and Dahech et al. (2013). The shifts obtained by the polymer are also similar to those observed with levan produced by Lev S from *L. mesenteroides* B-512F (Morales-Arrieta *et al.,* 2006), *S. mutans* (Shimamura *et al.,* 1987) and *Bacillus subtilis M* revise (Esawy et al., 2013). On the other hand, the traces of glucose unit in the polysaccharides could not be detected by $^{1}H$ NMR deduced the high molecular weight of levan polysaccharide with high degree of polymerization.

The findings presented in this study demonstrated the capacity of levan and its sulphated derivative to liquefy rapidly clotted fibrin of normal human plasma. This result introduced new approach in levan applications. Fibrinolytic activity of polysaccharides was reported by many authors (Al-Nahas *et al.,* 2011; Esawy *et al.,* 2013).

In this study, carboxymethylated levan was found to potentially support the growth of probiotics where the highest prebiotic activity scores was recorded when *L. acidophilus* grown on media with carboxymethylated levan (log10 (CFU/ml) = 2.43). This gave a good indication that carboxymethylated levan could be exploited further as a prebiotic. Use of polysaccharide as prebiotics was reported by (Bello *et al.,* 2001). (Jang *et al.,* 2003) stated that, the intake of the levan-containing diet by rats significantly increased the total cecal weight and wall weight. Also, the analysis of short-chain fatty acids (SCFAs) in the cecal and colonic contents revealed that levan was converted into acetate, butyrate, and lactate, which resulted in acidic conditions and the intake of levan significantly increased the number of microorganisms by 5 folds and lactic acid bacteria 30-fold in the feces. It could be concluded that different bacterial strains affected the different prebiotic activity score owing to the differences in the metabolic capacity of related strains apparently existed, and utilization of prebiotics by these related bacteria required the presence of specific hydrolysis and transport systems for the particular prebiotic (Hubner *et al.,* 2007). Many symbiotic relationships between probiotics and prebiotics have been studied in order to maximize their beneficial effects. Both bifidobacteria and lactobacilli were best known to utilize prebiotics in the gastrointestinal (GI) tract, based on the fact that they contain relatively high amount of β-fructosidase and glycotransferases, respectively which enable them to break down polymers (the prebiotic) into smaller units and make it available as a substrate during fermentation (Lee *et al.,* 2002; Vergara *et al.,* 2010). Organic acids will be produced by lactic acid bacteria as a result of fermentation, thus providing an acidic environment in the colon which indirectly suppresses the growth of pathogens. This mechanism allows prebiotics to manipulate the composition of colonic microbiota in human gut (Rycroft *et al.,* 2001), thus improving the host health (Roberfroid, 2002) in return. These include enhancement of immune function, improve digestion and elimination of faeces as well as reducing the potential of getting irresistible bowel syndrome (IBS) (Douglas & Sanders, 2008).

## Conclusion

From all previous results, it was concluded that the halophile *C.salexigens* was good levan producers. The second part gave us clear explanation of the levan structure and its carboxymethylated and sulphated derivatives. The bioactivity results reported that levan derivatives have fibrinolytic activities. Also, it recommended that carboxymethylated levan to be used as prebiotic.

## References

Al-Nahas, M. O., M. M. Darwish, A. E.Ali, and M. A. Amin, 2011. Characterizations of an exopolysaccharide-producing marine bacterium isolate *Pseudoalteromonas sp*. African Journal of Microbiology Research, 5, 3823–3831.

Anprung, P., and S. Sangthawan, 2012. Prebiotic Activity and Bioactive Compounds of the Enzymatically Depolymerized Thailand-Grown Mangosteen Aril. Journal of Food Research, 1, 1927-0887.

Arias, S., A. E. Del Moral, M. R. Ferrer, R. Tallon, E. Quesada, and V. Bejar, 2003. Mauran, an exopolysaccharyde produced by the halophilic bacterium *Halomonas maura*, with a novel composition and interesting properties for biotechnology. Extremophiles, 7, 319–326.

Barrow, K. D., J. G. Collins, P. L. Rogers and G.M. Smith, 1984. The structure of a novel polysaccharide isolated from *Zymomonas mobilis* determined by nuclear magnetic resonance spectroscopy, European Journal of Biochemistry. 145, 173–179.

Belghith, K.S., I. Dahech, H. Belghith, and H. Mejdoub, 2012. Microbial production of levan sucrase for synthesis of frutooligosaccharides and levan. International Journal of Biological Macromolecules. 50:451-458.

Bello F.D, Walter J., Hertel C., and W.P. Hammes, 2001. In vitro study of prebiotic properties of levan-type exopolysaccharides from Lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis. Systematic and Applied Microbiology*, 24(2):232-7.*

Block, R. J., E. L. Durrum and G.Zweig, 1995. A manual of paper chromatography and paper electrophoresis. New York, NY: Academic Press International concept, pp 127.

Bouchotroch, S., E. Quesada, I. Izquierdo, M. Rodriguez and V. Bejar, 2000. Bacterial exopolysaccharides produced by newly discovered bacteria belonging to the genus *Halomonas*, isolated from hypersaline habitats in Morocco. Journal of Industrial Microbiology and Biotechnology, 24, 374–378.

Dahech, I., K.S. Belghith, K. Hamden, A. Feki, H. Belghith, and H. Mejdoub, 2011. Antidiabetic activity of levan polysaccharide in alloxan-induced diabetic rats. International Journal of Biological Macromolecules, 49:742-6.

Dahech, I., J. Fakhfakh, M. Damak, H. Belghith, H. Mejdoub and K. S. Belghith, 2013. Structural determination and NMR characterization of a bacterial exopolysaccharide. International Journal of Biological Macromolecules, 59, 417– 422.

Donot, F., A. Fontana, J.C. Baccou and S. Schorr-Galindo, 2012. Microbial exopolysaccharides: Main examples of synthesis, excretion, genetics and extraction. Carbohydrate Polymer*, 87, 951-962*.

Douglas, L.C., and M.E. Sanders, 2008. Probiotics and prebiotics in Dietetics Practice. Journal of American Dietetic Association*.* 108, 510–521.

Duboc, P., and B. Mollet, 2001. Applications of exopolysaccharides in dairy industry. International Dairy Journal, 11:759–768.

Dubois, M., K.A. Gillis, J.K. Hamilton, P.A. Rebers and F. Smith, 1956. Colorimetric method for determination of sugar and related substance. Analytical Chemistry, 28, 350–356.

Esawy, M. A., H. Amer, A. M. Gamal-Eldeen, H. A. El Enshasy, W. A. Helmy, M. A. M. Abo-Zeid, R. Malek, E. F. Ahmed and G. E. A. Awad, 2013. Scaling up, characterization of levan and its inhibitory role in carcinogenesis initiation stage. Carbohydrate Polymer, 95, 578–587.

Esawy, M.A., E.F. Ahmed, W. A. Helmy, N.M. Mansour, W.M. El- Senousy and M.M. El-Safty, 2011. Production of levansucrase from novel honey *Bacillus subtilis* isolates capable of producing antiviral levans. Carbohydrate Polymer*,* 36, 823-830.

Gonzalez-Domenech, C. M., F. Martinez-Checa, E. Quesada and V. Bejar, 2008. *Halomonas cerina* sp. nov., a moderately halophilic, denitrifying exopolysaccharide-producing bacterium. International Journal of Systematic and Evolutionary Microbiology, 58, 803–809.

Han, Y.W., and , M.A. Watson, 1992. Production of microbial levan from sucrose, sugarcane juice and beet molasses. Journal of Industerial Microbiology and Biotechnology, 9, 257–260.

Helmy, W. A., H. Amer and N. M. A. EL-Shayeb, 2007. Biological and anti-microbial activities of aqueous extracts from neem tree. Journal of Applied Sciences Research, 3, 739 1050–1055.

Hopkins, A., R. A. Day and A. Williams, 1983. Sulfate group transfer between nitrogen and oxygen: Evidence consistent with an open 'exploded' transition state*.* Journal of American Chemical Society, 105, 6062–6070.

Huebner, J., R. L. Wehling and R. W. Hutkins, 2007. Functional activity of commercial prebiotics. International Dairy Journal, 17, 770-775. http://dx.doi.org/10.1016/j.idairyj.2006.10.006.

Jang KI-Hyo, A.H.K. Soon, C. Yunhi, K. Yun-Young, L. Yun-Jung, H. Kyunghee, S. Kyung-Hwa, H.K. So, K. Chul-Ho, R. Sang-Ki, H. Sang –Do, and C. Ryowon, 2003. Prebiotic properties of levan in rats. Jornal of microbiology and biotechnology, *13(3), 348-353*.

Jathore, N. R., M. V. Bule, A. V.Tilay and U. S. Annapure, 2012. Microbial levan from *Pseudomonas fluorescens*: Characterization and medium optimization for enhanced production. Food Science and Biotechnology, 21(4), 1045–1053. doi:10.1007/s10068-012-0136-8.

Ko, S.H., H. S. Lee, S. H. Park, and H. K. Lee, 2000. Optimal conditions for the production of exopolysaccharide by marine microorganism *Hahellachejuensis*. Biotechnological Bioprocess Engineering, 5, 181–185. 28.

Küçükaşik, F., H. Kazak, D. Güney, I. Finore, A. Poli, O. Yenigün and E. T. Oner, 2011. Molasses as fermentation substrate for levan production by Halomonas sp. Applied Microbiology and Biotechnology, 89(6), 1729–40. doi:10.1007/s00253-010-3055-8.

Lee, H.S., S.H. Park, J.H. Lee and H.K. Lee, 2001. Effect of Aeration Rates on Production of Extracellular Polysaccharide, EPS-R, by Marine Bacterium *Hahella chejuensis*. Biotechnological Bioprocess Engineering, 6: 359-362.

Lee, H.W., Y.S. Park, J.S. Jong and W.S. Shim, 2002. Chitosan oligosaccharides, dp 2-8, have prebiotic effect on the *Bifidobacterium bifidum* and *Lactobacillus* sp. Anaerobe, *8*, 319–324.

Marx, S. P., S. Winkler and W. Hartmeier, 2000. Metabolization of β-(2, 6)-linked fructose-oligosaccharides by different bifidobacteria. FEMS Microbiology Letter 182:163-169.

Mata, J. A., V. Bejar, I. Llamas, S. Arias, P. Bressollier, R. Tallon, M. C. Urdaci and E. Quesada, 2006. .Exopolysaccharides produced by the recently described halophilic bacteria *Halomonas ventosae* and *Halomonas anticariensis*. Research in Microbiology, 157,827–835.

Matsunaga, T., H. Sudo, H. Takemasa and Y. Wachi, 1996.Sulfated extracellularpolysaccharide production by the halophilic cyanobacterium *Aphanocapsahalophytica* immobilized on light-diffusing optical fibers. Applied Microbiologyand Biotechnology, 45, 24–27.

Morales-Arrieta, S., M. E. Rodriguez, L. Segovia, A. Lopez-Munguia and C. Olvera Carranza, 2006. Identification and functional characterization of levS a gene encoding for a levansucrase from *Leuconostoc mesenteroides* NRRL B-512 F. Gene, 376, 59–67.

Park, F.S., 1971. Application of I.R Spectroscopy in Biochemistry, Biology and Medicine, Plenum, New York.

Poli, A., H. Kazak, B. Gürleyendağ, G. Tommonaro, G. Pieretti, E. Toksoy Öner and B. Nicolaus, 2009. High level synthesis of levan by a novel *Halomonas* species growing on defined media. Carbohydrate Polymer, 78:651–657. doi:10.1016/j.carbpol.2009.05.031.

Poli, A., V. SchianoMoriello, E. Esposito, L. Lama, A. Gambacorta and B. Nicolaus, 2004. Exopolysaccharide production by a new *Halomonas* strain CRSS isolated from saline lake Cape Russell in Antarctica growing on complex and defined media. Biotechnology Letters, 26, 1635–1638.

Rahul, R., U. Jha, G. Sen and S. Mishra, 2014. Carboxymethyl inulin: A novel flocculant for wastewater treatment. International Journal of Biological Macromolecules, 63, 1– 7.

Roberfroid, M., 2002. Functional food concept and its application to prebiotics. Journal of Digestive and Liver Disease. 34 (Suppl. 2), 105–110.

Rycroft, C.E., M.R. Jones, G.R. Gibson and R. A. A. Rastall, 2001. comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. Journal of Applied Microbiology. *91*, 878–887.

Satpute, S.K., I.M. Banat, P.K. Dhakephalkar, A.G. Banpurkar and B.A. Chopade, 2010. Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. *Biotechnology*, 28, 436–450.

Sehgal, S. N., and N. E. Gibbons, 1960. Effect of metal ions on the growth of *Halobacterium cutirubrum*. Canadian Journal of Microbiology, 6, 165–169.

Shih, I.L., L.D. Chen, and J.Y. Wu, 2010. Levan production using *Bacillus subtilis natto* cells immobilized on alginate. Carbohydrate Polymers, 82(1), 111–117.

Shimamura, A., K. Tsuboi, T. Nagase, M. Ito, H. Tsuori and H. Mukasa, 1987. Structural determination of D-fructans from *streptococcus* serotype b, c, e and f strains by $^{13}C$ NMR spectroscopy. Carbohydrate Research, 165, 150- 154.

Silbir, S., S. Dagbagli, S. Yegin, T. Baysal and Y. Goksungur, 2014. Levan Production by *Zymomonas mobilis* in Batch and Continuous Fermentation Systems. Carbohydrate polymer, *99*, 454–61. doi:10.1016/j.carbpol.2013.08.031

Sudipta, S., M. H. Navid, S. B. Shruti, S. Paul and B. Ray, 2012. Sulfated polysaccharides from *Laminaria angustata*: Structural features and in vitro antiviral activities. Carbohydrate Polymer, 87, 123–130.

Sun, Y., H. Liang, G. Cai, S. Guan, H. Tong and X. Yang, 2009. Sulfated modification of the water-soluble polysaccharides from *Polyporus albicans* mycelia and its potential biological activities. *International* Journal of Biological Macromolecules, 44, 14–17.

Tanaka, T., S. Oi and T. Yamamoto, 1980. The molecular structure of low and high molecular weight levans synthesized by levansucrase, Journal of Biochemistry, 87, 297–303.

Tanaka, T., M. OiLizuka and T. Yamamoto,1978. Levansucrase of *Bacillus subtilis*. Agricultural and Biological Chemistry, 42: 323–326.

Tomasic, J., H. J. Gennings and C. P. J. Glaudemans, 1978. Evidence for a single type of linkage in a fructofuranan from *Lolium perenne*. Carbohydrate Research, 62, 127–133.

Vergara, C.M.A.C., T.L. Honorato, G.A.Maia and S. Rodrigues, 2010. Prebiotic effect of fermented cashew apple (*Anacardium occidentale* L) juice. LWT-Food Science Technology. *43*, 141–145.

Wei, D., Y. Wei, W. Cheng and L. Zhang, 2012. Sulfated modification, characterization and antitumor activities of *Radix hedysari* polysaccharide. International Journal of Biological Macromolecules, 51, 471–476.

Yamamoto, Y., Y. Takahashi, M. Kawano, M. Iizuka, T. Matsumoto, S. Saeki and H. Yamaguchi, 1999. In vitro digestibility and fermentability of levan and its hypocholesterolemic effects in rats. Journal of Nutritional Biochemistry., 10, 13.

Yang, J. H., Y. M. Du, and R. H. Huang, 2002. Chemical modification, characterization and structure-anticoagulant activity relationship of Chinese lacquer polysaccharides. International Journal of Biological Macromolecules, 31, 55–62.

Yoo, S. Ho., E.J. Yoon, J. Cha and H.G. Lee, 2004. Antitumor activity of levan polysaccharides from selected microorganisms. International Journal of Biological Macromolecules. 34, 37–41.

Yoshida, Y., and R. Suzuki, 1990. Production of levan by a *Zymomonas* sp. Journal of Fermentation and Bioenginering, 70, 269–271.

Zhang, K., D. Peschel, E. Brendler, T. Groth and S. Fischer, 2009. Bioactivity of cellulose derivatives. Macromolecular Symposia, 280, 28–35.