World Academy of Science, Engineering and Technology
International Journal of Nutrition and Food Engineering
Vol:4, No:8, 2010

Microbial Production of Levan using Date Syrup and Investigation of Its Properties

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***Abstract*—**Levan, an exopolysaccharide, was produced by *Microbacterium laevaniformans* and its yield was characterized as a function of concentrations of date syrup, sucrose and the fermentation time. The optimum condition for levan production from sucrose was at concentration of 20% sucrose for 48 h and for date syrup was 25% for 48 h. The results show that an increase in fermentation time caused a decrease in the levan production at all concentrations of date syrup tested. Under these conditions after 48 h in sucrose medium, levan production reached 48.9 g/L and for date syrup reached 10.48 g/L . The effect of pH on the yield of the purified levan was examined and the optimum pH for levan production was determined to be 6.0. Levan was composed mainly of fructose residues when analyzed by TLC and FT-IR spectroscopy. Date syrup is a cheap substrate widely available in Iran and has potential for levan production. The thermal stability of levan was assessed by Thermo Gravimetric Analysis (TGA) that revealed the onset of decomposition near to 49°C for the levan produced from sucrose and 51°C for the levan from date syrup. DSC results showed a single Tg at 98°C for levan produced from sucrose and 206 °C for levan from date syrup.

Keywords*—**Date syrup, Fermentation, Levan, *Microbacterium laevaniformans

I. Introduction

LEVAN is a biopolymer in which fructose units are mainly linked by β(2→6)-glycosidic bonds, with some β(2→1) linked branch chains [1,2] (Fig 1). Levans are naturally found in many plants and microbial products. Plant levans found in certain grasses have low molecular weights and exhibit little branching [3, 4]. Microbial levans are different from plant levans and are produced from sucrose-based substrates by extracellular levansucrases and have high molecular weights and extensive branches [4].

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Different microorganisms can produce levans such as *Pseudomonas* sp., *Xanthomonas* sp., *Bacillus* sp., and *Streptococcus* sp. [5].

The enzyme that is responsible for synthesizing levan is levansucrase which transfer fructose moiety of sucrose to pre-existing acceptor molecules [6, 7].

Nutrient concentration is very important for levan production. Production of microbial levans as exopolysaccharides is largely affected by the concentration of nutrients in culturing medium and the environmental conditions. The chemical structure and physical properties of levans have been extensively characterized, in terms of molecular weight, linkage type, sugar components, and viscosity [8, 9]. All these differences influence the rheological properties of levan polysaccharides and affect the overall quality of foods [18].

Many carbohydrate polymers have been shown to express biological activities such as immunostimulating, antitumor, anti-inflammatory activities and cholesterol lowering effect [10, 11, 13, 14, 15, 16]. Levan polysaccharides have various potential applications for foods. They have been used as emulsifiers, stabilizers, and food coating materials [12]. Levan produced from *Zymomonas* as a potential antitumor agent was studied with Sarcoma-180 cell. They found that antitumor activity of *Zymomonas* levan was related to its molecular weight [17].

Levan may have a wide range of applications in medicine, food, printing, and cosmetics, thus optimization of levan production is an important research area [19, 20].

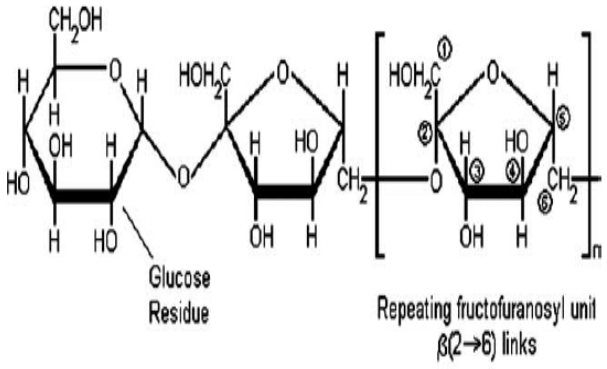

Fig. 1 Chemical structure of main type of fructosan (levan). [20]

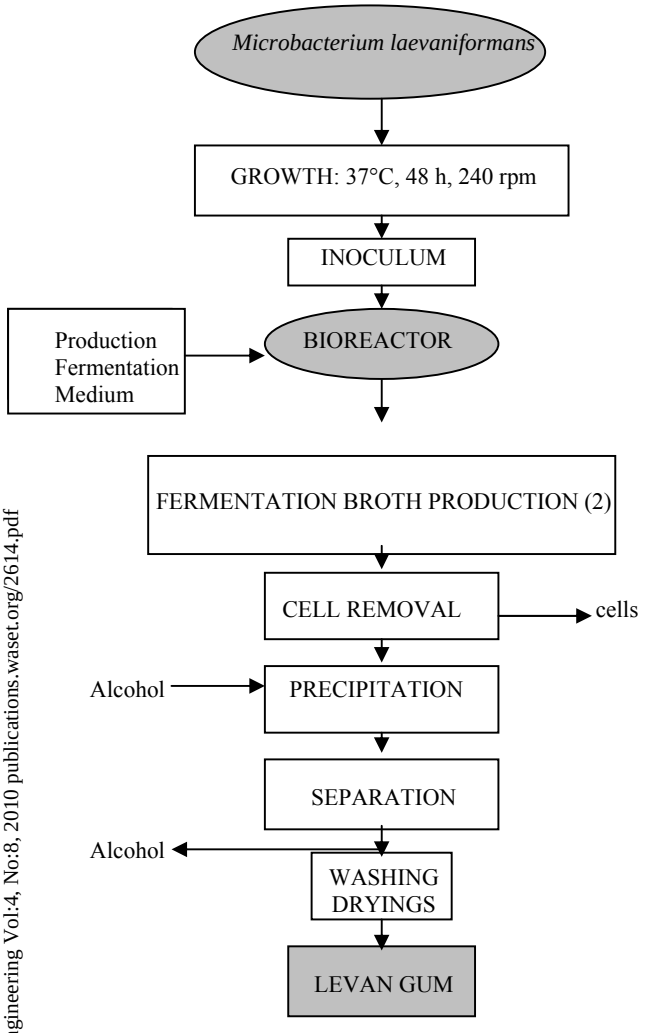

Fig.2 Schematic diagram of production, isolation and purification of *Microbacterium laevaniformans* levans.

II. Materials And Methods

A. Preparation of date syrup

The initial extract was prepared by soaking 200 g of stone free, low quality date fruits in 500 ml distilled water, then mixed for 1 min at low speed, and additional 3 min at high speed. The homogenized extract was maintained in hot water for 20 min, and then centrifuged at 10000 g for 30 min at room temperature to remove solids.

B. Microorganisms, growth medium and cultivation conditions

Microbacterium laevaniformans was obtained from the Persian Type Culture Collection (PTCC), strain number 1406. *M. laevaniformans* was grown aerobically at 37°C.The medium was autoclaved for 15 min at 121°C. Sucrose and date syrup were autoclaved separately. Inoculum preparation was by means of microorganism transfer from the stock solution to plates and incubation for 2 days at 37 °C. Following this period, single colonies were transferred into the activation medium, which contained 5 g glucose, 10 g peptone, 5 g yeast extract, and 5 g NaCl. Activation media was sterilized at 121 °C for 20 min. Following sterilization, the medium pH was 7.2. The cultures were incubated at 37 °C until the absorbance at 650 nm reached ≥ 0.6. Three flasks each containing 200 ml medium, were incubated with 20 ml of the culture and placed in a shaker incubator at 240 rpm and 37 °C for 2 days and were used as inoculum for the bioreactor. The composition of medium was as follows: 10 (BX) sucrose, 5 g yeast extract, and 5 g NaCl and for date syrup medium 10 (BX) date syrup, 5g yeast extract, and 5g NaCl (Fig.2). To determine the exopolysacharide (EPS) yield, samples were taken at different times and levan was purified from the culture supernatant by acetone precipitation. Results are averages of three different runs.

C. Isolation and purification of levan

Samples were collected every 24 h and kept at 80 °C for 10 min followed by centrifugation at 4 °C (2500 g, 20 min). The supernatants were decanted and treated with a 3:1 volume of acetone. The mixture were centrifuged again and dried at 50 °C for 24 h and waighted.(5)

III. Analytical Methods

Carbohydrate presence was analyzed by spot test on thin layer chromatography (TLC) plates. Levan samples (3% v/v) were acid-hydrolyzed with 15 ml of 0.1N HCl at 90°C for 3 h. The resulting acid hydrolyzates were used for TLC. For the TLC analysis, 10 µg of the hydrolyzed samples were spotted on a silica gel plate and a mixture of 1-buthanol: 2-propanol: water (1:3:1 v/v) was used as developing solvent. Detection of sugar was performed using 5% (v/v) sulfuric acid in methanol. Solution was sprayed on the plate, and the color was developed at 110 °C for identifying sugars in levan samples. Fourier transform-infrared (FT-IR) spectrum of EPS were obtained with Shimadzu 8300 at 400 to 4000 (cm^{-1}). The effect of date syrup and sucrose concentration on glass transition temperature (Tg) was assessed using a DSC-PL polymer laboratories(England) and TGA-PL 1500. For DSC small pinholes were punched into the lids to allow water to escape. An empty crucible was used as a reference. Calibration of the instrument was performed using indium as a standard. Two heating cycles were employed. Therefore, a first heating cycle from 0 °C to 160 °C was employed at a heating rate of 10 °C/min which evaporated the water. The sample was then cooled to 0 °C and a second heating cycle employed from 0 °C to 400 °C to assess true thermal properties. The assignment of peaks and integration of peak areas was performed according to DSC-PL polymer laboratories (England). Thermal decomposition temperature measurement was carried out in TGA-PL 1500, at a heating rate of 10 °C/ min from 30 to 400°C.

IV. Results And Discussion

A. Levan Yield

The results of levan production are shown in Fig 3. Maximum EPS concentration was attained after 48 h with both sugar sources.

An increase in fermentation time after 48 h, caused a decrease in levan production. This might be due to the pH reduction in the medium and also aging of the bacteria.

Although pure sucrose seems to be a better source of fructose for levan production, date syrup is apparently a useful source of raw material for this process (15).

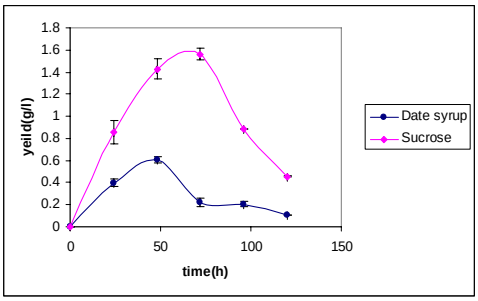

Fig. 3 Diagram of *Microbacterium laevaniformans* levans yield at different fermentation times.

B. pH

The pH of fermentation medium changed during fermentation period as shown in Fig 4. The effects of pH on the yield of the purified levan were examined, and the results are shown in Fig. 5. The optimum pH for levan production was 6.0.

During the first 24 h of fermentation, the pH of the inoculated medium decreased from 7.0 to about 6.8.

In the second day of fermentation, pH reduction was faster and was probably caused by acid production by bacteria which were in their log phase of growth. Results show that reduction in pH of the medium caused a decrease in levan production.

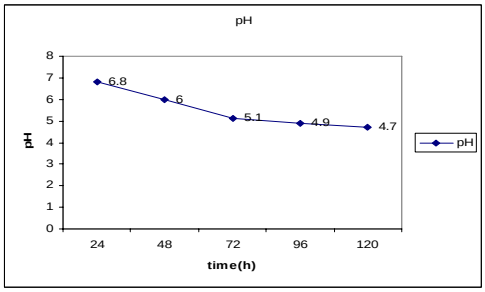

Fig. 4 Effect of fermentation time on the pH of fermentation medium.

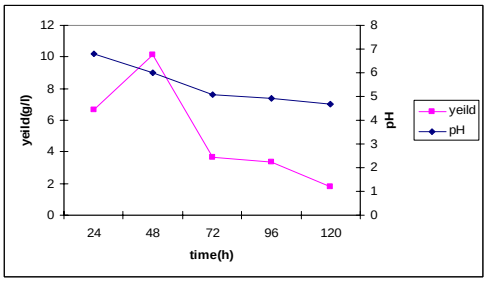

Fig. 5 Changes in yield and pH during fermentation period for production of levan by *Microbacterium laevaniformans* using date syrup.

C. Concentration

The optimum conditions for levan production in sucrose were at 20% concentration and a fermentation time of 48 h and for date syrup was at 25% concentration. Under these conditions in sucrose medium, levan production reached 48.9 g/L and for date syrup reached 10.48 g/l. The results show that an increase in fermentation time caused a decrease in levan production when date syrup was used.

Levan yield increased by an increase in date syrup concentration, however, levan production was higher in sucrose medium. In both media, sucrose was used as carbon source. In the first group, there were lower levan contents and date syrup was used as a carbon source. The low levan values obtained when date syrup was used possibly due to the presence of combination of several sugars in date syrup (sucrose, glucose, and fructose) that inhibited the cell growth and metabolites production (Fig. 6).

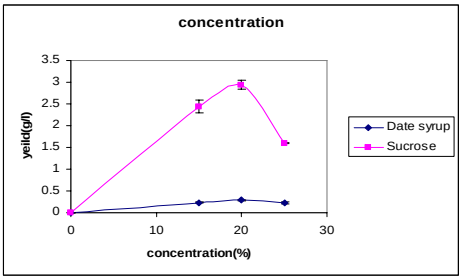

Fig. 6 Effects of concentration of sugars in production of levan by *Microbacterium laevaniformans*.

D. FT-IR Spectroscopy

Fourier transform-infrared (FT-IR) spectra of EPS were obtained with Shimadzu 8300 between 400 and 4000 wave number (cm^{-1}). Levan is hydroxyl-containing hydrocarbon with 5-membered ring fructose.

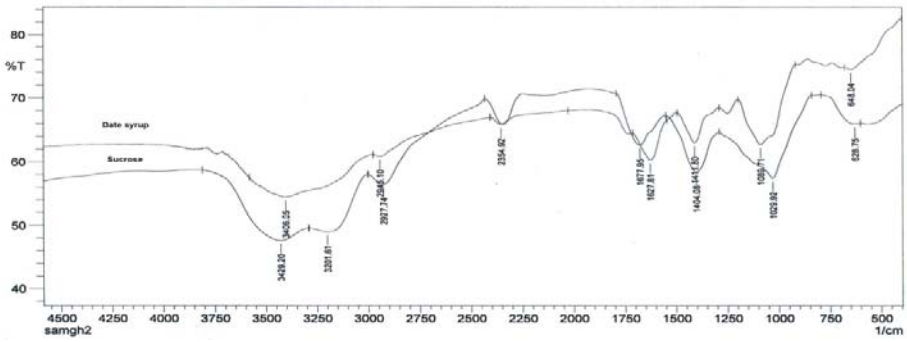

Fig. 7 FTIR spectra of *Microbacterium laevaniformans* levans powder.

The infrared spectrum of this biopolymer showed the characteristic peak signals of polysaccharides: broad stretching peak at around 3406 cm^{-1}, (O-H stretching), a weak C-H band at around 2945 cm^{-1}, C=O stretching at 1677 cm^{-1} and several sharp peaks around 1000 cm^{-1} typical of carbohydrates (Fig.7) (Table I). This demonstrates that date syrup, a cheap substrate widely available in Iran, has a potential to be used as a substrate.

E. Sugar composition of levan

Sugar components of levan were identified by TLC analysis. The R_f value of acid-hydrolyzed levan from *M. laevaniformans* PTCC 1406 was identical to that of fructose under our solvent ascending condition. These results indicated that levans were composed solely of fructose. Levan produced from sucrose hydrolyzed completely in 90 min but levan which was produced from date syrup hydrolyzed in 180 min. This difference might be due to the high molecular weight of levan in date syrup which takes more time to hydrolyze (Fig. 8).

F. Thermal Analysis

The thermal stability of levan was assessed by Thermo gravimetric analysis (TGA). Fig. 9 shows the onset of decomposition near to 49°C for levan produced from sucrose and 51°C for levan from date syrup which is due to the loss of water present in the scaffold. The second decomposition shows the breaking up of C-H bonds.

The glass transition temperature (Tg) of levan was measured using differential scanning calorimetry (DSC). The values were taken from the second run after heating and cooling. The DSC thermpgram (Fig. 10) showed single transitions at 98°C for levan from sucrose and 206 °C (13)for levan from date syrup. This may be attributed to the different sugar content and different structure of levan produced from sucrose or date syrup. *T*g of levans shows that even small changes in the chemistry of a carbohydrate change the thermal phase behavior of the system significantly.

TABLE I
BAND ASSIGNMENTS OF THE LEVANS FTIR SPECTRA

Peak position cm^{-1}	peak assignment
3466[a]	(OH) stretching
2945[a]	(CH) vibration
1677[a]	(CO) stertching
3429[b]	(OH) stretching
2927[b]	(CH) vibration
1627[b]	(CO) stertching

a. levan (date syrup) b. levan (sucrose)

G. ANOVA test analysis for levan

The yields from each sampling time were analyzed by Duncan test. The obtained results showed four non-significant groups which are shown in Table 3. These results demonstrate that the yield after 48h fermentation was different relative to all the other yields at the level of 0.05. Factorial test (time* treatment (date syrup and sucrose media)* repetition) results (Table 2) represented the significant difference for treatment and sampling times alone at the level of 0.001 while time* treatment interaction did not show any significant difference even at the level of 0.05.

TABLE II
ANOVA RESULTS BY FACTORIAL TEST FOR LEVAN YEILD

Source	df	Mean Square	F	Sig
Time	4	171.205	23.125	.000**
Treatment	1	1323.219	178.732	.000**
Time*Treatment	4	42.960	5.803	.003$^{n.s}$
Error	20	7.403		
Corrected Total	29			

** : significant difference at level of 0.001
n.s: Non-significant

TABLE III
RESULT OF DUNCAN TEST FOR YEILD OF LEVAN

Time (h)	Weight (g/l)
24	$12.5367^{bc}\pm 0.2214$
48	$20.3200^{d}\pm 0.3210$
72	$14.4567^{c}\pm 0.3566$
96	$10.9267^{b}\pm 0.2574$
120	$5.6400^{a}\pm 0.1882$

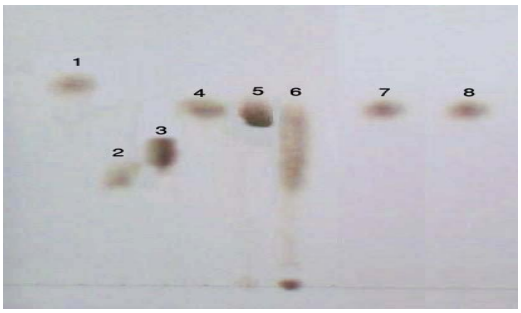

Fig. 8. TLC showing the process of levan hydrolysis at different times and other sugars (1. xylose 2. raffinose 3. sucrose 4. glucose 5. Levan (sucrose) after 1.50 h 6.(14) Levan (date syrup) after 1.50 h 7. Levan (date syrup) after 3 h 8. fructose

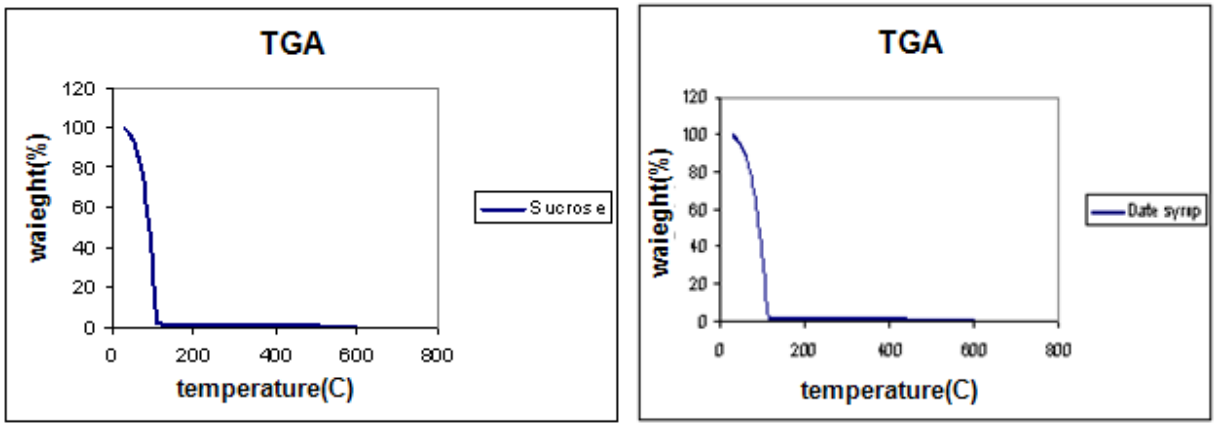

Fig. 9. Thermogravimetric analysis of levan

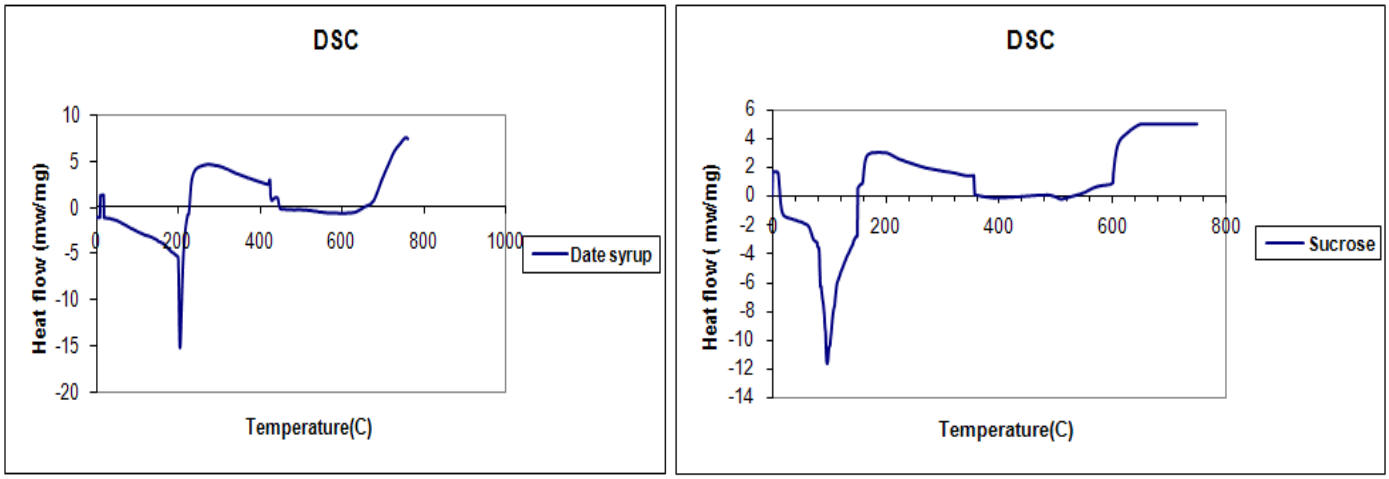

Fig. 10. Differential Scanning Calorimetry Thermograms of levans

V. Conclusions

Levan could be economically a produced through a microbial process, the molecule has potential to be formed into films and molded products for commodity polymer applications. Levan polysaccharides were produced from *Microbacterium laevaniformans*. Two culture (11)media were used, commercial sucrose and date syrups. (18)

Three successive factorial planning were used to assess the sugar concentration, fermentation time, carbon sources and pH of the medium.

The first factorial planning was carried out to optimize the sucrose concentration and fermentation time for levan production. In the second factorial planning the effect of pH of the sucrose medium on levan production were assessed. The fermentation time (5 days) and sucrose and date syrup concentration (15%, 20%, 25%) were defined from the results obtained in the first planning and the optimum pH for levan production was 6.0.

It would be necessary to study the practical performance of levan polysaccharides when incorporated into food formulations for the better understanding of its mouth feeling and perception by consumers. *T*g of levan from sucrose and levan from date syrup show that even small changes in the chemistry of a carbohydrate change the thermal phase behavior of the system significantly.

The results of this study indicate that levan can be produced by microbial growth in the presence of inexpensive raw materials such as date syrup which is abundantly available in Iran. By controlling pH of medium, it would be possible to increase the yield of production.

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