Production of Levan from *Paenibacillus polymyxa* in Date Syrup and analyzing of levan composition by TLC

* Shamim Naser Radhi, Shatha S. Hasan and Sanaa Burhan alden
Department of Biology, College of science, University of Baghdad
* E-mail address: shamam_bio@yahoo.com

Abstract

Levan, an exopolysaccharide, was produced by *Paenibacillus polymyxa* and its yield was characterized as a function of concentrations of date syrup and sucrose. The optimum condition for levan production from sucrose was in a concentration of 20% at 37°C, pH 7for 48 hrs and the same conditions for date syrup. Under these conditions in sucrose medium levan production reached to 48 g/L and total carbohydrates were 95.8% while for date syrup was reached to 24g/L and total carbohydrates were 69%. Levan was composed mainly of fructose residues when analyzed by thin layer chromatography (TLC). Date syrup is a cheap substrate widely available in Iraq and has potential for levan production.

Key words: Levan, date syrup, *Paenibacillus polymyxa*, TLC

الخلاصة

ينتج متعدد السكريات الليفان من بكتريا*Paenibacillus polymyxa* ويعتمد انتاجه على تركيز السكريات في كل من خلاصةالتمر ووسط الاملاح الحاوي على السكروز بتركيز 20% .

حددت الظروف المثلى لانتاج الليفان في وسط السكروزواظهرت النتائج ان الوسط الامثل للانتاج كان وسط الاملاح الحاوي على20% من السكروز وبدون اي مصدر نايتروجيني برقم هيدروجيني(pH) 7 وبدرجة حرارة 37 °م ولمدة حضانة 48 ساعة واستخدمت نفس الظروف لانتاج الليفان في وسط خلاصة التمر, حيث بلغت قيمة الوزن الجاف لليفان في وسط السكروز 4,8غم/100مل وكانت النسبة المئوية للسكريات الكلية95,8%اما في وسط خلاصة التمر فكان الوزن الجاف 24 غم/100مل ونسبة السكريات 69%

ان تحليل الليفان باستخدام تقنية Thin Layer Chromatography اظهرت الليفان كبقعة واحدة سوداء تمثل الفركتوز.

تم استخدام وسط خلاصة التمر في هذه الدراسة على اعتبار انه مادة اولية متوفرة في العراق بالاضافة لقابليته في انتاج الليفان.

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] Levans are naturally found in many plants and microbial products. 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 [3]. Different microorganisms can produce levans such as *Pseudomonas* sp., *Xanthomonas* sp., *Bacillus* sp., and *Streptococcus* sp. [4]. 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 [5, 6]. All these differences influence the rheological properties of levan polysaccharides and affect the overall quality of foods [7]. Levan polysaccharides have various potential applications for foods. They have been used as emulsifiers, stabilizers, and food coating materials [8]. Levan produced from *Zymomonas* as a potential antitumor agent [9]. Levan may have a wide range of applications in medicine, food, printing, and cosmetics [10].

MATERIALS AND METHODS

Sucrose mineral salts medium

It was prepared according to [11]; it was composed of 0.3%KH_2PO_4, 0.3%K_2HPO_4 and 0.05%$MgSO_4.7H_2O$ and 20% (w/v) sucrose. This medium was prepared as broth, and as solid medium by addition of 2% agar, pH was adjusted to 7.2 autoclaved at 121°C for 10 min.

Date extract medium

This medium was prepared by mixing 100gm of Al-Zahidi date (without pits) with 500ml tap water and boiling with constant stirring for 10min after that it was cooled and stored at 4°C overnight, then mixed again and filtered through many layers of cheese cloth and Whatman No.1 filter paper, the filtrate was centrifuged twice at 3000rpm [12]. Total carbohydrate in a supernatant was estimated using phenol-sulfuric acid method [13] then it was sterilized by autoclaving at 121°C for 15min.

Samples collection

Seventy five samples were collected from soil and water, soil samples were collected in plastic bags while water samples were collected in sterile bottles.

Isolation of bacterial sample

Seventy-five samples were collected from soil and water (50 samples of soil and 25 of water). 10 grams of each soil sample (after sieving) had been added to 90ml of distilled water, mixed vigorously and heated to 80°C for 10 min. Serial dilutions for each sample were set up from 10^{-1}-10^{-3}. 0.1 ml of appropriate dilution was spread on the surface of nutrient agar plates and incubated at 37°C for 24 hrs. One hundred millilitres was taken from water samples by sterile syringe centrifuged at 6000 rpm for 15 min. There after centrifugation most of supernatant (water) was discarded and the pellet was shaked to get suspension; the suspension was heated at 80°C for 10min. Then 0.1 ml of each sample was spreaded on the surface of nutrient agar plates and incubated at 37°C. Morphological features of the bacterial colonies (such as: size, margin and color) were observed after 24hrs of incubation.

Bacterial colonies were transferred and purified by sub-culturing on nutrient agar which was subjected for microscopic examination. The purified bacteria were then identified depending on microscopic examinations and biochemical tests [14].

Screening for Levan-producing *Bacillus* isolates

Bacillus isolates were activated in Brain Heart Infusion broth (B.H.I.) 0.1 ml of culture was streaked on sucrose mineral salt agar and incubated at 37°C for 48 hours. Mucoid consistence of bacterial colonies was the indicator of levan production. The highly mucoid isolates were selected and subjected to further step of screening [15].

Quantitative screening in liquid medium

Highest mucoid isolates on mineral salt agar were inoculated in B.H.I broth. After incubation the absorbency at 600nm for each culture was measured. Fifty ml of mineral salt broth containing 20% sucrose was inoculated with 1 ml of culture in 250ml conical flask and incubated at 37°C for 48 hours, bacterial culture was centrifuged at 6000rpm for 30 min. to remove bacterial cells levan was extracted by mixing the cell free supernatant with cold ethanol at a ratio of (1:4 v/v) and allowed to stand overnight, the aqueous layer was removed and the off white gummy precipitated layer (levan) was collected in a sterilized Petri dishes and dried at 60 °C [16] dry weight for the extracted levan was measured

Production of levan in date syrup and sucrose media

Date extract medium and mineral salts broth with 20% sucrose were used as for levan production media were inoculated with 1ml of activated bacterial culture broth and incubated at 37°C with shaking for 48 hrs. Levan was extracted, its dry weight was determined, total carbohydrates were estimated

Analysis of levan by thin layer chromatography

Separation and identification of levan was performed by thin layer chromatography (TLC) using silica gel coated plate (TLC coated with silica gel 60) (20×20) cm, this method was applied by [17] as following:

1- 0.01gm from collected levan was dissolved in 1N HCl (400 µl) and incubated at 70°C for 3hrs.

2- About 10 µl was taken from the sample suspension by capillary tube and spotted many times at about 2cm far from the lowest edge of TLC plate; sucrose, fructose and glucose solutions were also spotted separately in the same manner.

The plate was transferred to a closed jar containing separation system by mixing *n*-butanol : 2-propanol : distilled water : acetic acid at ratios of 7:5:4:2, v: v: v: v. and left to diffuse through silica gel plate to about 15 cm, after that the plate was dried at room temperature.

3-The dry plate was sprayed with specific reagent composed of ethanol and H_2SO_4 at a ratio of 9:1, v: v. [18] and placed in an oven at 90°C for 5-10min, to complete the reaction; levan components appeared as colored spot, the position and distance of the spots were determined. The relative flow (R_f) was estimated by dividing the distance of the sample mobilized across the plate on the distance of the solvent [19].

RESULTS AND DISCUSSION

Isolation and identification of bacteria

In this study 112 bacterial isolates were obtained from 75 soil and water samples. The morphological characteristics and microscopic examination revealed that 88 of these isolates (31 isolates from water and 57 from soil) were belonged to *Bacillus* group [15]. Soil and water are the original habitats from which the majority of *Bacillus* species have been isolated except some species that caused disease to human and animal such as *B.anthracis* which cause anthrax and can be isolated from infected animals and some other species which may isolated from infected insect, such as *Bacillus larvae* and *Bacillus popilliae* [20].The common habitat for *Bacillus* spp. is the soil [21]. *Bacillus* can be found in water contaminated with soil [22].

Screening of levan producing isolates

Pure cultures of *Bacillus* isolates were cultured in mineral salts agar media containing sucrose to detect their ability to grow with mucoid appearance as indicator for levan production. Twenty five *Bacillus* isolates (28.4%) revealed a mucous phenotype, "Fig.1".

Many *Bacillus* species produce levan. Levan production by *Bacillus* differ from species to species, the highest levan productivity species was *Paenibacillus polymyxa* (*Bacillus polymyxa*) [23]. Colonies of *Paenibacillus polymyxa* became gummy and adhered to the agar surface, when growing on agar medium with sucrose as a result for levan production as mentioned by [24].

Bacillus licheniformis when grown on agar medium containing sucrose, the colonies had a mucoid slimy appearance which indicated the production of the polysaccharide (levan) from sucrose [25].

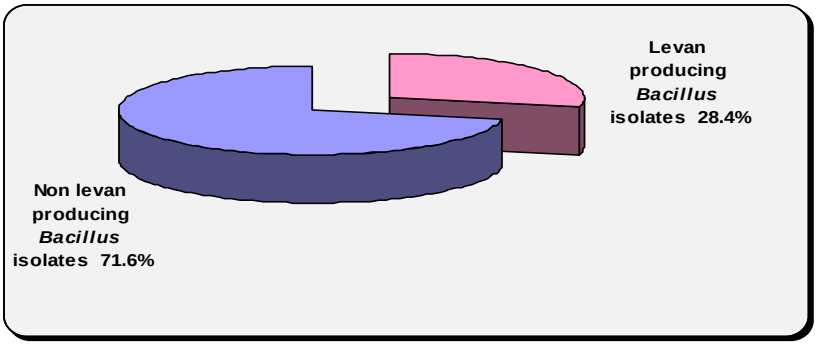

Figure1: Percentage of levan-producing *Bacillus* isolates on sucrose mineral salt agar at 37 for 48hrs

Quantitative screening of levan in liquid medium

Ten *Bacillus* isolates that had highest degree of mucoid growth on solid medium (mineral salts agar) were selected for further screening in mineral salt liquid medium containing sucrose as only carbon source. Maximum levan yield (4gm/ml) was achieved by B2 isolate "Fig. 2", thus it was selected for the further steps of this study.

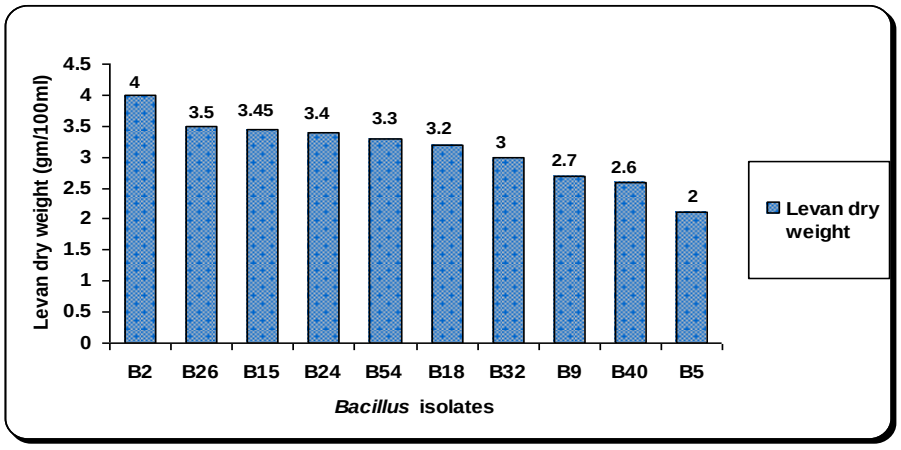

Figure2: Levan production by different *Bacillus* isolates cultured in mineral salts medium containing 20% sucrose, pH 7.2 and incubated at 37°C for 48 hrs.

Identification of bacteria

The highest levan producing isolate (B2) was identified depending on morphological and physiological characteristics the results of biochemical tests showed that this isolate is *Paenibacillus polymyx*a (*Bacillus polymyxa*) [14 and 26].

Production of levan in date syrup medium

The use of alternative regional low-cost substrates has become very interesting because in addition to the ease of acquisition it represents a relatively low cost, thus date palm syrup was used in this study. It was found that levan dry weight from date syrup was 2.4gm/100ml which was less than that produced from mineral salts medium 4.8gm/100ml the concentration of total carbohydrates in sucrose media 95.8% were higher than that produced from date syrup media "Fig. 3".

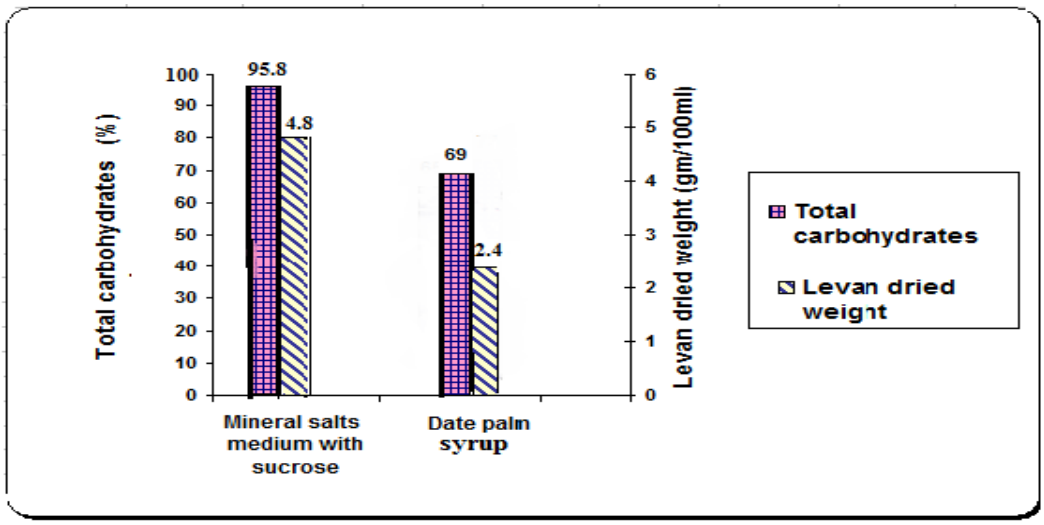

Figure 3: Production of levan from *Paenibacillus polymyxa* B2 in different media incubated at 37°C for 48 hrs and pH 7 in shaking incubator at 100 rpm and inoculum size 2%.

levan produced from date syrup differs in texture and appearance from levan produced from sucrose it was dark brown with gummy texture , devoid of the typical white, smooth, gummy properties of levan that produced from sucrose.

levan production reached 48.9 g/L while for date syrup reached 10.48 g/l there were lower levan contents when 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 [27]. Dates containing different chemical components such as sugars, proteins, sodium, potassium, calcium, magnesium, phosphorus, zinc, copper, iron and manganese ; the excess of these minerals may affect levan production(minerals influence levansucrase enzyme by changing catabolism pathway) causing inhibition in levan formation and produce a poor quality product [28].

Analysis of levan by TLC

Paenibacillus polymyxa B2 levan contents were analyzed by TLC to determine its components of monosaccharides. Levan extracted from *Paenibacillus polymyxa* B2 isolate was first hydrolyzed with HCl before application on TLC plate, the separation system composed of *n*-butanol: 2-propanol: distilled water: acetic acid at ratio of 7:5:4:2 v/v/v/v, standard sugars glucose, fructose and sucrose were used as markers. The spots were visualized after spraying with ethanol-H_2SO_4 (9:1 v/v) as dark spots. Levan appeared as a single dark spot, "Fig. 4". Rf value for levan and fructose was 0.57 and 0.586 for glucose, Rf = 0.5 for sucrose, Rf value for the same compound may differ at different conditions such as solvent, TLC matrix and dimension of container.

In the solvent system butanol-acetic acid - water (9:6:1), Rf = 0.58 for glucose and 0.71 for fructose, while in butanol-acetic acid - water (4:5:1.) Rf = 0.92 for glucose, Rf =0.95 for fructose. In propanol - ethyl acetate - water (7:2:2.) Rf =0.78 for glucose and 0.84 for fructose [29]. After acid hydrolysis of levan, followed by TLC analysis; fructose is identified as a single spot on TLC plates [30]. The Rf value of acid-hydrolyzed levan from *Microbacterium laevaniformans* PTCC 1406 was identical to that of fructose under solvent ascending condition. These results indicated that levans were composed solely of fructose [27].

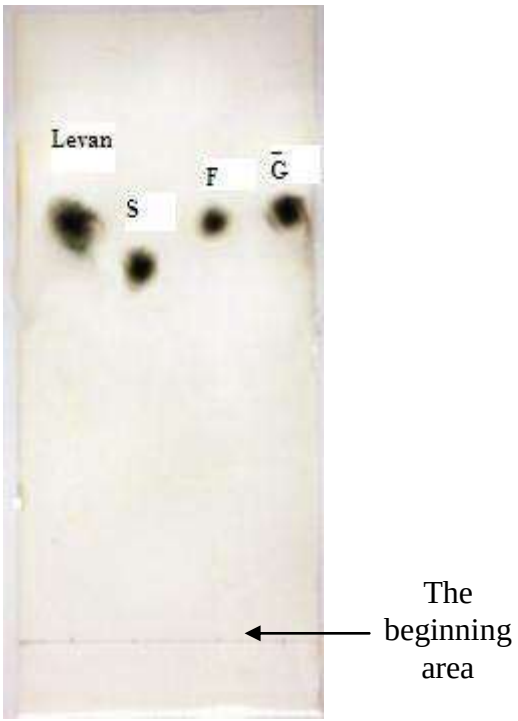

Figure 4: TLC analysis for detection of levan treated with ethanol-H_2SO_4 (9:1 v/v) using silica gel plate (20×20) cm with solvent system *n*-butanol: 2-propanol: distilled water: acetic acid at ratio of 7:5:4:2, v:v:v:v) at room temperature. S: sucrose, F: fructose, G: glucose.

CONCLUSIONS

Results of this study indicated that levan can be produced from *Paenibacillus polymyxa* in presence of inexpensive raw materials date palm syrup and its quantity was lower than that produced from sucrose as a carbon source in the same conditions. Acid hydrolyzed levan was analyzed by TLC on silica gel coated plate fructose is identified as a single spot on TLC plate after levan analyzing. This result indicates that hydrolyzed levan is mainly composed of monosaccharide fructose.

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