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ISOLATION AND IDENTIFICATION OF HALOPHILIC BACTERIA PRODUCING EXOPOLYSACCHARIDES FROM WHEY AND MILK PERMEATE

[123]

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ABSTRACT

The aim of this research was to study the utilization ability of the salt whey and milk permeate to produce exopolysaccharides (EPSs) from halophilic bacteria. Where cheese whey is simultaneously an effluent with nutritional value and a strong organic and saline content. However, it is drained in the sewers, the EPSs are highly heterogeneous polymers produced by different species of bacteria and have recently been attracting considerable attention from biotechnologists because of their potential applications in many fields. Thus, we have isolated some halophilic bacteria that showed the ability to produce EPS from whey and milk permeate. A total of 46 strains of moderately halophilic bacteria were isolated from two types of samples. The first type was dairy samples (Baramily cheese whey and mish cheese), while the second type was water samples (salty lake water and its sediments) of Wadi El-Natrun Valley (lakes Hamra, UmRisha, and Baida), Beheira Governorate, Egypt. From isolated strains there are twelve strains were having the ability to produce exopolysaccharides but only seven strains can produce EPS from whey and milk permeate. The growth conditions i.e. concentrations of NaCl, pH value and different incubation temperature, of isolates were determined. The effect of these conditions on the production of EPS was investigated. The obtained results indicated that the optimum conditions for the production of EPS by these strains were 10 % NaCl, pH 7 and the optimum incubation temperature was 37°C. Three strains showed the highest production of exopolysaccharides. These strains were identified using two methods the first method was biolog system and the second one was 16S rRNA sequence analysis method. It could be identified as *Alteribacillus bidgolensis* and *Bacillus licheniformis*. *Alteribacillus bidgolensis* (strain P4B) produced the highest amount of EPS (52 g/L) from whey followed by *Bacillus licheniformis* (DSM 13) (42 g/L), while the highest amount of EPS produced from milk permeate was (43 g/L) by *Alteribacillus bidgolensis* (strain P4B) followed by *Bacillus licheniformis* (DSM 13) (36 g/L).

Keywords: Halophilic bacteria, Exopolysaccharides, Whey, Milk permeate, *Alteribacillus bidgolensis, Bacillus licheniformis*.

INTRODUCTION

Cheese whey is the most polluted byproduct induced in the production of cheese. It can cause an overabundance of oxygen utilization, toxicity, impermeabilization, etc.., in the receiving environment conditions. The volume of effluents delivered in the cheese production industry has expanded with the expansion in cheese creation. World production of whey is about 2×10^8 tons per annum, containing ~9×10^6 tons of lactose and 1.4×10^6 tons of whey proteins **(Fox et al 2017)**.

Milk permeate got from the ultrafiltration of milk is free from proteins, however, contains the other dissolvable constituents of milk. The dairy industry is effectively looking for approaches to use this byproduct which will help increment the estimation of dairy industry. These byproducts still contain valuable components, i.e. amino acids, lactose, minerals and even little amounts of protein that could be utilized for the production of some valuable products such as fermented products, sports drinks, snacks, infant's food etc.

Several types of bacteria such *Xanthomonas campestris* can produce extracellular polysaccharides (EPS)**.** EPS are bound to the cell surface and after that release into the grown media. Microbial EPS offer a potential new wellspring of useful biopolymers for industrial, pharmaceutical medicinal applications and food. It can be used as thickeners, suspending operators, or gelling agents to improve the quality and structure of food **(Morris and Harding 2009)**.

Halophilic and halotolerant organisms were found in each of the three species of life: Archaea, Bacteria, and Eukarya. Halophiles are classified into three groups according to salt needs, slightly halophiles (2– 5 % NaCl), moderately halophiles (5– 20 % NaCl) and extremely halophiles (20– 30 % NaCl) while strains live in 0– 5 % saltiness are considered halotolerant microorganisms **(Abd Samad et al 2017)**. Some halophilic microorganisms can produce exopolysaccharides **(Bejar et al 1998)**. Their EPS's are of extraordinary physical properties, e.g. the capacity to emulsify at acidic pH **(Martinez-Checa et al 1996)**. Their chemical and physical structures are valuable in different industrial fields when using as thickening and binding agent **(Tombs and Harding 1998)**.

Wadi El Natrun, Beheira Governorate, Egypt, is a valley consists of seven substantial, basic, hypersaline, thalassic lakes and various fleeting pools. Salinities extending from 1.5 to 5.0 M NaCl, pH estimations of somewhere in the range of 8.5 and 11 and temperatures of up to 50 °C make Wadi El-Natrun lakes great environments for the confinement of extremophiles. A rich microbial assorted variety exists in both the waters and sediments of Wadi El Natrun lakes **(Mesbah et al 2007)**.

The objective of this study was to isolate some halophilic bacteria to select those that maximize utilization of lactose as a substrate to produce exopolysaccharides from salt whey and milk permeate.

MATERIALS AND METHODS

Materials

1- Samples collection and handling

The halophilic strains were isolated from two types of samples. The first type was dairy samples (Baramily cheese whey and mish cheese), while the second type was water samples (salty lake water and its sediments) collected during July 2016 from three lakes, i.e Hamra, UmRisha, and Baida, of Wadi El-Natrun.

2- Whey and milk permeate

Sweet whey and milk permeate samples were obtained from Arabian Food Industries Company (Domty), 6th October City, Giza, Egypt.

Methods

1- Determination of salt content

The salt content of the brine was determined according to Mohr titration **(James, 1995).**

2- Screening test for exopolysaccharide production

Screening test was carried out on Halobacteria medium (372) was prepared according to (German Collection of Microorganisms and Cell Cultures) DSMZ GmbH catalog 2007, whey agar and milk permeate agar media (with 10 % NaCl). The isolated strains were plated and incubated under aerobic conditions at 37°C for 3- 4 days. At the end of incubation period, mucoid of colonies was determined by visual appearance, while it's ropiness was evaluated by touching them using a sterile inoculation loop **(Welman et al 2003)** and confirmed by ethanol precipitation method **(Ruas-Madiedo and de los Reyes-Gavilan 2005)**.

3- Identification of selected strains

The strains were identified using physiological, biochemical tests **(Biolog, 2013)**. and 16S rRNA sequence analysis methods as following:

- DNA (Deoxyribonucleic acid) was extracted using protocol of GeneJet genomic DNA purification Kit (Thermo K0721)
- PCR (Polymerase Chain Reaction) was made using Maxima Hot Start PCR Master Mix (Thermo K1051)
- PCR cleanup was made to the PCR product using Gene JET™ PCR Purification Kit (Thermo K0701)
- Finally sequencing to the PCR product was made on GATC Company by use ABI 3730xl DNA sequencer by using forward and reverse primers.

RESULTS AND DISCUSSION

1- Characterization of isolated strains

Data presented in **Table (1)** indicate the sampling sites, types, pH, sodium chloride concentrations (%) and distribution of halophilic isolates. It could be noticed that the water of lakes Baida and

UmRisha are similar and they greatly differ from that of Lake Hamra. This result agrees with those obtained by **Mesbah et al (2007)**. The distribution of the isolated halophilic bacteria grown on halo-bacteria medium (DSMZ M 372) using 15 and 20% NaCl as well as those grown in whey and milk permeate and production of exopolysaccharides is illustrated in **Fig. (1)**.

Table 1. Samples sites, types, pH, NaCl (%) and distribution of halophilic isolates

Sampling site	Sample type	pH*	NaCl (% w/v)	Total number of isolates
Hamra	Salty water Salty sediments	8.5	10	11 13
UmRisha	Salty water Salt	9.8	29.1	2 9
Baida	Salty water Salt	9.3	30	0 3
Whey	Baramily whey	4.5	10.8	5
Mish	Mish cheese	4.1	8.2	3

*pH was measured at 25º C

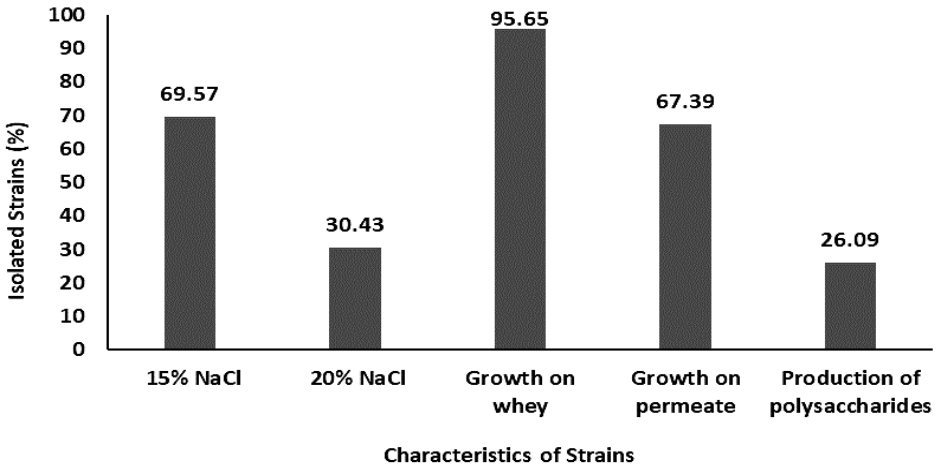

Fig. 1. Distribution of isolated strains at different growth conditions

The total number of isolated strains were 46 strains with identification numbers (1: 46). As shown in **Fig. (1)** it could be noticed that there are seven strains have the ability to grow on whey or milk permeate producing polysaccharides. Those strains were identified under numbers were 16, 36, 37, 39, 41, 44 and 45. The selected strains were used for further research work while the other strains that couldn't grow on salted whey and/or permeate were discarded.

2- Characterization of selected strains

Fig. (2) indicates that the strains number 37, 39, 41 and 44 grown from 0 to 20 % of NaCl with optimum concentration at concentration of 5 % NaCl except strain No. 44 was 10 % NaCl its optimum. The growth range of NaCl for the strains No. 16 and 36 were from 5 to 20 % with optimum concentration 10, 15 % NaCl, respectively. While the optimum salt concentration for the strain No. 45 was 15 % and growth range was from 0 to 15 % NaCl.

Data illustrated on **Fig. (3)** shows that pH 7 was optimum for growth of all strains except strains strain No. 36 and 44 where the optimum pH for them was 8 after 96 hr of incubation. The pH of the growth medium ranged from 5 to 9 for strains No. 37, 39,41 and 44. While it was from pH 6 to 9 for the other strains, i. e No. 16, 36 and 45.

The effect of the growth temperature was illustrated in **Fig. (4)** where the temperature range for all strains was from 25 ºC to 45 ºC and the optimum growth temperature was 37 ºC for the strains No. 16, 37 and 41, and 30 ºC for the strains No. 36, 39, 44 and 45.

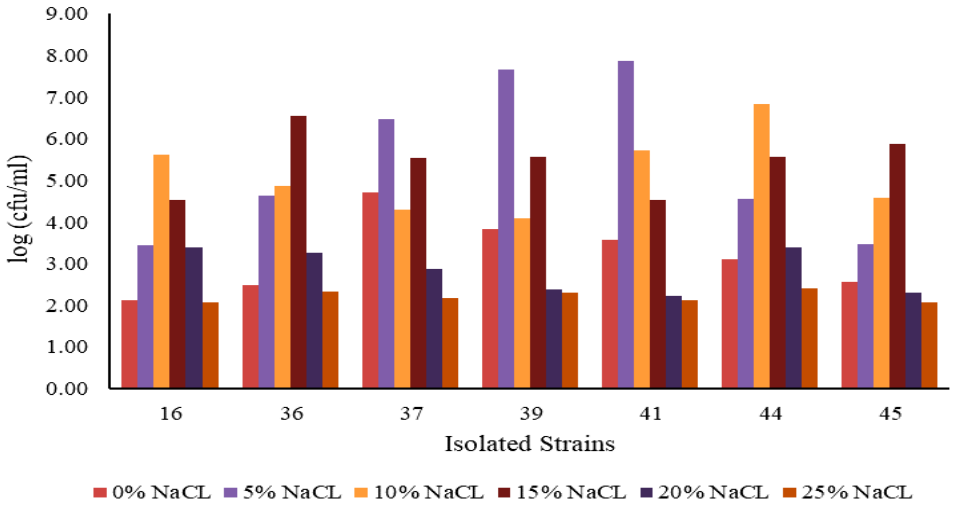

Fig. 2. Viable bacterial count of selected isolates at different concentrations of NaCl

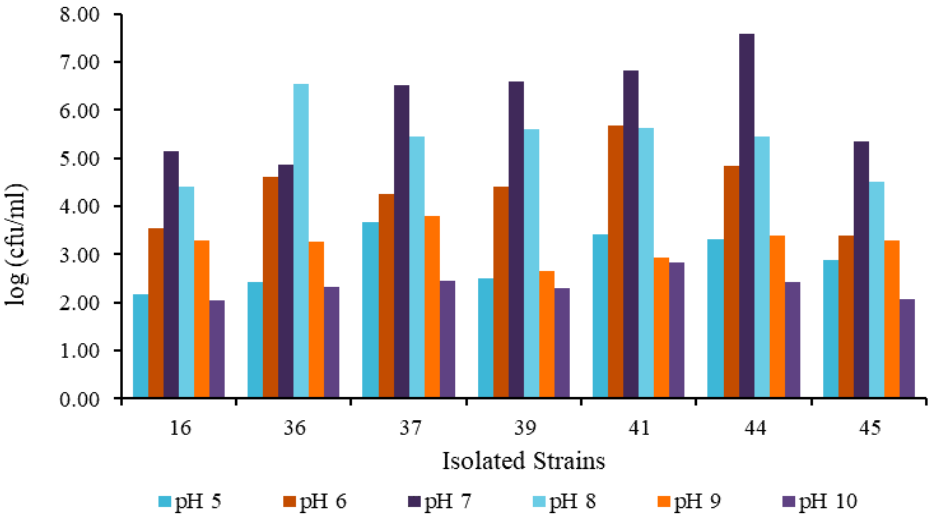

Fig. 3. Viable bacterial count of selected isolates at different pH values

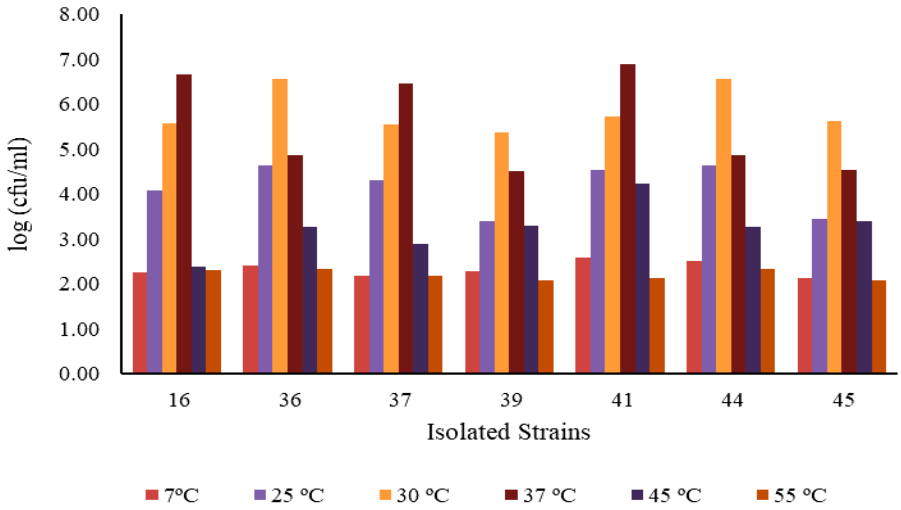

Fig. 4. Viable bacterial count of selected isolates at different incubation temperatures

The obtained results showed that the different concentrations of NaCl (0, 5, 10, 15 and 20%), different ranges of pH (5, 6, 7, 8 and 9) and different temperatures of incubation (25, 30, 37 and 45ºC) could be used for the growth of the selected strains and utilized whey and/or milk permeate for the production of exopolysaccharides.

3- Effect of some growth conditions on EPS production from selected isolates

Several studies have revealed that whey can be used as a fermentation medium to produce EPS using different strains **(Pantazaki et al 2009, Mozzi, et al 2001).**

Data presented in **Figs. (5)**, **(6)** and **(7)** indicate that the optimum concentration of NaCl was 10% to all the selected strains except strains No. 36 and 39 was 15 %. While grown at pH 7 give the best quantity of EPS for all the studied strains except strains No. 36 and 45 was pH 8. The optimum incubation temperature was 37º C for all the selected strains except strain No. 36.

The previous result show that strains No. 16, 37 and 41 were the best strains in EPS production from whey as it appears in **Fig (8).**

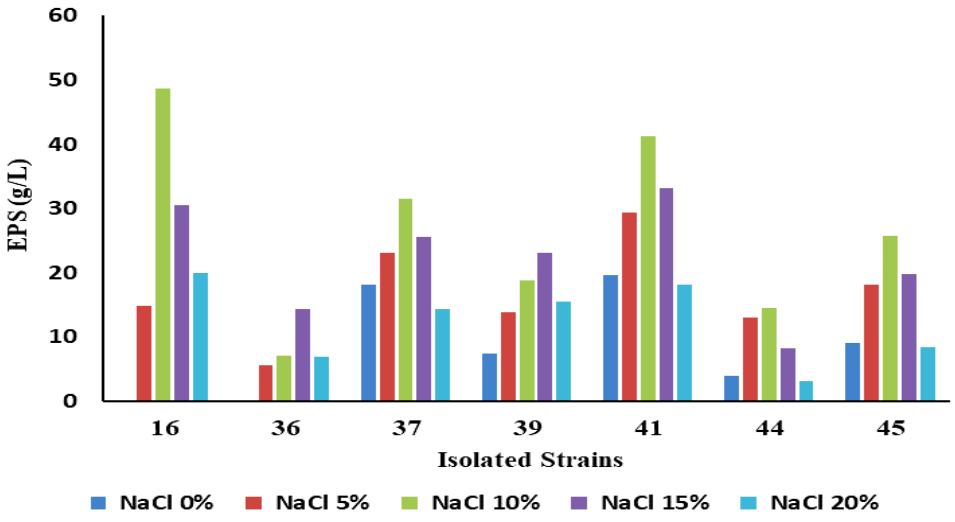

Fig. 5. Production of EPS from selected isolates grown on whey at different concentrations of NaCl.

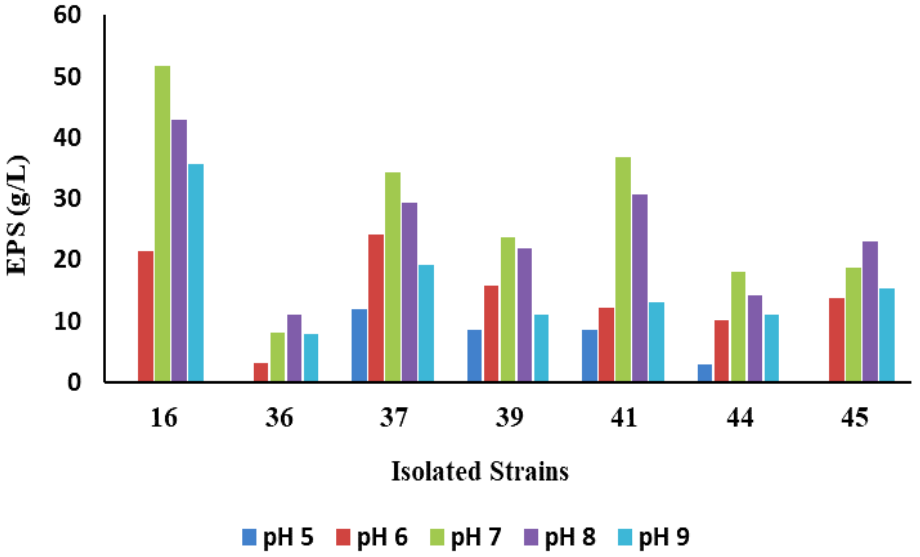

Fig. 6. Production of EPS from selected isolates grown on whey at different pH values

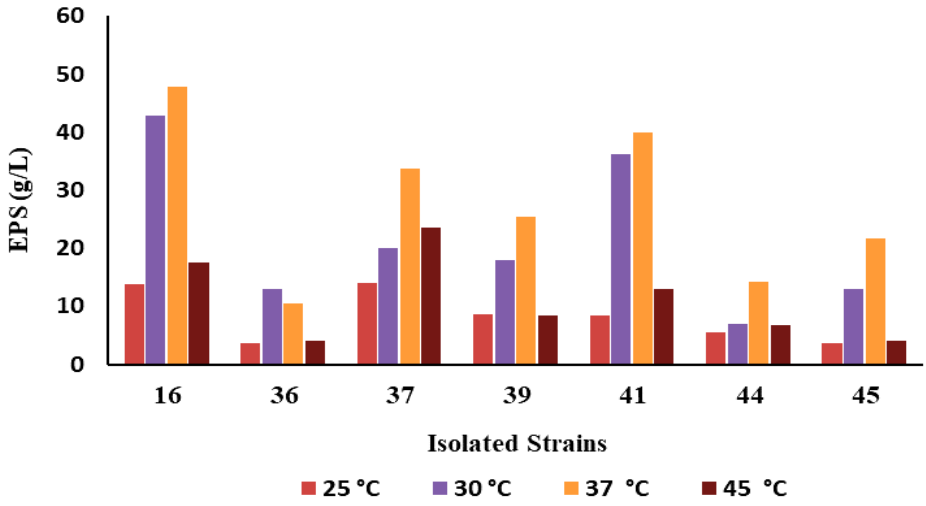

Fig. 7. Production of EPS from selected isolates grown on whey at different incubation temperatures

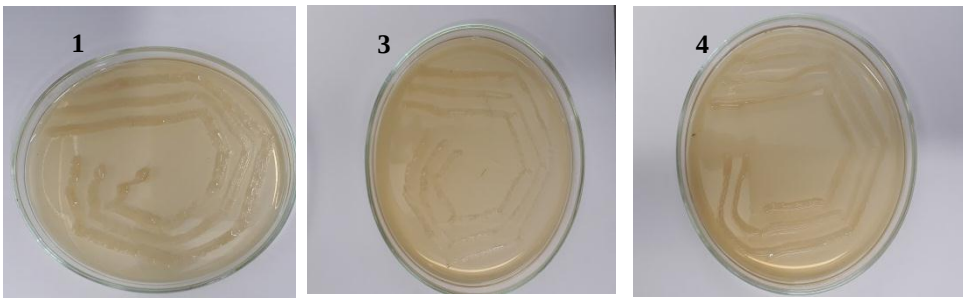

Fig. 8. Mucous colonies of isolated strains on whey medium

The effect of NaCl concentrations, pH and incubation temperatures on the production of EPS from selected isolates grown on milk permeate was illustrated in **Figs. (9)**, **(10)** and **(11)**. The obtained results show that the strains No. 16, 37 and 41 were also the best strains in the EPS production when grown on milk permeate. Therefore, these three strains were selected to produce EPS from whey and milk permeate.

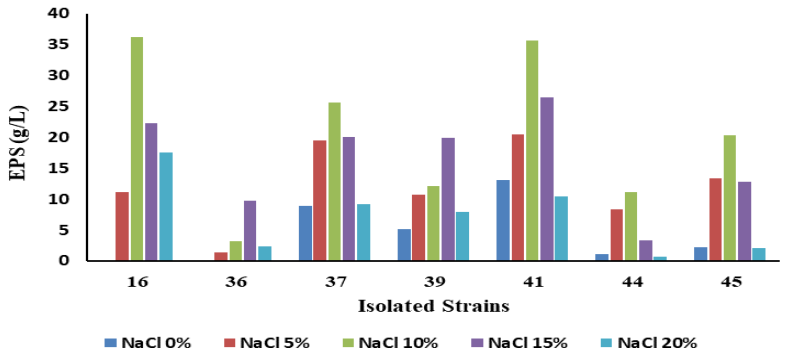

Fig. 9. Production of EPS from selected isolates grown on milk permeate at different concentrations of NaCl.

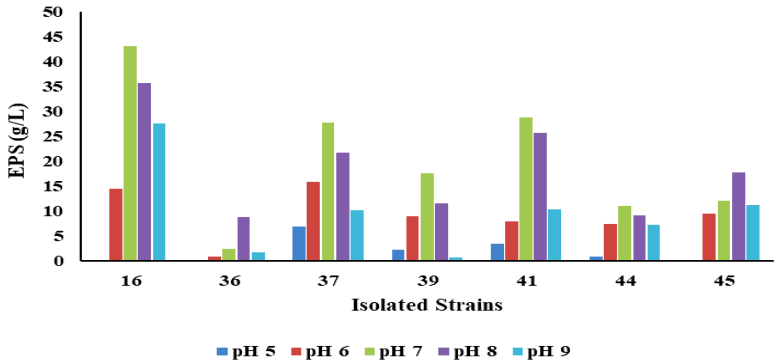

Fig. 10. Production of EPS from selected isolates grown on milk permeate at different pH values

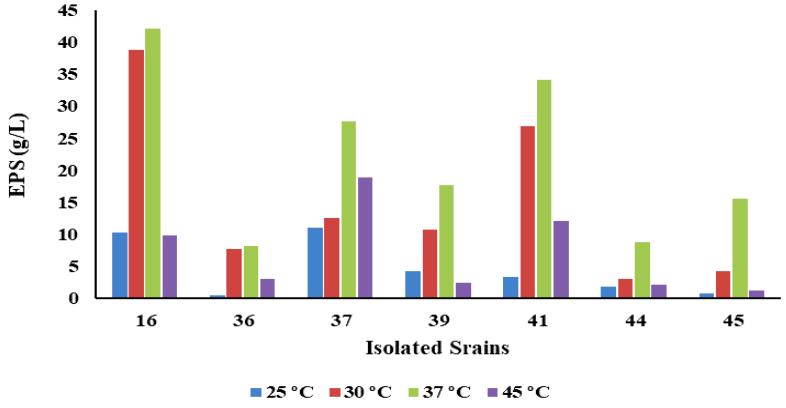

Fig. 11. Production of EPS from selected isolates grown on milk permeate at different incubation temperatures.

4- Identification of selected isolates (No. 16, 37 and 41)

Table (2) shows the taxonomical characteristics of the strains 16, 37 and 41. While **Fig's (12, 13 and 14)** represent the molecular identification of the isolated strains 16, 37 and 41 respectively, based on the partial sequence of 16S rRNA gene.

Table 2. Taxonomical characteristics of the strains No. 16, 37 and 41

Property	Characterization		
	16	37	41
Gram stain	G^+	G^+	G^+
Shape	Rod	Rod	Rod
Sporulation	+	+	+
Motility	-	+	+
Nitrate reduction	+	+	+
VP (Voges-Proskauer) test	+	+	+
Citrate utilization	+	+	+
Acid from D-glucose	+	+	+
Acid from D-xylose	+	+	+
Acid from mannitol	+	+	+
Urease production	-	+	+
Indole formation	+	+	+
Catalase test	+	+	+
Oxidase test	+	+	+
Lysine decarboxylase	-	+	+
Ornithine decarboxylase	-	-	-
H_2S Production	-	-	-
ONPG (hydrolysis of β-nitrophenyl-β-d-galactopyranoside)	+	+	+
TDA (production of indolpyruvate)	+	+	+

+: positive −: negative

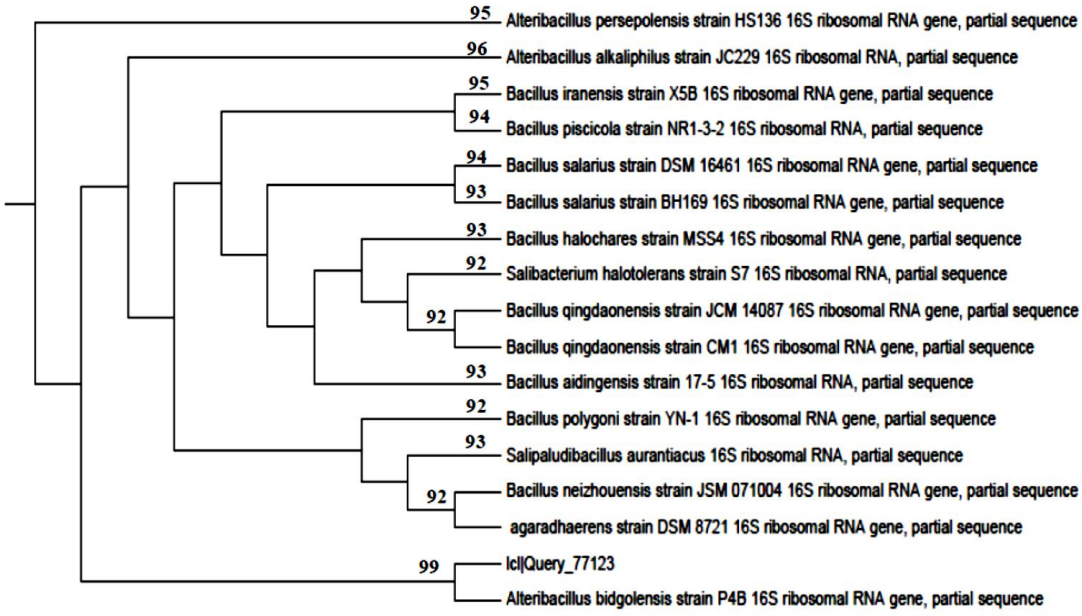

Fig. 12. Phylogenetic tree based on 16S rRNA sequences showing the position of strain 16 among its closely related organisms

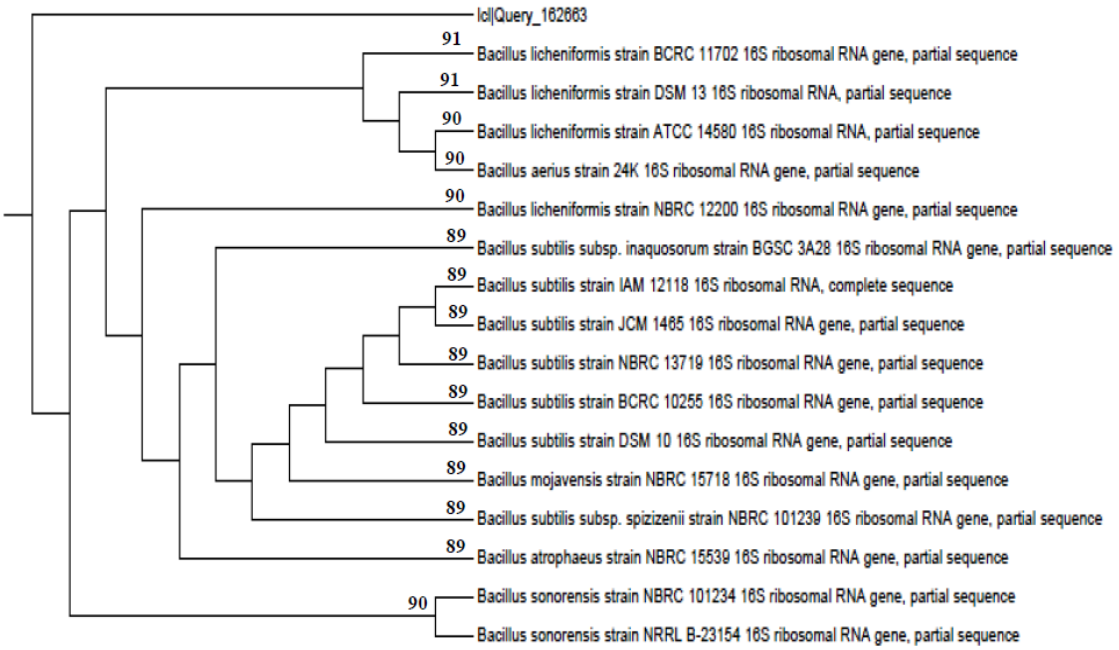

Fig. 13. Phylogenetic tree based on 16S rRNA sequences showing the position of strain 37 among its closely related organisms

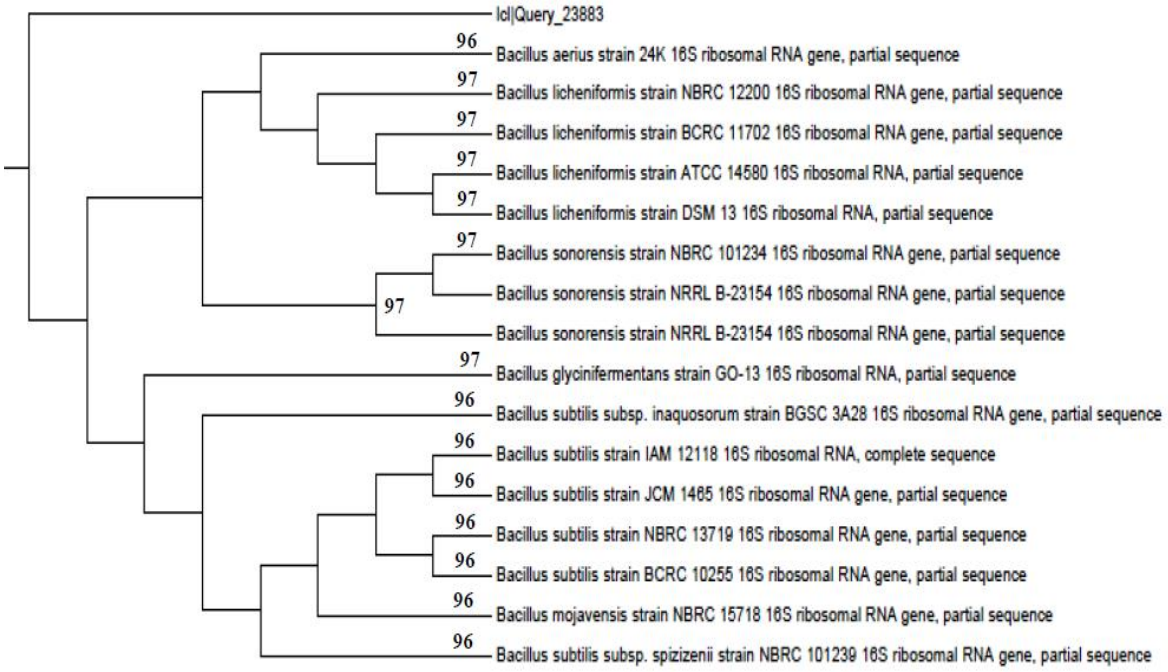

Fig. 14. Phylogenetic tree based on 16S rRNA sequences showing the position of strain 41 among its closely related organism

In conclusion, the isolated strains are identified as follow:

- **16**: *Alteribacillus bidgolensis* strain P4B 16S ribosomal RNA gene, partial sequence
- **37**: *Bacillus licheniformis* strain DSM 13 16S ribosomal RNA, partial sequence
- **41**: *Bacillus licheniformis* strain DSM 13 16S ribosomal RNA, partial sequence

These results are in a contrast with those reported by **(Song et al 2011)** for *B. licheniforms* which produced large amounts of EPS.

The genus *Alteribacillus* belongs to the family *Bacillaceae* within the phylum firmicutes. The genus includes two species with validly published names at the time of writing. The genus *Alteribacillus* was first proposed by **Didari et al (2012)** with *Alteribacillus bidgolensis* as the type species. The other species of the genus, *Alteribacillus persepolensis* **(Didari et al 2012)**, was previously classified as *Bacillus persepolensis*. The common characteristics of members of the genus *Alteribacillus* are: Gram-stain-positive, aerobic, rod shaped, endospore-forming, oxidase- and catalase-positive, chemo-organotrophic, moderately halophilic. Members of the genus *Alteribacillus* were isolated from sediment/water samples of hypersaline habitats **(Didari et al 2012; Amoozegar et al 2009).**

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[123]

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الموجـــــــز

الهدف من البحث دراسة مدي إمكانية إستخدام شرش اللبن المملح وراشح اللبن بغرض إنتاج سكريدات عديدة من البكتريا المحبة للملوحة، حيث يتميز الشرش الناتج من صناعة الجبن بقيمة غذائية عالية ومحتوي عالي من المواد العضوية والأملاح ومع ذلك يتم التخلص منه دون إستفادة ويمثل عبئ علي البيئة، السكريدات العديدة عبارة عن بوليمرات متجانسة أو غير متجانسة من الوحدات العضوية تنتجها أنواع مختلفة من الكائنات الحية الدقيقة ولقد زاد الأهتمام بها نظرا لتطبيقاتها المتعددة في مجالات التصنيع الغذائي والدوائي، لذا فقد تم في هذا البحث عزل بعض سلالات البكتريا التي لها القدرة علي إنتاج السكريدات العديدة من الشرش وراشح اللبن حيث تم عزل 46 سلالة من سلالات البكتريا المحبة للملوحة من نوعين من العينات. النوع الأول هو عينات الألبان (شرش جبن براميلي، مش) ، أما النوع الثاني فكان عينات من المياه عالية الملوحة والرواسب من منطقة وادي النطرون (بحيرات نبع الحمراء وأم ريشة والبيضاء) في محافظة البحيرة، مصر. وجد أن أثني عشر سلالة لها القدرة علي إنتاج السكريدات العديدة منها سبعة سلالات لها القدرة علي إنتاج السكريدات العديدة من الشرش وراشح اللبن. ولقد تم تحديد الظروف المثلي لإنتاج السكريدات العديدة من نسب كلوريد الصوديوم وقيمة الرقم الهيدرجيني ودرجات حرارة التحضين المختلفة. حيت أشارت النتائج إلي أن الظروف المثلي للإنتاج كانت 10 % كلوريد صوديوم، الرقم الهيدروجيني 7 ودرجة الحرارة المثلي للتحضين كانت 37°م. وقد أظهرت 3 سلالات أعلي إنتاجية للسكريدات العديدة. تم تعريف هذه السلالات بإستخدام طريقتين للتعريف الطريقة الأولي بإستخدام نظام الإختبارات البيوكيميائية (Biolog System)، الطريقة الثانية كانت بإستخدام نظام 16S rRNA. وقد عرفت السلالات علي إنها *Alteribacillus bidgolensis* و *Bacillus licheniformis*. ولقد كانت سلالة *Alteribacillus bidgolensis* الأعلى في إنتاج السكريدات العديدة حيث وصل إنتاجها إلي 52 جم/ لتر من الشرش يليها سلالة *Bacillus licheniformis* بإنتاجية 42 جم/ لتر. بينما كانت سلالة *Alteribacillus bidgolensis* أيضا الأعلي إنتاجيه من راشح اللبن حيث وصل إنتاجها إلي 43 جم/ لتر يليها سلالة *Bacillus licheniformis* بإنتاجية 36 جم/ لتر. لذلك توصي الدراسة بإمكانية إنتاج تلك المركبات بإستخدام سلالة *Alteribacillus bidgolensis* وذلك لرفع القيمة الأقتصادية للمخلفات السائلة لصناعة الجبن وتقليل العبئ البيئي.

الكلمات الدالة: البكتريا المحبة للملوحة، السكريدات العديدة، الشرش، راشح اللبن، *Alteribacillus bidgolensis, Bacillus licheniformis*.

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