

FUNCTIONAL PROPERTIES OF EXOPOLYSACCHARIDES PRODUCED BY *Halomonas eurihalina* F₂₋₇ AND *Xanthomonas campestris* pv. *campestris* IN SALTED WHEY

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ABSTRACT

Effect of salted whey medium after optimization as culture medium on the chemical composition and Functional properties of exopolysaccharide produced by *Halomonas eurihalina* F₂₋₇ and *Xanthomonas campestris* pv. *campestris* was studied . All polymers (EPSs) extracted from optimized reconstituted salted whey medium (ORSW) had lower contents of carbohydrates (29.34– 23.72%) but, were highly proteins (8.79–12.15%) than synthetic media-EPSs. Considerable quantities of uronic acid and sulfates were detected in *Halomonas*-EPSs (4.7 - 3.8% and 8.33–17.11%, respectively). All polymers produced viscous solutions with pseudoplastic behavior and emulsified sunflower oil. Aqueous solutions (1%, w/v) of *Xanthomonas*-EPS, especially, collected from synthetic medium(MM) were highly viscous (97.7cPs) at pH 7 and also were of the highest viscosity (122.4cPs), when heated at 70°C. The polymers extracted from ORSW medium, especially, *Halomonas*-EPS had the highest emulsifying activity at pH 3 and 7(80 and 71.25 – E₉₆ respectively) .The most interesting EPSs, was *Halomonas*-EPS which extracted from ORSW medium, where produced viscous solutions, their viscosity increased from 15.4 to 82.7cPs at pH 3 and also produced stable emulsion (80-E₂₄₀) till 240 hr at the same pH. These properties makes it potentially useful for various industrial applications.

Keywords: exopolysaccharides, Functional properties, *Halomonas eurihalina* F₂₋₇ , *Xanthomonas campestris* pv. *campestris*, salted .

INTRODUCTION

Currently , in the food and pharmaceutical industries , the stabilizers and emulsifiers from microbial sources such as microbial extracellular polysaccharide (EPS) have attracted attention because of their several advantages as compared with the synthetic products used in this respect. These advantages include non toxic, higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperature, pH, salinity and ability to be synthesized from renewable feed stocks (Lye *et al.*, 2006) . This polysaccharide is produced by numerous microorganisms such as *Xanthomonas*(Evans *et al.*, 1979) , *Pseudomonas* (Jarman,1979) , *Azotobacter* (Jarman *et al.*, 1978), *Sphingomonas* (Lobase *et al.*, 1992) *Alcaligenes* (Sutherland , 1990) , *Halomonas* (Quesada *et al.*, 1990), etc. *Xanthomonas*-EPS produced by *Xanthomonas campestris* pv. *campestris* has a variety of applications in the food industries as a stabilizing, viscosifying , emulsifying , suspending and thickening agent because of its viscosity, stable properties in extreme chemical and physical environments and pseudoplastic behavior (Kiosseoglou *et al.*,2003) . Also, the *Halomonas*-EPS produced by *Halomonas eurihalina* have interesting properties, such as, the ability to

emulsify hydrocarbons and increase the viscosity in acidic solution (Calvo *et al.*,1995 and Bejar *et al.*,1998) .This property would make it valuable for use in the food industry as an additive in yoghurt (Ali *et al.*, 2008),where the pH is usually acidic .

For the production of *Xanthomonas*- or *Halomonas*-EPS, the medium containing glucose or sucrose as a carbon source is normally used . Because of *X.campestris* and *H. eurihalina* had the low level of β -galactosidase . They cannot use lactose as an efficient carbon source. Consequently, these bacteria grow poorly and produces a little EPS in a medium containing lactose as the sole carbon source (Brandforde and Baird, 1983).

So, in previous report, Ali *et al.*,(2007) have studied the production of the EPS produced by *Xanthomonas campestris pv. campestris* and *Halomonas eurihalina* and established the fermentation conditions which, lead to optimum EPS production in optimized reconstituted salted whey medium .Because of the commercial use of these products, which depends on the rheological behavior of their solutions, it is necessary to know its physical properties as well as the influence of physical and chemical factors on this behavior (Kiosseoglou *et al.*,2003 , Calvo *et al.*,1998 and Martinez-Checa *et al.*,2002) . Therefore the present study aim to evaluate the functional properties of xanthomonas-EPS and Halomonas-EPS produced by *Xanthomonas campestris pv campestris* and *Halomonas eurihalina*, respectively in optimized reconstituted salted whey medium compared with EPSs of the same strains in their synthetic media .

MATERIALS AND METHODS

Dried EPS samples are produced by *Xanthomonas campestris pv campestris* and *Halomonas eurihalina* in optimized reconstituted salted whey medium (ORSW) and in the synthetic media (MM and MY medium, respectively) under the culture conditions described in our previous report (Ali *et al.*, 2007). Sunflower oil was obtained from Arma Food Ind., 10th of Ramadan city, Egypt.

Dry matter (DM) and total protein were determined as described in AOAC(1990). Total reduced sugars were determined by phenol sulphuric acid method according to Johan and Abd El-Twab(1957). Uronic acid was determined by colorimetric method according to Blumenkrantz and Asboe-Hansen(1973). Sulfate content was analyzed using advanced Microwave Digestion system (ETHOS 1 ; USA) then measurement by ICP Spectrometer (ICP 6000 Series ; USA) .

Determination of EPS solubility was based on the method of Briczinski and Roberts (2002) performed at pH 3.0 and 7.0. Briefly, a sample of EPS was suspended in a solution of 0.1 M NaCl and adjusted to the desired pH. The solution was transferred to a 50-ml volumetric flask, filled to volume with 0.1 M NaCl solution and then mixed by inverting. The carbohydrate content of this solution was determined in the same manner as above. A sample was then centrifuged for 30 min at 20,000 $\times g$. The

supernatant was analyzed for carbohydrate content. Solubility was expressed as the relative amount of carbohydrate in the supernatant compared with the original solution prior to centrifugation.

Dried EPS samples were dissolved in distilled water to measurement of viscosity by a Brookfield viscometer RTV fitted with a small sample adaptor (Brookfield Engineering Laboratories, MA). Determinations were made at 25°C and at a shear rate of 1 rpm, unless otherwise stated.

The influence of polysaccharide concentration, temperature and pH on the viscosity was studied.

Viscosity of polysaccharide aqueous solutions at concentrations of 0.1, 0.3, 0.5 and 1% (w/v) and at different shear rates (0.3, 0.6, 1, 1.5 and 6 rpm) was determined.

The effect of heat treatment on stability of viscosity of EPS solutions was studied compared with viscosity of non heated solutions. Heat treatment was carried out by heating different polysaccharide water solutions (1%, w/v) at temperatures 55, 75 and 90°C, for 20 min, then cooling until 25°C. Viscosity was determined at 1 rpm.

The influence of pHs 3 and 7 on the viscosity of EPS solutions (1%, w/v) was studied. The pH of the EPS solutions was adjusted by 1N HCL or NaOH and the viscosity of these solutions was determined at 1 rpm.

Emulsifying activity (EA) was measured using a modified method of Cooper and Goldenberg (1987). Dissolved EPS in 5 ml adjusted distilled water (0.5%, w/v) at pH 3 and 7 was mixed with 5 ml sunflower oil in test tube, which was vortexed vigorously for 2 min. and left to stand for 24 h at 4°C. EA was expressed as the percentage of the total height occupied by the emulsion. The formed emulsion was checked at different time intervals (24h, 96h, 240h) for stability.

All tests were carried out in triplicate. Averaged results are presented.

Statistical analysis for the obtained data was carried out using 2 × 3 factorial design. Duncan's test was used to make the multiple comparisons (Steel and Torri, 1980). Significant differences were determined at $P < 0.05$.

RESULTS AND DISCUSSION

The gross chemical composition of the EPSs is shown in Table 1. All polymers isolated from optimized reconstituted salted whey medium (ORSW) had lower contents of carbohydrates than the polymers produced in synthetic media (MM and MY medium). However, they had larger amounts of proteins, which might be due to the existence of some amino acids linked to the polymers (Bejar *et al.*, 1998). Uronic acid were also present in all the produced EPS. Halomonas-EPSs had the highest contents of uronic acid in both of these collected from MY and ORSW media [(4.7 and 3.8 %, respectively) Bejar *et al.*, 1998]. The large amount of uronic acid present in Halomonas-EPSs could be useful in biodegradation and water treatment as it happens with other microbial EPS (Geddie and Sutherland, 1993). Bacterial exopolysaccharides of industrial interest do not contain large amounts of uronic acid, with the exception of gelatin and the bacterial alginates (Guezennec *et al.*, 1994).

As for sulfates determination, Halomonas-EPSs contained considerable quantities of sulfates, especially that collected from ORSW medium. As reported in literatures, sulfates polysaccharides provide interesting applications for the pharmaceutical industry as antiviral (Okutani, 1992), antitumoral (Inoue *et al.*, 1988) and anticoagulant (Nishino *et al.*, 1989).

Table (1):Chemical composition of the EPSs synthesized by *Halomonas eurihalina* and *Xanthomonas campestris* pv *Campestris*.

| EPS composition | <i>Hal. eurihalina</i> | | <i>Xan. campestris</i> | |
|-----------------|------------------------|-------------|------------------------|-------------|
| | MY medium | ORSW medium | MM medium | ORSW medium |
| Dry matter | 91.88 | 92.11 | 90.36 | 90.49 |
| Proteins | 4.31 | 12.15 | 1.68 | 8.79 |
| Carbohydrates | 31.19 | 23.72 | 33.89 | 29.34 |
| Uronic acid | 4.70 | 3.80 | 0.11 | 0.13 |
| Sulfates | 8.33 | 17.11 | 0.20 | 0.84 |

Data are expressed as percentages of total dry weight of EPS. Each value is a mean of three determinations.

EPS solubility is often considered a prerequisite for functionality and therefore, it was determined. Solubility for the EPS of each sample was determined at pH 3 and pH 7 and summarized in Table (2). All polymers produced in synthetic media (MM and MY medium) or in ORSW medium were soluble at both pH levels, but, with different ratios (71 – 88%). The Halomonas-EPS whether collected from MY medium or collected from ORSW medium had the highest solubility at pH 3, compared with Xanthomonas-EPSs. Contrary, solubility of Xanthomonas-EPS collected from MM medium was the highest at pH 7, followed by Halomonas-EPS-MY , Xanthomonas-EPS-ORSW, then came Halomonas-EPS- ORSW. These differences in EPS solubility as a function of pH are due to nature of the EPS fraction in these powders (Briczinski and Roberts, 2002).

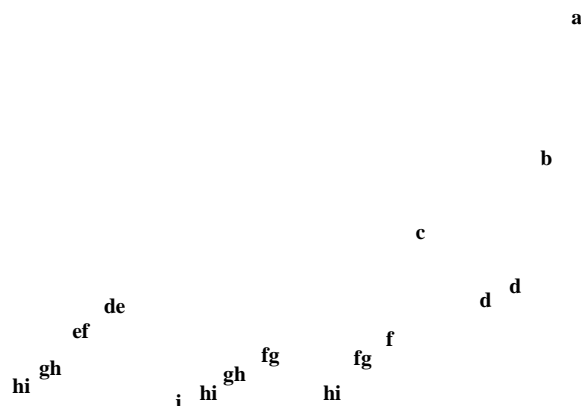
Table(2):Solubility (%)^a of Halomonas-and Xanthomonas-EPS at deferent pH values.

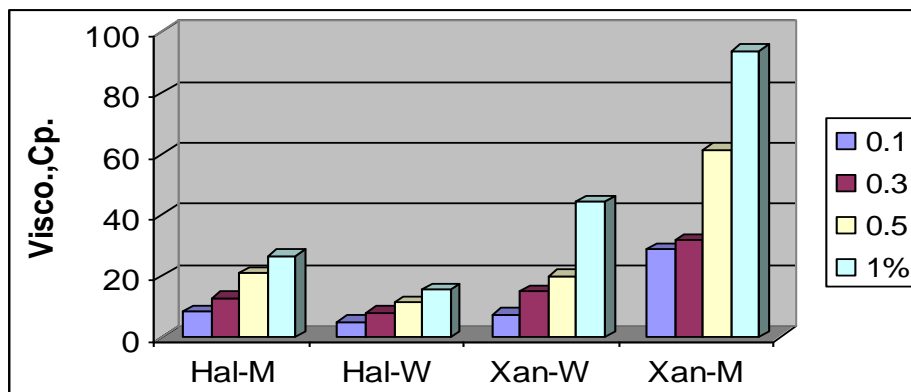
| pHs | Hal.-EPS | | Xan.-EPS | | LSD |
|-----|----------|-----------|----------|-----------|-------|
| | MY med. | ORSW med. | MM med. | ORSW med. | |
| 3 | 87.9 | 87.3 | 74.3 | 71.2 | 0.568 |
| 7 | 75.9 | 64.8 | 81.7 | 66.4 | 0.420 |

^a EPS solubility was determined by centrifuging a 1% EPS solution. EPS solubility is the fraction of original EPS quantified in the supernatant. LSD at 0.05%.

To study the effect of culture medium on the rheological behavior of EPS solutions, EPS was dissolved in distilled water at different concentrations and stored overnight at 4°C .The viscosity of these solutions was measured and the relationship between the polysaccharide concentration and the viscosity was shown in Fig (1). The viscosity of EPS solutions increased with the increase of the concentration.

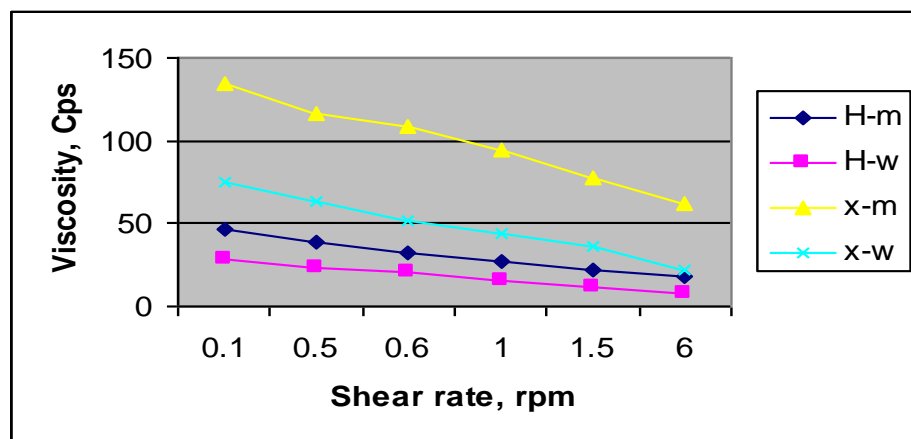
At the same time, solutions of all polymers exhibited an interesting pseudoplastic behavior, as the solution's viscosity was influenced by shear rate (Fig 2).





Fig(1): Viscosity for various concentrations(w/v) of EPSs synthesized by *Halomonas eurihalina* and *Xanthomonas campestris pv campestris*. Measurements were made at 25°C

LSD at 0.05% for Con. x EPSs = 6.31 .



Fig(2): Viscosity vs shear rate for 1% (w/v) concentration of EPSs synthesized by *Halomonas eurihalina* and *Xanthomonas campestris pv campestris*. Measurements were made at 25°C.

The culture media do not only affect the conformation, molecular mass, degree of decoration and monosaccharide ratios of EPS, but they do affect EPS content from sugar and other components , thus finally its functional properties(Orgambide *et al.*, 1991 and Sutherland 1994) . Therefore, the data in Table (3) show that the solutions of Xanthomonas-EPS had higher viscosity values than those of Halomonas-EPS, whether collected from synthetic medium or collected from ORSW .This is due to high carbohydrate content of Xanthomonas-EPSs (Table 1), which possibly explains why Xanthomonas-EPSs produced a more viscous solutions than other samples (Arias *et al.*, 2003). Moreover Halomonas-EPSs contain high

contents sulfates, especially these synthesized on ORSW medium, where whey is a source of amino acids sulfates, in addition *H. eurihalina* is a halophilic bacteria and produces of EPSs with high sulfate contents (Calvo et al. 1998 and Bejar et al. 1998). The results demonstrated that the increase of sulfate groups lead to a loss of viscosity. Also, EPSs extracted from synthetic media were of higher viscosity values than those extracted from ORSW, as shown in Fig (1) and Table (3). This due to the high salt content of ORSW-EPSs and its detrimental effect since the presence of cations lead to alteration in the conformation of the EPS molecules (Sutherland 1988). Nevertheless, Hal-W exhibited more resistance to the detrimental effect of salt than Xan-W (Table 3).

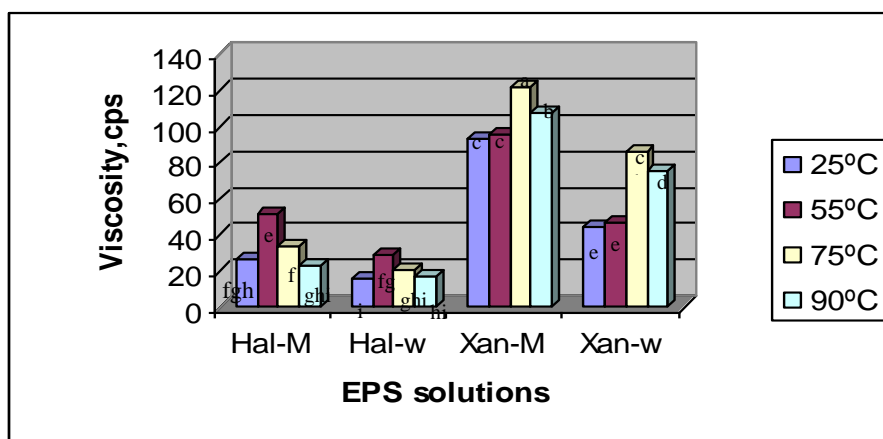
Table(3): Viscosity(cPs)^a of *Halomonas eurihalina* and *Xanthomonas campestris pv campestris* EPS solutions (1%,w/v) at deferent shear rats.

| shear rats (rpm) | Halomonas-EPS | | Xanthomonas-EPS | | Mean |
|------------------|---------------|-------|-----------------|-------|-------|
| | MY | ORSW | MM | ORSW | |
| 0.1 | 46.6 | 27.9 | 134.0 | 75.0 | 70.88 |
| 0.5 | 39.1 | 23.4 | 116.6 | 63.7 | 60.70 |
| 0.6 | 31.8 | 20.7 | 108.2 | 51.3 | 53.00 |
| 1.0 | 26.7 | 15.4 | 93.8 | 44.4 | 45.08 |
| 1.5 | 22.2 | 11.3 | 77.3 | 36.6 | 36.85 |
| 6.0 | 17.7 | 7.2 | 62.1 | 21.4 | 27.10 |
| Mean | 30.68 | 17.65 | 98.67 | 48.73 | |

LSD value at 0.05% for Sh.r= 8.05 & EPSs= 6.57 & Sh.r x EPSs= 16.09 ^a Measurements were made at 25°C .

One of the most striking properties of EPS is its stability under different stress conditions such as high temperature and acidic pH. From the obtained results in Fig (3), it could be observed gradual increase of viscosity of Halomonas-EPS solutions heated till 55°C, then decrease at high temperatures (over 55°C). While Xanthomonas-EPS solutions heated from 55 to 70°C had higher viscosity values than those reached by nonheat-treated Xanthomonas-EPS solutions (25°C). This might be attributed to thermal denaturation involving the dissociation of the double helix into two single strands (Arias et al., 2003). This phenomenon has been described in several polymers, particularly, in xanthan (Sutherland 1994; Capron et al., 1998 and Villain-Simmont et al., 2000).

Concerning the effect of pH on the solutions viscosity, Table (4) shows that Halomonas-EPS whether collected from MY medium or from ORSW medium have interesting property, which was their capacity to gelify at acidic pH, where viscosity of their solutions reached maximum values (117.4 and 82.7 cP, respectively) at pH 3, while was 25.9 and 14.4 cP respectively at pH 7. In this respect Calvo et al., (1995) reported that solution of exo-polysaccharide produced by strain F2-7 of *Halomonas eurihalina* extracted from MY medium its viscosity increased up to 800 cP at pH 3. On the contrary, the Xanthomonas-EPS, whether collected from MM medium or from ORSW exhibited remarkable decrease in viscosity of their solutions, as it decreased from 97.7 cP at pH 7 to 35.3 cP at pH 3 in Xan-M solutions and from 46.1 cP at pH 7 to 11.8 cP at pH 3 in Xan-W solutions.



Fig(3): Effect of heat treatment on viscosity of EPS *Halomonas eurihalina* and *Xanthomonas campestris pv campestris* EPS solutions 1%(w/v) . Measurements were made at 25°C. LSD at 0.05% for Heat x EPSs = 10.37

Table(4):Effect of pH on viscosity(cps)^a of Halomonas- and Xanthomona- EPS solutions (1%,w/v) .

| pHs | Halomonas-EPS | | Xanthomonas-EPS | | LSD |
|-----|---------------|-----------|-----------------|-----------|------|
| | MY med. | ORSW med. | MM med. | ORSW med. | |
| 3 | 117.4 | 82.7 | 35.3 | 11.8 | 8.32 |
| 7 | 25.9 | 14.4 | 97.7 | 46.1 | 7.06 |

LSD at 0.05% . ^a Measurements were made at 25°C and at shear rate of 1 rpm .

The emulsifying activity at pH 3 and 7 of the EPSs is shown in Table (5).All polymers isolated from ORSW medium had higher emulsifying activity than polymers produced in synthetic media. They showed high emulsification indices, especially Hal-W, as it was 80 and 71.25 till 240 h at pH 3 and 7, respectively. The ability of Halomonas-EPS to form stable emulsion may be attributed to the nature and concentration of its protein (Table 1).

Table (5): Emulsifying activity of Halomonas- and Xanthomonas-EPSs at different pHs.

| Emulsifying act.(%) ^a | E24 | | E96 | | E240 | |
|----------------------------------|-------|-------|-------|-------|-------|-------|
| | 3 | 7 | 3 | 7 | 3 | 7 |
| Halomonas-EPS | | | | | | |
| MY medium | 62.16 | 50.82 | 61.21 | 45.61 | 57.71 | 44.11 |
| ORSW medium | 95.00 | 75.00 | 80.00 | 71.25 | 80.00 | 71.25 |
| Xanthomonas-EPS | | | | | | |
| MM medium | 36.15 | 49.90 | 24.23 | 44.00 | 24.22 | 44.00 |
| ORSW medium | 68.60 | 65.75 | 62.35 | 64.80 | 62.00 | 64.50 |
| LSD at 0.05% | 5.35 | 2.92 | 11.59 | 4.24 | 5.09 | 5.61 |

^a Emulsifying activity was expressed as a percentage of the total height occupied by the emulsion after 24, 96 and 240 h.

These results are in agreement with Mata *et al.*,(2006) , who suggested that the protein content plays a crucial role in the emulsifying activity of EPS synthesized by *Halomonas ventosa* strain A112, since removing of protein from EPS lead to loss of its emulsifying activity.

In conclusion, our results suggest that the exopolysaccharide synthesized by *Halomonas eurihalina* and *Xanthomonas campestris pv campestris* growing on ORSW medium in this study, could be valuable for various industrial applications. Xanthomonas-EPSs produced highly viscous solutions at neutral pH and furthermore, its solution viscosities were more stable at high heat treatment. While, Halomonas-EPSs showed interesting emulsifying activity and had the highest solubility and viscosity at acid pH.

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الخواص الوظيفية للسكريات العديدة المنتجة بواسطة ميكروبي *Halomonas eurihalina* و *Xanthomonas campestris pv campestris* في الشرش المملح

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تم دراسة تأثير الشرش المملح كبيئة أستزراع ليكتريا

Xanthomonas campestris pv campestris و *Halomonas eurihalina*
على الخواص الوظيفية للسكريات العديدة المنتجة بواسطة هذه البكتريا مقارنة بالبيئات التركيبية
المتخصصة لكل سلالة

وقد دلت النتائج على ما يلي :

- إنخفاض محتوى السكريات العديدة المستخلصة من بيئة الشرش المملح من الكربوهيدرات بينما كان محتواها عاليا من البروتينات وذلك على عكس السكريات المستخلصة من البيئات التركيبية .
- وجود كميات كبيرة من الكبريتات بالسكريات العديدة المنتجة بواسطة *Halomonas eurihalina* وخاصة المستخلصة من بيئة الشرش المملح .
- تراوحت نسب ذوبان جميع السكريات العديدة المستخلصة سواء من بيئة الشرش المملح أو من البيئات التركيبية ما بين ٧١ إلى ٨٨ ٪ وكانت أعلاهم ذوباناً عند pH ٣ السكريات العديدة المنتجة بواسطة *Halomonas eurihalina* .
- أظهرت محاليل جميع السكريات العديدة المستخلصة سواء من بيئة الشرش المملح أو من البيئات التركيبية لزوجة عالية نسبياً وذات سلوك pseudoplastic وخاصة السكريات المنتجة بواسطة *Xanthomonas campestris pv campestris* والمستخلصة من بيئتها التركيبية عند pH ٧ ، كما زادت لزوجة محاليل *Xanthomonas* بعد معاملتها حرارياً على 70°C . إلا أن لزوجة محاليل السكريات المنتجة بواسطة *Halomonas eurihalina* في بيئة الشرش المملح زادت عند pH 3 و أضعاف لزوجتها عند pH 7 .
- أظهرت السكريات العديدة المستخلصة من جميع البيئات المستخدمة إستحلاباً لزيت عباد الشمس إلا أن أعلاهم في النشاط الإستحلابي كانت السكريات المنتجة بواسطة *Halomonas eurihalina* في بيئة الشرش المملح وخاصة عند الإستحلاب على pH 3 وقد حقق هذا المستحلب ثباتاً عالياً وصل إلى ٢٤٠ ساعة .
- ومن ثم فإن الخواص الوظيفية للسكريات العديدة المنتجة بواسطة *Halomonas eurihalina* في بيئة الشرش المملح تمكنها من الأستخدام في صناعة المنتجات الغذائية ذات الوسط الحمضى .

