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Production of Polysaccharides (Xanthan gum) by Xanthomonas campestris pv. Sesame

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ABSTRACT

Many microorganisms and plants were found produce polysaccharides which are widely varying in their composition and structure. The extracellular polysaccharide produced by *Xanthomonas campestris* pv. *campestris*, is chemically analyzed and used industrially. However, only the xanthan gum which was ranking as the best among the ten polysaccharides was used commercially in large amounts. The xanthan gum has numerous applications in food and other industries. The present study was aiming to investigate the capacity of *X; campestris* pv. *sesami* for the production of xanthan gum, using culture media containing different carbon sources. The properties of the produced material were investigated (pH, viscosity, and chromatographic analysis using thin layer chromatography). The results showed that the weight of the product was increasing with time reaching about 9.8g at the tenth day compared to only 2.1g on the second day. The results of the effect of different carbon sources indicated that sucrose was the best giving 9.0 g in the 7th day compared to only 1.5g by raffinose. The pH was changing from 6.7 at the beginning of the experiment to 4.2 in the tenth day. However, the viscosity of the inoculated sucrose medium was found to increase from 2.4 unit at the second day to 9.7 units after ten days. Analysis on paper chromatography showed that the produced polysaccharide contains mannose, glucuronic acid, glucose, rhamnose but no ribose nor trehalose or fructose were detected. The results indicated that the investigated bacterium was producing large amounts of xanthan gum. Although, Xanthan gum was discovered in 1950s its substantial commercial production began in 1964 and in 1969, the USA, Food and Drug Administration authorized its use in food. Xanthan gum, now has numerous uses in food and other industries. Further studies should be carried on other isolates of the bacterium *X. campestris* pv. *sesami* and more chemical analysis are needed.

INTRODUCTION

`Bacterial leaf spot of sesame plants have been reported from areas of production in different parts of the world (Rao, 1962). *Xanthomonas campestris* pv. *sesami* was reported by Sabet and Dowson (1960) to be the causal agent of the disease in Sudan. The bacterium was described by the authors as a Gram-negative rod, capsulated and motile by a polar flagellum. Further investigations have showed that the bacterium gave negative reactions with the following tests; methyl red, Voge-Proskauer, nitrite destruction, phenylalanine deaminase, ammonia from tryptone, indole from peptone and urease production. On the other hand, the bacterium reacted positively with catalase production, levan production, and ammonia from arginine (Abdel-Rahim and Adam, 1987). There is abundant evidence that bacteria and fungi are capable of producing metabolites. Slimy polysaccharides are one part of these metabolites that are produced in larger quantityes by bacterial cells (Feder and Ark, 1951). The extracellular polysaccharide produced by *X. campestris* pv. *campestris*, is chemically analyzed and used industrially. It was found to contain, D-glucose, D-mannose, D-glucuronic acid, and small amounts of pyruvic and acetic acids (Sloneker et al, 1964). Many other pathogenic Xanthomonads were reported to produce heteropolysacchides similar to that formed by *X. campestris* pv. *campestris* except that they

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contain galatose in place of mannose (Glazer and Nikaldo, 2014).). Polysaccharides are used to modify the flow characteristics of fluids, to stabilize suspensions, to encapsulate materials and to produce emulsions. They are also used in enhanced oil recovery and as drag-reducing agents for ships ((Sutherland, 1999; Becker and Vorholter, 2009). However, only one microbial polysaccharide, the xanthan gum, ranks among the ten industrial polysaccharides used in large amounts. Xanthan gum was discovered by Allen Rosalind Jeans and her research team at the USA Department of Agriculture ((FDA, 2013)). Xanthan gum is produced commercially by submerged aerobic fermentation by X. campestris pv campestris, using glucose, sucrose or starch as a source of carbon (Tortora *et al*., 2010). Xanthan gum, is a polysaccharide secreted by the bacterium *Xanthomonas campestris* pathovars. It is used as a food additive, rheology modifier and or as a food thickening agent (in salad dressing), to prevent ingredients from sedimentation (Garcia-Ochoa *et al*., 2000). The Xanthan gum is used to give the dough or batter a stickiness that would otherwise be achieved with gluten (Kim and Dale, 2005). The shear thinning and particle-suspension properties of xanthan solutions are important when it is included in paints and as a component of drilling mud used in drilling oil wells (Kurdikar et al., 2001). The widespread use of horizontal drilling and the demand for good control of the drilled solids, has led to the expanded use of the Xanthan gum in the oil industry. Xanthan gum is also added now to concretes poured underwater to increase its viscosity and prevent its washout (Alexander and Hiroshl, 2007). The discovery that many bacteria synthesize large amounts of biodegradable polymers of high molecular weight, which can be used to manufacture plastics, has arose considerable interest. There are more than hundred varieties of synthetic plastics in use. The annual production of these materials in the USA alone exceeds 30 billion pounds. Plastics are also manufactured from petrochemicals, but these are less degradable (Tortora *et al*., 2010). The molecular weight and even the composition of these extracellular polysaccharides may vary depending on the culture conditions (Sutherland, 1999 and 2002). The present study was conducted in order to test the ability of the bacterium *Xanthomonas campestris* pv. sesami, the causal organism of the leaf spot of the sesame plants, to produce polysaccharides (Xanthan gum) and to study the chemical composition of the product.

MATERIALS AND METHODS

The bacterium *Xanthomonas campestris* pv. *sesami* was isolated from leaves of sesame plant showing leaf spot symptoms. The nutrient agar medium was used for the isolation. The bacterium was tested for its ability to produce a high molecular weight polysaccharide by being grown on the following medium..

(NH4) 2SO4	1.30 g
Mg SO4 .7H2O	0.1g
Yeast extract	1.0 g
Casein hydrolysate	2.5 g
Sucrose	20.0g
Distilled water	1 L

The medium was dispensed into 100 ml batches in 250 ml flasks and 0.5 g $CaCo_3$ was added to prevent growth inhibition then inoculated and incubated. After incubation the $CaCO_3$ was removed by filtering the culture through No .I whatsman filter paper in a Buchner funnel and the filtered liquid was then centrifuged at 15.000 xg for 15 min to remove bacterial cells .The

supernatant liquid was treated with three volumes of acetone and the resulting material was precipitated by centrifugation at 5.000 xg for 10 min .The pellet was dissolved in 50 ml of water and centrifuged at 15.000 xg for 10 min to remove undeserved particles .

Viscosity of polysaccharide solution and culture :-

Increase in viscosity of solutions of polysaccharide with increase in time of incubation were measured .Ten ml samples of the solutions of polysaccharide were placed in " cannon – fenske " size 200 viscometers in a water bathe at 30 ^{0}C . The time for 10 ml of the solutions to pass maried points on the viscometers was recorded. The corresponding value for 10 ml distilled water was 0% polysaccharide.

Chromatographic analysis of polysaccharide :-

To determine the monosaccharide of the extracellular polysaccharides, acid hydrolysis followed by paper chromatography was used. 20 ml of 0.5N H_2SO_4 were added to 100 mg of polysaccharide and hydrolyzed overnight at 100 ^{0}C. The mixture was cooled, diluted with distilled water and neutralized with excess barium chloride and was again hydrolyzed overnight at 100 ^{0}C .The mixture was then cooled and the Barium chloride was removed by centrifugation and the supernatant liquid was collected and reduced to dryness prior to chemical analysis. Separation and identification of sugar residues in the acid hydrolyzed fraction was done using paper chromatography. Aliquots (50 ml) of the hydrolysate were spotted on .Whatsman NO 3 chromatography paper (25 x 60 cm). Hydrolysate spots were eluted alongside authentic monosaccharide markers with butanol : acetic acid : water (4 : 1 : 5) for 30 h. After that, the spots were visible on the developed chromatograms as green fluorescent areas under a U.V. light source at 366 nm

RESULTS

The effect of the incubation on the polysaccharide production:

The effect of the incubation time on the production of the polysaccharides was investigated by growing the bacterium in the salt medium supplemented with sucrose as a substrate. The medium was distributed in 100 ml batches in conical flasks (250 ml) and after inoculation the flasks were incubated at room temperature (25 ^{o}C), on a rotary shaker for 10 days. Samples were taken every two days and the polysaccharides were precipitated by acetone as mentioned previously. The precipitated polysaccharide was weighed then dried and reweighed. The results (Table, 1) showed that the amount was increasing with the incubation time reaching 9.6 g dry weight at the 10th day. However, an almost similar amount was produced on the 8th day, although, an appreciable amount was produced on the 4th and the 6th days (Table, 1).

The pH change in the culture medium:

The pH of the inoculated culture medium and incubated at 25 0C, was measured every two 2 days for ten days using a pH meter. The results on Table (2) were showing that the acidity of the culture medium was increasing with the incubation time. The pH value was 6.7 at the zero time, it was 5.5 at the second day while, it was 4.2 at the 10th day.

The effect of the substrate on the polysaccharide production:

The effect of different sugars as carbon sources on the production of the polysaccharide was also investigated in the present study, using the same salt medium, supplemented with

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different sugar (sucrose, glucose, mannose. trehalose, ribose, cellulose, maltose and raffinose). After inoculation and incubation for 7 days the polysaccharide was precipitated with acetone as above, dried and weighed. The results (Table, 3) showed that sucrose was the best sugar as a carbon source, for the production of the polysaccharides, giving 9.0 g, however; large amounts were also produced with glucose, mannose, maltose, cellulose and trehalose. In contrast, ribose and raffinose gave poor amounts of the polysaccharides (Table, 4).

Viscosity determination:

After inoculation and incubation at 25 ^{0}C and at intervals of 2 days for ten days, 10 ml samples were drawn from the culture and their viscosity was determined using a Cannon – Fenske viscometer. The viscosity was expressed as a viscosity relative to that of water. The results (Table, 4) showed that the viscosity of the cultures was increasing continuously with the incubation time. At the 10th day the culture was very viscous, it was 9.8 relative viscosity units (RVU).. It was almost ten times that of the first day and compared to only 2.4 units at the second day.

Table (1). Effects of different incubation times on the production of polysaccharides by X. campestris pv. sesami.

Time (days)	Fresh weight (g)
0	0.0
2	2.1
4	5.0
6	6.8
8	9.2
10	9.8

Table (2). The pH changes in the culture medium during The incubation time.

Incubation time (days)	pH value
6.7	0
5.5	2
5.2	4
5.0	6
4.6	8
4.2	10

Table (3.). Effects of different sugars on the production of the polysaccharides by *X. campestris* pv. *sesami*.

Sugars	Fresh weight (g)
Sucrose	9.0
Glucose	7.2
Trehalose	6.2

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Mannose	**6.0**
Cellobiose	**5.5**
Maltose	**5.0**
Galactose	**3.0**
Ribose	**2.0**
Raffinose	**1.5**

Monosaccharide composition of the polysaccharide

To determine the monosaccharide of the extracellular polysaccharides, acid hydrolysis followed by paper chromatography was used. The results showed that the polysaccharide produced by the bacterium *X. campestris* pv. *sesami* was separated into into four spots, corresponding with, glucose, mannose, glucuronic acid and rhamnose. However, few traces close to the origin no other spots were detected. However, no ribose trehalose or fructose was detected (Table, 5).

Table (4). Viscosity determination of the polysaccharides produced by *X. campestris* pv. *sesami* with time.

Incubation time (days)	Viscosity (RVU) *
1.0	**0**
2.4	**2**
4.6	**4**
6.9	**6**
8.8	**8**
9.7	**10**

*** RVU = Relative Viscosity Units**

Table (5) Monosaccharide composition of the polysaccharide produced by *X. campestris* pv. *sesami*.

Presence	Sugars	Presence	Sugars
+	**Glucose**		
Mannose		+	**Glururonic acid**
+			
+	**Rhamnose**		
-	**Ribose**		
		-	**Trehalose**
		-	**Fructose**

+ = present **- = absent**

DISCISSIONS

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Slimy polysaccharides are one part of the metabolites that are produced in larger quantities by bacterial cells, around which they form a slime layer varying in thickness. The organic polymers are commonly referred to as capsules or slime layer. Many Xanthomonads were found to produce polysaccharides, but only that of *X. campestris* pv. *phaseoli* has been correlated with pathogenesis (Dumitriu, 2005). Polysaccharides or xanthan gums are used to modify the flow characteristics of fluids, to stabilize suspensions, to encapsulate materials and to produce emulsions. They are also used in enhanced oil recovery and as drag-reducing agents for ships ((Sutherland, 1999). However, only one microbial polysaccharide, the xanthan gum, ranks among the ten industrial polysaccharides used in large amounts (Cuisine, 2014). Xanthan gum, is used as a food additive, rheology modifier and or as a food thickening agent (in salad dressing), to prevent ingredients from sedimentation (Garcia-Ochoa *et al*., 2000). The shear thinning and particle-suspension properties of xanthan solutions are important when it is included in pants and as a component of drilling mud used in drilling oil wells (Tortora *et al*., 2010).

In the present study the bacterium *X. campestris* pv. *sesami* was found to produce large amounts of polysaccharides or xanthan gum. However, the amount was found to increase with the incubation time. Similar results were also reported by other authors working with different bacterium. The effect of different sugars on the production of the polysaccharides was investigated in this study. The results showed that sucrose was the best sugar as carbon source. Large amounts were also produced with glucose, mannose, maltose, cellulose and trehalose. On the other hand,, ribose and raffinose gave poor amounts of the polysaccharides. According to Glazer and Nikaldo (2014), glucose and sucrose gave large amounts and can be used for the production. However, they added that the yield from glucose in X. campestris pv. campestris, is influenced by the growth limiting nutrient, with the highest yield under the conditions of nitrogen limitation (Glazer and Nikaldo (2014). The pH changes in incubated culture medium were measured with time, the results showed that the acidity of the medium was increased. The pH values were 6.7 and 4.2 at the zero time and the tenth day, respectively. Similar results were also reported by Abdel-Rahim (1980). Increase in the viscosity of the incubated medium of the polysaccharides was found to increase with increasing time. Researchers who were studying the production of the polysaccharides using different bacteria were reaching to the same conclusions (Internet, 2016). To determine the monosaccharide of the extracellular polysaccharides, acid hydrolysis followed by paper chromatography was used. The results revealed that the polysaccharides produced by the bacterium *X. campestris* pv. *sesami* was separated into four spots, corresponding with, glucose, mannose, glucuronic acid and rhamnose. Except for few traces close to the origin no other spots were detected. However, no ribose or fructose was detected. The polysaccharides produced by the bacterium *X. . campestris* pv. *campestris* were chemically analyzed by Sloneker *et al*. (1964) and were found to contain; glucose, mannose, glucuronic acid and small amounts of pyruvic and acetic acids. Other pathogenic Xanthomonads were also found to produce similar polysaccharides except that their polysaccharides were contained galatose in place of the mannose. These bacteria include; *X. campestris* pv. *malvacearum*, X. campestris pv. vesicatoria, *X. campestris* pv. *carotae* and *X. campestris* pv. *translucens* (Sutton and (Sutton and Williams, 1971; Camesano and Wilkinson, 2001, Becker and Vorholter, 2009 and Cuisine, 2014).

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إنتاج عديدات التسكر (صمغ الذانثان) بواسطة البكتيريا *Xanthomonas campestris* pv. *sesami*

ملخص الدراسة

وجد أن العديد من الكائنات الحية الدقيقة والنباتات تنتج عديدات التسكر التي لها محتوى وتراكيب مختلفة. هذا ويعتبر صمغ الذانثان الأفضل من بين عشرة من عديدات التسكر التي تستخدم تجارياً وبكميات كبيرة ولصمغ الذانثان استخدامات كثيرة في مجال الأغذية وصنعات أخرى.. هدفت هذه الدراسة لاختبار قدرة البكتيريا *Xanthomonas campestris* pv. *sesami* على إنتاج عديدات التسكر وذلك باستخدام أوساط غذائية تحتوي على مصادر مختلفة للكربون وفترات تحضين مختلفة. كما تمت دراسة خصائص المادة المنتجة وذلك بقياس درجة الأس ودرجة، اللزوجة ودراسة محتواها الكيميائي بكروماتوجرافيا الطبقة الرقيقة. أوضحت النتائج أن وزن المنتج قد زاد مع زيادة فترة التخزين حيث وصل الوزن إلى 9.8 ملجم في اليوم العاشر مقارنة مع 2.1 ملجم لليوم الثاني. أشارت نتائج استخدام مصادر مختلفة للكربون إلى أن سكر السكروز هو الأفضل حيث أعطى 9.0 ملجم مقارنة بسكر الرافينوز الذي أعطى 1.5 ملجم فقط. أما درجة الأس الهيدروجيني فقد تراوحت بين 6.7 في بداية التجربة إلى 4.2 في اليوم العاشر. وهذا وقد زادت درجة لزوجة الوسط الغذائي المحتوي على سكر السكروز من 2.4 إلى في اليوم الثاني إلى 9.7 في اليوم العاشر. التحليل الكيميائي بواسطة بكروماتوجرافيا الطبقة الرقيقة أوضح أن عديد التسكر المنتج يحتوي على سكريات المانوز والجلكوز الرامنوز وحمض الجلوكويرونيك ولم توجد املاح الرايبوز ولا تريهلوز أو الفراكتوز. هذه الخصائص مثيلة لخصائص صمغ الذانثان المنتج من طرز مرضية أخرى. أفادت الدراسة أن البكتيري المختبرة تنتج كميات كبيرة من صمغ الذانثان. على الرغم من أن صمغ الذانثان قد تم اكتشافه في الخمسينات إلا أن استخدامه تجارياً قد بدأ في العام 1964م وفي عام 1969م تم اعتماده من قبل الوكالة الأمريكية للأغذية والأدوية لاستخدامه في الأغذية. هذا ولصمغ الذانثان في الوقت الراهن استخدامات عديدة في مجال الأغذية وفي صناعات أخرى. يجب إجراء مزيد من الدراسات على عزلات أخرى من البكتيريا *Xanthomonas campestris* pv. sesami كما ولا بد من إجراء المزيد من التحاليل الكيميائية.