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Production of Extracellular Polysaccharide by *Agrobacterium tumefaciens* MSI from Date Molasses

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ABSTRACT. The nutritional requirements of *Agrobacterium tumefaciens* in production of extracellular polysaccharides (EPS) were studied in a chemically defined medium containing 10% centrifuged date molasses as a sole carbon source. The maximum EPS yield was obtained at pH 7, in presence of 7 g/L KNO_3, with 5% of 12 hour old inocula and incubated statically in Kole flaks at 30°C. As in secondary metabolite production, EPS production started late in the exponential phase and the maximum yield was obtained at the stationary phase. Among the inducers tested, succinic acid proved to be the best for the yield of EPS. The rheological properties of polymer solutions were also studied. Viscosity was not affected by pH and temperature. The pure polysaccharide was not toxic and the polymer solution showed a good emulsifying activity.

A wide range of bacteria are known to produce copious quantities of extracellular polysaccharides EPS of commercial interest (Anton *et al.* 1988). Production of polysaccharides by bacteria, instead of plants, affords greater potential control over production conditions and greater consistency of the final products (Wachenheim and Patterson 1988). These water soluble polymers have many applications in food, pharmaceutical, cosmetics, paper and paint industries, amongst many others. Bacterial polysaccharides have been recently reviewed (Sutherland 1985, Graber *et al.* 1988, Whitfield 1988). With the increased demand for microbial polysaccharides, it is important to consider agricultural and industrial waste as the substrates for the production of these biopolymers. Only a few such studies have been done (Sutherland 1983, Schwartz and Bodie 1984, Sabry *et al.* 1992). Date molasses is a rich carbohydrate waste product of microbial gums, available in Saudi Arabia. The selection of this waste product is based on the fact that polysaccharide formation requires an excess supply of carbon and energy (Graber *et al.* 1988). The work reported here is a part of an investigation undertaken to asses the possible use of date molasses for EPS production. Factors affecting maximal polymer productivity by *Agrobacterium tumefaciens,* the rheological properties of the polymer and some of its possible uses are discussed.

Materials and Methods

Organism

The bacterium used throughout the present work was isolated from soil and identified as *Agrobacterium tumefaciens* MSI by staff of the National Collection of Industrial Bacteria at Torry Research Station, Scotland. It was maintained on agar slants of the following composition (g/L): glucose, 20; $NaNO_3$, 2; K_2HPO_4, 0.8; KH_2PO_4, 0.6; $MgSO_4$. $7H_2O$, 0.5. Microelements were provided (in p.p.m.): Fe, 0.1; Zn, 0.09; Mn, 0.01, and prepared from 974.8 mg $FeCl_2$; 57 mg H_3BO_3; 790 mg ZnSO, $7H_2O$ and 81 mg $MnSO_4$. $4H_2O$ and make up with distilled water to 1 liter; 0.5 ml of 2% (v/v) yeast extract and 20 g/L agar.

Cultures were stored at –10 °C in an incubator and a monthly sub-culture of inoculum to freshly prepared slants was carried out.

Molasses

The crude date molasses (DM) was kindly supplied from Saudi Arabia Dates company, El Kasseim – El Bbadaee Riyadh. Molasses for present study was prepared by dilution with a known amount of water and the muddy precipitate was removed by centrifugation.

Cultivation media and culture conditions

The growth medium used in this study has the following composition (g/L): molasses, 60; NH_4NO_3, 2.5; KH_2PO_4, 0.5; $MgSO_4$. $7H_2O$, 0.01; Peptone, 2; yeast lower script extract, 2; $FeCl_3$, 0.05, unless otherwise indicated. Seed cultures were prepared by transferring from a single slant of a 7 day old culture into 50 ml aliquots of the basal medium, finally dispensed in 250 ml Erlenmeyer flasks to initiate growth. A standard inoculum of 2% (w/v) was taken from the previous liquid culture, after growth for 48 h at 30 °C, continuous shaking was made to start growth unless otherwise indicated.

Measurement of relative viscosity

The viscosity of the culture fluid was measured by using a conventional Ostwaid Viscometer at 30 °C (Tanaka *et al.* 1974). Apparent relative viscosity (app) was determined as follows: $(\text{app}) = \frac{t_s}{t_o}$

where t_s is the falling time of sample at 30 °C.

Cell growth determination

Cells were removed by centrifugation at 10,000 r.p.m. for 20 min. The cells were thoroughly washed, centrifuged and dried at 60 °C untill constant weight was obtained.

Recovery, determination and purification of the extracellular polysaccharide (EPS)

Polysaccharide was precipitated from the viscous supernatant after removal of cells by addition of 5 volumes of cold ethanol (Anton *et al.* 1988) and left at 4 °C overnight. The mixture was then centrifuged, washed with alcohol, acetone, ether and finally dried and weighed. Purification of the polysaccharide was carried out according to the method of Amin and Awad (1968).

Toxicity test

The crude and pure polysaccharides obtained were tested for their degree of toxicity before being recommended as blood plasma expander or in food preparation by the determination of lethal dose 50 (LD_{50}) of the polysaccharide following the Carlos Sevick method (Sevick 1987).

Immunization and emulsification

Immunization was carried out according to the methods discribed by Adlam *et al.* (1984) and Ouchterlony (1953) while emulsification activity was measured according to the method summerized by Robert *et al.* (1989).

Analysis of polysaccharide

The ash content of the pure polysaccharide was estimated by the sulfated ash method (Browne and Zerban 1948). The total nitrogen content was determined using the micro-Kjeldahl technique. Yield coefficient (Y p/c) is calculated as gram extracellular polysaccharide/g carbon.

Results

The centrifuged date molasses (DM) is rich in sugars and many of the minerals needed for growth and activity of a bacterium, yet the addition of complementary nutritive constituents may be quite necessary. Therefore, the centrifuged DM solution was separately supplemented with different media to select a basal medium most favourable for growth and polysaccharide formation. Medium I supported the highest growth and polysaccharide yield (Table 1). Growth and polymer yields were estimated during different phases of growth under both static (in Kole flasks) and shaken cultures (in 250 ml Erlenmeyer flasks), (Figs. 1a and 1b). Under both conditions, the time course of extracellular polysaccharide formation paralleled growth. The polymer was formed during the exponential phase.

Table 1. *Agrobacterium tumefaciens* growth, polysaccharide production and yield coefficient as influenced with the composition of the fermentation media

Medium + (g/l)	Dry weight (D.Wt.) mg/100 ml	Extracellular polysaccharide (EPS) mg/100 ml	Yield Coefficient (Y p/c)
I (basal Medium)	408	230	0.17
II	310	220	0.16
III	260	218	0.16
IV	40	150	0.11
V	130	110	0.08
VI	240	170	0.13
VII	140	150	0.11
VIII	180	180	0.08

+I: NH_4 NO_3, 2.5; KH_2 PO_4, 0.5; Mg, SO_4, $7H_2O$, 0.01; Peptone, 2: Yeast extract, 2; $FeCl_3$, 0.05; Centrifuge DM, 60.

II: $NaNO_3$, 2; K_2HOP_4, 0.8; KH_2PO_4, 0.6; $MgSO_4$, $7H_2O$, 0.6; Centrifuged DM, 60.

III: $(NH_4H_2PO_4)$, 2; KH_2PO_4, 1; KCl, 0.5; $MgSO_4$, $7H_2O$, 0.5; $FeSO_4$, $7H_2O$, 0.01; Centrifuged DM, 60.

IV: Peptone, 5; KH_2PO_4, 1; $MgSO_4$. $7H_2O$, 0.5; Centrifuged DM, 60.

V: $(NH_4)_2$ HPO_4, $MgSO_4$. $7H_2O$, 1; Yeast extract, 1; $ZnSO_4$, $7H_2O$, 0.005; $FeCl_3$, 0.05; Centrifuged Dm, 60.

VI: NH_4NO_3, 2.5; K_2HPO_4, 5; $MgSO_4$. $7H_2O$, 0.1; Centrifuged DM, 60.

VII: NH_4NO_3, 2.5; K_2HPO_4, 5; $MgSO_4$. $7H_2O$, 0.1, Centrifuged DM, 60.

VIII: Tryptic Soy broth medium: Tryptone, 17.0; Soy peptone, 3.0; NaCl, 5.0; K_2HPO_4. 2.5; Glucose, 2.5

The pH was initially adjusted to 5.5 in all media.

Incubation Time; 4 days.

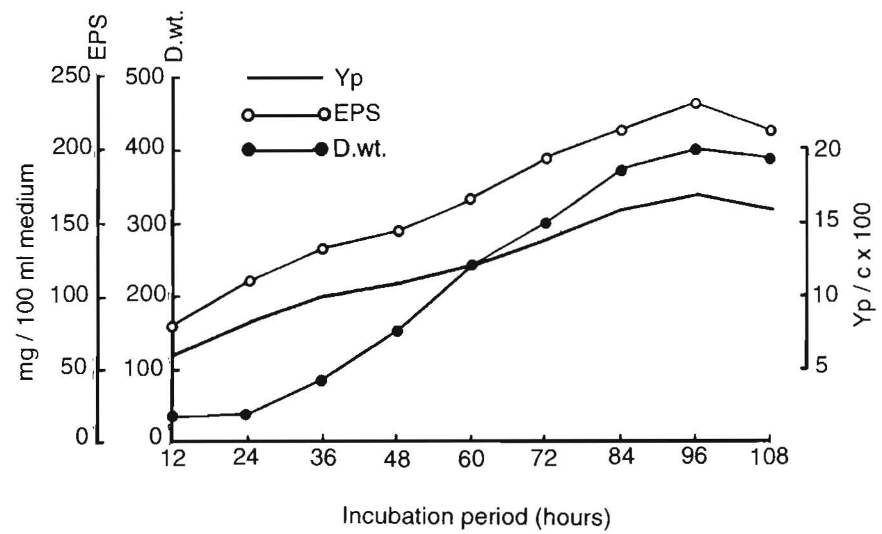

Fig. 1a. *A. tumefaciens* growth, EPS production and yield coefficient during 108 hours of growth under static culture condition.

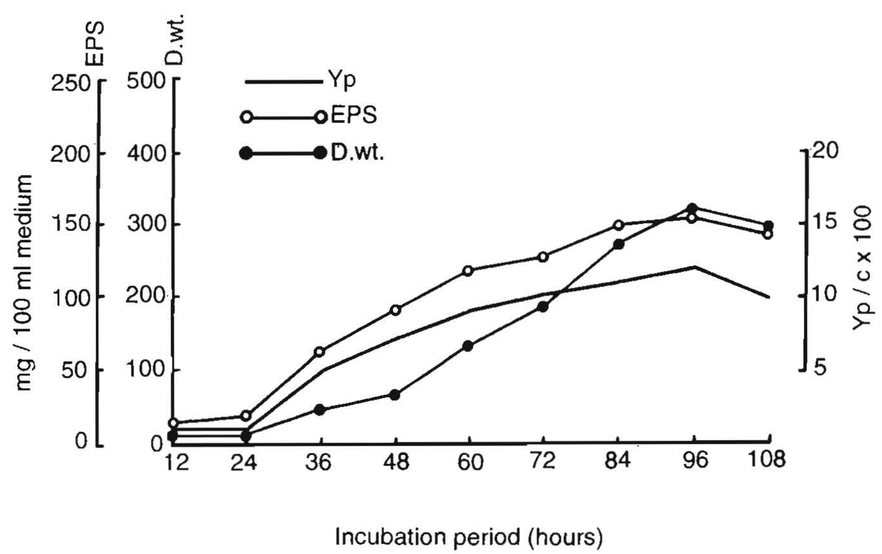

Fig. 1b. *A. tumefaciens* growth, EPS production and yield coefficient during 108 hours of growth under shaked culture condition.

Polysaccharide yield increased with increasing initial molasses level up to 10%, but did not increased above this concentration, although bacterial growth continued to increase (Fig. 2). The analysis of DM revealed the presence of glucose and fructose in equimolar proportion (46% each) as major constituents while sucrose was a minor component.

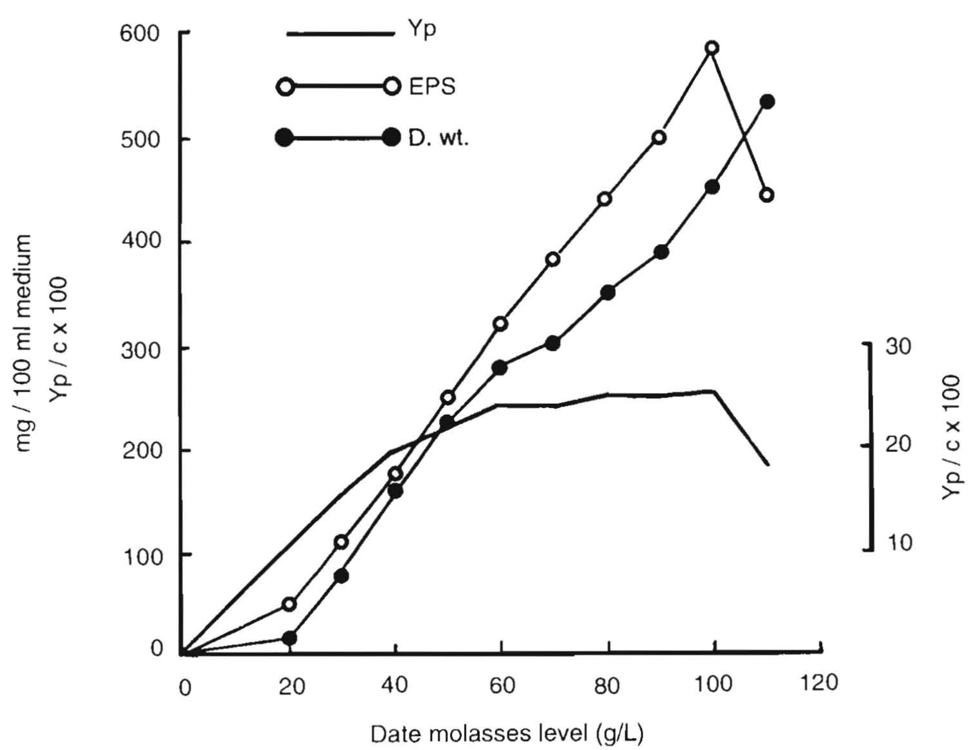

Fig. 2. *A. tumefaciens* growth, EPS production and yield coefficient as influenced with date molasses level.

The inclusion of a nitrogen source in the medium was essential as molasses is poor in nitrogen content. Nitrate was best assimilated and the highest polysaccharide yields were obtained with 7 g/L KNO_3 (Table 2). Maximum polymer levels were obtained between pH 6-8 with a definite optimum at pH 7, while pH 3 and 10 were inhibitory to the growth of the bacterium (Fig. 3). Both cell and polymer yields were markedly accelerated as the inoculum size increased from 1 to 4%. At the levels of 4% and 5% maximum growth and EPS productivity were achieved, respectively.

Table 2. *Agrobacterium tumefaciens* growth and polysaccharide production as influenced with variation of nitrogen source of the medium

Nitrogen source	Dry weight (D.Wt.) mg/100 ml	Final pH	Extracellular polysaccharide (EPS) mg/100 ml	Yield Coefficient (Y p/c)
$(NH_4)_2SO_4$	300	5.5	450	0.20
$NaNO_3$	510	5.0	588	0.26
NH_4Cl	400	5.0	540	0.24
$(NH_4)_2HPO_4$	430	5.0	540	0.24
NH_4NO_3	460	5.0	580	0.26
KNO_3	520	5.0	600	0.27

Molasses level 10%.
Initial pH value of the medium = 5.5.
Incubation period 4 days.

The volume of the culture medium exerted an effect on growth and polymer formation. Dispensing 200 ml of the modified basal medium into the Kole flasks proved to be the most favourable effect for EPS formation and yield coefficient, due to the low oxygen level available. The optimal temperature of *A. tumefaciens* for growth and EPS production was 30 °C. The addition of some supplements (Table 3) did not enhance the bacterial growth or EPS formation, except for succinic acid which was found to be the best inducer used for growth and EPS production.

Figures 4a, and 4b show the rheological properties of the EPS formed by *A. tumefaciens.* The viscosity increased parallel with an increase in polymer concentration and good temperature stability was observed. In addition, the polymer showed a high solubility in 30% (w/v) NaCl solution with an increase in viscosity in salt solutions.

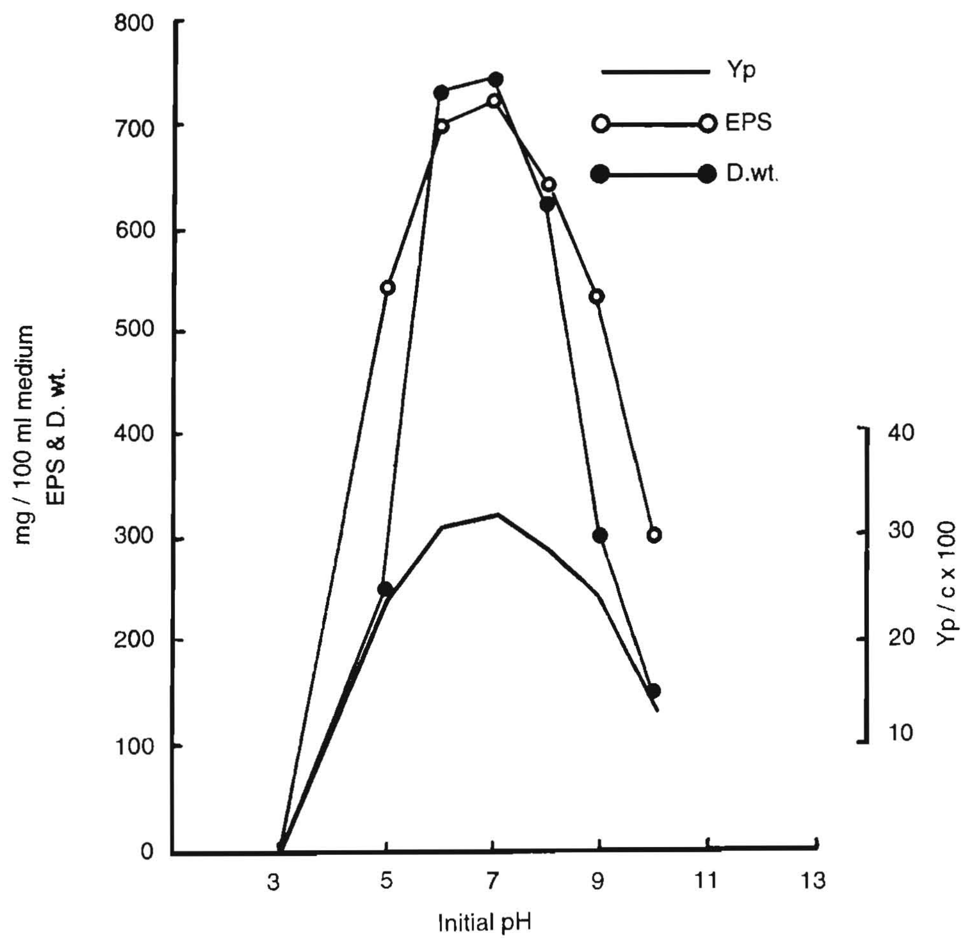

Fig. 3. *A. tumefaciens* growth, EPS production and yield coefficient as influenced with the initial adjusted pH of the medium.

Table 3. *Agrobacterium tumefaciens* growth and polysaccharide production yield as influence with separate addition of different inducers to the growth medium

Inducers (5 g/l)	**Dry weight (D.Wt.) mg/100 ml**	**Extracellular polysaccharide (EPS) mg/100 ml**	**Yield Coefficient (Y p/c)**
Control	1350	910	0.41
Ribose	1520	900	0.40
Glucose	1420	910	0.41
Galactose	1320	890	0.40
Rhammnose	1300	890	0.39
Sucrose	1390	890	0.40
Mannitol	1280	800	0.36
Succinic acid	1550	1100	0.49
Malic acid	1390	890	0.40
Sod. pyruvate	1370	910	0.41
Sod. acetate	1360	908	0.41
Sod. citrate	1330	810	0.36

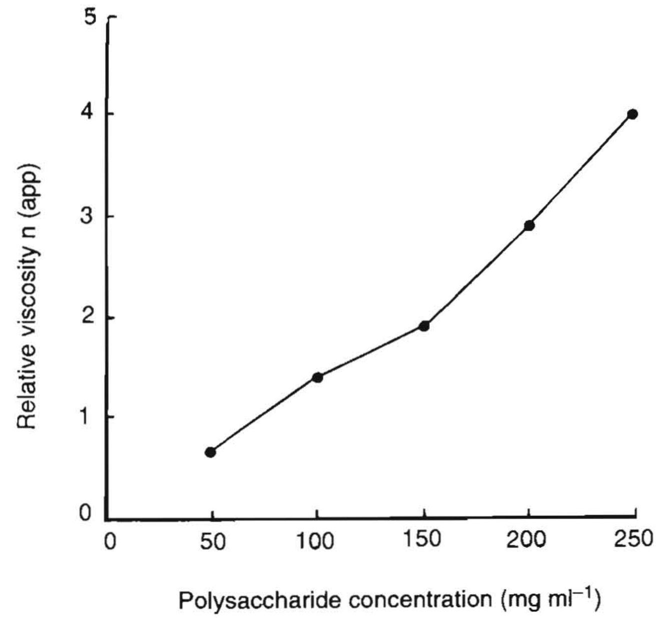

Fig. 4a. Concentration of polysaccharide versus relative viscosity.

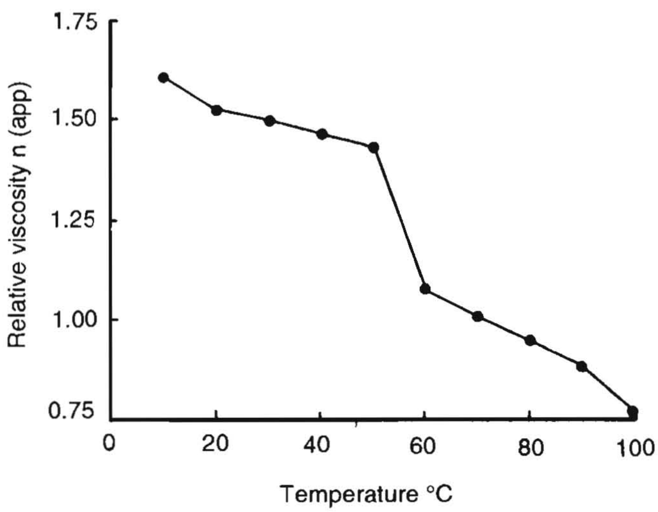

Fig. 4b. Effect of temperature on relative viscosity.

Discussion

The results in the present study revealed that a high growth of *A. tumefaciens* and high yield of polysaccharide were obtained on medium 1. Under static and shaken culture conditions the time course for extracellular polysaccharide (EPS) formation was concomitant with growth. This might be due to the rapid exhaustion of nutrients during the exponential phase leading to glucose deficiency which induces the release of the polysaccharide into the medium. This type of behaviour has been reported to be common in many other polysaccharide producing bacteria (Chan *et al.* 1984, Marques *et al.* 1986, Leberkuhne and Wagner 1986, Zevenlizen and Bertochi 1989). It is also noted that the EPS yield was 33% higher in static compared to shaked culture. This might be explained by assuming the EPS production as a process requiring limited aeration (Cadmus *et al.* 1982), Tully and Terry 1985). This might be due to restricted aeration provided by the Kole flasks according to its surface volume ratio.

It is also noted, in the present study, that EPS yield was limited to certain level (10%). This may be explained by limitations of sufficient carbon and energy which is required for EPS formation as reported in other organism (Souw and Demain 1979, Sabry and Olama 1990).

As indicated in Table 2, nitrate as the best nitrogen source for highest EPS yield. This may be due to their high permeability through the cell wall and their involvement into reactions such as oxidative assimilation of organic compounds. These findings are in accordance with Pfiffner *et al.* (1986).

pH levels affected differently the growth of the organism and EPS production similar to the previous studies by Moraine and Rogovin (1966) and Goto *et al.* (1971). They explained that the optimum pH for the polymer formation depends on the individual species and near neutrality is favourable for most bacteria. The inculum size also affected the EPS yield and this is also in accordance with Moraine and Rogovin (1973).

The results of rheological properties establish that the polysaccharide, is similar to PS-7 gum, of which variety of applications are suggested, particularly in the oil field drilling (Kang and McNeely 1976). The filtrate containing the polysaccharide exhibited an emulsification of 45% when shaked with kerosene for 2 min. The toxicity level of the crude polysaccharide (900 mg/kg) allows for the application in some fields. Pure samples proved to be non-toxic, allowing its use as blood plasma expanders and in food preparations. Antigen-antibody reactions were measured by the formation of insoluble complex by Ouchterlony (1953) double diffusion method. The precipitation in case of the crude acetone preparation (crude polymer) proved that the polymer was immunogenic, while the pure one did not elicit a specific antibody response.

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إنتاج عديدات السكاكر من بكتيريا *Agrobacterium tumefaciens* MSI باستخدام دبس التمر

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استخدم الإنسان منذ آلاف السنين عديدات السكاكر التي استخرجها من نباتات اليابسة ومن الأعشاب البحرية . واليوم اتجهت الأنظار إلى الكائنات الدقيقة كمصادر لهذه المواد نظراً للطلب المتزايد على استخدام عديدات السكاكر ، كما أن البعض من تلك الكائنات يفرز من هذه المواد ما تتفوق في صفاتها وتكون تكلفة انتاجها أقل ولقد اتيحت فرصة استخراج دبس التمر الناتج أثناء تجفيف البلح في مصنع تمور المملكة العربية السعودية بالقصيم ، حيث تؤدي هذه العملية إلى تراكم كميات كبيرة من الدبس . وتستهدف هذه الدراسة الحالية استغلال هذا المخلف الجديد (دبس التمر) كمصدر كربوني بديل ورخيص وغني بالمواد الكربوهيدراتية في انتاج عديدات السكاكر البكتيرية .

واستخدمت في هذه الدراسة بكتيريا *Agrobacterium tumefaciens* واتضحت قدرتها على افراز عديد السكاكر بوفرة في المنبت الغذائي . وكان الهدف من البحث التالي هو دراسة المتطلبات الغذائية لبكتيرية *Agrobacterium tumefaciens* والتي تحفزها لإنتاج أكبر قدر ممكن من عديدات السكاكر .

ولهذا أختير تأثير الوسط الغذائي على إنتاج عديدات السكاكر في البكتيرية المختارة تبين أن أكثر هذه الأوساط ملائمة هو الوسط الغذائي (I) للنمو (٤٠٨ ملليجرام/ ١٠٠ ملليتر وسط غذائي) ولعديدات السكاكر (٢٣٠ ملليتر وسط غذائي) ، اختلفت قدرة البكتيرية على تمثيل المصادر النيتروجينية في المنبت الغذائي وكانت نترات البوتاسيوم هي أفضل المصادر عند استخدامها بتركيز ٧ جرام/ لتر عند ضبط تفاعل المنبت الغذائي في البداية عند رقم هيدروجيني ٧ .

كما كان لعمر اللاقحة الثانوية المستخدمة وحجمها تأثير فعال في انتاج البوليمر السكري حيث أمكن الوصول إلى أفضل النتائج باستخدام لاقحة عمرها ١٢ ساعة وحجمها ٥٪ بالنسبة للوسط الغذائي ، عند التحضين في دوارق كحول كل منها يحتوي على ٢٠٠ ملل منبت غذائي وتحضينها عند درجة ٣٠ درجة مئوية في الظروف الساكنة .

كما وجد أن إضافة حمض السكسينيك قد أدى إلى تحسين ملموس في إنتاج البوليمر السكري . وقد وجد أن اللزوجة النسبية للمحلول تزداد بازدياد تركيز عديدات السكاكر وتقل درجة اللزوجة النسبية بارتفاع درجة الحرارة من ١٠ إلى ١٠٠ درجة مئوية ، كما أظهر اختبار درجة سمية المادة المنتجة بعد تنقيتها من الشوائب صلاحيتها في عمليات تعويض الفاقد من بلازما الدم وفي التجهيزات الغذائية .

وأثبتت النتائج أن لعديد السكاكر المنتج نشاط استحلاب . وقد أمكن الحصول على المصل المضاد لعديدات السكاكر بواسطة الطرق الصناعية ، وقد أمكن رصد الاتحاد بين مولد المضاد والجسم المضاد بظهور الراسب بواسطة الانتشار المزدوج لاخترلوني في حالة استخدام عديدات السكاكر الخام . وقد حللت النتائج كلها تحليلاً احصائياً وثبت أن جميعها ذات دلائل معنوية عالية .