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PRODUCTION OF EXOPOLYSACCHARIDE FROM LOCAL ISOLATES OF Rhizobium leguminosarum biovar viciae

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ABSTRACT

Fourteen local isolates of *Rhizobium leguminosarum* biovar *viciae* were isolated from root nodules of *Vicia faba* plants collected from agricultural soils of different regions of Nineveh Governorate/Iraq. Six isolates with mucoid colonies were selected as follows: WS14, WS15, WS18, WS21., WS23 and WS26. These local isolates were incubated for periods 1, 2, 3 and 4 days. Dry biomass weight and exopolysaccharide (EPS) (g/L) were recorded. Results revealed that WS18 isolate was the best among the six mentioned isolates of *R. leguminosarum* biovar *viciae*, which it gave the maximum production of EPS (6.31 g/L) after two days of incubation. Maximum dry biomass w1.22 g/L after three days of incubation by the same isolate. The effect of addition of different carbon sources to yeast extract mannitol (YEM) broth medium on EPS production by WS18 isolate showed that mannitol, as carbon source was the best for production of EPS (6.36 g/L), after two days of incubation. Two percent was the optimal concentration of mannitol which supported the maximum production of EPS (9.67 g/L) after two days of incubation. The effect of addition of different nitrogen sources at 0.10 % concentration to YEM broth medium (supplement with 2.0 % mannitol) revealed that $NaNO_3$ gave the best production which reached to 12.94 g/L after two days of incubation. The effective concentration of $NaNO_3$ support the maximum production was 0.1 %.

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INTRODUCTION

Rhizobium produce different types of polysaccharides, these polysaccharides found on surface of rhizobial cell that important in establish specific symbiotic relationship between *Rhizobium* and leguminous plants (Janczarek *et al*., 2015; Ghosh and Maiti, 2016; de Oliveira *et al.*, 2018; Castellane, *et al*., 2019 and Castellani *et al*., 2021). Also bacterial exopolysaccharides play an importance role in plant abiotic stress (Bhagat *et al*., 2021). Types of polysaccharides as follows: Exopolysaccharides (EPS), capsular polysaccharides (KPS), lipopolysaccharides (LPS), and cyclic glucans (Cglu). Some of them are secreted into cultural media, whereas another was present around the rhizobial cells and another were present in the space around the plasmic membrane (Ribeiro and Burkert, 2016; Castellane *et al*., 2017). EPS production is very complexly modulated and frequently, co-regulated with Nod factors, but the type of regulation varies depending on the rhizobial strain (Acosta-Jurado *et al*., 2021). Polysaccharides like xanthan and algenes were used for thickness of food texture and making pastes and creams (Roberts, 1995).

The medical importance of microbial exopolysaccharides was in using as agents that inter in producing vaccines (Abdalla *et al.*, 2021). Polysaccharides also used as plant growth regulators as well as insecticides and fungicides (Sayyed *et al*., 2011). Muknerjee *et al*. (2011) reported that polysaccharides used to increase field capacity of water in soil and chelating agent for different enzymes that important for plant growth. Fast growing rhizobia can produce different types of high molecular polysaccharides (Duta *et al*., 2006).

In vivo experiments results revealed that rhizobia can produce neutral gluconate β-1,4 that consider micro cellulose fibers with low molecular weight and act as inhibitor growth factor against H1V1 virus (Jagodzinski *et al*., 1994). Polysaccharides has activity against tumor, microbial disease and inhibits sarcoma tumors (Bohn and Bemiller, 1995). Tavernier *et al.* (1997) reported that polysaccharides used in production of oils and woven industry.

MATERIAL AND METHODS

Vicia faba plants collection:

Plants of *Vicia faba* were collected from fields of different area in Nineveh Governorate/Iraq. Isolates of *Rhizobium leguminosarum* biovar *viciae* were obtained from root nodules of collected plants according to Vincent (1970).

Cultural media:

Media used in this study:

Tryptone Yeast Extract (TY):

This medium used to growth and purification rhizobial isolates as well as maintenance the isolates. The medium composed of: Tryptone, 5.0; Yeast Extract, 3.0; $CaCl_2$, 0.12 (g/L), complete the volume with D. W. to 1 L. pH adjusted to 7.0 (Cha *et al*., 1998).

Yeast Extract Mannitol (YEM):

This medium used to prepare rhizobial inoculum and production of exopolysaccharide. The medium compassion is (g/L): Yeast Extract, 1.0; K_2HPO_4, 0.5; $MgSO_4$, 0.2; NaCl, 1.0, complete the volume with D. W. to 1 L. pH adjusted to 6.8 (Vincent, 1970).

Isolation of rhizobial bacteria and purification:

R. leguminosarum biovar *viciae* bacteria were isolated from root nodules of *Vicia faba* plants according to Vincent (1970)**.** Purification of rhizobial bacteria were done by streaking on TY solid medium. Petri plates placed in incubator with temperature 28±2 ºC for 1-2 days. Transparent colony was picked up for later experiments.

Reverse test:

Two days *Vicia faba* seedlings were inoculated with rhizobial strains on nitrogen free slants to confirm of infection of *Vicia faba* root hairs to give proof the isolated strains are *R. leguminosarum* biovar *viciae*.

Cultural conditions for exopolysaccharide production:

Rhizobial inoculum preparation:

Inoculum of rhizobial strains were prepared with transfer a loopfull of rhizobial bacteria growth from TY solid plates to 100 ml conical flask containing 20 ml of YEM liquid medium. Incubation was done in shaker incubator with irrigation 150 rpm with temperature 28±2 ºC for 24 hrs.

Preparation of cultural medium for production of exopolysaccharide:

YEM broth medium for growing rhizobial bacteria and production of exopolysaccharide were prepared, pH was adjusted to pH 6.8. 50 ml of the broth medium distributed to each 250 ml conical flasks. Triplicates were done for each treatment. Each flask was sealed with cotton plug. Autoclaving of conical flasks with broth medium were done with 1bar in 121 ºC for 20 minutes. After sterilization the conical flasks left to cold, and then 2% inoculation were done with 24 hours old rhizobial inoculum. Flasks were transferred to shaker incubator (New Brunswick scientific) with average shake 150 rpm. Temperature were adjusted to28±2 ºC with incubation periods 1, 2, 3, 4 days (Sayyed *et al*., 2011).

Biomass determination:

After suitable incubation period, flasks containing fermented broth medium were taken for centrifugation with 6000 rpm for 20 minutes. Supernatant were left a side for determination of exopolysaccharide. Pellet were collected for determination of biomass in small plates with known weights. Plates were dried in oven with temperature 80 ºC for 1 hr. Then determination the biomass with sensitive balance (Duta *et al*., 2006).

Isolation and determination of Exopolysaccharide:

To 5 ml supernatant two velum (10 ml) of acetone were added in order to precipitate the exopolysaccharide. Mixture were stir and exopolysaccharide was separated by spooling and then spooled samples were oven dried at 50 ºC till the constant weight and estimated them.

Selection of a local rhizobial strain with a maximum exopolysaccharide production:

Growing single colonies on solid YEM after 2 days incubation were noticed with naked eye and the selection of the best local rhizobial strain according to colony appearance with a maximum sticky mucous (Sridevi and Mallaiah, 2007).

Effect of different incubation periods on growth and production of exopolysaccharide in a selected rhizobial strains:

After selection the best exopolysaccharide producer strains, inoculation of YEM broth medium with these strains and incubation for 1, 2, 3 and 4 days were done. Triplicates for each treatment were followed. Flasks were removed from shaker incubator after a suitable incubation period.

Effect of different carbon sources on growth of *R. leguminosarum* biovar *viciae* WS18 and production of exopolysaccharide:

Effect of 1.0 % (w/v) of deferent carbon sources on growth of *R. leguminosarum* biovar *viciae* strain WS18 and production of exopolysaccharide were studied. The added carbon sources were as follows: glucose, galactose, fructose, mannose, mannitol, lactose and sucrose. pH adjusted to 6.8. Replicates were done for each treatment. Conical flasks with YEM broth medium were autoclaved and inoculated. Results were taken after two days of incubation.

Effects of different concentrations of mannitol on growth of *R. leguminosarum* biovar *viciae* WS18 and production of exopolysaccharide:

Deferent concentrations of mannitol support the production of exopolysacharide were studied. Prepared concentrations of YEM medium were as follows: 1.0, 2.0, 3.0 and 4.0 gm/L. pH was adjusted to 6.8. Results of dry biomass and EPS were recoded after 2 days of incubation.

Effects of different nitrogenous sources on growth of *R. leguminosarum* biovar *viciae* WS18 isolate and EPS production:

Deferent nitrogenous sources were added for each alone to fermentation medium containing mannitol (2.0%) as carbon source to study their effect on dry biomass and EPS. Added nitrogenous sources with 0.1% (w/v), as follows: $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, KNO_3, glutamic acid and casamino acid.

Effects of different concentrations of $NaNO_3$ on growth of *R. leguminosarum* biovar *viciae* WS18 isolate and EPS production:

Sodium nitrite with concentrations: 0.05, 0.1., 0.15 and 0.20% (v/w) were added to fermentation medium containing mannitol 2.0% to know the best concentration of $NaNO_3$ which give the best EPS production.

Statistical analysis

The result values were statistically analyzed for averages and standard deviation (S. D.) using computer program (SAS, 2009).

RESULTS AND DISCUSION

Isolation of rhizobial bacteria:

An attempt was done to isolate *R. leguminosarum* biovar *viciae* local strains from root nodules of *Vicia faba* plants collected from agricultural soils of different regions of Nineveh Governorate/Iraq. After purification of fourteen local rhizobial strains which obtained according to their colony appearance, i. e., regular circular and transparent colonies. Reverse test showed the success symbiosis relation and confirm that these strains are *R. leguminosarum* biovar *viciae*. By naked eye examination, six colonies were chosen according to gummy texture of colonies grown on TY solid medium. These six isolates were chosen for further studies. These isolates are as follows: WS14, WS15, WS18, WS21, WS23 and WS26. Sridevi and Mallaiah, (2007) recorded ability of isolation of 16 stains of *Rhizobium* from root nodules of *Sesbania sesban* grown in deferent area in India and they found that the strain SS5 was the best among the isolated strains in exopolysaccharide production. Ten strains of *Rhizobium* isolated from *Vigna mungo* L.

Effect of different incubation periods on growth of *Rhizobium leguminosarum* biovar *viciae* and production of exopolysaccharide in a selected isolates:

To study the effect of incubation on growth of selected rhizobial isolates and production of exopolysaccharide, an incubation for 1 to 4 days were done. Results revealed that incubation periods have effects on rhizobial growth and production of exopolysaccharide (Table 1). WS18 gave a maximum production, which reached to 6.31 g/L after two days of incubation. Incubation to more than two days led to reduction in production, which reduced to 5.09 g/L after four days of incubation. EPS production from WS21 isolate reached to 4.35 g/L after 3 days of incubation. Minimum EPS production was from WS14 isolate (2.19 g/L) after one day of incubation. Among six selected rhizobial isolates, a maximum growth was with WS18 isolate, which average dry biomass was 1.22 g/L after three days of incubation (Table 1). Less dry biomass obtained from WS21 which reached 0.83 g/L after three days of incubation. Minimum dry biomass was obtained from WS14 (0.31 g/L) after one day of incubation. As the WS18 isolate gave the maximum exopolysaccharide production among the other rhizobial isolates, this isolate were chosen for farther experiments. Increasing in biomass led to decreasing in exopolysaccharide

production due to consumption by bacteria itself under hydrolysis condition because of consumption of carbon source in cultural medium. Decrease in viscosity improved this hypothesis (Sayyed and Chincholkar, 2008). Chai (1980) showed that *Rhizobium leguminosarum* biovar *viciae* gave a maximum exopolysaccharide productivity (4.30 g/L). According to the many researches, exopolysaccharides quantities are highly dependent on microorganisms, under specific conditions, media composition, as well as the genus and species of bacteria (Ali and Orf, 2022).

Table (1): Effect of different incubation periods on growth and production of exopolysaccharide in a selected rhizobial isolates.

Isolate	Incubation (Day)	Dry Biomass (g/L)	EPS td hg,s'(g/L)
WS14	1	0.31 ±0.07*	2.19 ±0.08
	2	0.64 ±0.03	2.34 ±0.06
	3	0.92 ±0.09	3.36 ±0.10
	4	0.87 ±0.14	3.30 ±0.13
WS15	1	0.66 ±0.08	4.19 ±011
	2	0.79 ±0.09	4.30 ±0.05
	3	0.70 ±0.12	5.32 ±0.07
	4	0.62 ±0.05	4.30 ±0.09
WS18	1	0.90 ±0.12	5.21 ±0.05
	2	1.01 ±0.08	6.31 ±0.08
	3	1.22 ±0.06	5.34 ±0.10
	4	0.94 ±0.11	5.09 ±0.05
WS21	1	0.69 ±0.09	3.27 ±0.11
	2	0.78 ±0.04	4.38 ±0.09
	3	0.83 ±0.10	4.35 ±0.06
	4	0.80 ±0.15	3.88 ±0.05
WS23	1	0.65 ±0.13	3.23 ±0.11
	2	0.73 ±0.09	4.35 ±0.06
	3	0.70 ±0.07	3.44 ±0.09
	4	0.66 ±0.14	3.41 ±0.05
WS26	1	0.99 ±0.11	3.35 ±0.03
	2	0.73 ±0.09	4.26 ±0.07
	3	0.64 ±0.07	3.33 ±0.09
	4	0.60 ±0.12	3.34 ±0.06

* Each value presents triplicates, ± values presents S.D.

Effect of different carbon sources on growth of *R. leguminosarum* biovar *viciae* WS18 and production of exopolysaccharide:

Results of the effect of different carbon sources on growth of *R. leguminosarum* biovar *viciae* WS 18 isolate and production of exopolysaccharide after two days of incubation revealed that a best carbon source achieved a maximum production of exopolysaccharide was mannitol (Table 2), which production reached to 6.36 g/L. Less production of exopolysaccharide was from glucose as a carbon source (5.40 g/L), whereas a minimum EPS production (3.20 g/L) when lactose used

as a carbon source. The best carbon source support the a maximum growth were mannitol and glucose, the dry biomass were 1.20 and 1.11 g/L, respectively. A minimum growth were 1.19 g/L, when lactose used as sole carbon source (Table 2). These results agree with results of Nirmala *et al.* (2011), which they found that mannitol supported a maximum EPS production which reached to 1530 µg/L. Also Breadveld *et al.* (1993) found that mannitol supported a maximum EPS production from *Rhizobium ciceri* strain CRC5.

Table (2):Effect of different carbon sources on growth of *R. leguminosarum* biovar *viciae* WS18 and production of exopolysaccharide.

Carbon source 1% (w/v)	Dry Biomass (g/L)	EPS (g/L)
Glucose	1.11 ±0.05*	5.40 ±0.05
Galactose	0.65 ±0.08	3.27 ±0.09
Fructose	0.72 ±0.03	4.21 ±0.11
Mannose	0.63 ±0.10	3.38 ±0.09
Mannitol	1.20 ±0.13	6.36 ±0.10
Lactose	0.59 ±0.09	3.20 ±0.06
Sucrose	0.78 ±0.07	4.43 ±0.12

* Each value presents triplicates, ± values presents S.D.

Effects of different concentrations of mannitol on growth of. *leguminosarum* biovar *viciae* WS18 and production of exopolysaccharide:

The effects of different concentrations of mannitol as carbon source on growth and EPS production by *R. leguminosarum* biovar *viciae* isolate WS18 after two days of incubation reveled that increase of mannitol concentration to 2% (Table 3) led to increase in exopolysaccharide production, which reached to 9.67 g/L. Increase of mannitol concentration to more than 2% led to decrease in EPS productivity, the amount decreased to 7.33 g/L with 4% concentration. Increase of mannitol concentration up to 3% led to increase of dry biomass which reached to 1.59 g/L after two days of incubation, whereas increase of mannitol concentration more than 2% led to decrease in dry biomass which decreased to 1.43 g/L with concentration 4% after two days of incubation. It was found that the best productivity of EPS from *Rhizobium* sp. strain DL10 and strain HGR12 occurred when mannitol used as carbon source with 2% concentration (Chosch *et al.*, 2005; Prabhavati and Mallaiah, 2009). Sethi *et al.* (2019) reached to the same results when they studied the production of exopolysaccharide by *Rhizobium* species.

Table (3): Effects of different concentrations of mannitol on growth of *R. leguminosarum* biovar *viciae* WS18 and production of exopolysaccharide.

Mannitol % (w/v)	Dry Biomass (g/L)	EPS (g/L)
1.0	1.20 ±0.07*	6.29 ±0.10
2.0	1.33 ±0.11	9.67 ±0.09
3.0	1.59 ±0.03	8.85 ±0.05
4.0	1.43 ±0.06	7.33 ±0.07

* Each value presents triplicates, ± values present S.D.

Effects of different nitrogenous sources on growth of *R. leguminosarum* biovar *viciae*
WS18 strain and EPS production:

The addition of different of nitrogenous sources with 0.1% concentration to yeast extract broth medium supported with 2% mannitol (Table 4) revealed that the best nitrogenous source support the maximum EPS production was sodium nitrate which productivity reached to 12.94 g/L. Glutamic acid gave 11.42 g/L of exopolysaccharide. A minimum EPS production was 10.51 g/L when potassium nitrate used as nitrogen source. Casamino acid supported a maximum growth which dry biomass reached to 2.61 g/L. These results agree with results of Ghosh and Basu (2001), and Sridevei and Mallaiah (2007) which show that the best nitrogenous source supported a maximum EPS production by *Rhizobium* sp. strain SS5 was sodium nitrate.

Table (4): Effects of different nitrogenous sources (supported with mannitol) on growth of *R. leguminosarum* biovar *viciae* WS18 strain and EPS production.

Nitrogen source 0.10 % (w/v)	Dry Biomass (g/L)	EPS (g/L)
$NaNO_3$	2.32 ±0.07*	12.94 ±0.05
NH_4Cl	2.36 ±0.11	11.39 ±0.09
$(NH4)_2SO_4$	2.41 ±0.09	11.28 ±0.06
KNO_3	2.47 ±0.13	10.51 ±0.08
Glutamic acid	2.58 ±0.08	11.42 ±0.10
Casamino acid	2.61 ±0.10	11.39 ±0.07

* Each value presents triplicates, ± values presents S.D.

Effects of different concentrations of $NaNO_3$ on growth of *R. leguminosarum* biovar *viciae* WS18 isolate and EPS production:

To study effect of different concentrations of sodium nitrate on growth of *R. leguminosarum* biovar *viciae* WS18 isolate and EPS production table 5 elucidate that addition of sodium nitrate with concentration of 0.1% gave a maximum EPS production which reached to 12.89 g/L, whereas more concentrations than 0.1% led to decrease in EPS productivity which decreased to minimum (10.88 g/L) with concentration 0.20%. A concentration of 0.10% also supported the maximum growth which reached to 2.35 g/L. These results agree with results of Sridevi and Mallaiah (2007). Increase in exopolysacharide production by the *R. leguminosarum* biovar *viciae* isolate WS18 it has potential role in industries and it is recommended that exposure this strain to different mutagens may resulted in extreme in exopolysaccharide production.

Table (5):Effects of different concentrations of $NaNO_3$ on growth of *R. leguminosarum* biovar *viciae* WS18 strain and EPS production.

$NaNO_3$ % (w/v)	Dry Biomass (g/L)	EPS (g/L)
0.05	2.07 ±0.07*	11.90 ±0.10
0.10	2.35 ±0.11	12.89 ±0.08
0.15	2.19 ±0.03	11.50 ±0.05
0.20	2.08 ±0.06	10.88 ±0.07

* Each value presents triplicates, ± values presents S.D.

CONCLOSION

According to the results of this study, the local isolate *R. leguminosarum* biovar *viciae* WS18 which accumulate high levels of EPS may be a promise strain in industrial biotechnology for exopolysaccharide production when grown in optimal conditions.

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CONFLICT TO INTEREST

Author declare no conflict of interest regarding the publication of this study.

انتاج السكر المتعدد الخارجي من عزلات محلية من *Rhizobium leguminosarum* biovar *viciae*

وجدان سالم قاسم

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الخلاصة

تم في هذه الدراسة عزل اربعة عشر عزلة محلية من بكتريا *Rhizobium leguminosarum* biovar *viciae* من العقد الجذرية لنباتات الباقلاء جمعت من ترب زراعية من مناطق مختلفة من محافظة نينوى/العراق. ستة عزلات وبمستعمرات مخاطية تم اختيارها وكالاتي: WS14, WS15, WS18, WS21, WS23 وWS26. تم تحضين العزلات المحلية لفترات 1 , 2 , 3 و4 ايام. تم تسجيل وزن الكتلة الحية الجافة والسكر المتعدد الخارجي (غم/لتر). اظهرت النتائج ان العزلة WS18 كانت الافضل من بين ستة عزلات من بكتريا *R. leguminosarum* biovar *viciae* , والتي اعطت اعلى انتاجية (6.31 غم/لتر) بعد يومين من التحضين. اعلى كتلة حيوية جافة كانت (1.22 غم/لتر) بعد ثلاثة ايام من التحضين ولنفس العزلة. اظهرت دراسة اضافة مصادر كاربونية مختلفة الى وسط مستخلص الخميرة والمانيتول السائل على انتاجية السكر المتعدد الخارجي بواسطة العزلة WS18, ان المانيتول كمصدر وحيد للكاربون كان الافضل لانتاج السكر المتعدد الخارجي (6.36 غم/لتر) بعد يومين من التحضين. 2.0% كان افضل تركيز للمانيتول, حيث دعم اقصى انتاجية للسكر المتعدد الخارجي حيث بلغت الانتاجية 9.67% غم/لتر بعد يومين من التحضين. اظهرت دراسة

اضافة مصادر نايتروجينية مختلفة عند التركيز 0.10% الى وسط مستخلص الخميرة والمانيتول السائل (والمدعّم بـ 2.0% مانيتول) ان $NaNO_3$ اعطى أفضل انتاجية سكر متعدد خارجي (EPS) حيث بلغت الانتاجية 12.94 غم/لتر بعد يومين من التحضين. ان التركيز الفعّال من $NaNO_3$ والذي يدعم اقصى انتاجية كان 0.1%.

الكلمات الدالة: السكر المتعدد الخارجي, أنتاجية, رايزوبيوم , مصادر كاربونية, مصادر نايتروجينية.

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