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## Isolation, Characterization and Optimization of Wild Type *Sinorhizobium meliloti* to Produce High Concentrations of Indole Acetic Acid

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### Abstract

*Rhizobium* bacteria was isolated from the root nodules of *Medicago sativa* plants and, based on morphological and some biochemical properties, it was characterized as *Sinorhizobium meliloti*. We studied the ability of this isolate, as well as that of *Agrobacterium rhizogenes* R1601, to produce the auxin indole acetic acid (IAA). For purposes of control, both isolates, in the absence of tryptophan-L, were similarly tested. The identification of IAA was achieved by checking the colour reactions with Salkowski's reagent. Low amounts (23, 69 and 26,77 µg/ml) of IAA were produced by *S. meliloti* and *A. rhizogenes* after 24 and 72 hours of incubation, respectively. *S. meliloti* was distinguished by the high production of this auxin (612 µg/ml) when adding 0.1% tryptophan to the growth medium (YEM), as compared to the amount of its production by the other bacteria. Therefore, this isolation was used to determine the highest production of IAA at optimal conditions, which reached to 553,550 and 610,662 µg/ml in liquid YEM medium supported with tryptophan-L (both at 0.5 and 1.0%) and a medium supported with glucose and lactose sugar (10%), after 72 hours of incubation, respectively. The 72-hour incubation period was better than that of 24-hour in obtaining an additional amount of IAA, which ranged between 0.6 and 0.7g/l. A spot of IAA produced by rhizobium bacteria was created corresponding to the standard spot of IAA in the thin layer chromatography (TLC) detection experiment.

**Keywords:** *Sinrhizobium meliloti*, Tryptophan-L, indole acetic acid (IAA), TLC.

عزل وتوصيف وتحسين النوع البري *Sinrhizobium meliloti* لإنتاج تركيز عالٍ من الاندول

### حامض الخليك

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### الخلاصة:

عزلت بكتيريا الرايزوبيوم من العقد الجذرية المتكونة على نباتات الجت *Medicago sativa* النامية في الحقل، عرفت انها *Sinorhizobium meliloti*، استناداً إلى الخصائص المورفولوجية وبعض الخصائص البيوكيميائية، وذلك لدراسة قابليتها و *Agrobacterium rhizogenes* R1601 على إنتاج الأوكسين، اندول حامض الخليك (IAA) بغياب التريبتوفان (كعينة مقارنة)، تم التحقق من ذلك بتفاعلات اللون مع كاشف

سالكوفسكي، وسجلت كمية منخفضة منه 23.69 و 26 و 77 ميكروغرام/ مل من قبل *S.meliloti* و *A.rhizogenes* بعد 24 و 72 ساعة من التحضين في وسط (YEM) Yeast Extract Mannitol السائل، على التوالي، وقد تميزت بكتيريا *S.meliloti* بانتاجها العالي من هذا الاوكسين 612 ميكروغرام/مل عند إضافة 0.1% تريبتوفان إلى وسط النمو (YEM) مقارنة بكمية إنتاجه من البكتيريا الأخرى، لذلك تم استخدام هذا العزلة لتحديد أعلى إنتاج من IAA ضمن الظروف المثلى وبلغ 553.550 و 610 و 662 ميكروغرام/مل في وسط YEM السائل المدعم بالتريبتوفان بتركيز 0.5 و 1.0% وإلى الوسط المدعم بسكري الجلوكوز واللاكتوز بتركيز 10% بعد 72 ساعة من التلقيح، على التوالي. كانت فترة التحضين 72 ساعة افضل من 24 ساعة في الحصول على كمية من هذا الاوكسين (IAA) تراوحت ما بين 0.6 الى 0.7 غم/ لتر، كما تم ملاحظة ظهور بقعة من IAA المنتجة من قبل هذه البكتيريا منظاراً لبقعة IAA القياسية في تجربة الكشف عنه بتقنية كروماتوغرافيا الورقة الرقيقة (TLC).

## Introduction

IAA is one of the plant growth hormones that fall into the group of auxins [1]. The researchers increased their interest in it due to its physiological significance to the plant as it controls the growth and division of cells as well as the differentiation of tissues into plant organs such as roots and fruits [2]. Soil bacteria belonging to the Rhizobacteriaceae family, especially *Rhizobium* bacteria, are of special importance from an agricultural point of view, as they work to increase soil fertility by fixing atmospheric nitrogen [3]. In addition, auxins, especially indole acetic acid, are produced from the amino acid tryptophan by this bacteria during its symbiotic relationship with the legume plant that is specialized in bacterial attraction by roots exudates [4]. Bacterial IAA stimulates the growth of roots and the division of cells infected with the bacteria to form the root nodules. Within these nodules, the atmospheric nitrogen is fixed to ammonia, and thus increasing the protein content of these plants [5,6]. IAA also has a role in modifying the root hairs of the legume plants infected with the *Rhizobium* bacteria, whose shape changes from the straight to the deformed shape, as one of the indications bacterial infection [7]. On the other hand, this auxin is produced by the bacteria *A. tumefaciens* and *A. rhizogenes*, known to be pathogenic to the plant, due to the encoding genes located on its plasmids called Ti-plasmid. The genes are encoded to form crown galls and Ri-plasmid, which encode genes to form hairy roots [8].

On the other hand, this auxin is produced by *A. tumefaciens* and *A. rhizogenes*, known to be pathogenic to the plant, due to the encoding genes located on Ti and Ri plasmid to form crown galls and hairy roots, respectively [8].

Given this importance, this study came to verify the production of auxin IAA by the isolated and characterized bacteria belonging to the Rhizobacteriaceae family and to optimize the amount of its production, in the presence or absence of the tryptophan base material and different sugars. In addition, we aimed at confirming its production, by using TLC technique, in terms of its appearance as a visible spot similar to the standard auxin spot.

## Materials and Methods

### Isolation and diagnosis of rhizobium

The bacteria were present in the root nodules of the one month-old alfalfa (*Medicago sativa*) plant, grown in the greenhouse of the Department of Biology, College of Education for Pure Sciences, University of Mosul. The nodules were sterilized, mashed and then inoculated on the solid YEM medium and incubated for 24 hours [9, 10]. Growing colonies were diagnosed according to phenotypic and cultural characteristics as well as biochemical tests. Their ability of infecting alfalfa plant seedlings and the formation of root nodules on it was also tested under laboratory condition [11].

While, the bacterium *Agrobacterium rhizogenes* strain R1601, known to be pathogenic to plants and genetically modified for the resistance to kanamycin and carbenicillin antibiotics, were obtained from Prof. Nester, E.W., Washington University, USA.

### Detecting the ability of *A. rhizogenes* and *S.meliloti* to produce IAA

Suspensions of *A.rhizogenes* and *S.meliloti* bacteria were prepared separately. A volume of 100  $\mu$ l was used to inoculate test tubes containing 5 ml of YEM liquid medium supported with L-tryptophan (0, 0.1%) at a rate of three repeats/ isolation. After inoculation, the tubes were incubated at a temperature of 28 ° C with shaking at a speed of 100 rpm/ min for a time period ranging from 24 to 72

hours from the date of inoculation. The bacterial suspension was taken and the bacteria were discarded by centrifugation at 10,000 g for 5 minutes for two times at a temperature of 4°C. A volume of 1 ml of the supernatant was then used to detect the ability of the studied isolates to produce IAA.

In order to determine the optimum conditions for the production of IAA from *S. meliloti*, different concentrations of tryptophan-L (0.5,1.0,1.5 and 2.0%) were added to the YEM liquid medium. Different types of sugars (glucose, sucrose, lactose and mannitol) were then added with a concentration of 10% to the YEM liquid medium. Then, we inoculated these media with bacteria which were incubated under the same conditions mentioned above to test the amount of the production of IAA.

#### Preparation of the standard curve for IAA

Gradient concentrations of the standard growth regulator (10-100 µg/ ml) with a volume of 1 ml were prepared. Then, 2 ml of Salkowski reagent was added, which was prepared according to Harley and Prescott method [12] by dissolving 23.2 g of FeCl<sub>3</sub> in 25 ml of distilled water, adding 1 ml of the solution to 52 ml HClO<sub>4</sub> (35%), then leaving the mixture for 30 minute in dark. The absorbance was read at the wavelength of 530 nm at a rate of three replicates/ concentration, where the relationship between the IAA concentration and the absorbance values for each concentration was estimated [13].

#### Estimation of the produced IAA

The production ability of the growth regulator IAA was quantified based on the standard curve using Salkowski color detector; 1 ml of the liquid culture medium was taken, then the bacterial growth was separated by centrifuging 2 ml of Salkowski's reagent was added. Next, the mixture was left for 30 minutes and the absorbance was read at a rate of three replicates/ isolation and a wavelength of 530nm. The most efficient isolation in terms of production was determined by reference to the standard curve for IAA. The samples with high absorbance values were diluted ten times, then the total value of IAA concentration was calculated.

#### Detection of IAA by TLC

The bacterial filtrate (supernatant portion) was taken and adjusted up to 2.5 mL by using 1M HCl. The same volume of ethyl acetate was added at a rate of 4 times for both lower and upper water phases. The mixture was dried with a rotary evaporator at 40°C and dissolved with methanol. Then, 10µl it was transferred and IAA standard to the line about 2 cm away from one side of the plate. Then, 10µl of it and of standard IAA was transferred to the line away from one side of the plate about 2cm.

After complete drying, the plate was placed in a solvent system consisting of ethyl acetate: chloroform : formic acid (55:35:10). The spots were detected by spraying the plate with Salkowski's reagent. The method described by Warsia *et al.* [14] was used with modifications. The value of retention factor (R<sub>f</sub>) was calculated as in the following equation [15]:

$$R_f \text{ Value} = \frac{\text{distance traveled by sample}}{\text{distance traveled by solvent}}$$

## Results

After isolating the bacteria from the root nodules of the alfalfa plants, it became clear to us that their individual cells are bacillus-shaped, negative to Gram stain, and not forming spores. Their growing colonies appeared on the solid YEM medium in the form of convex, mucoid, semitransparent, white-colored colonies with smooth edges. The results of biochemical tests are summarized in Table-1. The results reveal that the isolated bacteria are *S. meliloti*, with the ability to form root nodules on seedlings of alfalfa plants after being re-inoculated.

**Table 1- Results of biochemical tests of bacteria isolated from nodules of alfalfa plant.**

Bacteria	Motility	Congored	Enzymes				Sodium citrate	IMViC test			
			Oxidase	Catalase	Urease	Gelatinase		I	M	V	C
<i>S. meliloti</i>	+	Pink	+	+	-	+	-	-	-	-	-

I: Indole, M: Methyl red, V: Voges Proskauer, C: Cimon citrate

Table-2 displays the estimated production of IAA by bacteria in terms of its concentration in  $\mu\text{g/ml}$ . The results show that *S.meliloti* produced a higher concentration of IAA than *A.tumifaciens* R1601, with percentages of 90 and 89% higher than that of the control sample when the bacteria was grown in YEM supported by tryptophan at a concentration of 0.1%, after 24 and 72 hours of incubation, respectively. Therefore, *S. meliloti* was approved to be employed by the subsequent experiments.

**Table 2-**The amount of IAA produced by different preparations of *A.rhizogenes* and *S.meliloti* after 24 and 72 hours of incubation periods.

Bacteria	Bacterial count				Concentration of IAA ( $\mu\text{g/ml}$ )			
	24 h. ( $\times 10^8$ )		72 h. ( $\times 10^9$ )		24 h.		72 h.	
	Contr ol	Try(0.1 %)	Contr ol	Try(0.1 %)	Contr ol	Try(0.1 %)	Contr ol	Try(0.1 %)
<i>A.rhizogenes</i> R1601	31	45	99	144	26	37(30%)*	77	109(29%)
<i>S.meliloti</i>	38	135	85	235	23	220(90%)	69	612(89%)

\*=The increase rate compared to the control.

From Table-3, we can see a steady increase in the number of *S. meliloti* bacteria with the increase in the incubation period, especially when adding the different sugars at a concentration of 10%.

**Table 3-** Counts of *S. meliloti* in YEM medium supplemented with different concentrations of tryptophan alone or sugars (10%) with tryptophan (0.1%) after 24 and 72 hours of incubation periods.

Tryptophan (%)	Bacterial counts ( $\times 10^8$ )		Sugars (10%)	Bacterial counts ( $\times 10^8$ )	
	24 h.	72 h.		24 h.	72 h.
0.5	63	180	Glucose	73	256
1.0	87	195	Sucrose	93	221
1.5	120	215	Lactose	122	280
2.0	125	277	Mannitol	54	140

In order to achieve the goal of reaching the optimal laboratory conditions for the production of IAA by *S.meliloti*, the amino acid tryptophan was added in different concentrations of 0.5, 1.0, 1.5 and 2.0%. As compared to the control, improvements in the amount produced were detected, with increasing rates of 88, 87, 86, and 57% after 24 h of incubation and 88, 88, 80, and 61% after 72 h of incubation respectively (Table 2). However, the produced amount of IAA was reduced by increasing the tryptophan-L concentration to more than 1% (Table 4).

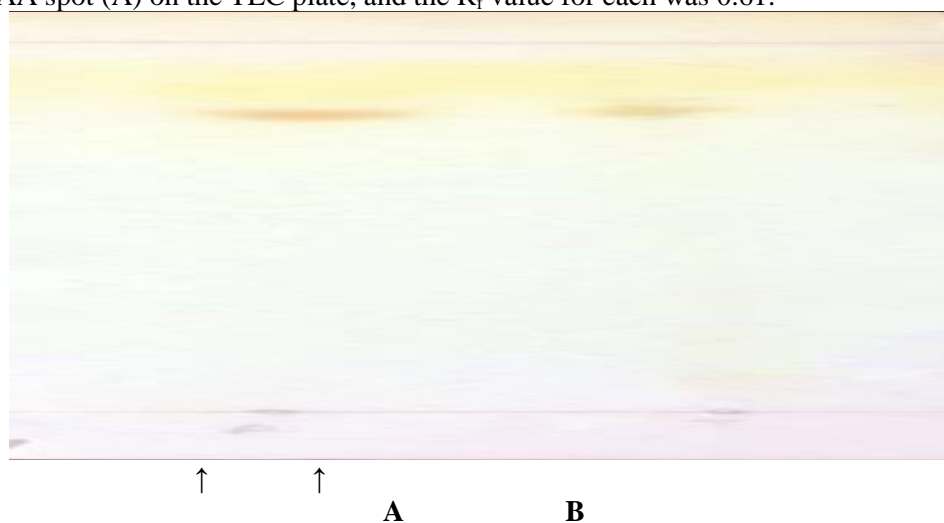
On the other hand, we added 10% of different sugars instead of 1% mannitol sugar, found within the leaflet of this medium, to the liquid YEM medium. More positive effects on the growth of *S.meliloti* bacteria and the production of IAA were observed when adding glucose and lactose sugars, ; the concentration was increased by 85 and 90% after 24 hours of incubation and by 89 and 90% after 72 hours of incubation, respectively, as compared to the control (Table-4).

**Table 4**-Effects of different concentration of tryptophan-L and sugars on the growth and production of IAA by *S. meliloti*

Tryptophan (%)	Concentration of IAA ( $\mu\text{g/ml}$ )		Sugars (10%)	Concentration of IAA ( $\mu\text{g/ml}$ )	
	24 (h.)	72 (h.)		24 (h.)	72 (h.)
0.5	189 (88%)*	553 (88%)	Glucose	216 (85%)	610 (89%)
1.0	175 (87%)	550 (88%)	Sucrose	68 (66%)	139 (50%)
1.5	159 (86%)	351 (80%)	Lactose	230 (90%)	662 (90%)
2.0	54 (57%)	176 (61%)	Mannitol	27 (15%)	108 (36%)

\*=The increase rate compared to the control.

As shown in Figure-1, a colored spot of IAA produced by *S. meliloti* (B) was identical to the standard IAA spot (A) on the TLC plate, and the  $R_f$  value for each was 0.61.

**Figure 1**-The results of TLC, A) standard IAA, and B) sample IAA extracted from *S. meliloti*.

### Discussion

Morphological and cultural characteristics of the isolated bacteria were found to be similar to those reported by Hussain *et al.*[16]. The results of the biochemical tests were consistent with the results provided by Al-Shakarchi [11], who also isolated *S. meliloti* from the root nodules of alfalfa plants.

Several laboratory studies indicated that tryptophan-L is the main substrate for the production of indole-3-acetic acid in bacteria [17], especially as related to symbiosis and the causing of plant diseases [18]. The production of IAA by *S. meliloti* and *A. rhizogenes* in the control sample is often due to the fact that the medium contains yeast extract which has tryptophan in its chemical composition [19].

The addition of 0.1% tryptophan to the nutrient medium led to an increase in the concentration of IAA produced by *S. meliloti* than that by *A. rhizogenes*. This is often explained by the response of the rhizobium bacteria, usually found in the soil, to the presence of tryptophan which is secreted by the

roots of the legume plants that are specialized in its conversion to IAA. This process is involved in converting the straight shape of the root hairs to the deformed shape in the early stages of the symbiotic relationship [20]. However, in the case of the plant pathogen *Agrobacterium sp.*, this auxin exhibits the behavior of virulence factors. Also, it is not produced in abundance by the presence of tryptophan in the medium, because it is encoded by special genes present on the plasmid that combines with the plant's genetic material and causes disease [21].

On the other hand, the increase of IAA production by adding sugars to the medium, especially glucose and lactose, may be due to the ability of this bacteria to hydrolyze lactose to glucose and galactose as well as the transformation of galactose during the metabolism process into glucose [22], which is known to be a simple monosaccharide that can be metabolized by *Rhizobium* [23].

Our isolate of *S.meliloti* produced a significant concentration of growth regulator IAA (662 µg/ml). This result is not in agreement with that reported by Seridevi and Mallaiah [24] and KÜÇÜK and Cevher [25] who indicated that the rhizobium produced highest levels of IAA (28 and 165 µg/ml, respectively) after 72 hours of inoculation. The incubation period was also reported to have positive effects on IAA production and the growth of *Rhizobium* strains [26]. The reason for this difference is often due to the asymmetry of other bacterial species in gene expression that includes the pathway for the synthesis of auxine, along with the inability to stimulate production using some other factors [27]. Other reasons can include the inconsistency of different studies in their use of the same bacterial type, primary inoculate concentration, or culture media components [28].

On the other hand, Mohite [29] indicated the similarity of the  $R_f$  values (0.57) in both spots of pure and standard IAA in the results of TLC. Similarly, our results showed  $R_f$  value of 0.61 for the standard and raw IAA spots. This state may be due to the free nature of this auxin [30]. Accordingly, studies on other isolated [31] reported similar  $R_f$  values for spots of the raw-extracted IAA and standard IAA.

After presenting and discussing the results of this study, it is clear to us that the specialized *S. meliloti* which infected with legume plant (*Medicago sativa*) has a high ability to produce IAA when adding tryptophan-L to the culture medium. The optimal laboratory conditions for the production of the highest concentration of IAA were the support of the YEM medium with a concentration of 0.1% tryptophan-L, as well as adding 10% of glucose or lactose. The 72-hour incubation period was better than that of 24-hour in obtaining an additional amount of IAA, with the produced amounts ranging between 0.6 and 0.7 g / l.

Through what was obtained from the significant results of this study, we recommend the production of the growth regulator indole acetic acid (IAA) at the commercial level and exclusively from the wild type of the bacteria *S.meliloti*, because of its excellent ability to produce IAA in huge quantities as compared to other studies published in this field.

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