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Analysis of Indole-3-acetic Acid (IAA) Production in *Klebsiella* by LC-MS/MS and the Salkowski Method

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[Abstract] Many rhizobacteria isolated from plant rhizosphere produce various phytohormones in the form of secondary metabolites, the most common of which is Indole-3-acetic acid (IAA). Here, we detail analytical protocols of IAA detection and quantification, *in vitro* and *in situ*, as recently applied to *Klebsiella* SGM 81, a rhizobacterium isolated from the rhizosphere of *Dianthus caryophyllus* (a commercially important flower across the globe). Specifically, we describe a detailed protocol for a colorimetric assay using the Salkowski reagent method, which can be used to screen for the presence of Indole compounds. To further detect and quantify IAA, a highly accurate analytical approach of LC-MS/MS is used. To detect the presence of IAA around the root system of *Dianthus caryophyllus*, *in situ* staining of plant roots is done using Salkowski reagent.

Keywords: Indole-3-acetic acid, *Klebsiella*, Salkowski reagent, LC-MS/MS, Tryptophan, Spectrophotometer

[Background] The bacterial auxin in the form of Indole-3 Acetic Acid (IAA) is a product of L-tryptophan metabolized by bacteria (Lynch, 1985). The group of bacteria known as plant growth promoting rhizobacteria (PGPR) specifically residing in the vicinity of the roots depend on tryptophan being present in the root exudates of plants (Kravchenko *et al.*, 2004; Kamilova *et al.*, 2006). These PGPR use IAA as a signal to interact with plant roots and to colonize the plant parts. This signaling feature of IAA is thought to effect on the physiology of the bacteria (Spaepen *et al.*, 2007).

Different methods are found in the literature to detect the biosynthesis of IAA. Gordon and Weber (1951) were the first to provide a colorimetric assay using Salkowski reagent for the detection of IAA. This method has since been widely used for detecting IAA from microorganisms. Salkowski reagent is a mixture of 0.5 M ferric chloride ($FeCl_3$) and 35% perchloric acid ($HClO_4$) which upon reaction with IAA yields pink color, due to IAA complex formation with and reduction of Fe^{3+} (Kamnev *et al.*, 2001). The color developed by positive reaction indicates the presence of various indole compounds as a product of tryptophan metabolism. Apart from the colorimetric assay, other methods for IAA estimation from bacteria and plant are High Performance Liquid Chromatography (HPLC) (Perrig *et al.*, 2007), Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC-ESI-MS/MS) (Chiwocha *et al.*, 2003), and by High Performance Thin Layer Chromatography HPTLC (Goswami *et al.*, 2015). Liquid Chromatography (LC) is the preferred approach to determine the concentration of IAA and to confirm its purity with high accuracy and standardization. LC coupled with various mass spectrometry

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detectors are powerful tools for IAA analysis. Because of the high sensitivity and selectivity, Mass Spectrometry detectors are most commonly coupled with LC. One of the important benefits of LC-MS is that analysis and separation of compounds can be achieved in a continuous manner eliminating the step of purification (Kallenbach *et al.*, 2009).

Materials and Reagents

1. 100-1,000 µl pipette tips (Sigma-Aldrich)
2. Test tube racks (Sigma-Aldrich, catalog number: Z334146)
3. Screw-cap tubes and caps (Sigma-Aldrich, catalog number: AXYSCT10MLS)
4. Pyrex test tube 20 ml (Sigma-Aldrich, catalog number: CLS980016)
5. UV quartz cuvette semi-micro square, 1.4 ml, PTFE stopper (Sigma-Aldrich, catalog number: Z276707)
6. Sterile syringe filters with a pore size of 0.2 µm (Fisher Scientific, catalog number: 720-1230)
7. Centrifuge tube with screw cap, capacity 10 ml (Sigma-Aldrich, catalog number: SIAL301NN10R)
8. Pyrex glass serological pipettes, capacity 5 ml (Sigma-Aldrich, catalog number: Z653829)
9. Amber storage bottles (Sigma-Aldrich, Supelco, catalog number: 23230-U)
10. *Klebsiella* SGM 81 strain (isolated from the rhizosphere of *Dianthus caryophyllus*)
11. Distilled water
12. Nutrient broth (Microbiology grade) (Sigma-Aldrich, catalog number: 70122-500G), its composition and final pH see below:

Grade	For microbiology
Composition	D(+)-glucose, 1 g/L
	Peptone, 15 g/L
	Sodium chloride, 6 g/L
	Yeast extract, 3 g/L
Final pH	7.5 ± 0.2 (25 °C)

13. $FeCl_3$ reagent grade (Sigma-Aldrich, CAS: 7705-08-0)
14. Perchloric acid 70% (Sigma-Aldrich, CAS: 7601-90-3)
15. Methanol (laboratory grade) (Sigma-Aldrich, CAS: 67-56-1)
16. HCl (ACS grade) (Sigma-Aldrich, CAS: 7647-01-0)
17. L-tryptophan (traceCERT grade) (Sigma-Aldrich, CAS: 73-22-3)
18. Acetic acid glacial (Sigma-Aldrich, CAS: 64-19-7)
19. Ethyl acetate (grade anhydrous) (Sigma-Aldrich, CAS: 141-78-6)
20. Indole-3-Acetic acid (IAA) (Sigma-Aldrich, CAS: 87-51-4)

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21. Murashige and Skoog medium (Sigma-Aldrich)
22. Acetonitrile
23. Ammonium formate
24. CH_3CN
25. Formic acid
26. Ethanol
27. Agarose
28. Culture media (see Recipes)
29. Salkowski reagent (see Recipes)

Equipment

1. Spatula (Thermo Fisher, catalog number: F36711-0012)
2. Nichipet Eco pipette, 100-1,000 µl volume (Sigma-Aldrich, catalog number: Z710199)
3. Measuring cylinder (Sigma-Aldrich, catalog number: Z324361-2EA)
4. 250 ml conical flask (Sigma-Aldrich, catalog number: Z723088-1EA)
5. Rotary Vacuum Flash Evaporator (Buchhi Type) (Jain Scientific Glass, 1188)
6. Balance
7. Agilent 1100 LC system and an ABSciex 6500 Qtrap MS [Chromatography was on a Phenomenex Luna C18 column (100 mm x 2 mm x 3 µm)]
8. UV-spectrophotometer (Systronics, 166)
9. Microcentrifuge (Thermo scientific, Pico 17)
10. Magnetic stirrer (Remi, 1 MLH)
11. Vortex cyclo mixer (Remi, CM 101)

Software

1. Analyst 1.6.1 (AB Sciex)

Procedure

A. IAA screening and quantification from *Klebsiella* SGM 81 using Salkowski reagent method
   1. Culture preparation
      a. Take preculture medium (without tryptophan) and inoculate it with a single colony of SGM 81 strain, or a loopful of glycerol stock of the strain.
      b. Incubate the inoculated pre-culture medium at 30 °C, 120 rpm overnight. This will result in the young culture of the *Klebsiella* SGM 81 strain which can be used for inoculating IAA production medium (test media).
      c. Add 100 µl of the young culture in test medium and vortex it to get a uniform suspension.

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d. Incubate it under dark conditions (by wrapping the container with newspaper/aluminum foil) at 30 °C in shaking condition at 120 rpm.
e. Incubation lasts for 24-96 h in dark condition depending on IAA production ability of the strain. *Klebsiella* SGM 81 produces IAA till 72 h and subsequent decrease in the production in recorded after that.

2. Indole quantification using Salkowaski reagent
   a. After 24 h, take out 1.5 ml of sample culture broth and transfer to an Eppendorf tube.
   b. Centrifuge at 16,278 *x g* for 5 min using a microcentrifuge.
   c. Carefully withdraw 1 ml supernatant and transfer to a new test tube.
   d. Mix equal volume (1 ml) of Salkowski reagent, vortex gently and incubate the reaction at 30 °C in a dark condition for 30 min.
   e. In a new test tube replace the supernatant with 1 ml of control uninoculated medium and mix with 1 ml of Salkowski reagent. This serves as blank.
   f. The presence of IAA is detected by measuring pink color development after 30 min (some strains may develop red color as shown in Figure 1).

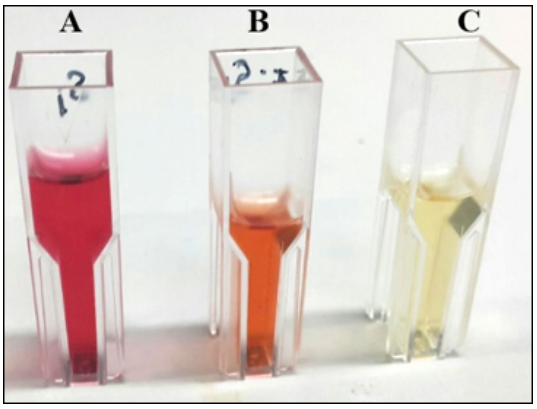

Figure 1. Color development due to Indole-Salkowski reagent reaction. A. Klebsiella SGM 81. B. SGM09. C. Control uninoculated media.

   g. Use uninoculated medium to set the blank. Measure the color intensity spectrophotometrically at 536 nm using cuvette.
   h. Compare the optical density of the test sample with a standard IAA curve (10-100 $\mu g \cdot ml^{-1}$) to calculate the concentration.
   i. Repeat the process at similar intervals until the optical density begins to decrease, indicating no further production of IAA.

B. Analysis of IAA by liquid chromatography-mass spectrometry (LC-MS/MS)
   1. Sample preparation
      a. Prepare 100 ml nutrient broth medium supplemented with 0.5 g of L-tryptophan in a 250 ml Erlenmeyer flask.

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b. Add 100 µl of the young culture in test medium and vortex it to get a uniform suspension.
c. Incubate it under dark conditions (by wrapping the container with newspaper/aluminum foil) at 30 °C in shaking condition at 120 rpm for 72 h.
d. To separate the cells, centrifuge at 4 °C for 20 min at 2,800 *x g* and collect 1,000 ml supernatant.
e. Transfer the supernatant to a 250 ml screw-cap glass bottle. Acidify it by adding 2-3 drops of 1 N concentrated HCl to reach pH 2.5-3.
f. To extract IAA, add double the volume of ethyl acetate to the acidified supernatant and shake vigorously for 5 min (alternatively, a separating funnel can be used). Let the mixture stand at room temperature for 10 min to get the top layer of ethyl acetate. Use this layer for further treatment.
g. In a rotary evaporator, set water bath temperature to boiling temperature of ethyl acetate *i.e.*, 77.1 °C (alternatively, ethyl acetate phase can be vacuum dried in a rotational evaporator at 40 °C). The time for drying of the solvent system varies according to the sample volume.
h. Transfer the ethyl acetate layer to the round bottom flask and adjust its rotation to avoid any bumping of the liquid sample. “Bumping is the phenomenon in chemistry where homogenous liquids boiled in a test tube or other container will super heat and, upon nucleation rapid boiling will expel the liquid from the container (Wikipedia)”–This would leave the IAA escaping the flask into the condenser tube attached with the round bottom flask.
i. Upon complete evaporation of the liquid ethyl acetate, pure IAA is left behind in the crystalline form attached to the bottom of the rotary flask.
j. Switch off all the units of the rotary evaporator and remove the round bottom flask. Re-dissolve the crystalline IAA in 5 ml of 20% methanol and store at -20 °C for future use.

2. LC-MS/MS analysis
   a. Filter the stored methanol extract (100 µl) using sterile syringe filters of 0.2 µm so as to separate insoluble particles and larger compounds if any. This is the final analysis sample.
   b. Phenomenex Luna C18(2) column (100 mm x 2 mm x 3 µm) at temperature of 50 °C is used as chromatography system.
   c. A gradient solvent system of 10% solvent A (2% acetonitrile, 10 mM ammonium formate, pH 4.2) to 90% solvent B (94.9% CH_3CN, 5% H_2O, 0.1% formic acid) is used for the column over 10 min at a flow rate of 250 ml/min.
   d. Wash the column with 90% solvent B for 3 min and then re-equilibrate with 90% solvent A for 6 min.
   e. Use 10 µl injections to load the sample for analysis.
   f. The MS is configured with a Turbo Spray Ion Drive source where the source temperature is set to 500 °C and the ion spray voltage to 5,500 V.
   g. Analyze IAA by Multiple Reaction Monitoring (MRM) in positive mode using a transition of 176 > 130 with collision energy of 20 eV. Transition of 176 > 130 means the specific pairs

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of mass to charge (m/z) values associated to the precursor and fragment ions. In our case the Q1 is set to a value of 176 amu and the Q3 to 130 amu, the collision energy was 20 mV.

h. Set the declustering potential, exit potential and collision cell exit potential at 30 V, 10 V and 10 V respectively.

C. *In Situ* Salkowski staining

1. Prepare Murashige and Skoog (MSM) basal salt medium with 0.8% agarose, devoid of plant hormone supplements.
2. Sterilize the *D. caryophyllus* seeds using three-step procedure: a 1 min wash in 70% ethanol, followed by a 4 min wash in 20% NaClO, and a final rinse in sterile distilled water 3 times.
3. Allow the *D. caryophyllus* seeds to germinate on the MS media by incubating the plates at 25 °C in 14 h light and 10 h dark cycles in a plant culture room, until the root length reaches 2 cm (approximately six days).
4. Treat the germinated plant roots by immersing the root tips into 5 ml of the bacterial suspension with titre 10^5 CFU ml^{-1} in the universal tube.
5. Transfer the treated plants onto new MS agar medium devoid of IAA and sucrose in square Petri plates (120 mm x 120 mm x 15 mm).
6. Again, incubate the plates at 25 °C in 14 h light and 10 h dark cycles in a plant culture room.
7. After two weeks of infection, stain the treated roots with Salkowski reagent.
8. To stain the roots, add 400 µl of Salkowski reagent on each root and observe any visible color change.
9. The development of pink color as shown in the figure below indicates the presence of proximal IAA on and around the roots (Figure 2).

Figure 2. *In situ* Salkowski staining on plant roots treated with *Klebsiella* SGM 81 of 10^5 CFU ml^{-1} titre

Data analysis

1. Take the optical density of the samples at 536 nm and graphically calculate the concentration of IAA based on the standard curve. IAA estimation from *Klebsiella* SGM 81 strain, using

Salkowski reagent shows a maximum yield to 215 µg·ml^{-1} detected after 72 h with 0.15% tryptophan (Figure 1a in Gang *et al*., 2018).

2. For LC-MS/MS analysis, confirmation of the identity in the samples is done by Enhanced Product Ion scans.
3. Data acquisition and analysis is done with Analyst 1.6.1 (AB Sciex).
4. Quantification is carried out by comparing the test sample data with control standard IAA.
5. Overlapping retention times during LC (5.90 min and 5.92 min) and identical m/z ratios (130.1) between a commercial IAA standard and sample supernatant showed *Klebsiella* SGM 81 to produce substantial amounts (960 µg·ml^{-1} after 72 h) homogeneous IAA (Gang *et al.*, 2018).
6. Development of pink color on the Salkowski treated roots indicates the presence of bacterial IAA proximal to the roots (Figure 3a in Gang *et al.*, 2018).

Notes

1. The Salkowski reagent method discussed in this paper is shown using nutrient broth liquid medium (supplemented with L-tryptophan) which is non-specific liquid medium for most gram-positive and gram-negative bacteria. However other media like Yeast extract mannitol (YEM), Pikovskaya's broth (supplemented with L-tryptophan) can be used if IAA production and quantification is to be done from Rhizobium or phosphate solubilizing bacteria respectively.
2. The preculture preparation is important to normalize the initial cell count especially when a comparative study of IAA quantification is carried out for the optimization process.
3. Salkowski reagent method quantifies the overall Indole present in the sample. To get the purity and conjugants of Indole-3-acetic acid (IAA), other analytical methods like HPLC, LC-MS/MS, HPTLC *etc*. can be used.

Recipes

1. Culture media
   a. Making use of measuring cylinder, measure 100 ml distilled water and transfer it into a 250 ml Erlenmeyer flask or screw-capped glass bottle. Two other flasks of the same media, with and without tryptophan will be used as control and pre-culture media (used to activate the SGM 81 culture to yield young culture) respectively
   b. For simple liquid nutrient broth medium, weigh 1.3 g of Nutrient broth and dissolve it in 100 ml distilled water. Stir it properly so as to mix the powder in distilled water properly
   c. Weigh 0.15 g of L-tryptophan (0.15% w/v) and add to the prepared nutrient broth flask. This is liquid nutrient broth with tryptophan supplement
   d. Autoclave the prepared media at 121 °C for 15 min
   e. Allow the media to cool down and come to room temperature before inoculation so as to ensure the survival of inoculum

2. Salkowski reagent
   a. 0.5 M of 100 ml ferric chloride (dissolve 8.125 g of $FeCl_3$ in 100 ml of distilled water)
   b. To dilute readily available perchloric acid, measure 24.5 ml (v/v) of distilled water in a measuring cylinder and add 24.5 ml (v/v) of concentrated acid to it
   c. Add 1 ml (v/v) of 0.5 M ferric chloride solution to 49 ml of 35% perchloric
   d. Mix well and store in a dark brown bottle at room temperature. It can be used for further experiments if stored under described conditions

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Competing interests

We declare no conflict of interest.

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