IJMPR 2022, 6(8), 51-57

International Journal of Modern Pharmaceutical Research
www.ijmpronline.com

ISSN: 2319-5878
IJMPR
Research Article

SJIF Impact Factor: 5.273

ISOLATION OF *AZOTOBACTER* FROM DROUGHT PRONE AREA AND STUDY ON ITS PLANT GROWTH PROMOTING ACTIVITIES

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Received on: 12/07/2022
Revised on: 02/08/2022
Accepted on: 22/08/2022

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ABSTRACT

As global warming is increasing, the world is facing the problem of water scarcity. This affects the crop yield. Large area of India is under drought condition so giving low crop yield. Crop yields are more dependent on adequate water supply than any other single environmental factors. The plants found in arid and semiarid area require low water for their growth. Some drought resistant microorganisms may yet established in such region. Isolation of such microorganisms, its mass production and inoculation in drought region may definitely help to increase the crop yield. Such microorganisms may produce plant growth promoting compounds near rhizosphere, which help for growth of plant and crop yield. Plant growth promoting properties of these organisms, like, Nitrogen fixation, production of IAA, gibberline, cytokinins, exopolysaccharide, phosphate solubilization, nitrogen fixation, production of siderophore and antifungal compounds can be studied. Present study is for isolation of free living Azotobacter from drought prone area of Dist. Sangli (MH), and its plant growth promoting activities, phosphate solubilization, IAA production and its media optimization. Isolates obtained show increasing IAA production at pH 9 in presence of arabinose and at tryptophan conc. 400µg/ml.

KEYWORDS: Drought, IAA, *Azotobacter*, nitrogen fixation.

INTRODUCTION

Drought is one of the major problems related to agricultural productivity. Water stress in arid and semiarid regions of the world is associated with plant growth which results in less growth as well as low product yield. As the world's population is increasing constantly, the demand of food is also increasing. To fulfill this increasing demand, new methods to increase crop yield are studied by many workers in the world. Many bacteria colonize in rhizosphere and remain attached to plant roots and promote plant growth. Bacteria can survive under water stress conditions by some mechanisms like production of capsule. Capsules are made up of exopolysaccharide, which retain water and regulate diffusion of organic carbon sources (Hepper, 1975, Wilkinson, 1958, Roberson and Firestone, 1992). Exopolysaccharides possess unique water holding and cementing properties, thus play a vital role in the formation and stabilization of soil aggregates and regulation of nutrients and water flow across plant roots through biofilm formation (Roberson and Firestone, 1992; Tisdall and Oadea, 1982)

Besides, these bacteria can also offer plant protection against desiccation by maintaining moist environment. These bacteria are helpful for plant growth by many mechanisms like production of plant growth hormones, phosphate solubilization, nitrogen fixation, production of siderophore and antifungal compounds. Phytohormones like, indole acetic acid, gibberline, cytokinins are helpful for increasing plant growth and product yield. Microorganisms such as phosphate solubilizers and potash mobilizer can play a vital role in release of available forms of phosphorous and potash from insoluble forms present in natural pool (Sajid Mohammad). Out of the three forms of potassium found in the soil, soil minerals make up more than 90 to 98 per cent of soil potassium (Ahmad, 2010). The soil microorganisms play an important role in making these minerals in the available form for plants.

Present study is aimed to isolate *Azotobacter* from drought prone area and its characterization for plant growth promoting activities. *Azotobacter* is free living nitrogen fixing diazotrophic bacteria producing phytohormones, IAA and many biochemicals which are important for plant growth. It can solubilize phosphate and potash. Alginate production is another property of *Azotobacter*. It was postulated that the compact alginate layer formed around the cell acts as a diffusion barrier for oxygen, which limits its transfer to the extremely oxygen sensitive nitrogenase enzyme.

Isolated *Azotobacter* is studied for its potential for plant growth promotion like in vitro production of IAA, solubilization of phosphate and potash so that it can be used as a biofertilizer in drought prone area. Optimization of media for in vitro IAA production was also studied.

MATERIAL AND METHOD

1. Collection of soil sample: Soil samples were collected from drought prone area of Sangli, (MH), India. These samples were collected from rhizosphere zone of sorghum crop at harvesting stage. Samples at different locations are mixed together in sterile container and immediately stored at cool temperature.

2. **Isolation of *Azotobacter* from soil samples**: For isolation of *Azotobacter*, Nitrogen free isolation broth (Atlas manual) was inoculated and incubated at R.T. (28-29^{o}C) for 7 days. Enriched broth was serially diluted up to 10^{-7} and spread inoculated on *Azotobacter* isolation media plates (Atlas manual) and incubated at R.T. for 3-4 days. After incubation, off-white, mucoid, glistening colonies appeared on the media plates. The isolated colony was purified by sub culturing and confirmed for colony morphology, Gram's nature, capsulation and motility. The pure cultures were retained on N_2-free Maintenance agar slope (Atlas manual). Amongst obtained isolates, four isolates were used for current study.

3. Biochemical characterization: Biochemical characters of isolated organisms were studied as per Bergey's Manual of Systematic Bacteriology Vol. II (2nd edition).

Sugar fermentation (glucose, maltose, dextrose, mannitol, sucrose, arabinose), urease, starch hydrolysis, antibacterial activity of streptomycin (0.2µg/ml), neomycin (1µg/ml), kanamycin (1µg/ml) and $HgCl_2$ (10µg/ml), catalase, ammonia production.

4. Phosphate solubilization ability of isolates

4.1 Phosphate solubilization test

Pikovskaya's (PK) media plates were spot inoculated with isolates under study and incubated at R.T. for 3 days and observed for phosphate solubilizing clear zone around colony. Efficiency was studied by recording diameter of zone.

4.2 Phosphate solubilization assay

50ml of PK media in 250ml flask were inoculated using 100µl inocula (3×10^{8}CFU/ml) and incubated at 30^{0}C for 5 days on rotary shaker at 180 rpm. Un-inoculated media was used as control. After incubation, centrifugation was done at 6000rpm for 15 min, and 500 µl supernatant was taken in separate tube, to which, 500 µl trichloroacetic acid (10% v/v) & 4ml color reagent (3M H_2SO_4, 2.5% ammonium molybdate, 10% ascorbic acid, distilled water 1:1:1:2) was added and incubated at R.T. for 15min and optical density was recorded at 820 nm. The results were compared with Standard curve of KH_2PO_4.

5. Potash solubilization activity: Aleksandrov Agar media (Hi-Media) plates were spot inoculated by isolates and incubated at R.T. for 3days and observed for clear zone of potash solubilization. Efficiency was studied by recording diameter of zone.

6. Alginate production and cell dry mass

6.1 Alginate production

Alginate production was done by using following media: (g/l) sucrose, 20; $(NH_4)_2SO_4$,0.6; $MgSO_4.7H_2O$,0.3; yeast extract, 6.0; pH was adjusted at 7.2 Sterile broth media (50ml) was inoculated by 10% (v/v) fresh culture (10^{4}CFU/ml), incubated at 28^{0}C at 180 rpm for 96 hrs on rotary shaker. Alginate production and dry cell mass was determined by method described by Husan Saba (2013) and E. Parente (1998) with some modification. After incubation, 1ml NaCl (5.0M) and 2ml disodium salt of EDTA was added and shaken for 5min. Then centrifugation was done at 10,000 rpm at15^{0}C for 15 min. Precipitate obtained was used for determination of dry cell mass. Supernatant was taken in separate tube and three volumes of ice cold isopropanol was added and centrifuged as above. The precipitate thus obtained was collected on Whatman filter paper no.1, dissolved in water. Precipitation step was repeated and final precipitate was dissolved in water.

6.2 Determination of cell dry mass: Precipitate obtained in above first step was used for determination of cell dry mass. Precipitate was washed with water, re-centrifuged and dried at 80^{o}C for 24hrs and dry weight was measured.

7. IAA production and media optimization

7.1 IAA production

IAA produced by isolates was determined by the method described by Mathurot C. (Curr.Microbiol. 2011). N2 free broth medium containing 0.2% L-tryptophan was inoculated with 1% suspension (10^{8}CFU ml^{-1}) of isolates separately and incubated at R.T. (28-29^{0}C) for 7 days on rotary shaker. Ability of isolates to produce IAA was determined by Salkowski's method. Briefly, incubated broth was centrifuged at 3000 rpm for 30 min. Then 2ml of supernatant was mixed with 2 drops of ortho-phosphoric acid & 4 ml of Salkowski reagent, kept in dark at R.T. for 30 min., absorbance was taken at 530nm. The amount of IAA produced was extrapolated from the standard curve.

7.2 Preparation of standard curve

For standard curve 100µg/ml standard stock solution of IAA was prepared in 50% ethanol. Standard concentrations of IAA used were 10-100µg/ml.

7.3 Extraction of crude IAA: The supernatant obtained after centrifugation was used to extract the crude IAA from broth. Supernatant was acidified to 2.5 to 3 with

HCl (1N) (Vikram Patil, Recent Research in Science and Technology, 2011) and extracted with ethyl acetate. Ethyl acetate was taken at double the volume of supernatant. After extraction, ethyl acetate fraction was separated and dried at 40^0C and dissolved in methanol (Mathurot C. 2011, Curr. Microbiol).

7.4 Effect of conc. of tryptophan on IAA Production: N2 free media containing different conc. of tryptophan (200, 400, 600, 800, 1000 µg/ml) was used for this study. 1% culture broth (10^8CFU ml^{-1}) was inoculated and incubated at R.T. for 72 hrs and production of IAA was determined by Salkowski's method as described above. Standard graph of IAA was done (10-100µg/ml).

7.5 Effect of various C sources on IAA production: Effect of various sugars was studied by using N2 free media containing sugars like sucrose, lactose, arabinose, mannose, maltose, glucose in separate flasks. Each flask was amended with 1% tryptophan. All the flasks were inoculated with 1% of 48hrs old culture suspension ($O.D._{600}$=1). The flasks were incubated at R.T. for 7 days on rotary shaker at 180rpm. IAA produced was determined by Salkowski's method as described above.

7.6 Effect of various pH on IAA production The effect of pH was studied by adjusting pH at 5,6,7,8 & 9 in each flask amended with 1% tryptophan and sucrose as carbon source. All the flasks were inoculated with 1% of 48hrs old culture suspension ($O.D._{600}$=1). The flasks were incubated at R.T. for 7 days on rotary shaker at 180rpm. IAA produced was determined by Salkowski's method as described above.

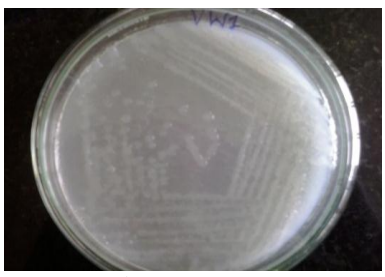
RESULT AND DISCUSSION

Isolation and identification of Isolates

Isolation of *Azotobacter* from soil samples was carried out by serial dilution and streak plate technique on nitrogen free Ashbey's broth and agar media respectively. After 3-4 days of incubation, on N2 free media plates white, mucoid water drop like colonies were observed, characteristically of *Azotobacter*. Isolated colony was used to study colony morphology and biochemical characterization as per Bergey's Mannual of Systematic Bacteriology. This gives the tentative identification of isolates as *Azotobacter*. Four isolates showing characteristic *Azotobacter* morphology were used for further study.

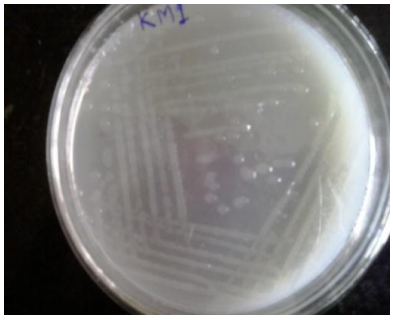
Table 1: Colony morphology & Biochemical characterization.

Character/Test	**Vw1**	**Vw2**	**KM1**	**KM3**
Color of colony	Off white	Off white	milky white	milky white
Gram nature	Gram negative	Gram negative	Gram negative	Gram negative
Motility	Motile	Motile	Motile	Motile
Glucose	+	+	+	+
Maltose	+	+	+	+
Dextrose	+	+	+	+
Mannitol	+	+	+	+
Sucrose	+	+	+	+
Arabinose	+	+	+	+
Urease	+	+	+	+
Starch hydrolysis	+	+	+	+
Antibacterial activity				
1)Streptomycin (0.2µg/ml)	+	+	+	+
2)Neomycin (1µg/ml)	+	+	+	+
3)Kanamycin(1µg/ml)	+	+	+	+
4)HgCl2 (10µg/ml)	+	+	+	+
Catalase	+	+	+	+
Ammonia production	+	+	+	+
Nitrate reduction	+	+	+	+
Phosphate solubilization	+	+	+	+
Potash solubilization	+	+	+	+

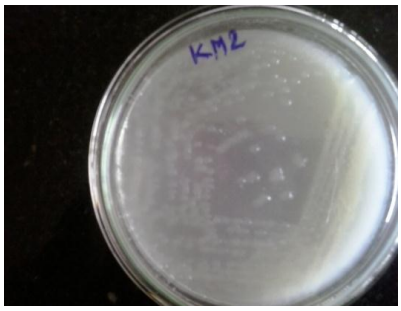


a) Vw1

b) Vw2



c) KM1



d) KM2

Fig.1 Isolation of *Azotobacter* on N2 free media plates.

Phosphate solubilization Test

PVK media plates were spot inoculated using isolates & incubated at R.T. for 3 days & observed for clear zone surrounding to growth. All the four isolates show clear zone of phosphate solubilization. Highest phosphate solubilization activity was shown by VW2 (2.25mm) followed by VW1 (2mm). KM1 and KM2 show less phosphate solubilization activity, 1.3mm & 1.2mm respectively. It indicates that all the four isolates have phosphate solubilization capacity. The insoluble phosphate in soil can be made available to the plants by the activity of these isolates.

No.	Zone diameter (mm)(D)	Diameter of growth(mm)(d)	Ratio(D/d)
VW1	18	9	2
VW2	18	8	2.25
KM1	12	9	1.3
KM2	12	10	1.2

Phosphate solubilization assay

The measurement of phosphate solubilization in broth gives more reliable results than plate method (Johri et al 1990), so the isolates were tested for their ability to solubilize tri-calcium phosphate in broth. For this, PVK broth was inoculated using 100µl culture inocula (3×10^8 CFU/ml) separately and incubated at 30^0C for 5 days on rotary shaker at 180 rpm and then phosphate solubilization was studied spectrophotometrically & compared with std. KH_2PO_4. Phosphate solubilization activity of VW1 was 176.56µg/ml and that of VW2 was 162.34µg/ml.

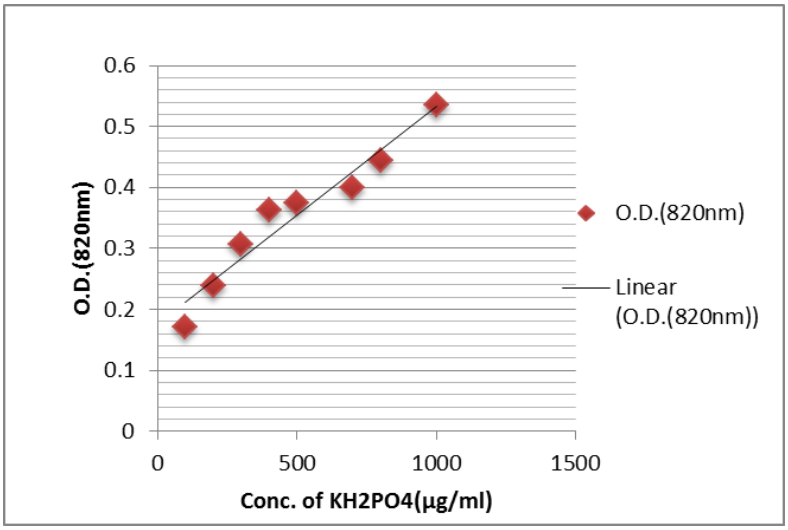


Fig. Standard curve of KH_2PO_4

Absorbance of cultures at 820nm

Isolates	O.D. (820nm)
VW1	0.211
VW2	0.194
Uninoculated	0.088

Potash solubilization activity

Aleksandrov media plates were spot inoculated by isolates and incubated at R.T. for 3days and observed for clear zone of potash solubilization. It was found that, both the isolates can solubilize potash. Both the isolates Vw1, Vw2 show almost equal activity of potash solubilization, VW2 show slightly larger zone of potash solubilization(1.4mm) than VW1(1.37mm). The isolates can provide three of important nutrients, NPK, for plant growth.

Isolate	Diameter of clear zone (mm) (D)	Diameter of growth(mm) (d)	Ratio D/d(mm)
Vw1	11	8	1.37
Vw2	13	9	1.4

Alginate production and cell dry mass

	After24 hrs. (g L^{-1})	After 5 days (g L^{-1})
Alginate VW1	0.0290	7.6
Dry cell mass VW1	0.020	0.82
Alginate VW2	0.0304	7.2
Dry cell mass VW2	0.016	0.8

Alginate was produced by inoculating N2 free broth & incubating on shaker incubator. Separation of alginate was done by using EDTA & NaCl, after centrifugation precipitated by ice cold isopropanol. After five days of incubation, alginate produced and cell dry mass by VW1 was 7.6 g L^{-1} & 0.82 g L^{-1} and that of VW2 was 7.2 g L^{-1} & 0.8g L^{-1} respectively.

7. IAA production

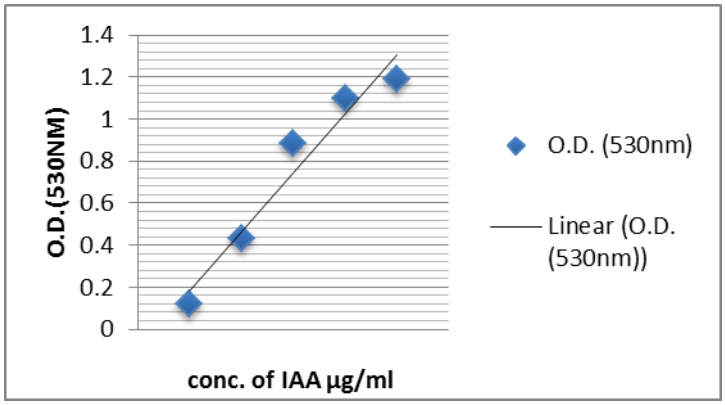
For IAA production N2 free broth was inoculated using 1% suspension (10^8CFU ml^{-1}) amended with 0.2% tryptophan and estimated by Salkowski's method. Standard curve was prepared using conc. of tryptophan ranging from 20-100 µg/ml. From the standard graph, conc. of IAA produced by VW1 was 8µg/ml and that of VW2 was 43µg/ml when media was supplied with 0.2% tryptophan. Tryptophan is a precursor for IAA production.

Effect of conc. of tryptophan on IAA production

It was found that media amended with 400µg/ml tryptophan show highest IAA by VW1 (44 µg/ml) followed by1000 µg/ml (29 µg/ml). Concentration of tryptophan like 800 µg/ml, 600 µg/ml and 200 µg/ml show sequentially decreasing IAA production; 12, 11, 8 µg/ml respectively. This reveals that VW1produce highest IAA in vitro if 400 µg/ml tryptophan is added. In case of VW2, highest IAA production was 37µg/ml if 1000 µg/ml tryptophan is added in broth. This was followed by 600 µg/ml(20 µg/ml IAA), 400 µg/ml(15 µg/ml IAA), 800 µg/ml(14 µg/ml IAA) and 200 µg/ml(10 µg/ml IAA)

Conc. of tryptophan (µg/ml)	O.D.at 530nm VW1	O.D. at 530nm VW2
200	0.102	0.120
400	0.517	0.183
600	0.130	0.235
800	0.147	0.178
1000	0.340	0.445

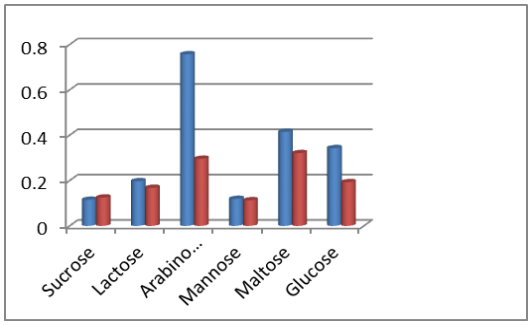
Standard graph of IAA



Effect of carbon source

Effect of various carbon sources on IAA production by isolates VW1 & VW2 was studied. For this study, N2 free broth media containing different sugars as a carbon source like sucrose, lactose, arabinose, mannose, maltose, glucose was prepared. After incubation produced IAA was estimated by Salkowski's method. It was found that VW1 produces highest conc. of IAA utilizing arabinose as a carbon source (0.63 g/L IAA). It produces moderate conc. of IAA using maltose & glucose; 0.35 & 0.29 g/L respectively, while lowest IAA using sucrose and mannose; 0.09 g/L each. VW2 produces highest IAA using mannose: 0.9 g/L followed by maltose and arabinose; 0.27 & 0.25 g/L resp. Lowest IAA produced by VW2 using glucose, lactose and sucrose; 0.16,0.14,0.1 g/L resp.

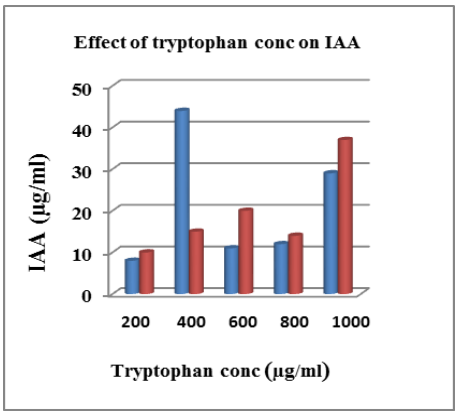
Effect of carbon source on IAA production



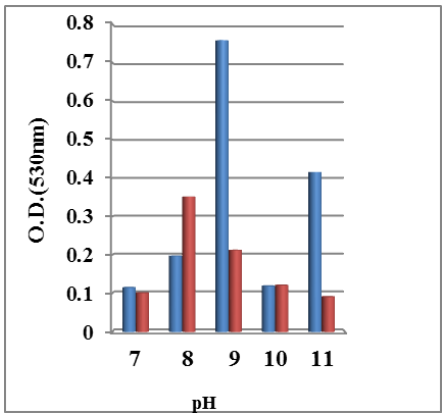
VW1: blue, VW2: red

Effect of tryptophan concentration on IAA

N2 free media containing different conc. of tryptophan (200, 400, 600, 800, 1000 µg/ml) was used for this study. After incubation produced IAA was estimated by Salkowski's method as mentioned above. It was found that VW1 produces highest conc. of IAA (0.044µ g/L IAA) using 400 µg/ml tryptophan and that VW2 produces highest conc. of IAA (0.037µg/L IAA) using 1000 µg/ml tryptophan**.**



Effect of various pH on IAA production The effect of pH was studied by adjusting pH at 5,6,7,8 & 9 in each flask amended with 1% tryptophan and sucrose as carbon source. After incubation, produced IAA was estimated by Salkowski's method. It was found that VW1 produces highest conc. of IAA at pH 9 and VW2 at pH 11.



CONCLUSION

Characterization of *Azotobacter* sp. Isolated from drought aera was done. It was found that isolate VW1 produces highest amount of IAA by utilizing arabinose as carbon source at pH 9 in presence of tryptophan conc. 400µ/ml while, isolate VW2 produce highest amount of IAA by utilizing maltose as carbon source at pH 8 in presence of tryptophan conc. 1000µ/ml. According to present study, isolate VW1 is more efficient for production of plant growth promoting components which help to increase crop yield in drought area. VW2 is also producing plant growth promoting components. Both the isolates can solubilize phosphate and potash which are made available to plant. Large production and inoculation of these isolates as biofertilazer can increase the growth of crop in drought prone area.

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