International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 11, Issue 7, 2019, pp.-1645-1650.
Available online at https://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

Research Article

INDUCTION OF AGGREGATION IN PLANT GROWTH PROMOTING *RHIZOBACTERIA*

SAMATHA G.[1], KUMUTHA K.*[1], ANANDHAM R.[2], KOKILA DEVI E.[3], KRISHNAMOORTHY R.[4] AND SAI APARNA N.[2]

[1]Department of Agricultural Microbiology, Agricultural College and Research Institute, Madurai, 625 104, Tamil Nadu Agricultural University, Coimbatore, 641003, India
[2]Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, 641003, India
[3]Department of Biotechnology, Tamil Nadu Agricultural University, Coimbatore, 641003, India
[4]Department of Crop Management, Vanavarayar Institute of Agriculture, Pollachi, 642 103, Tamil Nadu Agricultural University, Coimbatore, 641003, India
*Corresponding Author: Email - kkumuthatnau@gmail.com

Received: June 30, 2019; Revised: July 24, 2019; Accepted: July 26, 2019; Published: July 30, 2019

Abstract- Bio-inoculants have to overcome several biotic and abiotic stresses for their successful colonization on the rhizosphere region. Such adverse conditions drive the researchers to exploit the abilities of the aggregated bacterial cells in enhancing their survivability in rhizosphere. The main objective of this study is to develop aggregated cells by preparing aggregation inducing medium using fructose and ammonium chloride as carbon and nitrogen sources respectively which resulted in aggregation in high C:N medium visible to the naked eye after 24 hours of inoculation. *Methylobacterium, Azospirillum, Pseudomonas* and *Pantoea* formed aggregated cells whereas no aggregation was noticed in *Bacillus* cultures. Poly-β-hydroxybutyrate granules or inclusion bodies were observed higher in aggregated cells than the non-aggregated cells. Microbial load of the aggregated cells was relatively higher than non aggregated cells which was 4×1011 CFU ml^{-1} with more biomass. Microbial adhesion was also observed more in aggregated *Methylobacterium* cells. Plant growth promoting traits such as IAA, ACC deaminase, phosphate, zinc and silica solubilization were observed more in the aggregated cells than the non aggregated cells. The results revealed the effectiveness of aggregated cells than non aggregated cells on PGP traits.

Keywords- *Methylobacterium, Fructose, Ammonium chloride, Aggregated cells, Plant growth promoting traits*

Citation: Samatha G., *et al*., (2019) Induction of Aggregation in Plant Growth Promoting *Rhizobacteria*. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 11, Issue 7, pp.-1645-1650.

Introduction

Development of bioinoculants for agricultural usage requires strategies that can prevent the rapid decline of inoculant populations and extend the shelf life of formulations for a longer period both under storage conditions and after inoculation into soil [1]. It is desirable to study the physiological state of bioinoculants and its role in survival of the inoculants under stress environment [2]. Various physiological states of bio-inoculants include sporulation, aggregation or flocculated cells [3], accumulation of exopolysaccharides (EPS) [4] *etc*. Among these physiological states, cell aggregation of inoculants can be a promising approach for large scale production and easy harvest from the culture medium. Bacterial aggregation or auto-agglutination is a wide spread phenomenon in the microbial world. It can be defined as the gathering or clumping of bacterial cells in liquid culture to form a stable, contiguous, multicellular association or a large unit under certain physiological conditions. The surface of the bacteria plays an important role in the bacterial aggregation. Aggregation in turn plays a positive role in affecting the bacterial dispersion and survival in soil and their ability to adhere to plant surfaces [5]. However, the production of aggregated cells is influenced by various chemical and physical factors [6, 7]. Inoculants with PGP traits can induce plant growth and development by increasing the availability of nutrients to plants, release of plant growth substrates, production of antibiotics, siderophore and can induce resistance to abiotic and biotic stress and promoting beneficial plant-microbe symbiosis [8]. Plant growth promoting rhizobacteria like *Azosprillum, Methylobacterium* and *Pseudomonas* were used in this study to produce aggregated cells. Aggregated cells offer a high degree of resistance to dehydration and the survivability was higher. Use of aggregated cells for agricultural purposes is novel and its use in delivery of plant beneficial microorganisms should sustainably enhance crop productivity [9]. Many studies have reported that flocculated cells or aggregated cells maintain higher survival rate of bio-inoculants during storage and inoculation on the spermosphere of the plant. This research was mainly focused on the optimization of the cultural conditions to achieve maximum aggregation and the interaction between carbon and nitrogen sources to bring out aggregation.

Materials and methods

Bacterial strains and growth conditions

Seven bacterial strains such as *Bacillus altitudinis* (SSB4), *Bacillus aryabhattai* (KSBN2K7), *Pseudomonas gessardii* (SSB7), *Pantoea dispersa* (PRPB12), *Azospirillum brasilense* (CW903), *Methylobacterium thiocyanatum* (DSM11490) and *Pseudomonas psychrotolerans* (KTMV7-6) were used. Bacterial growth was estimated by taking optical density values, at different time intervals using spectrophotometer at 600 nm and growth curves were drawn in graph. The cell growth was estimated to determine the log phase of the bacterial culture for inducing aggregation.

Induction of cell aggregation and quantification

For inducing aggregation, bacterial cultures were grown in 250 ml Erlenmeyer flask in 100 ml nutrient broth. Aggregation inducing medium contained (g 1-l) D-fructose, $MgSO_4$, NaCl, $CaCl_2$, K_2HPO_4, KH_2PO_4, yeast extract (Difco), NH_4Cl and microelements as described by [10]. The liquid medium was adjusted to pH 6.8 [11]. Ten ml (8.00 log CFU ml^{-1}) of exponential phase (log phase) bacterial cultures with an initial OD of 0.05 was transferred to a sterile broth separately in

order to produce aggregated cells and incubated in a rotary shaker of 150 rpm at 30º C for 48 h. Aggregates which are present in the liquid culture were transferred to a conical tube and allowed to stand for 15 min. Aggregated cells were settled at the bottom of the conical tube while most of the free cells remained in the liquid suspension. The supernatant or the top layer which contains free cells were collected and its turbidity was measured using spectrophotometer at 540 nm (ODs). The aggregated cells were then mechanically dispersed by treating in a homogenizer (vortex) for 1 min. and the total turbidity was measured (ODt). The aggregation percentage was calculated as follows:

$$\% \, Aggregation = \frac{ODt - ODs}{ODt} \times 100$$

Where ODt = Total optical density after mechanical dispersion and ODs = Optical density of the supernatant after aggregates had settled.

Estimation of aggregated bacterial cell dry weight and cell load

To measure aggregated bacterial dry cell weight (DCW), the weight of the empty centrifuge tube was taken. Then ten ml of the aggregated cells were centrifuged and washed with 0.85% saline solution and fresh weight of the culture was taken (Fw). After that the centrifuge tube was kept in hot air oven at 50±1º C overnight, dry weight (Dw) was taken the next day and recorded as mg ml^{-1} of broth [12]. Simultaneously the population contained in the cell pellet was estimated in nutrient agar medium by spread plate technique.

Quantification of biofilm formation

Biofilm formation assay of bacterial cultures was performed by following [13] on pre-sterilized 96 well flat bottom polysterene microtitre plate in triplicates. 10 µl of the cell suspension was taken from high C:N and low C:N medium having 0.5 OD was inoculated in each well containing 150 µl of nutrient broth and incubated at 30 ± 2ºC for 48 h. Initial cell turbidity in each well was monitored by using a microtitre plate reader at 590 nm. After incubation of 48 h the broth was poured out from the wells and the wells were washed with saline water to remove loosely attached bacteria. Then the plates were air dried and each well was filled with 150 µl of 1% crystal violet solution and kept aside for 45 min. After staining, the plates were rewashed with sterile water for 2-3 times. Formation of purple rings on the side of the wall indicates the formation of biofilm. Quantitative analysis of the biofilm formation was assessed by adding 200 µl of 95% ethanol to destain the walls and the colour intensity of crystal violet present in destained solution was measured in microtitre plate reader at 550 nm.

Quantification of cell surface hydrophobicity

Cell surface hydrophobicity of the bacterial cells was determined by measuring the microbial adhesion using three solvents such as xylene, ethyl acetate and chloroform. Microbial adhesion to xylene solvent shows the cell surface hydrophobicity or hydrophilicity while ethyl acetate was regarded as electron donor (basic) and chloroform was regarded as electron acceptor (acidic) respectively. The bacterial cells grown in high C:N media were centrifuged at 5000 rpm for 15 min. and washed twice with 0.1 M KNO_3 (pH 6.2) and resuspended in 10 ml of 0.1 M KNO_3 and maintained as two sets. 3 ml of the cell suspension was taken and added with 1 ml of solvent. After 10 min. incubation two phase system was formed which was vortexed for 2 min. and again kept for 20 min. incubation. After 20 min. of incubation the aqueous phase was removed and initial absorption (A0) was recorded at 600 nm. The other set of tubes were kept for 24 h incubation (A1) and after that the absorbance was recorded at 600 nm. The percentage of microbial adhesion to different solvents was calculated by using the formula (1-A1/A0)×100.

Screening for PHB production

The smears of the aggregated bacterial cells were prepared on glass slides and heat fixed and were stained with 0.3% (w/v in 70% ethanol) solution of Sudan Black B for 10 min. The smears were decolorized by immersing the slides in xylene for 2 min. and then counterstained with safranin (5% w/v in sterile distilled water) for 10 sec. The bacterial cells appearing black under microscope were PHB producing strains [14].

Assessment of plant growth promoting traits

Preparation of starter culture for assessing plant growth promoting traits

24 h bacterial culture was inoculated in high C:N and low C:N medium and kept for incubation in rotary shaker at 150 rpm for 48 h. The aggregated cells produced in high C:N and non aggregated cells produced in low C:N medium were then centrifuged at 5000 rpm for 10 min. and the pellets were collected and washed twice with 0.85% saline solution and resuspended in sterile water. This suspension was used as the starter culture for assessing the plant growth promoting traits of the tested bacterial cultures.

Estimation of Indole Acetic Acid (IAA) production

The in vitro Indole acetic acid production assay was performed in nutrient broth supplemented with tryptophan [15]. Aggregated and non aggregated cell suspension was inoculated separately in nutrient broth with tryptophan, which were then wrapped with black sheet and incubated at 28±2°C in rotary shaker at 100 rpm for 72 h. The cultures were then centrifuged at 12000rpm for 10 min. and the supernatant was used for the estimation IAA. 500 µl supernatant was taken in eppendorf tube to which 50 µl of 0.1 mM orthophosphoric acid and 2 ml of Salkowaski reagent (1 ml of 0.5 M $FeCl_3$ dissolved in 50 ml of 35% perchloric acid) was added. The obtained mixture was kept in dark for 30 min for the development of pink to red colour. The colour intensity was measured by using spectrophotometer at 530 nm and IAA production was calculated using standard curve which was expressed as µg ml^{-1}.

Aminocyclopropane-1-carboxylic acid deaminase activity

ACC- deaminase activity was performed by following [16] to determine whether the aggregated cells use ACC as a sole nitrogen source. 0.5 ml of the aggregated and non aggregated cell suspension was inoculated separately in 5 ml of N-free malic acid broth and incubated for 24 h and were again inoculated (1% v/v) into the fresh tubes containing 5 ml N-free malic acid broth supplemented with 30 µl 50 mM ACC and incubated at 30±2ºC for 48 h. Cultures were centrifuged at 10000 rpm for 10 min at 4ºC. The pellets were harvested and washed two to three times with 1 ml of 0.1 M Tris-HCl (pH 7.6) and stored at -20ºC for 30 min. after suspending in 1 ml of 0.1 M Tris- HCl (pH 7.6), and again centrifuged and the pellet was resuspended in 600 µl of 0.1 M Tris -HCl (pH 8.5). Then 30 µl of toluene was added and vortexed for 30 sec. The obtained supernatant or suspension was divided into two parts in eppendorf tube each with 200 µl and 230 µl.230 µl present in centrifuge tube was stored at 4ºC for protein estimation. To this 200 µl, 20 µl of 50 mM ACC was added and the tubes were vortexed and incubated at 30ºC for 15 min. Then it is added with 1 ml of 0.56 M HCl and centrifuged for 5min at 10000 rpm at room temperature. 300 µl 2,4-Dinitrophenyl hydrazine (DNPH) was added to the supernatant, vortexed and incubated for 30 min at 30ºC. Finally, 2ml of 2N NaOH was added, mixed well and OD was observed at 540 nm using Spectrophotometer. ACC deaminase activity was measured using α-ketobutyrate released min^{-1} mg^{-1}protein h^{-1}.

Quantification of phosphate solubilization

The phosphate solubilization was quantitatively determined by following [17]. 0.5 ml of the aggregated and non aggregated cells were inoculated separately into 5ml of Pikovaskaya' s broth which was supplemented with 0.5% tricalcium phosphate and incubated at 30±2ºC for 4 days. The 4 days old culture was centrifuged at 10000 rpm for 5 min. and 1ml of the supernatant was collected in the test tube and 1 ml distilled water was added. Then 2 ml of color reagent (Solution A and Solution B) was added and the volume was made upto 6ml by adding distilled water and incubated for 15 min to develop dark blue color. The absorbance was measured at 882 nm in spectrophotometer. KH_2PO_4 was used as the standard and the amount of phosphate solubilization was expressed as total P release mg l^{-1}.

Quantification of silica solubilization

Silica solubilization assay was performed by following Tank & Saraf (2010) [18]. 0.5 ml of the aggregated and non aggregated cells were inoculated in Bunt and Rovira liquid medium which was supplemented with sodium metasilicate and

Table-1 *Physiological characters of aggregated and non aggregated cells*

S	Culture	Aggregation (%)		Microbial load (CFU ml^{-1})		Cell dry weight (mg ml^{-1})		Microbial adhesion(%)						Biofilm formation	
								Xylene		Ethyl acetate		Chloroform			
		High C:N	Low C:N	High C:N	Low C:N	High C:N	Low C:N	High C:N	Low C:N	High C:N	Low C:N	High C:N	Low C:N	High C:N	Low C:N
1	*Pseudomonas gessardii*	5.7± 0.1^{d}	2.1±0.1^{c}	4.7×10^{6}	3.9×10^{6}	2.6±0.1^{c}	1.4±0.0^{c}	34.0±0.61^{a}	20.1±0.01^{c}	-	-	18.83±0.08^{b}	10.43±0.16^{c}	+++	+++
2	*Pantoea dispersa*	4.6± 0.0^{e}	1.8± 0.0^{c}	1.2×10^{6}	1.4×10^{5}	1.5±0.1^{d}	0.9±0.0^{d}	13.0±0.25^{c}	9.0±0.12^{d}	-	-	-	-	-	-
3	*Azospirillum brasilense*	30.6± 0.0^{b}	24.6± 0.6^{b}	3.8×10^{11}	2.6×10^{7}	5.5±0.1^{b}	2.0±0.1^{b}	33.05±0.29^{a}	25.4±0.30^{b}	-	-	-	-	+	+
4	*Methylobacterium thiocyanatum*	33.5± 0.6^{a}	26.8± 0.4^{a}	4×10^{11}	4.9×10^{7}	6.0±0.1^{a}	1.5±0.0^{c}	45.10±0.87^{a}	36.20±0.68^{a}	-	-	49.70±0.62^{a}	25.43±0.24^{b}	++	+
5	*Pseudomonas psychrotolerans*	14.8± 0.3^{c}	25.3 ±0.2^{b}	2.5×10^{8}	3×10^{11}	0.55±0.0^{e}	3.0±0.0^{a}	24.35±0.08^{b}	35.37±0.70^{a}	-	-	19.45±0.23^{b}	30.27±0.77^{a}	++	+
	SEd±	0.46	0.44			0.12	0.05	3.73	0.64			0.42	0.51		
	CD (0.05)	1.03	0.98			0.26	0.13	8.32	1.44			0.93	1.15		

Values are mean (± standard error) (n=3) and column values followed by different letters are significantly different from each other at 5% LSD; +++ = Strong; ++ = Moderate; += Weak; - = Negative;

Table-2 PGPR traits of aggregated and non-aggregated cells

Culture	EPS Production (µg/ml)		IAA (µg/ml)		PO_4- Solubilization (µg/ml)		ACC deaminase activity (n moles)		Zinc Solubilization (mg/l)		Silica Solubilization (mg/l)	
	Aggregated cells	Non aggregated cells	Aggregated cells	Non aggregated cells	Aggregated cells	Non aggregated cells	Aggregated cells	Non aggregated cells	Aggregated cells	Non aggregated cells	Aggregated cells	Non aggregated cells
Pseudomonas gessardii	14.3 ±0.51^{b}	8.1 ±0.18^{b}	9.00±0.08^{d}	8.20 ±0.14^{d}	312 ±0.28^{d}	200±7.75^{c}	20±0.50^{b}	12.30±0.48^{d}	50.41±0.37^{b}	28.41± 0.22^{c}	251 ±0.39^{a}	218 ±3.41^{a}
Pantoea dispersa	3.7 ±0.09^{d}	4.6 ±0.02^{d}	9.20 ±0.31^{d}	8.60 ±0.30^{c}	522 ±2.82^{b}	188±7.46^{c}	12.10±0.15^{d}	10.30±0.35^{e}	0.18 ±0.00^{d}	-	80 ±0.95^{d}	53 ±1.11^{d}
Azospirillum brasilense	3.8 ±0.04^{d}	4.8 ±0.09^{d}	20.60±0.30^{a}	9.30 ±0.37^{b}	260 ±11.25^{d}	232±2.51^{b}	19.20±0.33^{b}	15.10±0.19^{c}	-	-	79 ±1.76^{d}	44 ±0.78^{e}
Methylobacterium thiocyanatum	17.1 ±0.37^{a}	16.0 ±0.17^{a}	19.00 ±0.24^{b}	8.00 ±0.21^{a}	456 ±15.62^{c}	446±16.48^{a}	26.80±0.68^{a}	18.40±0.05^{a}	68.88± 1.25^{a}	50.47 ±0.60^{a}	117 ±2.20^{b}	102 ±0.58^{c}
Pseudomonas psychrotolerans	9.1 ±0.05^{c}	3.2 ±0.04^{c}	13.50 ±0.49^{c}	10.40 ±0.06^{d}	548±0.49^{a}	310 ±5.03^{a}	17.40±0.77^{c}	13.80±0.20^{b}	44.41± 0.21^{c}	38.65 ±0.76^{b}	108± 0.03^{c}	95± 0.20^{b}
SEd±	0.26	0.34	0.22	0.09	5.23	8.68	0.51	0.35	0.96	0.51	2.17	2.44
CD (0.05)	0.59	0.77	0.51	0.21	11.66	19.34	1.31	0.78	2.15	1.15	4.83	5.45

Values are mean (± standard error) (n=3) and column values followed by different letters are significantly different from each other at 5% LSD

Fig-1 Aggregation of *Methylobacterium thicyanatum*

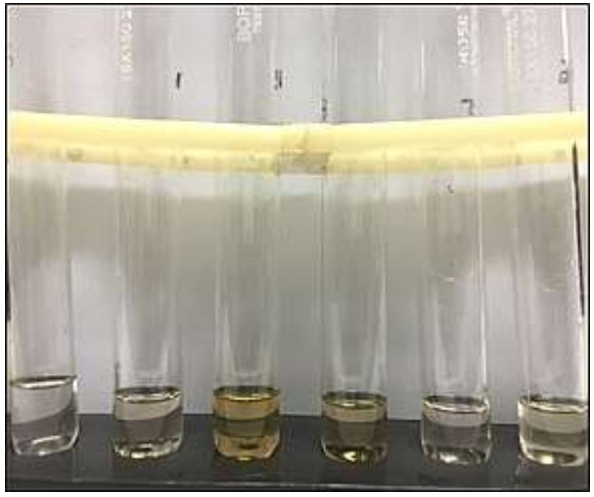

Fig-2 EPS Production

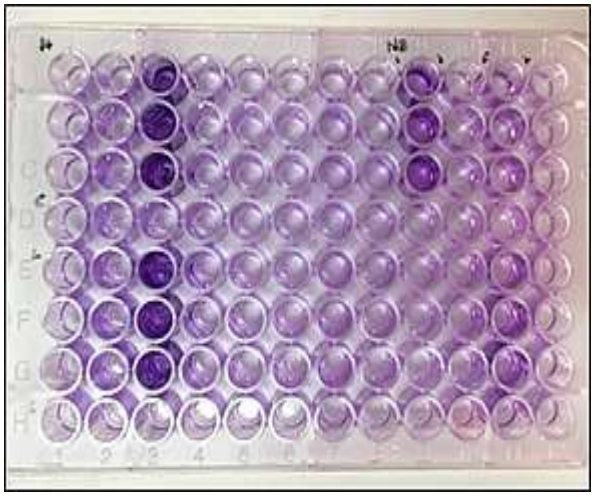

Fig-3 Biofilm formation

incubated for 2 days. After incubation, the broth was centrifuged at 10000 rpm of 10 min. and the supernatant was collected. To this supernatant 0.5 ml of Reagent A (50 ml of conc. HCl mixed with 50 ml of distilled water) and 1ml of Reagent B (10 g Ammonium molybdate was dissolved in 100 ml of distilled water with pH between 7 and 8) were added. After 10 min. of incubation 1.5 ml of Reagent C (10 g of Oxalic acid dissolved in 100 ml of distilled water) was added and mixed thoroughly. The yellow colour developed was read at 420 nm in UV- VIS Spectrophotometer.

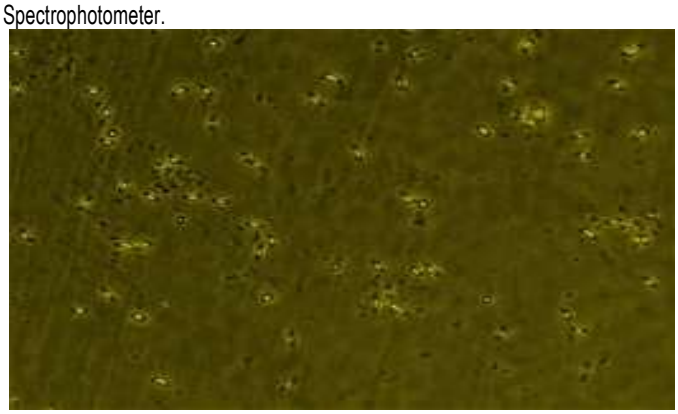

Fig-4 PHB production in aggregated cells

Quantitative assay for Zinc solubilization

1 ml of aggregated and non aggregated cells were inoculated into 10 ml of Bunt and Rovira liquid medium separately which was supplemented with 0.01% Zinc Oxide and kept under incubation for 2 days. After incubation the cells were centrifuged at 10000 rpm for 10 min and the supernatant was collected. The concentration of Zinc in the supernatant was estimated through Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES).

Quantification of exopolysaccharide production

Bacterial Exopolysaccahride (EPS) content was estimated by phenol sulphuric acid method using glucose as standard [19] to determine the exopolysaccharide production by the aggregated cells. EPS was extracted from 3 days old culture grown in high C:N and low C:N medium and centrifuged at 10,000 rpm for 10 min. From this 1 ml of the supernatant was collected to which 2 ml of 90% ethanol was added and incubated at -20º C for 24 h. The next day the suspension was centrifuged at 8000 rpm for 15 min. and the precipitate was collected. Then the precipitate was dissolved in 2ml of water and then 200 µl of 5% phenol and 1 ml of 93% sulphuric acid was added and kept under incubation for 10 min. to form yellow colour which indicates the production of EPS. The OD value was observed at 490 nm using Spectrophotometer. By using standard stock solution of glucose at different concentrations, calibration curve was prepared and the EPS production was calculated and expressed in release of reducing sugars µg ml^{-1}.

Results and discussion

Induction of cell aggregation and quantification

Aggregation was mostly observed in *Methylobacterium thiocyanatum*, *Azosprillum brasilense*, *Pseudomonas gessardii* and *Pantoea dispersa* cultures in high C:N medium whereas *Pseudomonas psychrotolerans* culture showed more aggregated cells in low C:N medium than high C:N medium [Fig-1]. *Bacillus* cultures did not show any aggregation either in high C:N or low C:N medium. The results infer that the microorganisms when provided with rich carbon source and limited nitrogen source i.e., high C:N, they tend to form aggregates. Production of aggregated cells is influenced by various chemical and physical factors and among these factors, nutrient limited conditions are the major factor for inducing aggregation of microorganisms. Among the 5 bacterial cultures (*Methylobacterium thiocyanatum*, *Azosprillum brasilense*, *Pseudomonas psychrotolerans*, *Pseudomonas gessardii* and *Pantoea dispersa*) evaluated, percentage of aggregation was significantly higher in *Methylobacterium thiocyanatum* (33.5%) than other cultures followed by *Azospirillum brasilense* (30.6%) and *Pseudomonas psychrotolerans* (25.3%) in high C:N medium. The aggregation percentage of *Pseudomonas psychrotolerans* in high C:N was on par with *Azospirillum brasilense* (24.6%) in low C:N. Similar findings were observed by Joe, *et al*., (2013)who reported that aggregation occured in *Methylobacterium* strains in high C:N conditions and among the strains studied CBMB12 showed highest aggregation of 53.4% at 72 h.

Physiological states of aggregated and non-aggregated cells

Observations on physiological states of the cultures such as bacterial cell dry weight, microbial load and biofilm formation were presented in [Table-1]. Bacterial cell dry weight of *Methylobacterium thiocyanatum* (6 mg ml^{-1}) was significantly higher than all other cultures followed by *Azospirillum brasilense* (5.5 mg ml^{-1}) and *Pseudomonas gessardii* (2.6 mg ml^{-1}). *Pseudomonas psychrotolerans* and Pantoea *dispersa* showed least dry weight of 2.6 mg ml^{-1} and 1.5 mg ml^{-1} respectively in high C:N. In low C:N condition, dry weight of *Pseudomonas psychrotolerans* (3.0 mg ml^{-1}) was significantly higher than all other cultures. The results indicates that cell dry weight of bacteria grown in high C:N medium was higher than low C:N, this might be due to the starvation for N. Burdman *et al*., (1998) reported that under nitrogen limiting conditions the growth of the bacterial strains resulted in lower cell yields than the high C:N bacterial strains. Microbial load was estimated by spread plate method after serial dilution of the aggregated cells. Among the 5 aggregated bacterial cultures microbial load was observed more in *Methylobacterium thiocyanatum* (4×1011 cfu ml^{-1}) followed by *Azosprillum brasilense* (3.8×1011 cfu ml^{-1}) and *Pseudomonas psychrotolerans* (3×1011 cfu ml^{-1}). *Pseudomonas gessardii* and *Pantoea dispersa* showed least microbial load of 4.7×106 cfu ml^{-1} and 1.2×106 cfu ml^{-1}.

From the present study, the cell load of high C:N media was much higher compared to the low C:N media. The results indicated that cell number as well as cell biomass were observed higher in high C:N medium than low C:N medium and this may lead to aggregation of cells in high C:N medium. Biofilms are the surface attached microbial communities where in microbial cells are embedded in self produced extracellular polymeric substances. In the present study aggregated cells and non aggregated cells of *Pseudomonas gessardi* had formed stronger biofilm [Fig-3] whereas *Methylobacterium thiocyanatum* and *Pseudomonas pychrotolerans* showed moderate biofilm formation and weak biofilm was formed in *Azospirillum brasilense*. No biofilm was formed in both aggregated and non aggregated cells of *Pantoea dispersa*. The results showed that biofilm provides aggregation of microbial cells which increases the population at a specified area. *Azospirillum brazilens* AZO1 was observed to form biofilm which helped to increase the cell mass around the root of *Arabidobsis*. The cell surface of bacteria plays a major role in aggregation of bacteria that in turn plays a positive role in affecting the bacterial dispersion and their survivability in soil and ability to adhere to plant surfaces. Microbial adhesion of aggregated bacterial strains to different solvents was studied to evaluate the cell surface properties. Among the 5 aggregated bacterial cultures microbial adhesion to xylene was significantly more in *Methylobacterium thiocyanatum* (45.10%) followed by *Pseudomonas psychrotolerans* (35.37%), *Pseudomonas gessardii* (34%) and *Azospirillum brasilense* (33.05%) and they were on par. Aggregated bacterial cultures didn't show any affinity to ethyl acetate which is a acidic solvent and electron acceptor and *Methylobacterium thiocyanatum* had shown more adherence of 49.70% to chloroform (basic solvent and electron donor) indicating it as a strong electron acceptor. Similar findings were observed by Joe, *et al*., (2013) that *Methylobacterium* strains under aggregated conditions showed increased hydrophobicity compared to the cells under standard grown conditions. Strain CBMB12 was observed with relatively higher hydrophobicity of 50.1 % adhesion with xylene under aggregated condition. PHB production was observed more in *Methylobacterium thiocyanatum*, *Pseudomonas psychrotolerans* and Azospirillim brasilense among the 5 aggregated bacterial cultures [Fig-4]. *Pseudomonas gessardii* and *Pantoea dispersa* did not show any PHB ganlues when observed under light microscope. Aggregated cultures have more poly-β-hydroxybutyrate granules or inclusion bodies than the non-aggregated cultures. Kumar, *et al*.,(2016) [20] reported that nutrient limited conditions mimic the soil rhizosphere where carbon sources are rich but nitrogen sources are limited for bacterial growth. Under such conditions, soil microorganisms accumulate intracellular food reserve in the form of PHB which enhances their survivability.

Evaluation of plant growth promoting traits of aggregated cells

Observations on plant growth promoting traits such as IAA, ACC deaminase, phosphate, zinc and silica solubilization of the aggregated cells was presented in [Table-2]. The aggregated as well as non-aggregated cells of all the bacterial cultures were evaluated for IAA production. Of all the cultures tested for IAA production, *Azospirillum brasilense* (20.60 µg ml^{-1}) recorded significantly higher values than all the other cultures, followed by *Methylobacterium thiocyanatum* (19.00 µg ml^{-1}) and *Pseudomonas psychrotolerans* (13.50 µg ml^{-1}). The aggregated cells of *Pseudomonas gessardii* (9 µg ml^{-1}) and *Pantoea dispersa* (9.20 µg ml^{-1}) are on par. The non-aggregated cells of *Pseudomonas psychrotolerans* (10.40 µg ml^{-1}) showed significantly higher levels of IAA production than all other non-aggregated cells.

In the present study IAA production was also comparatively higher in aggregated cells than the non-aggregated cells [Table-2]. IAA helps in the production of auxins, an important hormone which helps in cell division. Dimkpa, *et al*., (2009) [21] reported that IAA producing bacteria enhances the formation of lateral root and root hairs consequently improving water and nutrient uptake. When looking for ACC deaminase activity, among the bacterial cultures *Methylobacterium thiocyanatum* was found to release 26.80 n mol of α-ketobutyrate/mg of protein/hour under aggregated condition which was significantly higher than all the other cultures followed by *Azospirillum brasilense* (19.20 n mol) and *Pseudomonas gessardii* (20 n mol). *Pseudomonas psychrotolerans* was found to release 17.40 n mol of α-ketobutyrate/mg of protein/hour. ACC deaminase activity was observed higher in aggregated cells than non aggregated cells. Tank & Saraf (2010) reported that ACC deaminase producing PGPB can enhance plant growth and induce plant tolerance to stress through a reduction in the ethylene content in the root, and inhibition of the ACC biosynthesis in plants. Phosphate solubilization was observed in all the aggregated and non aggregated cells at varying levels. Aggregated cells of *Pseudomonas psychrotolerans* (548 µg ml^{-1}) was observed to solubilize more phosphate significantly than others followed by *Pantoea dispersa* (522 µg ml^{-1}), *Methylobacterium thiocyanatum* (456 µg ml^{-1}), and *Pseudomonas gessardii* (312 µg ml^{-1}). Non aggregated cells of *Methylobacterium thiocyanatum* (446 µg ml^{-1}) was observed to be significantly good in PO_4 solubilization than others. Phosphate solubilization efficiency was significantly lower in *Azospirillum brasilense* (260 µg ml^{-1}). Silicate solubilization was noted in all the aggregated and non aggregated cells at varying levels. Solubilization efficiency was found to be significantly higher in *Pseudomonas gessardii* (251 mg l^{-1}) than all the other aggregated and non aggregated cells. Aggregated cells of *Methylobacterium thiocyanatum* and *Pseudomonas psychrotolerans* were found to solubilize 117 mg ml^{-1} and 108 mg ml^{-1} respectively. Further the results showed that significant variation among non aggregated cells were observed for silicate solubilization and the solubilization was in lower levels than the aggregated cells. Earlier it was reported that majority of silicate solubilizers in the palm rhizosphere were *Bacillus* sp. and Pseudomonas sp. and the community population was as high as 3.6 per cent of total bacterial population [22]. The amount of solubilized Zinc was assayed by using (ICP-OES) Inductively Coupled Plasma Optical Emission Spectroscopy. Zinc solubilization efficiency was significantly higher in *Methylobacterium thiocyanatum* (68.88 mg l^{-1}) followed by *Pseudomonas gessardii* (50.41 mg l^{-1}) and *Pseudomonas psychrotolerans* (44.41 mg l^{-1}). *Pantoea dispersa* was found to solubilise very little (0.18 mg l^{-1}) but no solubilization was found in *Azospirillum brasilense*. From the results it can be attributed that zinc solubilization ability of aggregated cells may be due to production of organic acids in the culture broth which might have helped in the solubilization of the zinc oxide. Also, the solubilization of Zinc by bacteria might be due to other mechanisms which include proton extrusion and production of chelating agent [23,24].

All the aggregated cells produced more exopolysaccharides. Among them the aggregated cells of *Methylobacterium thiocyanatum* produced 17.1 µg ml^{-1} whereas *Pseudomonas psychrotolerans* and *Pseudomonas gessardii* produced 9.1 µg ml^{-1} and 14.3 µg ml^{-1} respectively [Fig-2]. In contrast, all the non aggregated cells have produced significantly lower levels of EPS. Extracellular polysaccharides play an important role in aggregation and surface colonization of plant associated bacteria.

Conclusion

All the studied strains showed aggregation ability at varying levels when grown under high C:N medium and *Methylobacterium thiocyanatum* as well as *Azospirillum brasilense* showed high degree of aggregation compared to other cultures. Aggregated cells of these cultures showed high cell load, dry weight as well as expressed high degree of adhesion to xylene as well as PHB production. When the cultures are grown under aggregated condition, EPS production and PGP characteristics of the cultures are positively influenced. Hence the aggregation property of these organisms could be exploited for the development of inoculant formulations with better survival in the rhizosphere.

Application of research: Study aimed to test the PGP traits of aggregated cells.

Research Category: Agriculture Microbiology

Abbreviations: PGP- Plant growth promoting, PHB- Poly hydroxy butyrate, EPS - Exopolysaccharides, OD- Optical Density, ACC deaminase- Aminocyclopropane 1- carboxylic acid, DCW- Dry Cell Weight

Acknowledgement / Funding: Authors are thankful to Agricultural College and Research Institute, Madurai, 625 104, Tamil Nadu Agricultural University, Coimbatore, 641003, Tamil Nadu, India

***Research Guide or Chairperson of research: Dr K Kumutha**
University: Tamil Nadu Agricultural University, Coimbatore, 641 003, Tamil Nadu
Research project name or number: MSc Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Madurai and Cuddalore district

Cultivar / Variety / Breed name: Nil

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

References

[1] Bashan Y., de-Bashan L. E., Prabhu S. R. & Hernandez J. P. (2014) *Plant and Soil*, 378(1-2), 1-33.
[2] Kadouri D., Jurkevitch E., Okon Y., & Castro-Sowinski S. (2005) *Critical reviews in microbiology*, 31(2), 55-67.
[3] Burdman S., Jurkevitch E., Soria-Díaz M. E., Serrano A. M. G. & Okon Y. (2000) *FEMS Microbiology Letters*, 189(2), 259-264.
[4] Bahat-Samet E., Castro-Sowinski S. & Okon Y. (2004) *FEMS microbiology letters*, 237(2), 195-203.
[5] Joe M. M., Saravanan V. S. & Sa T. (2013) *Archives of microbiology*, 195(3), 219-225.
[6] Joe M. M., Jaleel C. A., Sivakumar P. K., Zhao C. X., & Karthikeyan B. (2009) *Journal of the Taiwan Institute of Chemical Engineers*, 40(5), 491-499.
[7] Rathi N., Singh S., Osbone J., & Babu S. (2015) *Biocatalysis and Agricultural Biotechnology*, 4(3), 304-308.
[8] Bhattacharyya P.N., & Jha D.K. (2012) *World Journal of Microbiology and Biotechnology* 28(4), 1327-1350.
[9] Neyra C.A., Arunakumari A. & Olubayi O. (1997) *U.S. Patent and Trademark Office.*

[10] Okon Y., Albrecht S. L., & Burris R. H. (1977) *Applied and Environmental Microbiology*, 33(1), 85-88.
[11] Burdman S., Jurkevitch E., Schwartsburd B., Hampel M. and Okon Y. (1998) *Microbiology*, 144(7),1989–1999.
[12] Kumar P., Singh M., Mehariya S., Patel S. K., Lee J. K., & Kalia V. C. (2014) *Indian Journal of Microbiology*, 54(2), 151-157.
[13] Djordjevic D., Wiedmann M., & McLandsborough L. A. (2002) *Applied and Environmental Microbiology*, 68(6), 2950-2958.
[14] Nehra K., Jaglan A., Shaheen A., Yadav J., & Lathwal P. (2015) *International Journal of Microbial Resource Technology*, 2(3), 38-48.
[15] Glickmann E., & Dessaux Y. (1995) *Applied and Environmental Microbiology* 61(2), 793-796.
[16] Penrose D.M., & Glick B. R. (2003) *Physiologia plantarum*, 118(1), 10-15.
[17] Murphy J.A.M.E.S. & Riley J. P. (1962) *Analytica chimica acta*, 27, 31-36.
[18] Tank N. & Saraf M. (2010) *Journal of Plant Interactions*, 5(1), 51-58.
[19] Albalasmeh A. A., Berhe A.A., & Ghezzehei T.A. (2013) *Carbohydrate polymers*, 97(2), 253-261.
[20] Kumar P., Ray S., & Kalia V. C. (2016) *Bioresource technology*, 200, 413-419.
[21] Dimkpa C., Weinand T., & Asch F. (2009) *Plant, Cell & Environment*, 32(12), 1682-1694.
[22] Gopal M., Gupta A., & Nair R. V. (2005) *Current Science*, 1922-1927.
[23] Ramesh A., Sharma S. K., Sharma M. P., Yadav N., & Joshi O. P. (2014) *Applied Soil Ecology*, 73, 87-96.
[24] Saxena M.M. (1989) *Agrobotanical publishers, India*, 786