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# Strategy of Salt Tolerance and Productive Effects of *Bacillus tequilensis* Under Specific Conditions

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**Abstract**

Phosphorous (P) is the most commonly limiting the growth of plants and is the second important element after nitrogen as a mineral nutrient. Although present as both organic and inorganic forms in soil. It occurs mostly in insoluble forms; on average, the P content of soil is about 0.05% (w/w) but only 0.1% of this P is available for plant use. Naturally there are numerous microorganisms that make phosphorous available to plant by solubilization and mineralization. These group of microorganisms are called Phosphate Solubilizing Microorganisms (PSM). They solubilize insoluble inorganic phosphorous. After study, the given isolate showed optimum growth in 20% NaCl containing medium, produced IAA, exopolysaccharides, ammonia and solubilized phosphate, do not produce hydrogen cyanide. Hence, the use of plant growth promoting bacteria may represent an important biotechnological approach to increase production of beneficial compounds and survive under salinity stress condition. One option is to isolate and utilize salinity resistant microorganisms from the rhizosphere of plants.

***Keywords: NaCl, Phosphorous(P), Phosphate solubilizing bacteria, Phosphate solubilization, Salinity stress***

## 1. INTRODUCTION

Phosphorous (P) is one of the essential elements that are necessary for plant development and growth; it makes up about 0.2% of a plant dry weight [1]. It is a major growth-limiting nutrient after nitrogen and unlike the case for nitrogen, there is no large atmospheric source that can be made biologically available [2]. Root development, stalk and stem strength, flower and seed formation, crop maturity and production, N-fixation in legumes, crop quality, and resistance to plant diseases are the attributes associated with phosphorous nutrition. Although microbial inoculants are in use for improving soil fertility during the last century [3]. Organic matter is also an important reservoir of immobilized P [4]. On average, the phosphorous content of soil is about 0.05% (w/w%); however only 0.1% of this phosphorous is available for plant use [5], because of its fixation into an unavailable form due to P-fixation. Traditionally, the challenge of soil phosphorous deficiency is addressed by the application of phosphorous fertilizers.

Soil microorganisms enhance plant nutrient acquisition. They are involved in a wide range of biological processes including the transformation of insoluble soil nutrients [6]. Some are capable of solubilizing and mineralizing insoluble soil phosphorous for the growth of plants. Apart from chemical fertilization, microbial P-solubilization and mineralization is the only possible way to increase plant-available phosphorous [7]. Efficiency of P fertilizers throughout the world is around 10-25% [8]. This group of microbes are referred to as Phosphorous Solubilizing Microorganisms (PSM). Many species of soil fungi and bacteria are able to solubilize phosphorous *in vitro* and some of them can mobilize phosphorous in plants [5]. The inoculation of soil or crop with phosphate solubilizing/mineralizing microorganisms is therefore a promising strategy for the improvement of plant absorption of phosphorous and thereby reducing the use of chemical fertilizers that have negative impact on the environment [9].

The main P solubilization mechanisms employed by soil microorganisms include: (i) release of complexing or mineral dissolving compounds e.g., organic acid anions, siderophores, protons, hydroxyl ions, $CO_2$. (ii) liberation of extracellular enzymes (biochemical P mineralization) and (iii) the release of P during substrate degradation (biological P mineralization) [10]. Additionally, PSM in the presence of labile C serve as a sink for P, by rapidly immobilizing it even in low P soils;

therefore, PSM become a source of P to plants upon its release from their cells. Release of immobilized P by PSM primarily occurs when cells die due to changes in environmental conditions, starvation or predation [11]. Inorganic P solubilization by PSM occurs mainly by organic acid production either by: (i) lowering the pH, or (ii) by enhancing chelation of the cations bound to P, (iii) by competing with P for adsorption sites on the soil, (iv) by forming soluble complexes with metal ions associated with insoluble P (Ca, Al, Fe) and thus P is released. The lowering in pH of the medium suggests the release of organic acids by the PSM [12], [13]. Organic acids produced by the PSM are seen in Figure 1. The organic acids are produced in the periplasmic space [14], by the microbial metabolism mostly done by oxidative respiration or fermentation of organic carbon source (e.g., glucose) [15], [16]. Of all the organic acids, gluconic acid is the most frequent agent of mineral phosphate solubilization; it chelates the cations bound to phosphate, thus making the phosphate available to plants. Gram negative bacteria solubilize mineral phosphate by direct oxidation of glucose to gluconic acid [17]. Organic acids produced by the PSM can be detected by high performance liquid chromatography (HPLC) and enzymatic methods. However, acidification does not seem to be the only mechanism of solubilization, as the ability to reduce the pH in some cases did not correlate with the ability to solubilize mineral P [18]. Pyrroloquinoline quinone (PQQ) acts as a redox cofactor in glucose dehydrogenases (GDH) resulting in phosphate solubilization [19]. An alternative mechanism to organic acid production for solubilization of mineral phosphates is the release of $H^+$ to the outer surface in exchange for cation uptake or with the help of $H^+$ translocation ATPs [20]. The monovalent anion phosphate $H_2PO^{-4}$ is a major soluble form of inorganic phosphate [21].

Other mechanism of mineral phosphate solubilization by microorganisms are the production of inorganic acids such as sulphuric acid, nitric acid, and carbonic acid and, the production of chelating substances. It has, however, been reported that the effectiveness of the inorganic acids and the chelating substances in the release of phosphorous in soil is less than that of the organic acids [22]. Bacteria of the genera Nitrosomonas and Thiobacillus species can also dissolve phosphate compounds by producing nitric and sulphuric acids [23].

It was also reported that the assimilation of $NH^{+4}$ within microbial cells is accompanied by the release of protons and this results in the solubilization of phosphorous without the production of any organic acids [24]. One important theory proposed by Halvorson et al, (1990) [25], for the solubilization of organic P is the Sink theory. This refers to continuous removal of P that result in the dissolution of Ca-P compounds from the liquid culture medium. Consequently the decomposition of P in organic substrates is consistently correlated with the P content in the biomass of PSM [26]. This biological process plays an important role in phosphorous cycling. Organic P may constitute 4-90% of the total soil P [27]. Organic P solubilization is also called mineralization of organic phosphorus. Such P can be released from the organic compounds in soil by enzymes like Non-specific acid phosphatase (NSAPs), Phytases, Phosphatases and C-P lyases [28].

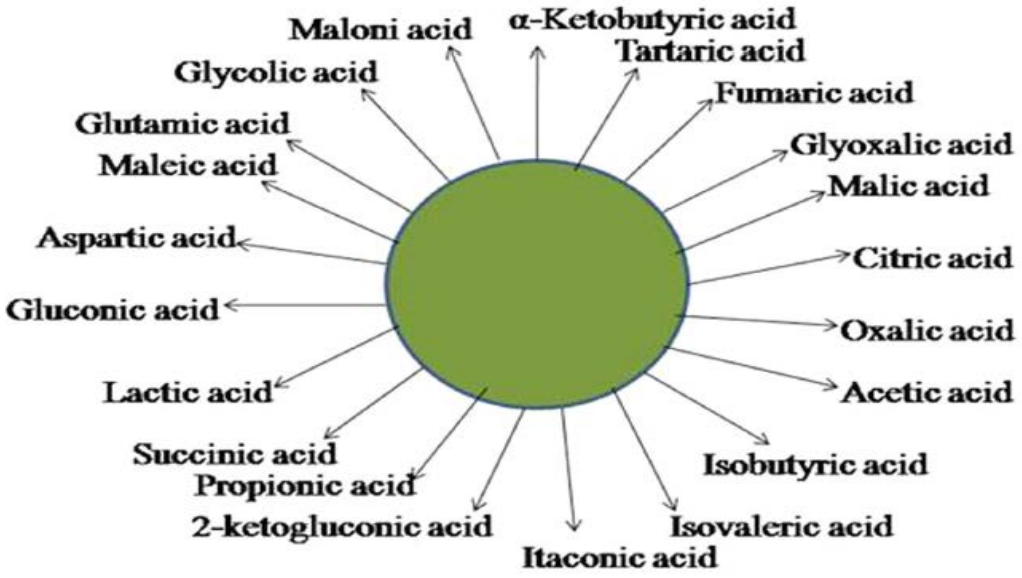



**Figure 1: Schematic representation of the organic acids that may be produced by PSM and used to solubilize inorganic forms of phosphate [29].**

The salt-tolerant or halophilic soil microorganisms that also exhibit the ability to solubilize insoluble phosphorous facilitate the development of saline-alkali soil-based agriculture [5]. Soil salinity is one major limiting factor that directly

affects the plant growth and crop productivity [30]. Sodium chloride is the predominant salt species in most of saline soils and its effect can be observed by decreased productivity or plant death [31]. Moreover, there is also an abnormal increase in saline soils due to several reasons, including some unfavorable agricultural practices, irrigation with saline water, and high surface evaporation rate [32]. Soil salinity causes plant stress in two ways: (i) making water uptake by the roots more difficult, and (ii) causing plant toxicity via accumulation of high salt concentration in the plant [31]. Several biochemical processes can be affected by salinity, including protein synthesis, photosynthesis, and lipid metabolism [33]. However, most plants posses several mechanisms to decrease the negative effects of salinity including regulation and compartmentalization of ions, synthesis of compatible solutes, induction of antioxidative enzymes, induction of plant hormones, and changes in photosynthetic pathway [34], [33]. Several strategies have been developed in order to decrease the toxic effects caused by high salinity on plant growth, including plant genetic engineering [35], and recently the use of plant growth-promoting bacteria (PGPB) [36]. The production of hormones in plant growth promoting rhizobacteria in numerous studies reports the importance of indole acetic acid (IAA) in the root development [37]. The effect of exogenous IAA in the crops can stimulate or inhibit growth and is often depends on the concentration of hormones available [38]. The evaluation of bacterial isolates, ACC02 and ACC06 for production of IAA discovered that both are significant producers of IAA suggesting that they could be used as PGPB [39].

Endophytes are organisms that exist in plant tissues. They colonize into the internal tissues of the crops and make a symbiotic relationship [40]. Endophytes are used as plant growth promoters and help crops adapt to better environment [41], [42]. Classification of these microorganisms are going from harmonious to somewhat pathogenic [43], [44]. A plant and endophytic bacterium association can produce secondary bioactive compounds which are helpful in many ways [45].

## 2. MATERIALS AND METHODS

### 2.1 Isolate collection

In this study, isolate N was procured from our laboratory .This isolate N was endophyte in nature and was collected from leaf of *Citrus limon* tree. Isolate N was 48 h old. By the use of this isolate N, all the following study were performed. Isolate N was transferred on Nutrient Agar plate and Nutrient agar slant at 28±2°C for further studies.

### 2.2 Bacterial culture characterization

Each species of bacteria exhibits morphological and cultural characteristics that, when taken together, can be used to differentiate the bacterial species.

### 2.3 Isolate N evaluation by temperature

Isolate N was streaked on Nutrient agar media and incubated at different temperature containing incubators like 4°C, 25°±3°C, 30°C, 37°C, 45°C, 55°C for 24 h with the control in each. Results were observed.

### 2.4 Effect of NaCl of isolate N

Tolerance of isolate N to NaCl was evaluated on Nutrient broth medium, supplemented with increasing NaCl concentration like 5%, 10%, 15%, 20%, and 25%. Flasks (100 ml) were inoculated with bacterial culture and each flasks with different NaCl concentration kept uninoculated and marked as a control. Incubated for 120 h at 28±2°C on the shaker with 150 rpm. The bacterial growth was monitored by measuring optical density at 600nm up to 120 h (5 d) at regular time interval of 24 h.

### 2.5 Production of Indole acetic acid (IAA) by isolate N

IAA production was performed in the nutrient broth medium containing 1% tryptophan. Inoculate 60 ml of media (250 ml flask) with 0.5 ml of isolate N culture and incubate in the dark condition for up to 8 days at 30±2°C on shaker with 150 rpm. Keep one flask uninoculated and mark as a control. Withdraw 5ml sample from $5^{th}$ d to $8^{th}$ d from both the flasks. Centrifuge the samples at 10,000 rpm for 15 min and take 2 ml of supernatant in a test tube. Add 2 ml of freshly prepared Salkowski's reagent [46], into test tubes and keep it in dark for 30 min. Then take OD at 536 nm. The amount of IAA production was calculated from standard graph of IAA [47].

### 2.6 Ammonia production by isolate N

Ammonia production was evaluated in peptone water broth and kept in 28±2°C for 4 to 8 d. Kept one tube uninoculated and marked as control. The activity was measured by supplying Nesseler's reagent. From 4$^{th}$ d to 8$^{th}$ d, withdraw 2 ml of sample aseptically and add 0.2 ml of Nesseler's reagent in both the tubes. Intensity was measured by development of brown to yellow color [48].

### 2.7 Exopolysaccharide (EPS) production by isolate N

EPS production was studied in the Nutrient broth media containing 5% of different sugars like sucrose, maltose and dextrose [49]. Prepared 30 ml of media and inoculate them with the isolate N and incubated at 28±2°C on shaker with 150 rpm. 2 ml of media was collected under aseptic condition and add 6 ml of chilled acetone and keep them overnight in freezer. EPS will be extracted from the media in the form of a slimy precipitates. EPS production was observed and recorded.

### 2.8 Qualitative phosphate solubilization by isolate N

Qualitative phosphorous solubilization assay of isolate N was determined on Pikovskaya's agar plate medium, having tricalcium phosphate as insoluble phosphate source and bromophenol blue dye. Plate is inoculated as dot or straight line with isolate N and kept one plate uninoculated, marked as a control. Incubated at 28±2°C for 6 d. Isolate N having capacity to solubilize phosphorous gives a clear yellow zone. Diameter of clear zone was measured from 4$^{th}$ d to 6$^{th}$ d [50].

## 3. RESULT AND DISCUSSION

### 3.1 Isolate N on Nutrient agar medium

Isolate N was endophyte and isolated from leaf of *Citrus limon* and transferred on Nutrient agar plate by four flame method and Nutrient agar slant by direct method for further study and incubated at 28±2°C. Isolate N was transferred at every 4$^{th}$ d on fresh medium to remain cells viable.

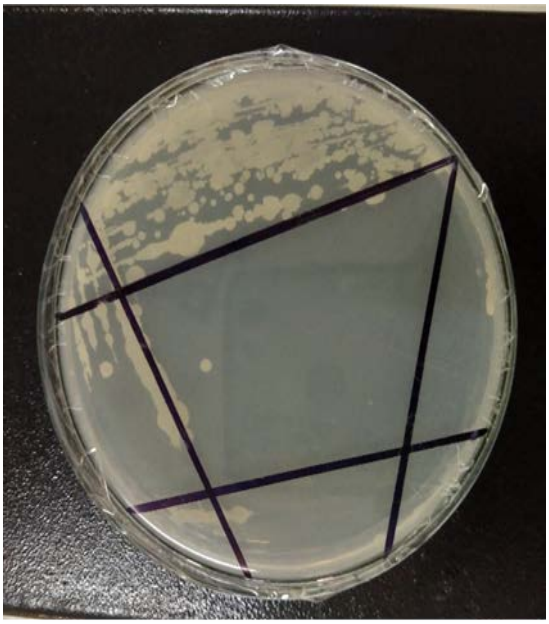


**Figure 2(a)**


**Figure 2(b)**

**Figure 2 (a)(b): Growth of isolate N on Nutrient agar medium**

### 3.2 Characterization of isolate N

Morphological characterization of colony was done by microscopic examination by perform Gram staining. Gram staining technique was developed by the Danish bacteriologist Hans Christian Gram in (1884). Gram staining differentiate

organism on the basis of Gram's reaction either Gram positive or Gram negative. Gram staining was done by Gram staining reagents like Crystal violet, Gram's iodine, decolorizer, and saffranin and observed by light microscope under oil immersion lens and cultural characteristics was writen by eye visulization. Results are as below:

**Table 1: Morphological characteristics of isolate N**

| Morphological characteristics | Organism |
|---|---|
| Size | Big |
| Shape | Rod |
| Arrangement | Single, Chain |
| Gram's reaction | Gram positive |

**Table 2: Cultural characteristics of isolate N**

| Cultural characteristics | Colony |
|---|---|
| Size | Big |
| Shape | Irregular |
| Edge | Uneven |
| Texture | Rough |
| Elevation | Flat |
| Opacity | Opaque |
| Pigmentation | - |

From table 1 and 2, it is observed that this isolate N can be *Bacillus* sp**.** Observation was done with the help of Experimental Microbiology Volume 1, 9$^{th}$ edition [51].

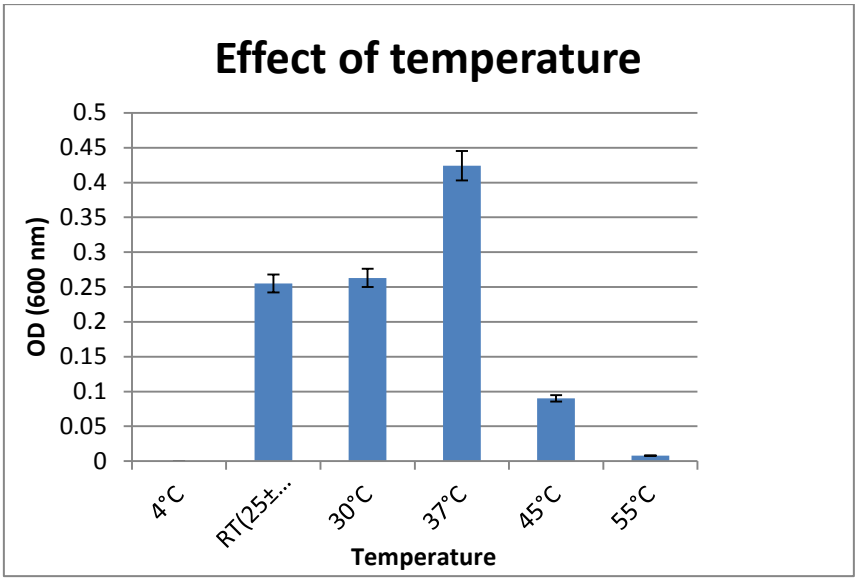
### 3.3 Isolate N evaluation by temperature

Isolate N was streaked on Nutrient agar media and incubated at different temperatures containing incubators. Isolate N show optimum growth at 37°C, at 30°C defined excellent growth, at room temperature defined good growth, at 45°C and 55°C organism remain viable and there was no growth at temp 4°C, and in control too. Results are given below:

**Table 3: Effect of temperature of isolate N**

| Temp. | Growth |
|---|---|
| Control | - |
| 4°C | - |
| RT (25±3°C) | ++ |
| 30°C | ++ |
| 37°C | +++ |
| 45°C | + |
| 55°C | + |

(Key: - = No growth, + = Viable growth, ++ = Visible growth, +++ = Optimum growth)

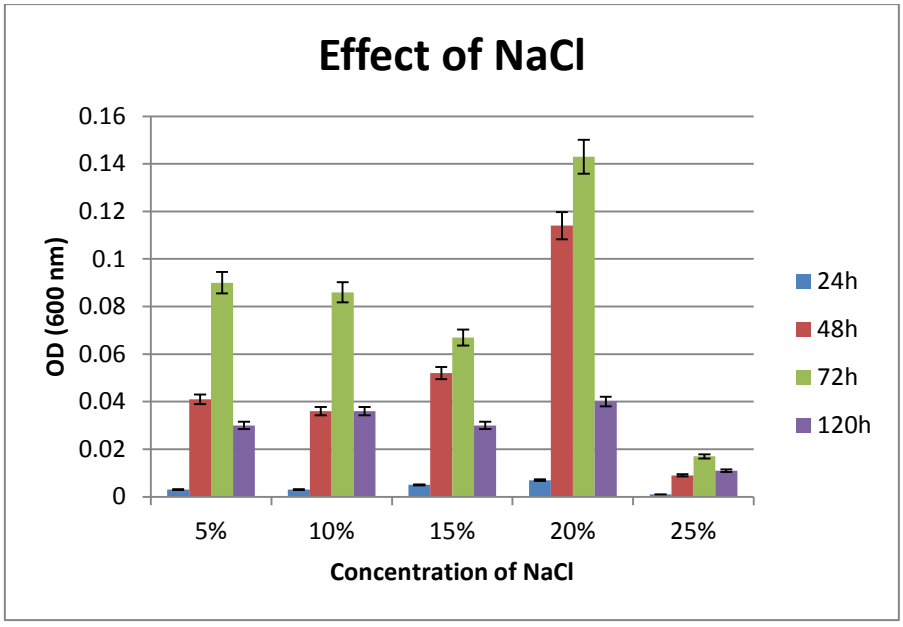



**Graph 1: Effect of temperature on bacterial growth**

In the experimental work done by Zalak Rathod (2016) [52], there was visible growth at RT (25±3°C), 30°C and excellent growth at 37°C. There was no growth found at 4°C, 45°C, 55°C in *Bacillus* sp.

### 3.4 Effect of NaCl of isolate N

There was 5%, 10%,…25% concentration of NaCl containing Nutrient agar plates prepared and streaked with isolate N, kept one plate uninoculated for each concentration and marked them as a control; incubated all plates at 28±2°C for 24 h. The isolate N was remain viable up to 25% NaCl concentration and show optimum growth in 20% NaCl at 72 h; at 48 h and 120 h there was good growth observed.




**Graph 2: Effect of NaCl on bacterial growth**

In the experiment done by Zalak Rathod (2016) [52], there was viable growth in 5.5% NaCl and no growth observed in 20.5% and 25.5% NaCl concentration.

### 3.5 Production of Indole acetic acid (IAA) by isolate N

IAA production was done in nutrient agar broth containing 1% tryptophan. Nutrient broth was inoculated with isolate N and one broth kept uninoculated and marked as a blank.Results of IAA production by using Salkowsky's reagent [46], are shown in table 4 and graph 3. Two ml of culture supernatant was mixed with 2 ml of Salkowsky's reagent [46] and incubated in dark for 30 min for the development of pink colour, which was then estimated on Systronic spectrophotometer 166 at 536 nm up to 8 d. Isolate N produce maximum (51.36 ± 2 µg/ml) IAA on 7 d [47].

**Table 4: Amount of IAA produced by bacteria from 5$^{th}$ d to 8$^{th}$ d**

| Test | Conc.(µg/ml) |
|---|---|
| Control | 0.0 |
| 5th d | 25.63±2 |
| 6th d | 40.81±2 |
| 7th d | 51.36±2 |
| 8th d | 35.18±3 |

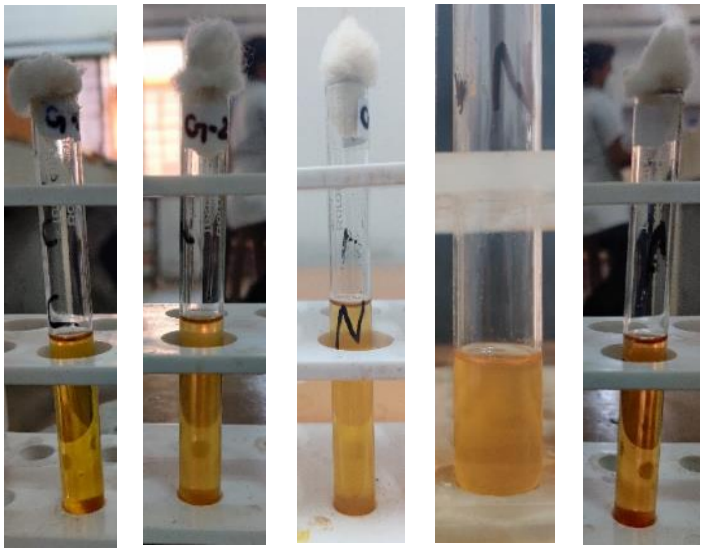


**Figure 3(a)** **Figure 3(b)** **Figure 3(c)** **Figure 3(d)** **Figure 3(e)**

**Figure 3: IAA production**

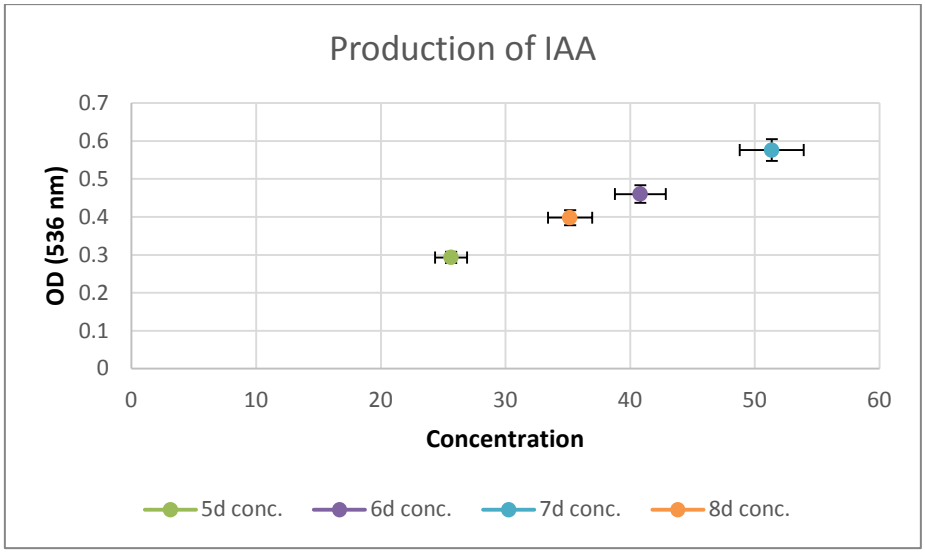
**(a) control test,**

**(b) Test of 5$^{th}$ d,**

**(c) Test of 6$^{th}$ d,**

**(d) Test of 7$^{th}$ d,**

**(e) Test of 8$^{th}$ d**




**Graph 3: Production of IAA from 5th d to 8th d**

It was estimated that the given bacterial strain posseses IAA producing potential. IAA production reaches maximum after 120 h (5 d) of incubation by the reports of Zimmer and Bothe (1988) [53], but our isolate N did not follow this pattern and showed maximum IAA production on 168 h (7 d).

### 3.6 Ammonia production by isolate N

Ammonia production was studied in peptone water broth. Medium was inoculated with isolate N and one tube kept uninoculated and marked as a control. Ammonia production was studied up to 8 d of incubation at 28±2°C. Optimum production of ammonia was observed in the isolate N on 8th d as compared to 4th and 5th d (Figure 6, Table 5) [48].

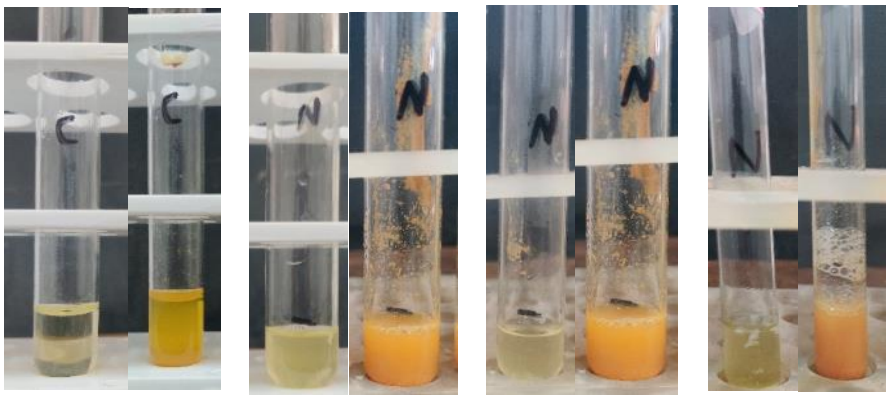



**Figure 4(a)** **Figure 4(b)** **Figure 4(c)** **Figure 4(d)**

**Figure 4: Ammonia production with (right-side test tubes) and without (left-side test tubes) addition of Nesseler's reagent**

**(a) Control test,**

**(b) Test of 4th d,**

**(c) Test of 5th d,**

**(d) Test of 6th d**

**Table 5: Qualitative estimation of ammonia from 4th d to 6th d**

| Day | Growth |
|---|---|
| Control | - |
| 4th | ++ |
| 5th | ++ |
| 6th | +++ |

(Key: - = No growth, ++ = Visible growth, +++ = Optimum growth)

Production of ammonia by PGPR in soil creates alkaline condition in soil and increase pH up to 9.5. It disturbes microbial community and inhibits growth of fungi and nitrobacteria because it has effective inhibition effect [54]. Joseph et al., (2012) [55], reported ammonia production in 95% of *Bacillus* spp.

### 3.7 Exopolysaccharide (EPS) production by PSB

EPS concentration was studied in nutrient agar medium containing 5% different sugars like dextrose, maltose and sucrose. These media were inoculated with isolate N and each tube from different sugars kept uninoculated and marked as a control. Optimum amount of EPS was produced at 96 h (4 d) in all sugars in the isolate N. There was good amount of EPS produced at 48 h and 72 h. A slight production was observed at 24 h and 120 h (Table 6) [49].

**Table 6: Qualitative estimation of EPS from 24 h to 120 h**

| Sugars | 24 h | 48h | 72h | 96h | 120h |
|---|---|---|---|---|---|
| Control | - | - | - | - | - |
| 5% Sucrose | + | ++ | ++ | +++ | + |
| 5% Lactose | + | + | ++ | +++ | ++ |
| 5% Dextrose | + | + | + | ++ | + |

(Key: - = No growth, + = Viable growth ++ = Visible growth, +++ = Optimum growth)

Optimum EPS production arise during early stationary phase than in the late stationary phase of culture [49]. The highest EPS production was recorded in *Pseudomonas aeruginosa* (226 µg/ml), *Streptococcus mutans* (220 µg/ml) and *Bacillus subtilis* (206 µg/ml) in N-free medium after 7 d of incubation at 28±2°C [56].

### 3.8 Phosphate solubilization by isolate N

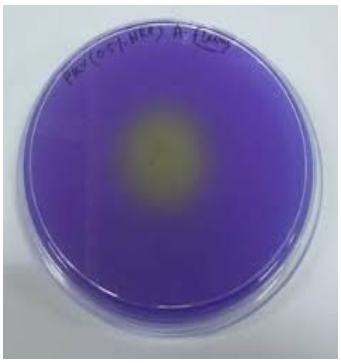


**Figure 5(a)**

**Figure 5(b)**

**Figure 5(c)**

**Figure 5: Result of phosphate solubilization on PSK plate,**
**(a) Test of 4th d,**
**(b) Test of 5th d,**
**(c) Test of 6th d**

Pikovskaya agar medium was inoculated with isolate N and one plate kept as uninoculated and marked as a control, for the estimation of phosphate solubilization. By the use of Pikovskaya's agar plate containing bromophenol blue, zone of phosphate solubilization was measured up to 6d of incubation at 28±2°C. Phosphate solubilization zone was obeserved in the isolate N and the diameter of zone of solubilization was 39 mm (Figure 7, Table 8) [50].

**Table 7: Zone of Phosphate solubilization of given isolate**

| Day | Zone |
|---|---|
| Control | 00 mm |
| 4th d | 18 mm |
| 5th d | 25 mm |
| 6th d | 39 mm |

In the experiment done by Zalak Rathod (2016) [52], there was 24 mm and 22 mm of phosphate solubilization zone observed in *Bacillus* spp.

## 4. CONCLUSION

In this study, from the isolate characterization it is observed that this isolate N is *Bacillus* sp. and further examination of this *Bacillus* sp. by 16s rRNA nucleotide gene expression study it is observed that this Bacillus sp. is *Bacillus tequilensis*. This experimental work was done thrice and average results were mentioned. Production of various kinds of plant hormones like IAA, exopolysaccharides, ammonia; and effect of NaCl, phosphate solubilization, etc. was shown by *B. tequilensis*. The all above mentioned results documented that *B. tequilensis* produced IAA, ammonia, exopolysaccharides, etc. which are beneficial to plants and shows phosphate solubilization. *B. tequilensis* showed the maximum growth under higher rate of salinity stress e.g., 20% NaCl containing medium and didn't produce any harmful compound which is toxic for us. This plant growth promoting bacteria play a positive role and do not show any harmful effect. This is beneficial for us and can be used as a biofertilizers. PSM gives excellent opportunity to develop eco-friendly P-biofertilizers to be used as supplements and/or alternative to chemical fertilizers.

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**Conflict of interest -** The authors have no conflicts of interest in preparing of this research article.

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