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Augmentation of salt tolerance by halotolerant *Bacillus amyloliquefaciens* as highly phosphate solubilizer

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Abstract: The vitality, growth and survival of microorganisms are highly affected because of salinity in the environment. Salinity results in osmotic stress and toxic ions, which in turn hinder the soil microbial activity. The salt tolerant *Bacillus* spp. showed Optimum growth at 20% salt concentration. While the salt tolerance observation, other test revealed the ability of organism to exhibit phosphate solubilization. Under salt stress, various mechanisms such as exopolysaccharides production, indole acetic acid production, ammonia production were stimulated. The salt stress was alleviated by these various mechanisms for the means of survival.

Index Terms **- Ammonia, Exopolysaccharide, Halotolerant, Phosphate solubilizing bacteria, Plant growth promoting bacteria.**

I. INTRODUCTION

It is expected that the human population will reach 8 billion by 2025. To avert or decrease food shortage, saline soils have to be improved and administered to meet the food demand of an ever growing human population (Ladeiro, 2012). Salinity stress is responsible for various negative effects in plant growth such as decrease in leaf water potential, because of the presence of sodium ions closure of stomata in turn inhibiting the leaf expansion and disrupting the photosynthesis. The plant growth is disrupted because of the presence of sodium and chloride ions. The salinity disrupts the nutrient uptake as well as water. These toxic ions accumulate in older leaves, reduce the yield and they cause the death of the plant (Carillo et al., 2011, Patel et al., 2021). Salinity affects microbes through two crucial mechanisms which are osmotic effects and specific ion effects (Oren, A., 1999; Chhabra R., 1996). Another factor having an influence on microbes is soil water content. Soil water potential which concerns to the energy level by which the water is held in the soil, also closely relates to soil salinity, it is influenced by osmotic potential in the soil solution (Yan et al., 2015). In soil, microorganisms contribute in oxidation, nitrification, ammonification, nitrogen fixation, sulphur oxidation, phosphorous mineralization and some other processes which in turn consequently partakes in the decomposition of soil organic matter and nutrient transformation (Amato & Ladd, 1994; French et al., 2009; Yan et al., 2015), which would cause distress in presence of high salt concentrations, in turn impairing soil fertility and agriculture. Salinity over time leads to sodicity, which is the accumulation of sodium salt relative to other salt cations (Shi & Wang, 2005; Wiesman, 2009). These cations include Na+ (sodium), Ca2+ (calcium), Mg2+ (magnesium) and K+ (potassium), and the anions Cl- (chloride), SO42- (sulfate), HCO3- (bicarbonate), CO32- (carbonate) and NO3- (nitrate) (Shi & Wang, 2005). Soluble salts amplify the osmotic potential (more negative) of the soil water, extorting water out of cells which may eradicate microbes and terminate roots through plasmolysis. Low osmotic potential also makes it more difficult for roots and microbes to eliminate water from the soil (Oren, 1999). Plants and microbes can adapt to low osmotic potential by accumulating osmolytes; however, synthesis of osmolytes requires large amounts of energy and thus resulting in reduced growth and activity (Oren, 1999; Wichern, Wichern, & Joergensen, 2006). At high-concentrations, certain ions, including Na+, Cl-, and HCO3-, are toxic to many plants (Chhabra, 1996). Studies have designated that hypersaline habitats tend to harbour unique and ancient microbial life capable of surviving or thriving under this harsh environment; such microorganisms are known as halophiles. Halophiles are salt loving and have adapted to thrive in extreme conditions of salinity (Akcay & Kaya, 2019). The term endophyte was first established by (De Bary, 1866), defined as any organism that grows within plant tissues, but now they are more specifically described in terms of their types (bacterial and fungal) and plant-microbe relationships (obligate or facultative with the host plant) (Petrini, 1991; Cabral et al., 1993; Hallmann et al., 1997; Rosenblueth and Martínez-Romero, 2006; Khare et al., 2018). According to Fesel and Zuccaro (2016), an inclusive definition of endophytes does not specify their functional relationship and despite commensalistic symbionts, they can survive as latent pathogens or saprotrophs to mutualistic associations. The mutualistic association by inhabiting plant tissues both intercellularly and/or intracellularly is a well-versed component of their lifestyle and most of the

modern researches clearly show that survival and health of plants are greatly dependent upon these microorganisms (Hardoim et al., 2015; Potshangbam et al., 2017). The environmental conditions under which the host plant is growing effects the endophytic population and the endophytic profile (Tan and Zou , 2001). They are also reported to supply essential vitamins to plants (Rodelas et al., 1993; Mehta et al., 2020) For example, in rhizobia-legume symbiosis, which is also considered as one of the best-described endophytic relationships, the bacterial endosymbiont presides over plant's need for nitrogen (Santoyo et al., 2016). According to Hallman et al. (1997), the endophytic bacteria involved in biological control showed advantages of having the same ecological niche of the pathogen and could be protected from diverse abiotic influences (Rathod Z., 2016). The microbial exopolysaccharides (EPS) are polymers that principally comprise of carbohydrates and they are excreted by some bacteria outside of their cell walls. It may also contain a number of different organic and inorganic constituents (Llamas et al., 2010; Sutherland, 1990).The microbial EPS are used in several applications in food, textile, pharmaceutical, agricultural, paint and petroleum industries where emulsifying (Calvo et al., 2002), viscosifying, suspending and chelating agents are required (Cojoc et al., 2009;Martines et al., 2005;Okutani, 1992). There are number of reports on EPS production by rather halophilic species e.g., Halomonas eurihalina, Halomonas maura, Halomonas ventosae, Halomonas anticariensis, Alteromonas hispanice, Idiomarina rambicola and Idiomarina fontisalpitosi (Nanjani & Soni, 2012). Protein can be converted back into microbial central metabolism through deamination of amino acids. This catabolic reaction produces free ammonia. Free ammonia is reassimilated and utilized for synthesis of all nitrogenous metabolites (Wernick & Liao, 2013). Bacteria that colonize the rhizosphere and plant roots, and enhance plant growth by any mechanism are referred to as PGPR. PGPR can exhibit a variety of characteristics responsible for influencing plant growth. The common traits include production of plant growth regulators (like auxin, gibberellin, and ethylene), siderophores, HCN and antibiotics (Arshad et al., 1992). Bacteria synthesize auxins in order to perturb host physiological processes for their own benefit (Shih-Yung, 2010). Indole acetic acid (IAA) is a common product of L- tryptophan metabolism produced by several microorganisms including Plant Growth- Promoting Rhizobacteria (PGPR). IAA is one of the most physiologically active auxins (Lynch, 1985). Cyanide is a prospective inhibitor of enzymes involved in major plant metabolic processes such as respiration, CO2 and nitrate assimilation, and carbohydrate metabolism, and can also obstruct photosynthetic electron transport (Grossman, 1996; Kremer & Souissi, 2001). Activity of ACC-deaminases enzyme that degrade the ACC, the precursor of ethylene is stimulated by IAA produced by bacteria(Glick et al., 2005)under salt affected soil condition, ACC-deaminase activity of bacteria can be helping for better growth and yield of crops(Metaliya et al., 2021). 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 (Azziz et al., 2012). 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 (Ezawa et al., 2002). Phosphorous can be found abundantly in soil in two forms which are organic and inorganic. However, until converted into a form that aids for root uptake, it is not accessible to the plant. Recurrent application of chemical fertilizers can result in formation of complexes of inorganic phosphorous that is insoluble, which cannot be absorbed by plants (Rengel and Marschner, 2005). Organisms with P-solubilizing ability increase the availability of soluble phosphate and make them available to the plant, and can enhance plant growth by increasing the efficiency of biological nitrogen fixation or enhancing the availability of other trace elements such as iron, zinc, etc. and by production of plant growth promoting regulators (Sattar and Gaur, 1987; Kucey etal., 1989; Ponumurugan and Gopi, 2006; Bhadaniya et al., 2021). Phosphate solubilizing bacteria present in soil are capable of transforming soil P to the forms available to plant. Microbial biomass assimilates soluble P, and prevents it from adsorption or fixation (Khan and Joergesen, 2009). Citrus Limon separates have shown a strong antimicrobial activity against *Salmonella enteritidis*, *E. coli* and *S. aureus* (Ahmed et al., 1998; Akinyemi et al., 2006; Rathod Z., 2020).

II. MATERIALS AND METHODS

2.1 Isolation of bacterial strain

Isolate A was isolated leaf of Citrus lemon. The isolate had been extracted from inside the plants and thus it is an endophyte. The isolate was streaked onto a nutrient agar plate and nutrient agar slant for further studies and preservation respectively.

2.2 Cultural and morphological characteristics

Colonies of isolate A on nutrient agar comprising 20% salt concentration were examined for cultural characteristics. From a well-isolated colony Gram staining was performed for determination of morphological characteristics under microscope. (Patel & Patel, 2015)Results were recorded.

2.3 Effect of temperature on growth

The isolate A was streaked on nutrient agar and were incubated at different temperatures including 4°C, 25±3°C (room temperature), 30°C, 37°C, 45°C and 55°C. The isolate was also inoculated in nutrient broth at these same temperatures. These were incubated for 24 hours along with a control at each temperature. Results were recorded.

2.4 Effect of salinity on growth

The salt tolerance of isolate A was examined on different salt concentrations including 5%, 10%, 15%, 20% and 25% containing nutrient agar medium as well as nutrient broth containing these same salt concentrations were incubated for five days and keep a control of each salt concentration.. Growth was examined and results were recorded.

2.5 Indole acetic acid production

60 ml of nutrient broth added with 1% tryptophan in a 250 ml flask was inoculated with the isolate A and incubated for 8 days in dark condition at 30±2°C on orbital shaker at 150 rpm. Another flask was kept uninoculated as a control. 5ml sample was withdrawn from day 5 to 8 from the test and control flasks. Both samples were centrifuges at 10,000 for 15 minutes and 2ml supernatant was taken in a test tube. Evaluation of the production of indole acetic acid was done using freshly prepared salkowsky reagent. Salkowski reagent was prepared by mixing two ml of 0.5 M FeCl3 with 98 ml 35% perchloric acid (Gordon and Weber, 1951). For this test the ratio of reagent to culture supernatant is 1:1. So, 2ml reagent was added in both the test tubes and they were kept in dark for 30 minutes and OD was taken at 536 nm spectrometrically. Results were recorded.

2.6 EPS production

For EPS production examination, nutrient broth having three different sugars namely sucrose, maltose and dextrose, 5% concentration of sugars was added in media. They were inoculated with the isolate A and incubated at 28±2°C on orbital shaker at 150 rpm for 5 days. A control was kept for each sugar. Each day from the 2nd day to the 5th day, 2 ml media was withdrawn aseptically and 6 ml chilled acetone was added and the mixtures were kept in the freezer overnight. The precipitates formed were separated through centrifugation at 10,000 rpm for 20 minutes (Mody et al., 1989). The pellet was taken on a previously weighed

aluminium foil and wet weight was recorded. After that for 40 minutes the foil was kept in an oven at 65±3°C and weight was recorded every 10 minutes.

2.7 Phosphate solubilization

For phosphate solubilization estimation, a spot was streaked on Pikovskaya's agar medium (Pikovskaya, 1948) incorporated with bromophenol blue and tricalcium phosphate as the source of insoluble phosphate. They were incubated for 6 days at 28±2°C with a control. Results were recorded.

2.8 Ammonia production

For ammonia production freshly grown culture of isolate A was inoculated in 10 ml peptone water. A control was kept. Both tubes were incubated for 4 to 8 days at 36±2°C. 0.5 ml Nessler's reagent was added in each tube. Development of brown to yellow colour is checked (Cappuccino and Sherman, 1992).

III. Results and Discussion

3.1 Isolate on nutrient agar plate and nutrient agar slant

Bacterial isolate A was isolated from the leaves of *Citrus lemon*. Thus, the isolate was an endophyte. Following isolation, the isolate was transferred onto a nutrient agar for further tests to determine various capabilities of the organism. The isolate was also transferred onto a nutrient agar slant for preservation.

Figure 1(a)

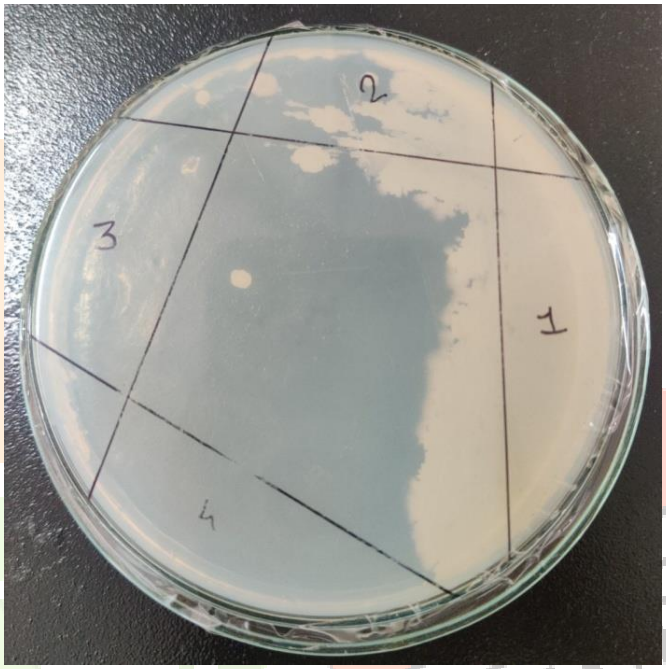


Figure 1(b)

Figure 1: Isolates on nutrient agar media

3.2 Morphological and cultural characteristics

Morphological characteristics were examined (using Gram staining technique) through microscope and cultural characteristics were examined directly from the plate as well as microscopically. The results are as follows:

table 1: morphological characteristics

Morphological characteristics	Organism
Size	Large
Shape	Rod
Arrangement	Single, Chain
Gram's reaction	Gram positive

table 2: cultural characteristics

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

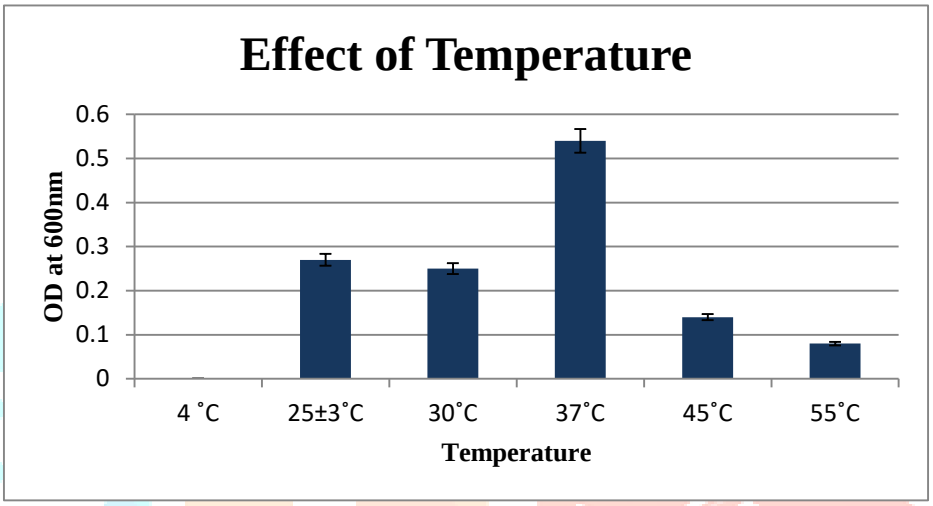
3.3 Effect of temperature on growth

The isolate A was streaked onto nutrient agar medium & inoculated in nutrient broth and incubated at different temperatures. The results are as follows:

table 3: effect of temperature

Effect of temperature	
Temperature	Growth
4℃	-
25±3℃	++
30℃	++
37℃	+++
45℃	+
55℃	+
Control	-

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

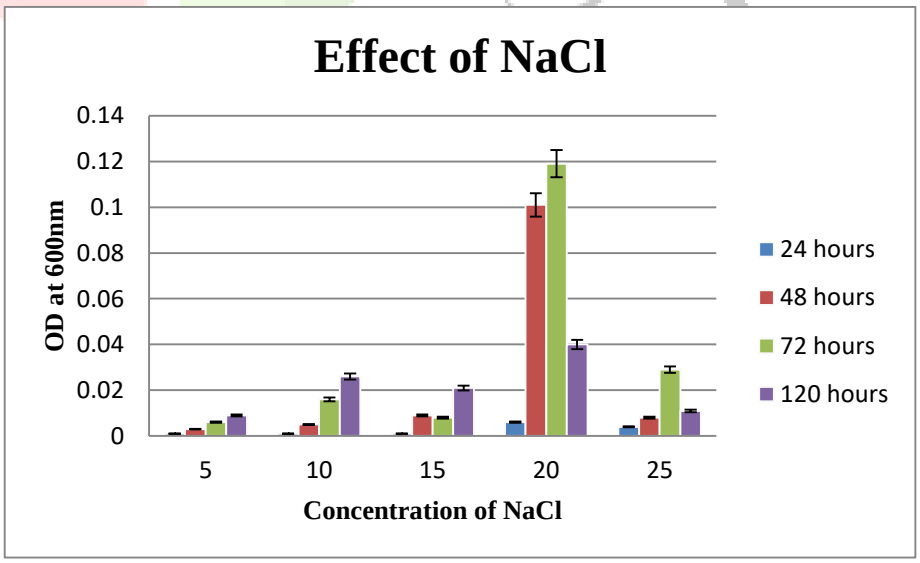


graph 1: effect of temperature on growth

As per the results, the isolate A gave Optimum growth at 37℃, excellent growth was observed at 30℃and room temperature (25±3℃), good growth was observed at 45℃ and viable growth was seen at 55℃. At 4℃ and in control, no growth was observed.

3.4 Effect of salinity on growth

Isolate A was streaked onto nutrient agar having different NaCl concentrations that include 5%, 10%, 15%, 20%, 25% and nutrient broth was also inoculated having these different NaCl concentrations. They were incubated at 28±2℃ for 120 hours.



graph 2: effect of salinity on growth

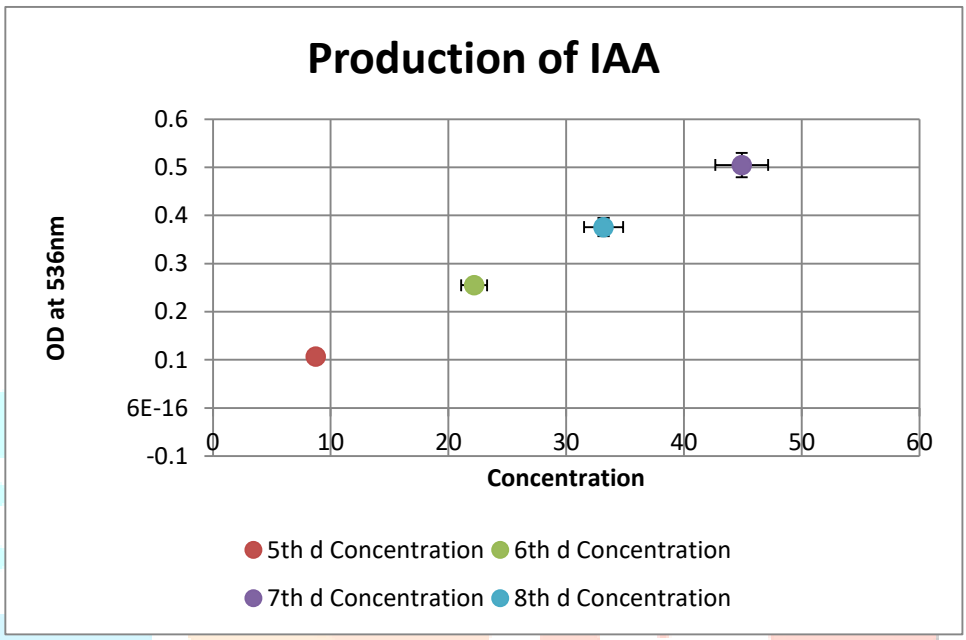
Isolate A showed Optimum growth at 20% NaCl concentration after 72 hours incubation. Good growth was observed after 48 hours and 120 hours. Viable growth was present at 25% NaCl concentration. Control showed no growth.

3.5 Indole acetic acid production

Isolate A was inoculated in nutrient broth containing 1% tryptophan and incubated for 5 to 8 days at 30±2℃. 5 ml of sample was withdrawn from day 5 to day 8. After centrifugation, 2 ml supernatant was mixed with 2 ml freshly prepared Salkowski reagent and kept in dark for 30 minutes and the OD was taken at 536 nm spectrometrically.

table 4: iaa production

Test	Concentration(µg/ml)
5th day	8.72
6th day	22.18
7th day	44.90
8th day	33.18



graph 3: production of iaa

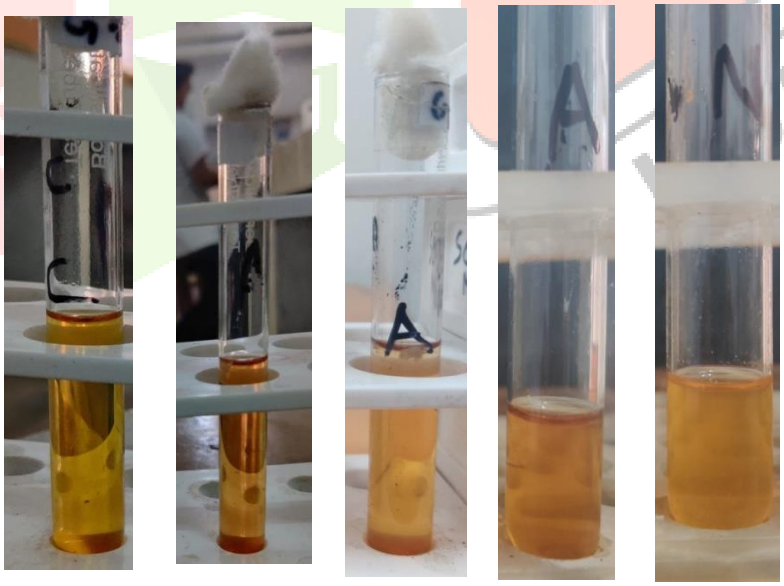


Figure 2(a) Figure 2(b) Figure 2(c) Figure 2(d) Figure 2(e)

figure 2 iaa production

figure 2 (a): test of control,

figure 2 (b): test of 5th day test,

figure 2 (c): test of 6th day test,

figure 2 (d): test of 7th day test,

figure 2 (e): test of 8th day test

3.6 EPS production

For EPS production, nutrient broth added with 5% sugar concentrations were inoculated with the isolate A and incubated for 5 days at 28±2℃. For every 2 ml of test sample withdrawn, 6 ml chilled acetone was added to be kept overnight. The precipitates were separated after centrifugation. The pellet was weighed and dried for 40 minutes. Results are as follows:

Table 5: EPS production

Sugars	24 hours	48 hours	72 hours	96 hours	120 hours
5% sucrose	+	+	+	++	+
5% lactose	+	+	+	++	-
5% dextrose	+	++	+++	+++	+

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

Optimum growth of the isolate A was observed after 96 hours of incubation in all the sugars. After 120 hours incubation no growth was observed in 5% Lactose containing nutrient broth. In media containing dextrose, visible growth was observed from 48 hours. Other media showed good to viable growth also. Control showed no growth.

3.7 Phosphate solubilization

The pikovskaya media was inoculated with the isolate A through spot-inoculation. The medium was incubated at 28±2℃ for 6days. Clear zone of phosphate solubilization was observed on 6th day.

figure 3(a) **figure 3(b)** **figure 3(c)**

figure 3: phosphate solubilization on pikovskaya medium
figure 3 (a) 4th day,
figure 3 (b) 5th day,
figure 3 (c) 6th day

table 6: zone of phosphate solubilization of isolate

Day	Zone
4th	20 mm
5th	29 mm
6th	37 mm

Clear zone of phosphate solubilization appeared on the 4th day of incubation with the diameter of 20 mm, on the 5th day zone became larger and reached diameter 29 mm and on the 6th day the zone gained the diameter of 37 mm. control showed no growth.

3.8 Ammonia production

Isolate A was inoculated in 10 ml peptone water and incubated for 4 to 8 days at 36±2℃. Control showed no growth. From day 4 to day 6, aseptically obtained 2 ml sample was mixed with 0.5 ml Nessler's reagent and colour change was observed.

Table 7: Ammonia production

Day	growth
4th	++
5th	++
6th	+++

(Key: + = Viable Growth, ++ = Visible Growth, +++ = Optimum Growth)

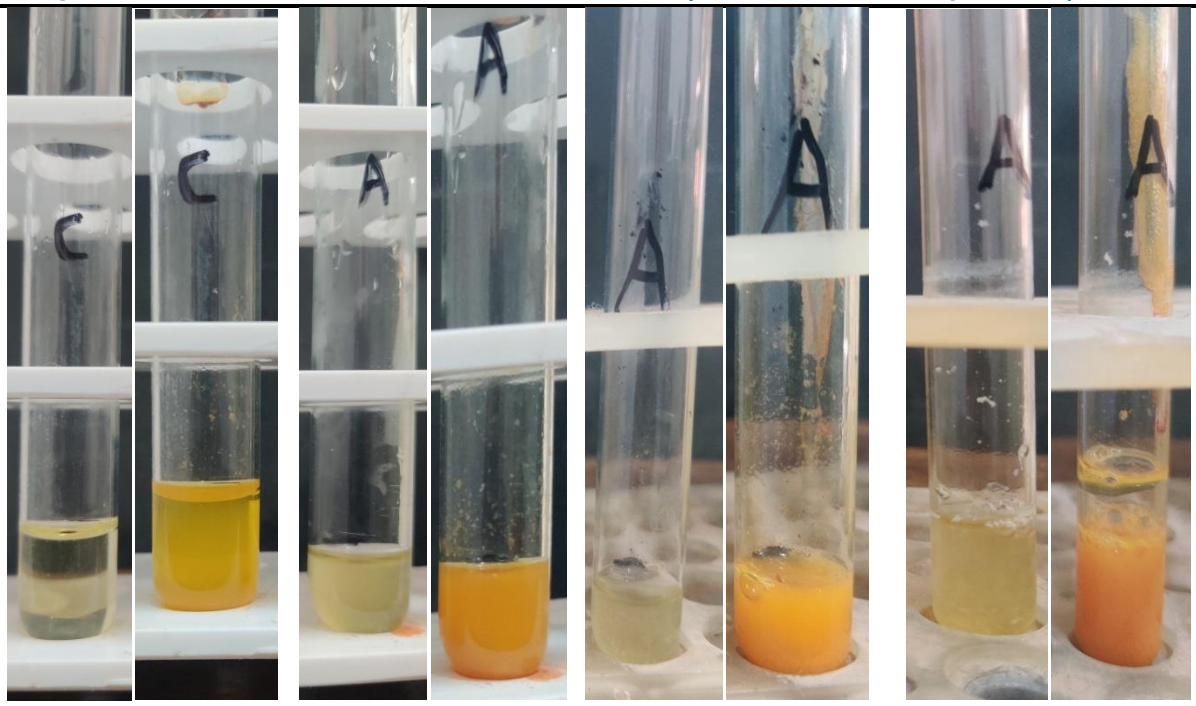


figure 4(a) **figure 4(b)** **figure 4(c)** **figure 4(d)**

figure 4: ammonia production

(Left side test tubes having creamish yellow media are without Nessler's reagent and right side test tubes having orange yellow media are with Nessler's reagent.)

figure 4 (a) test of control,
figure 4 (b) test of 4th day,
figure 4 (c) test of 5th day,
figure 4 (d) test of 6th day

As seen (in Figure 4), ammonia production was observed on 4th to 6th day. On the 4th and 5th day the production was observed in moderate amounts and on the 6th day Optimum ammonia production was observed.

IV. Conclusion

The present work aimed to study the role of isolate A to be able to tolerate the salt stress as well as perform various additional activities as a mechanism to alleviate the salt stress. Besides salt tolerance, it also possesses other plant growth promoting properties including IAA production, EPS production, phosphate solubilization and some others. The isolate A has been identified as *Bacillus amyloliquefaciens* by 16s rRNA analysis and nucleotide gene expression study. In conclusion, this study indicates the potential of *Bacillus amyloliquefaciens* to be used as biofertilizer for improve the saline stress conditions faced by the plants.

V. Acknowledgment

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VI. Conflict of Interest

The authors have no conflict of interest in preparing this research article.

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