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Evaluation of stress tolerance of *Azotobacter* isolates

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Evaluation of 50 *Azotobacter* isolates was done for tolerance to pH, high temperature, and salinity, and four isolates (Azo-48, Azo-137, Azo-144, and Azo-160) passed all three tests. Tolerance to pH (5.0) was shown by a large quota of 20 isolates, while most of them (75%) also showed temperature-tolerance ability. Analysis of colony characteristics, biochemical properties, nitrogen fixing capacity, and phytohormone production of 14 selected isolates was carried out. Finally, after an extensive evaluation and critical analysis, we were able to sort out the above-mentioned four isolates and one of them (Azo-144) exhibited tolerance to a reasonably high degree of salinity stress.

Keywords: stress, *Azotobacter*, tolerance, bio-fertilizers

INTRODUCTION

Biofertilisers are of paramount importance in agricultural production as they facilitate proportionate distribution of nutrients to plants (Wani et al., 2013). They are indeed a living entity that, when inoculated into the soil, helps in genesis of boundless beneficial microbes. In extreme climatic conditions like high and low temperatures, a high pH, etc., where crop production is extremely difficult, these microbes help in enhancing crop productivity without compromising the soil fertility status. Keeping this fact in mind, the main obstacle facing us lies in screening and isolation of stress-tolerant microbes. For development of an ideal microbial consortium capable of tackling a high degree of abiotic stress, our prime target should be well orchestrated in order to evaluate those microbes. In this sphere, some notable contributions have been made all over the globe by numerous researchers. For example, Kumar et al. (2014) identified several strains of *Pseudomonas* and *Bacillus* showing tolerance to high temperature (50°C) and salinity(14×10^2 dS/m); four bacterial strains of *Pseudomonas frederiksbergensis* OS211, *Flavobacteriumglaciei* OB146, *Pseudomonas vancouverensis* OB155, and *Pseudomonas frederiksbergensis* OS261 were found effective in countering chilling stress on the tomato plant by Subramanian et al. (2016). Similarly, Orhan (2016) evaluated 18 bacterial isolates for halophilic and halotolerant capability in the wheat plant under hydroponic conditions.

Azotobacter is an aerobic and free-living bacterium widely distributed throughout the world and characterised by an added advantage of nitrogen-fixation (Becking, 1981; Balow et al., 1979). Moreover, its propensity to utilise a wide array of substrates containing carbohydrates (Lineweaver, 1933), alcohols (Gomare et al., 2013), and organic

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acids (Wu et al., 1987) as a source of carbon augmented with several metabolic capabilities makes it an indispensible choice for scientists in the domain of biofertilizer research. Adding to its ability of potent nitrogen fixation by non-symbiotic means (Tejera et al., 2005), it is also noteworthy to mention that it has the highest metabolic rate among all organisms. Regardless of its vital and imperative nature in agriculture, the extent of its usage is still low due to its sensitiveness to acute abiotic stresses such as high temperature (Hogg, 2005), acidic pH (Raju et al., 1974; Khullar, Chahal, 1975), and high salinity (Ninawe, Paulraj, 2003). Therefore our approach in this experiment is subjected towards the evaluation of effective *Azotobacter* isolates competent enough for giving expected performance and withstanding harsh conditions of abiotic stress.

MATERIALS AND METHODS

Culture retrieval

Azotobacter slants were taken from the collection of the Department of Agricultural Microbiology under Indira Gandhi Krishi Viswavidhyalaya located at Raipur, Chhattisgarh, India. A total of 50 cultures were then revived by streaking them on Petri plates containing Jensen's medium having a pH 7.0; this was followed by incubation at 28°C in an incubator for 48–72 hours. Single distinctive bacterial colonies were then taken and inoculated in tube slants consisting of Jensen medium for further use.

Colony characteristics

Characteristics of all isolates were determined as per Bergey's Manual of Systematic Bacteriology (2001). The type of shape, arrangement of cells, and their Gram reaction were elaborately studied. Later on, after development of colonies was done on the ideal medium through streaking, a thorough examination of morphological characteristics (form, colour, elevation, margin, consistency) was carried out.

Biochemical analysis

The isolates were characterized using standard biochemical methods as given in Bergey's Manual of Systematic Bacteriology (2001). A series of biochemical test were done for selected 14 bacterial isolates after meticulous screening for pH and temperature tolerance capability in order to further determine their distinctive biochemical properties. The catalase test, the acid production test, the starch hydrolysis test, Winogradsky test, the urease production test, and the pigment test in iron-deficient medium were carried out. A very specialized test – the Triple Sugar Iron Agar test – was conducted in order to diagnose them for glucose, lactose, and sucrose fermentation along with peptone catabolization, gas and H_2S production ability.

Screening for pH, temperature, and salt tolerance

All the 50 cultures of *Azotobacter* isolates were grown in Jensen's medium on Petri plates with a range of pH of 5.0, 5.5, 6.0, 7.0, and 8.0. These plates were then incubated in an incubator at 28°C for a period of 48 to 72 hours. Based on their nature and the type of growth on the entire span of pH arrays, these isolates were screened for pH tolerance. The bacterial isolates were screened for temperature tolerance at distinct temperatures beginning at 28°C, 37°C, 40°C, 45°C, 50°C, and ending at higher temperatures of 55°C and 60°C. The cultures were first inoculated in a 5 ml Jensen's broth tube which were taken from the log phase culture and were subsequently incubated for 48–72 hours at 28°C. After appropriate growth in these tubes was observed, they were further incubated at respective above-mentioned temperatures for 30 min. To finally ascertain their growth, 100 µl of broth culture from each tube were then spread on agar plates containing Jensen's media and allowed for growing at 28°C for 48–72 hours. Signs of promising growth shown by isolates for temperature tolerance against control were selected. *Azotobacter* isolates were inoculated on 5ml nutrient broth medium with NaCl salt of 0%, 2%, 5%, and 10% concentration. It was then incubated at 28°C for 48–72 hours. Upon development of satisfactory growth at different concentrations of NaCl, they were compared with control for their salt tolerance capability.

Nitrogen-fixing capacity of *Azotobacter* isolates in pure culture

Establishing of N_2 fixing capacity of *Azotobacter* isolates was done by the micro-Kjeldhal method of Bergersen, 1980. Firstly, the isolates were allowed to grow in semisolid N_2-free Jensen's medium supplemented with L-glutamic acid (100 mg/l) and incubated for five days at a temperature of 28°C. Then 5 ml of well-homogenised culture were taken and digested with 5 ml concentrated H_2SO_4 along with 5 grams of digestion mixture (K_2SO_4 and $CuSO_4$ in a ratio of 10:1) until the content was clear enough. Following cooling, 5ml of aliquot was then transferred into a micro-Kjeldhal distillation unit and 10 ml of 40% NaOH was added, after which the distillation process was carried out. Upon completion, ammonia gas produced was collected in a conical flask containing 10 ml of 2% boric acid dissolved in mixed indicator (83.3 mg bromocresol green and 16.6 mg methyl red indicator dissolved in 10 ml of 95% alcohol) and titrated against 0.005N H_2SO_4. Using titre value and the formula of 1 ml of 0.005N H_2SO_4 = 0.00007 g of N, the nitrogen fixed *in vitro* was calculated and expressed in N fixed/g of malate supplied.

Calculation:

(sample titre – blank titre) × normality of standard × 14.007

nitrogen content (mg) = sample weight (gram)

Production of phytohormones

Estimation of IAA (indole acetic acid) production potential of selected bacterial isolates was tested in Jensen's or Bark's N_2-free broth further supplemented with 0.005 M tryptophan at 28°C. After an incubation of three days, the concentration of IAA in culture broth was established by the spectrophotometric method using Salkowski reagent. 1.5 ml of culture supernatant was mixed with 1 ml of Salkowski reagent. The amount of red colour intensity developed within 30 minutes and was checked at 530 nm using a scanning spectrophotometer. A standard curve was prepared from a standard solution of indole acetic acid (Mali and Bodhankar, 2009) for determining the concentration of IAA. In addition to this, the potential of isolates to produce gibberellins was also determined. The isolates were made to grow in Jensen's nitrogen-free broth at 28°C and incubated for three days after which the amount of gibberellins produced was estimated through the spectrophotometric method as described by Mali and Bodhankar, 2009.

RESULTS AND DISCUSSION

Screening of *Azotobacter* isolates for pH tolerance

All the 50 bacterial isolates showed encouraging positive growth at a neutral pH (7.0) from which we can infer that this is the epitome of pH for appropriate growth of all the bacterial isolates even though almost all of them, with the exception of nine (Azo-26, Azo-27, Azo-45, Azo-89, Azo-123, Azo-126, Azo-127, Azo-145, Azo-149), also grew at pH 8.0. In other words, these nine are the isolates that failed to grow on any other pH except the neutral one. The growth of some isolates started decreasing gradually as we progressed forward towards the acidic pH range thus showing their intolerance towards acidic medium and, in a way, giving us a chance for deciphering those tolerant isolates out of the pool of 50 isolates. Out of the remaining 41 isolates, seven of them (Azo-15, Azo-51, Azo-98, Azo-109, Azo-115, Azo-122, Azo-146) exhibited growth within pH range of 6.0 to 8.0, whereas only one isolate (Azo-65) grew at the pH 5.5. Meanwhile, as many as 20 isolates (Azo-18, Azo-32, Azo-33, Azo-35, Azo-38, Azo-47, Azo-48, Azo-91, Azo-108, Azo-121, Azo-125, Azo-129, Azo-133, Azo-137, Azo-144, Azo-154, Azo-156, Azo-160, Azo-162) succeeded in reaching the 5.0 stage, henceforth imprinting their ability to grow on a wide range of pH (5.0 to 8.0) (Table 1).

Screening of *Azotobacter* isolates for temperature tolerance

Transition in the temperature range has a profound impact on growth and development of *Azotobacter* bacteria which can be distinctly seen in the Table 2. Having perceived from our experiment that the ideal temperature for growth of all *Azotobacter* isolates is 28°C, from

Table 1. **Screening of *Azotobacter* isolates for pH tolerance**

No.	Isolates	pH 5.0	pH 5.5	pH 6.0	pH 7.0	pH 8.0
1.	Azo-11	–	–	–	+	+
2.	Azo-15	–	–	+	+	+
3.	Azo-18	+	+	+	+	+
4.	Azo-23	–	–	–	+	+
5.	Azo-24	–	–	–	+	+
6.	Azo-25	–	–	–	+	+
7.	Azo-26	–	–	–	+	–
8.	Azo-27	–	–	–	+	–
9.	Azo-28	–	–	–	+	+
10.	Azo-32	+	+	+	+	+
11.	Azo-33	+	+	+	+	+
12.	Azo-34	–	–	–	+	+
13.	Azo-35	+	+	+	+	+
14.	Azo-38	+	+	+	+	+
15.	Azo-39	–	–	–	+	+
16.	Azo-40	–	–	–	+	+
17.	Azo-44	–	–	–	+	+
18.	Azo-45	–	–	–	+	–
19.	Azo-46	–	–	–	+	+
20.	Azo-47	+	+	+	+	+
21.	Azo-48	+	+	+	+	+
22	Azo-51	–	–	+	+	+
23.	Azo-58	–	–	–	+	+
24.	Azo-65	–	+	+	+	+
25.	Azo-83	–	–	–	+	+
26.	Azo-89	–	–	–	+	–
27.	Azo-91	+	+	+	+	+
28.	Azo-98	–	–	+	+	+
29.	Azo-108	+	+	+	+	+
30.	Azo-109	–	–	+	+	+
31.	Azo-115	–	–	+	+	+
32.	Azo-121	+	+	+	+	+
33.	Azo-122	–	–	+	+	+
34.	Azo-123	–	–	–	+	–
35.	Azo-125	+	+	+	+	+
36.	Azo-126	–	–	–	+	–
37.	Azo-127	–	–	–	+	–
38.	Azo-129	+	+	+	+	+
39.	Azo-133	+	+	+	+	+
40.	Azo-137	+	+	+	+	+
41.	Azo-144	+	+	+	+	+
42.	Azo-145	+	+	+	+	–

Table 1. **(Continued)**

No.	Isolates	pH 5.0	pH 5.5	pH 6.0	pH 7.0	pH 8.0
43.	Azo-146	–	–	+	+	+
44.	Azo-149	–	–	–	+	–
45.	Azo-154	+	+	+	+	+
46.	Azo-155	–	–	–	+	+
47.	Azo-156	+	+	+	+	+
48.	Azo-159	–	–	+	+	+
49.	Azo-160	+	+	+	+	+
50.	Azo-162	+	+	+	+	+

Table 2. **Screening of *Azotobacter* isolates for temperature tolerance**

		Temperature, °C						
No.	Isolates	28°C	37°C	40°C	45°C	50°C	55°C	60°C
1.	Azo-11	+	+	+	–	–	–	–
2.	Azo-15	+	+	+	+	+	+	+
3.	Azo-18	+	+	+	+	+	+	+
4.	Azo-23	+	–	–	–	–	–	–
5.	Azo-24	+	–	–	–	–	–	–
6.	Azo-25	+	–	–	–	–	–	–
7.	Azo-26	+	–	–	–	–	–	–
8.	Azo-27	+	–	–	–	–	–	–
9.	Azo-28	+	–	–	–	–	–	–
10.	Azo-32	+	+	+	+	+	+	+
11.	Azo-33	+	+	+	+	+	+	+
12.	Azo-34	+	+	+	+	+	–	–
13.	Azo-35	+	+	+	+	+	+	+
14.	Azo-38	+	+	+	+	+	+	+
15.	Azo-39	+	–	–	–	–	–	–
16.	Azo-40	+	–	–	–	–	–	–
17.	Azo-44	+	–	–	–	–	–	–
18.	Azo-45	+	–	–	–	–	–	–
19.	Azo-46	+	–	–	–	–	–	–
20.	Azo-47	+	+	+	+	+	+	+
21.	Azo-48	+	+	+	+	+	+	+
22	Azo-51	–	–	+	+	+	+	–
23.	Azo-58	+	–	–	–	–	–	–
24.	Azo-65	+	+	+	+	+	+	+
25.	Azo-83	+	–	–	–	–	–	–
26.	Azo-89	+	–	–	–	–	–	–
27.	Azo-91	+	+	+	+	+	+	+
28.	Azo-98	+	+	–	–	–	–	–
29.	Azo-108	+	+	+	+	+	–	–

Table 2. **(Continued)**

		Temperature, °C						
No.	**Isolates**	**28°C**	**37°C**	**40°C**	**45°C**	**50°C**	**55°C**	**60°C**
30.	Azo-109	+	+	+	+	+	–	–
31.	Azo-115	+	+	+	+	+	–	–
32.	Azo-121	+	+	+	+	+	+	+
33.	Azo-122	+	+	–	–	–	–	–
34.	Azo-123	+	–	–	–	–	–	–
35.	Azo-125	+	+	+	+	+	+	+
36.	Azo-126	+	+	–	–	–	–	–
37.	Azo-127	+	+	+	+	+	–	–
38.	Azo-129	+	+	+	+	+	+	+
39.	Azo-133	+	+	+	+	+	+	–
40.	Azo-137	+	+	+	+	+	+	+
41.	Azo-144	+	+	+	+	+	+	+
42.	Azo-145	+	+	+	+	+	+	–
43.	Azo-146	+	+	+	+	–	–	–
44.	Azo-149	+	+	+	+	–	–	–
45.	Azo-154	+	+	+	+	+	+	–
46.	Azo-155	+	+	+	–	–	–	–
47.	Azo-156	+	+	+	–	–	–	–
48.	Azo-159	+	+	+	–	–	–	–
49.	Azo-160	+	+	+	+	+	+	+
50.	Azo-162	+	+	+	+	+	–	–

our experiment, it was not surprising to notice that the growth started to decrease progressively and ultimately ceased above that particular temperature. Having said that, 16 isolates (Azo-23, Azo-24, Azo-25, Azo-26, Azo-27, Azo-28, Azo-39, Azo-40, Azo-44, Azo-45, Azo-46, Azo-51, Azo-58, Azo-83, Azo-89, Azo-123) stalled their growth at 37°C, while three others (Azo-98, Azo-122, Azo-126) miserably failed to survive 40°C and along with them four isolates (Azo-11, Azo-155, Azo-156, Azo-159) broke down at 45°C. In the course of our experiment, we observed a very peculiar and inconsistent behaviour in Azo-51: it grew within the range of 40° to 50°C, however, it showed no signs of growth below and above that range. Among the left out isolates, two isolates – Azo-146 and Azo-149 – were unsuccessful in reaching 50°C, and isolates Azo-34, Azo-127, and Azo-162 met the same fate at 55°C. Finally, 16 isolates (Azo-15, Azo-18, Azo-32, Azo-33, Azo-35, Azo-38, Azo-47, Azo-48, Azo-65, Azo-91, Azo-121, Azo-125, Azo-129, Azo-137, Azo-144 and Azo-160) remained in our collection that were able to tolerate the temperature as high as 60°C. If we consider both the parameters (pH and temperature) from the angle of comparative vision, we will note an impressive correlation between them, that is, a 75% similarity can be observed between the isolates showing tolerance to pH (5.0–8.0) and temperature (28–60°C). There were 14 isolates of this kind which were selected for the remaining part of our experiment.

Colony characteristics

Colony morphology depicts the true nature of microorganisms. It is an imperative tool for their characterisation and an in-depth study of their behaviour in their natural habitat. The 14 selected isolates displayed negative reaction to Gram staining, possessing a circular form

when viewed on Petri plates with their colony displaying an entire margin which concluded that *Azotobacter* is a Gram-negative bacterium. Some degree of variation was seen in bacterial growth with respect to their texture or consistency. Azo-47 was showing a mucoid type texture while all other isolates showed gummy consistency, except Azo-32 and Azo-121, which were not gummy. Most of the isolates had a transparent colony, with few anomalies (Azo-33, Azo-47, Azo-144) revealing a translucent look. The colour of the colonies was in fact equally exchanged between them, with eight colonies displaying white colour while, in contrast to them, seven were showing off-white colour. Elevation of colony was mostly flat or convex type in the ratio of 8:7, respectively (Table 3).

Biochemical tests

A series of biochemical tests were carried out for a better understanding of the physiochemical functions going on within the cell. The hydrogen peroxide degrading enzyme catalase, which protects the cells from reactive oxygen species by converting it into water and oxygen, was present in all the selected 14 isolates. None of the isolates were found positive for utilising urea and starch due to the lack of urease and α-amylase enzyme, while all of them unambiguously passed the acid production and Winogradsky tests. Azo-47, Azo-91, Azo-137, and Azo-160 grew confidently in an iron-deficient medium, while the rest proved their inability. The triple sugar iron test was used for deducing the ability of the microbes of fermenting different types of sugars (glucose, lactose, sucrose) and producing H_2S and other gases. We observed that only three (Azo-47, Azo-48, Azo-144) of the 15 isolates succeeded in fermenting glucose, sucrose, and fructose, although two others (Azo-137, Azo-160) demonstrated ability of fermenting only glucose. Leaving behind only three isolates (Azo-47, Azo-48, and Azo-144), the remaining 12 exhibited their capability for catabolising peptones. None of the isolates produced any gas or H_2S (Tables 4 and 5).

Nitrogen-fixing capacity

Azotototobacter is a non-symbiotic, aerobic free-living bacterium capable of nitrogen fixation in plants which are devoid of extracting available nitrogen from the soil environment. The *Azotobacter* isolate with the strongest nitrogen-producing ability was Azo-47, which demonstrated unrivalled performance of 11.2 mg of nitrogen per gram of Jensen's medium. The whole spectrum of variation in nitrogen fixation ranged from the maximum of 11.2 to the minimum of

Table 3. **Colony characteristics**

No.	Isolates	Gram stain	Form	Colour	Elevation	Margin	Consistency	Density
1.	Azo-18	negative	circular	Off white	Convex	Entire	Gummy	Transparent
2.	Azo-32	negative	circular	white	flat	entire	not gummy	transparent
3.	Azo-33	negative	circular	white	flat	entire	gummy	not transparent
4.	Azo-35	negative	circular	white	flat	entire	gummy	transparent
5.	Azo-38	negative	circular	white	convex	entire	gummy	transparent
6.	Azo-47	negative	circular	white	flat	entire	mucoid	not transparent
7.	Azo-48	negative	circular	off white	flat	entire	gummy	transparent
8.	Azo-91	negative	circular	off white	convex	entire	gummy	transparent
9.	Azo-121	negative	circular	off white	flat	entire	not gummy	transparent
10.	Azo-125	negative	circular	off white	convex	entire	gummy	transparent
11.	Azo-129	negative	circular	white	flat	entire	gummy	transparent
12.	Azo-137	negative	circular	off white	convex	entire	gummy	transparent
13.	Azo-144	negative	circular	off white	convex	entire	gummy	not transparent
14.	Azo-160	negative	circular	white	convex	entire	gummy	transparent

Table 4. **Biochemical tests**

Serial No.	Isolate No.	Catalase	Starch hydrolysis	Iron deficient	Acid production	Winogradsky	Urease broth
1.	Azo-18	+	–	–	+	+	–
2.	Azo-32	+	–	–	+	+	–
3.	Azo-33	+	–	–	+	+	–
4.	Azo-35	+	–	–	+	+	–
5.	Azo-38	+	–	–	+	+	–
6.	Azo-47	+	–	+	+	+	–
7.	Azo-48	+	–	–	+	+	–
8.	Azo-91	+	–	+	+	+	–
9.	Azo-121	+	–	–	+	+	–
10.	Azo-125	+	–	–	+	+	–
11.	Azo-129	+	–	–	+	+	–
12.	Azo-137	+	–	+	+	+	–
13.	Azo-144	+	–	–	+	+	–
14.	Azo-160	+	–	+	+	+	–

Table 5. **Triple sugar iron test**

Serial No.	Isolate No.	Glucose fermentation	Lactose fermentation	Sucrose fermentation	Peptone catabolization	Gas production	H_2S production
1.	Azo-18	–	–	–	+	–	–
2.	Azo-32	–	–	–	+	–	–
3.	Azo-33	–	–	–	+	–	–
4.	Azo-35	–	–	–	+	–	–
5.	Azo-38	–	–	–	+	–	–
6.	Azo-47	+	+	+	–	–	–
7.	Azo-48	+	+	+	–	–	–
8.	Azo-91	–	–	–	+	–	–
9.	Azo-121	–	–	–	+	–	–
10.	Azo-125	–	–	–	+	–	–
11.	Azo-129	–	–	–	+	–	–
12.	Azo-137	+	–	–	+	–	–
13.	Azo-144	+	+	+	–	–	–
14.	Azo-160	+	–	–	+	–	–

2.8 by Azo-18. Concurrently, six out of 14 isolates crossed the convincing figure of above 8, which was observed in the case of two isolates (Azo-91 and Azo-144, with 8.4 mg/g capacity), while other three (Azo-32, Azo-33, and Azo-137) fixing 9.8 mg/g remained behind Azo-47 (Figure).

Production of phytohormones

Phytohormones, like auxins and gibberellins produced by plants, are well known but it is really interesting to know that they, too, are produced by microbes, in particular bacteria. These hormones serve as plant growth-promoting agents by enhancing root and shoot development

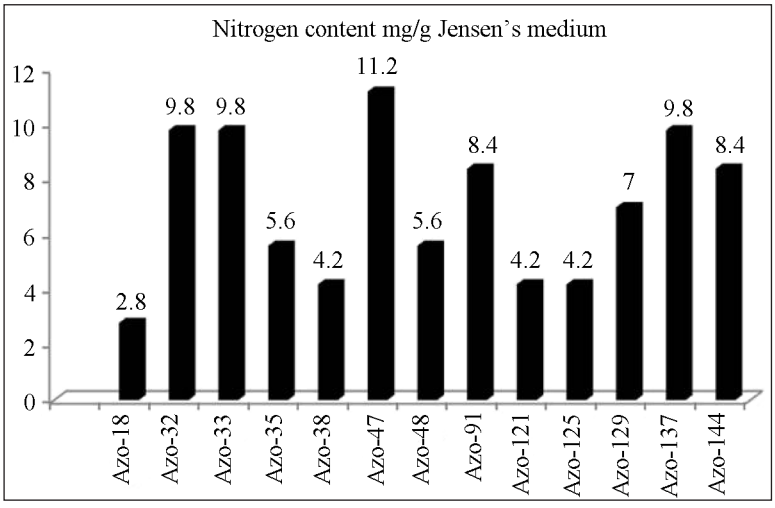

Figure. Nitrogen-fixing capacity

besides acting as anti-pathogenic agents. We found in our work that along with Azo-137, three other isolates (Azo-48, Azo-144 and Azo-160) produced indole acetic acid (IAA) in significant concentrations, but the performers to watch out for were Azo-137 and Azo-48. With OD value at 530 nm in a single-beam spectrophotometer, it was revealed that Azo-137 produced 96.35 µg/ml of IAA beating Azo-48 by a difference of 8.2. Intriguingly, after finding some encouraging results, we decided to check these four isolates (Azo-48, Azo-137, Azo-144, Az0-160) for their salinity tolerance based on the fact that there was some correlation between the amount of phytohormones produced with salinity tolerance, both by bacteria and plant (Tables 6 and 7).

Screening of *Azotobacter* isolates for salt tolerance

An extensive study was conducted for screening these four isolates at different levels of NaCl concentration. At 2% level, all four passed the test with flying colours, but the real challenge lied ahead when they were grown in 5% and 10% salinity

Table 6. **Production of phytohormones**

No.	Isolate No.	Indole acetic acid	Gibberellin
1.	Azo-18	–	–
2.	Azo-32	–	–
3.	Azo-33	–	–
4.	Azo-35	–	–
5.	Azo-38	–	–
6.	Azo-47	–	–
7.	Azo-48	+	–
8.	Azo-91	–	–
9.	Azo-121	–	–
10.	Azo-125	–	–
11.	Azo-129	–	–
12.	Azo-137	+	–
13.	Azo-144	+	–
14.	Azo-160	+	–

Table 7. **Concentration of phytohormones produced**

No.	Isolate No.	OD at 530 nm	Conc. of IAA in µg/ml
1.	Azo-48	0.346	88.15
2.	Azo-137	0.378	96.35
3.	Azo-144	0.136	34.04
4.	Azo-160	0.146	36.87

Table 8. **Screening of *Azotobacter* isolates for salt tolerance**

Serial No.	Isolated No.	0% NaCl	2% NaCl	5% NaCl	10% NaCl
1.	Azo-48	+	+	–	–
2.	Azo-137	+	+	–	–
3.	Azo-144	+	+	+	–
4.	Azo-160	+	+	–	–

mark, which only Azo-144 (at 5% NaCl) managed to cross. The other three isolates miserably failed to grow beyond the 2% level NaCl (Table 8).

DISCUSSION

There has been a tactical shift in the climate of the earth since the onset of the twenty-first century and the two great major causes are environmental pollution and exorbitantly high population. They complement each other in the phenomenon called global warming. The aftermath of such things correlates directly with agricultural productivity in the shadow of an exponential jump in abiotic stresses like pH, high temperature, and salinity. Nonetheless, these conditions also take a toll on microbes besides affecting plants directly (Kumar et al., 2014). Overall, all over the earth, around 40% of total surface accounts for the salinity problem affecting growth and productivity of plants at higher pH (Jadhav et al., 2010). *Azotobacter* sp. is well known to all researchers in the domain of biofertilizer research. A substantial bulk of study has been done in this bacterium endowed with multifarious qualities ranging from non-symbiotic nitrogen-fixing ability to potential use as a biofertilizer capable of plant growth promotion and synthesis of antibiotics and vitamins (Suliasih, 2013; Jiménez et al., 2011). Still, several constrains pose as a major hurdle in further research work, and one of them is its susceptivity to abiotic stresses like acidic pH, high temperature, and salinity (Jnawali et al., 2015); however, several stress-tolerant strains and isolates have been identified. Several species of *Azotobacter*, such as *A. chroococcum*, *A. berijerinkii*, and *A. vivelandii* with enhanced capacity of salt tolerance of up to 35 g/l have been reported by Ravikumar et al. (2004). Higher proline as well as malondialdehyde content in the rice plant inoculated with a novel isolate of *A. vinellandii* under 200 mM NaCl was observed by Sahoo et al. (2014). Drought tolerance ability was exploited by Viscardi et al. (2016) on the tomato plant shown by two strains of *Azotobacter chroococcum* (Strain 67B and 76A).

Our experiment found that the ideal pH and temperature required for proper growth of *Azotobacter* bacterium is a neutral pH (7.0) and a temperature of around 30°C, and it was supported by Alsamowal et al. (2016). It was reported that majority of the isolates (75%) showing pH tolerance at 5.0 were also found to tolerate a maximum temperature of 60°C. Having said this, a virtual correlation can be established between these two factors or, in other words, it can be said that isolates showing tolerance towards pH have the capability to withstand a higher temperature. Similar findings were reported by Chennappa G. et al. (2014), where a strain of *A. chroococcum* was found to simultaneously tolerate a pH of 8.0 and a temperature of 45°C. Jadhav et al. (2010) reported two *Azotobacter* isolates taken from different food samples that simultaneously tolerated a wide pH range (5.0–10.0) and a high temperature (60°C). Nitrogen fixation is one of the inherent features of this bacterium and studies have shown that it can fix up to 15 mg N per gram of glucose it had consumed (Sethi, Adhikary, 2012) besides fixing 20 kg N/ha annually (Rahmayani et al., 2017). From the above findings, an *Azotobacter* isolate (Azo-47) was reported to have the potential not only to fix nitrogen (11.2 mg/g of Jensen's medium utilised) efficiently but also was able to overcome high temperature (60°C) and pH (5.0).

Screening for salinity tolerance was done where we found that Azo-144 was able to resists 5% solution of NaCl and at same time produced a fair amount of IAA (34.04 µg/ml), much less than its other three competitors. Similar results were obtained by Omer et al. (2016), who reported production of IAA under 1% concentration of NaCl. Production of phytohormones activity was reduced drastically with increase in the level of salinity as was shown by Azo-144, which produced the lowest amount of IAA in comparison with other three isolates. Similar findings were reported by Paul et al. (2014) in *Azotobacter chroococcum* for three isolates when they were grown in 1.5 M concentration of NaCl.

CONCLUSIONS

Keeping in mind the rising obstacles in the form of abiotic stresses with respect to climate change,

we must formulate a decisive plan to sustain free-flowing production in the agricultural sector. To truly unleash the untapped potential of *Azotobacter* sp. in the shape of a well-configured physical matter called biofertilizer for harsh climatic conditions, we must explore novel strains. A great deal of study into the understanding of its biophysical and physiological properties when grown under stress conditions needs to be highlighted in order to attain fully-fledged sustainability in the agricultural sector.

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***AZOTOBACTER* IZOLIATŲ ATSPARUMO STRESUI VERTINIMAS**

Santrauka

Aukštos temperatūros, pH ir druskingumo tolerancijos vertinimas buvo atliktas 50 *Azotobacter* izoliatų, iš kurių keturi izoliatai (Azo-48, Azo-137, Azo-144 ir Azo-160) buvo tolerantiški visiems trims rodmenims. Toleranciją pH (5,0) parodė 20 izoliatų, dauguma jų (75%) toleravo ir temperatūrą. Atlikta 14-os atrinktų izoliatų kolonijų charakteristikų, biocheminių savybių, azoto fiksavimo pajėgumo ir fitohormonų produkcijos analizė. Galiausiai, atlikus išsamų vertinimą ir kritinę analizę, buvo atrinkti anksčiau minėti keturi izoliatai, iš kurių vienas (Azo-144) toleravo gana didelį druskingumą.

Raktažodžiai: stresas, *Azotobacter*, tolerancija, biotrąšos