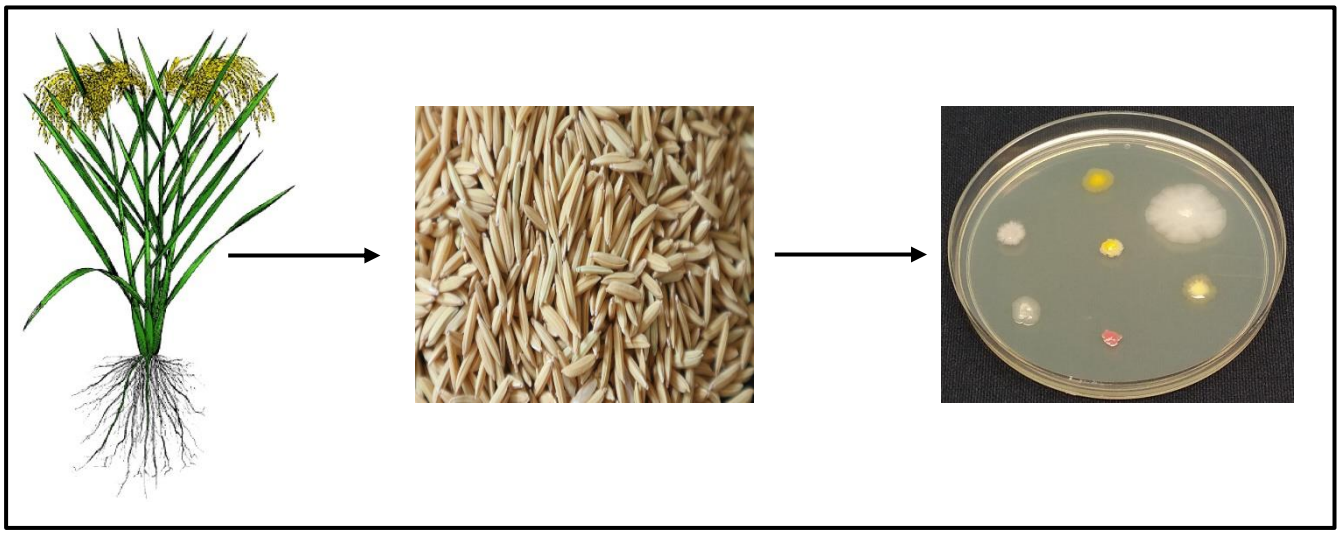
Characterization of Rice Seed Endophytes for their Abiotic Stress Tolerance Attributes



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Date:

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Abstract

Bacterial endophytes are non-pathogenic microorganisms internally associated with plants and are known to play a vital role in promoting plant growth and resistance to biotic and abiotic stress conditions. They are even capable of eliminating plant pathogens. Abiotic stresses, particularly salinity problems, heavy metal toxicity, low pH, and accumulation of xenobiotics in the soil due to harmful pesticides and fertilizers, are affecting plant health drastically. They constitute a significant threat to the agriculture sector in crop production and ecosystems' stability. Endophytic bacteria may hence be utilized as a substitute to lessen the use of toxic chemicals and pesticides. They can be used as a biological agent in plant growth promotion, including managing the global environment. Rice is an important food crop yet it is highly prone to damage due to abiotic stress. This report aimed to investigate the role of the endophytic microbes of rice seeds in tolerating various abiotic stress levels. Seven bacterial strains were isolated in this study, and their tolerance level was checked against different abiotic factors. As observed from the experiments the bacterial strain RS07 has shown the highest salt stress and temperature tolerance while the bacterial strain RS06 showed the highest pH and xenobiotics tolerance as inferred from the results thus making them the most potential endophytic bacteria. These results propose that the isolated bacteria can eventually be used to boost plant growth, combat extreme climatic conditions, harsh environment and benefit host plant to fight against fungal pathogens thereby creating a sustainable agriculture.

Keywords: rice seeds, endophytic bacteria, abiotic stress, agriculture, yield

1 Introduction

The plant microbiome is considered to be one of the essential causal factors for plant health and productivity. Lorenz Hiltner started in-depth research on this topic in 1901 (Hartmann et al., 2008). The study of microbial communities in close association with plants gives us insight into the mechanisms involved in their beneficial interactions. There is a great diversity of these plant microbiomes across the globe residing on plants both externally and internally. Among these, the group of organisms which inhabit a plant are referred to as endophytes. They can be either bacteria or fungi. Such beneficial bacteria living inside host plants that help improve nutrient uptake and enhance plant growth even under harsh conditions are termed endophytic bacteria. These bacteria have been found to colonize various parts of plants, such as seeds, roots, stems, leaves, fruits, flowers (Berg et al.,2014), and meristematic tissues. They do not cause any damage to their host plants and can combat plant pathogens (Mousa et al.,2015). Endophytic bacteria help plants sustain biotic and abiotic stress conditions by secreting certain enzymes, phytohormones, antagonistic substances, siderophores, and antimicrobial metabolites. Hence studies and research on bacterial endophytes are fundamental for further application as plant growth promoting factors and for controlling plant pathogens. This way, they can be used as a good substitute for chemical fertilizers and pesticides in the near future.

Population densities of bacterial endophytes are highly variable in different plants and tissues. Studies have shown that they can rise from hundreds up to as high as 9×10^9 bacteria per gram of plant tissue (Jacobs et al., 1985; Misaghi, Donndelinger, 1990; Chi et al., 2005). Typically, a higher density of endophyte populations is found in plant roots and other below-ground tissues than in above-ground tissues.

Rice (*Oryza sativa)* is one of the world's most widely grown food crops. It is an essential staple cereal for more than half of the world's population, West Bengal being India's leading rice producer. With the escalating population, the requirement for rice is increasing at a rapid rate. However, rice plant diseases such as bacterial leaf blight, sheath blight etc., are prevalent. According to some sources, about a 10–15% reduction in rice yield in tropical Asia (Gianessi et al., 2014) due to such diseases is being reported. The steadily growing global population has made food security a severe challenge. According to the 2020 Global Agricultural Productivity (GAP) Report, the GAP needs to increase by 1.73% annually to meet the food demand of 10 billion people in 2050 (GAP Report 2020). However, climate change and human activities have led to a remarkable decline in soil quality, with a subsequent loss in crop yield. Significant abiotic stresses such as drought, high salinity, cold weather,

submergence, and metallic content in soil from harmful fertilizers and pesticides result in low crop yield, decreased plant growth, pigment content, water relations, membrane integrity, photosynthetic activity and a lot more. Among these stresses, drought or high temperature is the first environmental stress responsible for decreased agricultural production globally. Salinity is the second most prevalent soil problem in rice-growing countries after the drought. Rice is a salt-sensitive crop, especially in its early seedling stages, limiting its productivity. Research have shown that the decline in rice yield under reasonably salt-affected soils is anticipated to be 68%. The next most destructive abiotic factor for plants is heavy metals. Plants growing on these soils show a reduction in growth, performance, and yield. To minimize the chances of rice plant diseases and to tolerate the extreme abiotic conditions, the use of bacterial endophytes could be of great importance. Although rice has complicated physiological and biochemical mechanisms to overcome the adverse effects of abiotic stresses (Singh et al., 2014; Zafar et al., 2018; Ganie et al., 2019; Moraes de Freitas et al., 2019; Oladosu et al., 2019), endophytes can be used to improve its responses. Phytohormone-producing endophytes improve plant performance and reduce abiotic stress-induced damage in rice.

In this study, rice seeds were used to extract the endophytic bacteria due to their easy availability, low cost, rapid growth rate and bulk production. Besides, seeds are considered to be the most mature form of a plant. At the dormant stage, there is an adequate amount of nutrients and food in the seeds allowing them to germinate efficiently. Also, bacterial endophytes present in the seed can be passed on to the next generations through heredity. Suppose these endophytic bacteria are successfully transmitted from one generation of rice to the next. In that case, it will not only allow the strain of rice to target and combat specific diseases but also help plant growth.

2 Objective

The purpose of this study was to isolate endophytic bacteria from rice seeds and investigate their ability to tolerate abiotic stress conditions such as salt stress, temperature, pH, heavy metals and xenobiotics tolerance.

3 Materials and Methods

3.1 Sample Collection

Rice seed provided by the lab was used as sample.

3.2 Isolation of bacterial endophytes

To isolate the endophytic bacteria, we undergo a series of tests to get the desired product.

At first, the seeds need to be surface sterilized. Surface sterilization is the most crucial part to get rid of any external bacteria or other microbes and avoiding any kind of contamination. Rice seeds were washed in running water for 10 mins and twice with double distilled water for 1 min. Next the seeds were dipped in fungicide (Bavistin). It was followed by dipping in (v/v) Mercuric chloride solution for 3 mins and 70% (v/v) ethanol for 3 mins and then washed five-seven times with sterile double distilled water for 2 mins. Few rice seeds from the last rinse were transferred to agar containing petri plate to check complete external disinfection process and incubated for 48 hours. The remaining rice seeds were then mixed with 0.9% NaCl solution and crushed thoroughly using autoclaved mortar and pestle. The sample solution containing those crushed seeds was then transferred to an Eppendorf tube. Tenfold serial dilution was performed and the aliquots were spread on agar plates in three replicates for each dilution. Three media were used in this experiment namely- LA (Luria Agar) media, TSA (Tryptic Soy Agar) media and R2A (Reasoner's 2A agar) media. The plates containing the sample were then allowed to incubate at 30℃ (5-7 days) for bacterial growth. After incubation period distinct colonies of bacteria appearing on the Petri plates were picked up using sterile loop.

3.3 Morphological characterization

For characterization of bacterial isolates, standard identification protocols were performed. Different colonies of endophytic bacteria that appeared on the petri plate were observed. Seven different colonies were found to grow on the petri plate. Based on the different colony morphology, these colonies were then separated to fresh petri plates and named as RS01, RS02, RS03, RS04, RS05, RS06, and RS07 respectively. Hence forth, they were allowed to multiply in the individual petri plates.

3.4 Bacterial genomic DNA isolation

Following standard protocols of the phenol-chloroform method (Marmur et al.,1961), the genomic DNA extraction was performed of each isolate from the 24hour fresh cultures. The fresh cultures were composed of each bacteria growing separately in 5ml LB media which was kept at 30℃ with shaking conditions at 120 rpm. Their extracted DNA were then subjected to agarose gel electrophoresis was to visualize the DNA bands. Isolated bacteria samples were taken and centrifuged at 6000 rpm for 7 to 8 mins. Next, the sample is washed and mixed thoroughly with 10:1 Tris HCl, EDTA. After that, it is centrifuged at 6000 rpm for 5 mins. After discarding the supernatant,

230 µl resuspending buffer, 20 µl lysozyme and 5 µl RNase are added to the pellet and appropriately dissolved. Next, it is allowed to incubate at 37℃ for 1hr. Then, after applying 450 µl lysing solution and 50 µl SDS, it is again allowed to incubate at 60℃ for 45 mins. Then 750 µl phenol is added and inverted, mixed 3-5 times. It is further centrifuged at 13000 rpm for 10 mins. The upper phase is then collected and mixed well with an equal volume of chloroform. Again, it is allowed to centrifuge at 13000 rpm for 10 mins. It is followed by adding 1/10th of sodium acetate and two volumes of chilled ethanol. The samples are then further kept at -20℃ for 15-20 mins. After centrifuging at 13000 rpm for 10 mins for one last time, the supernatant is discarded. Afterwards, it is air dried, and hence the sample is dissolved in 20 µl TE (10: 1). Finally, Gel is run, taking 5 µl DNA and mixing it with 1 µl dye.

4 Tolerance level of the bacterial endophytes

In this study we used the isolated bacteria to check various abiotic stress tolerance ability.

4.1 Abiotic stress condition

Abiotic stress is one of the detrimental factors that adversely effects plant growth and productivity. The negative impact of external non-living factors on living organisms in a certain environment is termed as abiotic stress. These may include conditions such as low or high temperatures, salinity, drought, heavy metal content or xenobiotics in soil etc. Too much salinity or imprecise levels of pH or extreme temperature may slow down plant growth. Heavy metals refer to metallic elements that have relatively high density and are poisonous to the environment. Excess accumulation of heavy metals in soil can hinder plant growth by affecting metabolism. Xenobiotics are chemical substances to which an organism is exposed that are extrinsic to the normal metabolism of that organism—for example- drugs, pollutants, insecticides, chemical carcinogens etc. An unrestricted pileup of xenobiotics can cause severe toxicity to the plant. In this research work, various abiotic stress factors were implied to check the tolerance levels of bacteria.

4.1.1 Salt tolerance

Different concentrations of salt were used in Luria-Bertani (LB) media and bacterial growth was observed. A total of seven bacteria were used in this case. Seven test tubes were taken for each bacterium and NaCl was added in 0%, 1%, 2%, 4%, 6%, 8% and 10% concentrations respectively to those test tubes containing Luria-Bertani (LB) nutrient broth. Seven bacterial strains namely RS01, RS02, RS03, RS04, RS05, RS06, and RS07 were then inoculated in the nutrient broth containing the different concentrations of NaCl followed by incubation for 24 hours. Corresponding OD values at 600 nm were then measured.

4.1.2 pH tolerance

The pH of Luria-Bertani (LB) media was adjusted to different levels and bacterial growth was observed. Seven test tubes were taken for each of 7 bacterium and pH of the Luria-Bertani (LB) nutrient broths were adjusted to 2, 4, 6, 8, 10, 12 and 14 respectively. Seven bacterial strains namely RS01, RS02, RS03, RS04, RS05, RS06, and RS07 were then inoculated in the nutrient broth containing different pH levels followed by incubation for 24 hours. Corresponding OD values at 600nm were then measured.

4.1.3 Temperature tolerance

Luria-Bertani (LB) media containing the bacteria were kept at different temperature and their growth was observed. Luria-Bertani (LB) media was poured in seven test tubes and bacterial strains namely RS01, RS02, RS03, RS04, RS05, RS06, and RS07 were then inoculated in the nutrient broth. These tubes were then allowed to incubate in 4℃, 15℃, 30℃, 40℃ and 60℃ respectively. After 24 hours of incubation their OD values were measured at 600 nm.

4.1.4 Heavy Metal tolerance

Arsenic was chosen to check the metallic tolerance of the endophytes. As was taken in concentrations 10µg/ml, 40 µg/ml, 80 µg/ml, 100 µg/ml, 150 µg/ml and 200 µg/ml and added to respective test tubes containing autoclaved Luria-Bertani (LB) broth. Then the bacterial strains were added. After incubation for 24 hours their OD values were measured at 600 nm. Another six test tubes were taken separately for preparing the control media which did not contain any bacterial strain. The purpose of this control media was to compare with the bacteria containing media and predict their tolerance capacity.

4.1.5 Xenobiotics tolerance

To check the tolerance of endophytes in terms of Xenobiotics effect, EtBr [Ethidium bromide] and AO [Acridine orange] have been used in the experiment. EtBr and AO were added in concentrations 10µg/ml, 40 µg/ml, 80 µg/ml, 100 µg/ml, 150 µg/ml and 200 µg/ml respectively to test tubes containing autoclaved Luria-Bertani (LB) broth. Thereafter the bacterial strains were added and kept in incubator for 24 hours. After incubation their OD values were measured at 600 nm. Another six test tubes were taken and the same concentrations of EtBr and AO were added separately in each case for the controlled media. This did not contain any bacteria strain and was used to compare the results with those media containing bacterial strains and comment on their tolerance ability.

5 Result and Interpretation

5.1 Morphological Identification and Genomic DNA Isolation

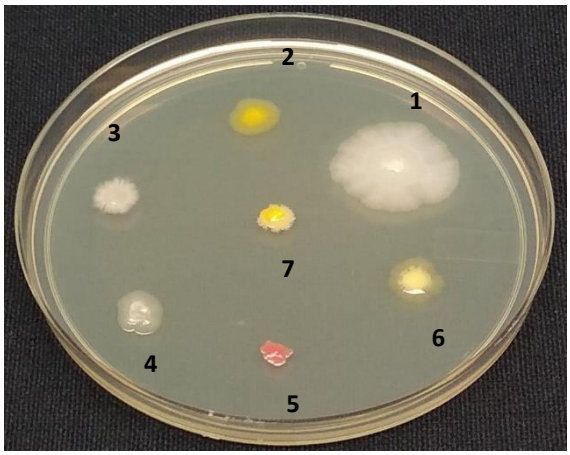

Fig. 1 showing colony morphology of the bacterial isolates

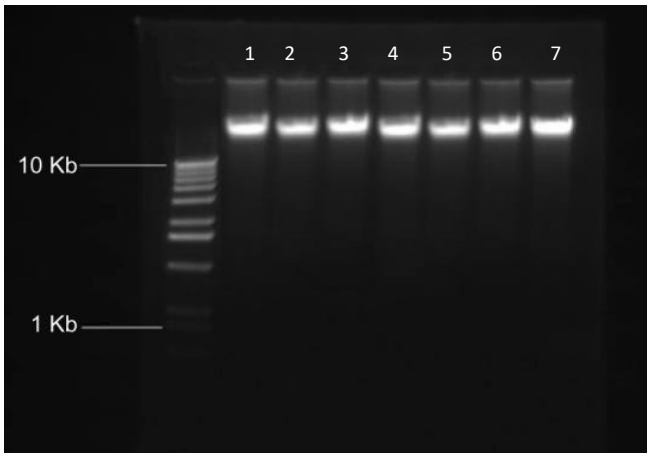

Fig. 2 showing respective DNA bands of the seven bacterial isolates

In the above figure (Fig. 1) we can see different colonies of endophytic bacteria after successful isolation i.e., free from any contamination. The difference in their colour and structure is clearly visible from the figure. They are labelled as 1, 2, 3, 4, 5, 6 and 7 which respectively denotes bacterial strains RS01. RS02, RS03, RS04, RS05, RS06 and RS07. It was observed that RS05 specifically couldn't grow on LB (Luria-Bertani) media. So, it was separately grown on R2A (Reasoner's 2A agar) media.

DNA was extracted from each of the seven bacterial isolates and agarose gel electrophoresis was performed. The figure above (Fig. 2) shows the respective DNA bands obtained from the gel run. These bands were visualized using ChemiDoc Imaging Systems. The lanes 1, 2, 3, 4, 5, 6 and 7 refers to the DNA of RS01, RS02, RS03, RS04, RS05, RS06 and RS07 subsequently. Ladder sequence was used against the respective DNA samples to check the band size.

Bar graphs were formulated based on the OD values corresponding to different concentrations of respective abiotic factors namely salt stress, pH, temperature, heavy metals and xenobiotics. The detailed description of the graphs regarding the endophytes abiotic stress tolerance are discussed below.

5.2 Salt tolerance

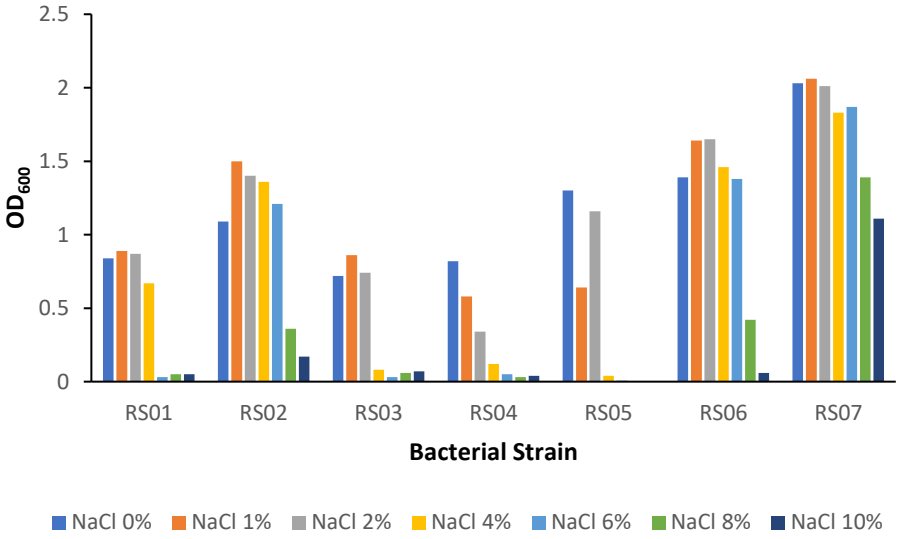

Fig. 3 showing different salt enduring capacity by rice seed endophytic bacteria

Usually, 1% NaCl is considered to be ideal for LB media. Compared to that the tolerance level of the bacteria in different concentrations of NaCl were observed. From the above figure (Fig. 3), we can see bacterial strain RS07 has a high capacity of tolerating salinity as it was found to survive in all the concentration of NaCl. Bacterial strains RS02 and RS06 were able to tolerate up to 6% NaCl content whereas RS01 was able to tolerate up to 4% NaCl content. RS03 and RS05 couldn't tolerate more than 2% NaCl. As mentioned earlier, although bacteria need 1% NaCl to grow in LB media yet all the seven strains survived at no saline conditions as well.

5.3 pH tolerance

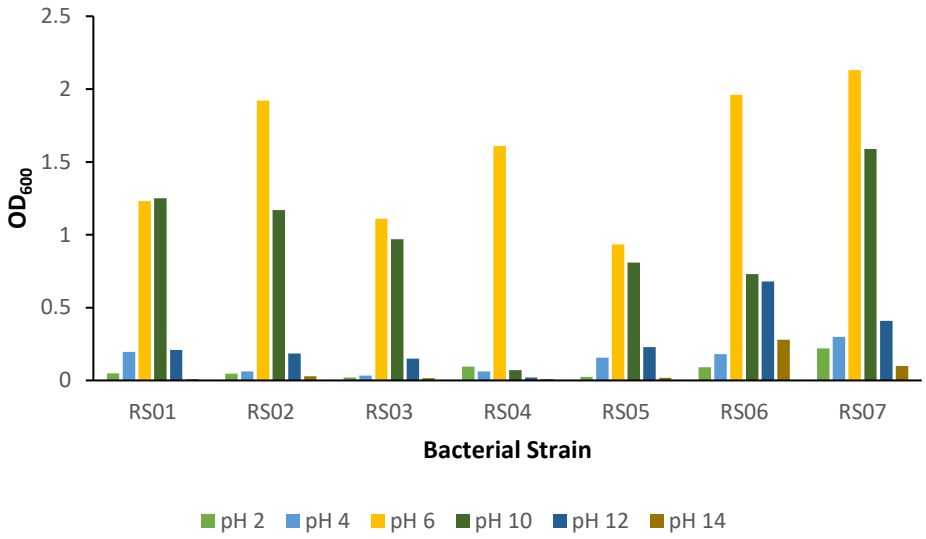

Fig. 4 showing different pH tolerance by rice seed endophytic bacteria

Traditionally the optimum pH for a bacterium to grow in a Luria-Bertani (LB) media is 7. The closest pH level to the standard one is pH 6. The diagram (Fig. 4) shows that all the strains i.e., RS01, RS02, RS03, RS04, RS05, RS06 and RS07 were found to grow in pH 6. In alkaline medium, the strains RS01, RS02, RS03, RS05, RS06 and RS07 could grow maximum up to pH 10. Strains RS01, RS05, RS06 and RS07 somewhat struggled to live at acidic condition i.e., at pH4. RS04 couldn't thrive at any pH other than pH6. No other bacteria other than RS07 existed at highly acidic condition which is pH2. So, we can claim that amongst all the strains the potential of RS07 to tolerate acidic as well as alkaline pH is the highest.

5.4 Temperature tolerance

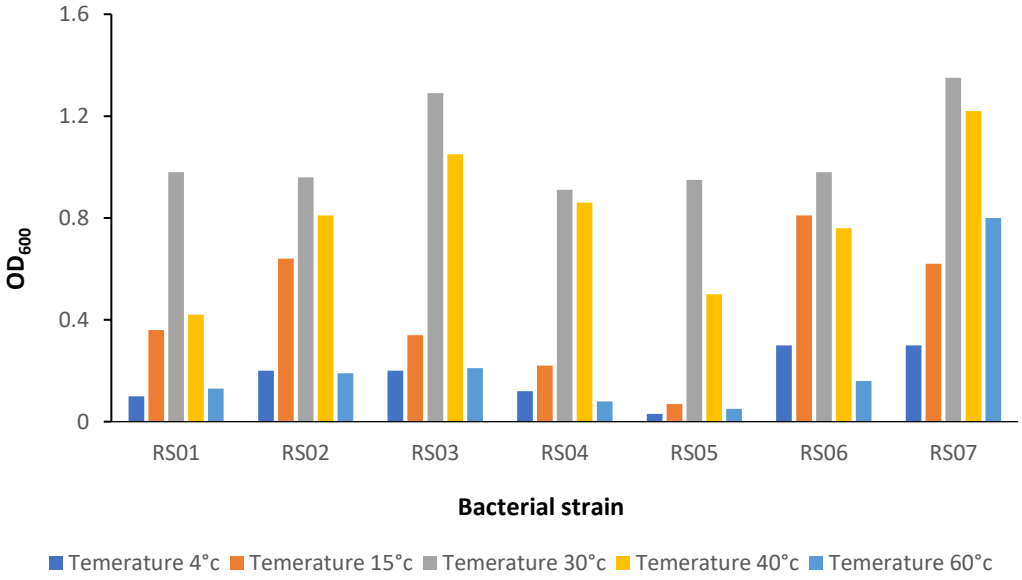

Fig. 5 showing temperature tolerance of the respective bacterial strains

The optimum temperature for bacteria to grow in a Luria-Bertani (LB) media is 30℃. Referring to the optimum temperature the tolerance level of the seven bacterial strains at different temperatures were observed. The bar graph (Fig. 5) shows that the strain RS07 could thrive in all the temperatures as high as 60℃ and even low as 4℃. Strains RS02, RS03, RS04 and RS06 endured up to 40℃. Strains RS01 and RS05 also survived up to 40℃ but their tolerance capacity was not so good. At very low temperature i.e., at 4℃ strains RS02, RS03 and RS06 were able to pull through whereas RS01 couldn't. However, strain RS01 sustained 15℃ so as the strains RS02, RS03, RS04, RS06 and RS07. The strain RS05 could neither tolerate 4℃ nor 15℃.

5.5 Heavy Metal tolerance

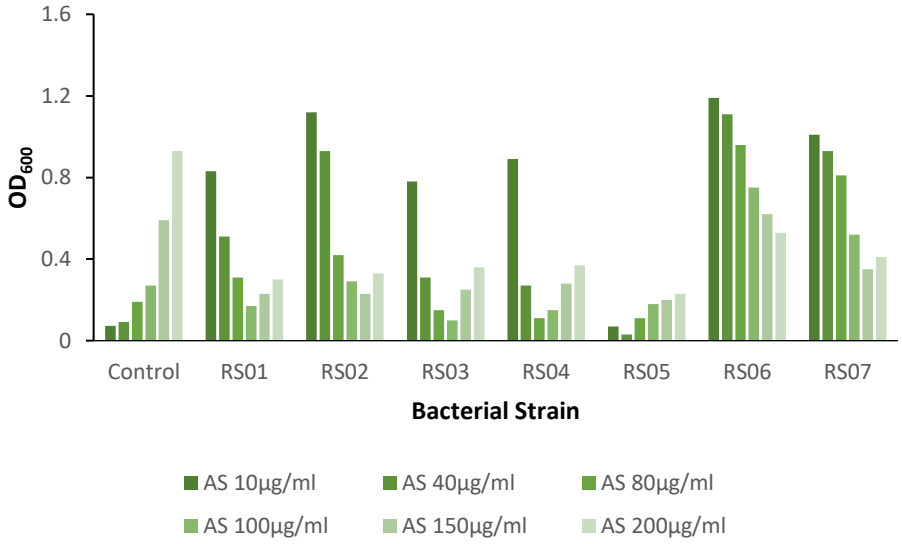

Fig. 6 showing Arsenic tolerance by rice seed bacterial endophytes [In this fig. AS represents Arsenic]

This bar graph (Fig. 6) shows us the results of heavy metal tolerance by the different bacterial strains against their corresponding OD values. From the graph it is clearly evident that bacterial strain RS05 was not able to tolerate any concentration of Arsenic in the media not even the minimum concentration i.e., 10µg/ml. The strains RS02, RS06 and RS07 were capable of tolerating Arsenic up to 100µg/ml. RS06 could exceptionally sustain at 150 µg/ml making it potentially stronger for heavy metal tolerance. Bacterial strains RS03 and RS04 could maximum tolerate up to 40 µg/ml Arsenic concentration in media. None of them survived at 200 µg/ml.

5.6 Xenobiotics tolerance

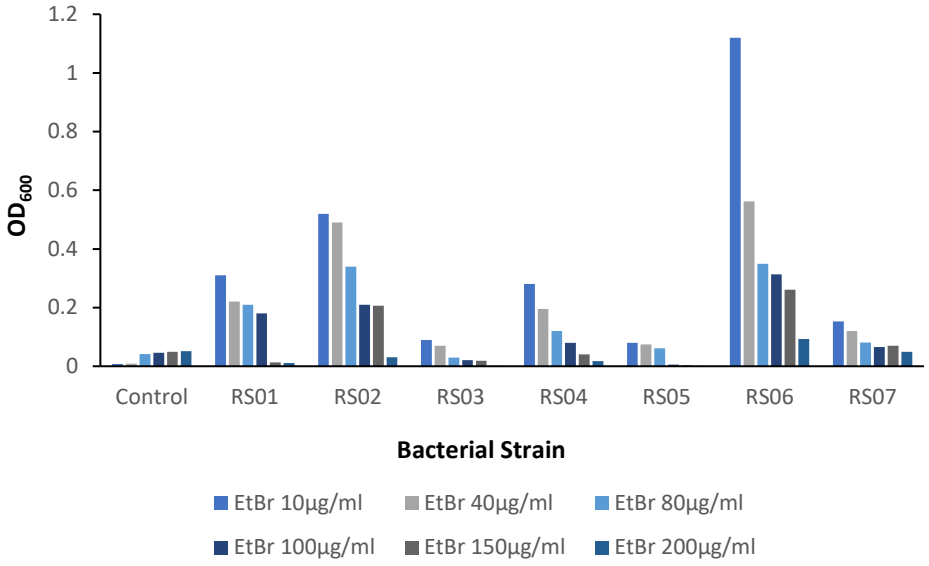

Fig. 7 showing different concentrations of EtBr tolerance by rice seed endophytic bacteria [Here, in this fig. EtBr represents Ethidium Bromide]

This bar graph (from the Fig. 7) shows the capacity of different bacteria to tolerate EtBr content in media compared to the control. The control only contains respective OD values with refer to different EtBr concentrations. Here, we can see the strains RS02 and RS06 to be more efficiently sustaining up to 150 µg/ml of EtBr than the other strains. Strain RS01 and RS04 shows survival capacity up till 100 µg/ml EtBr concentration. Strain RS03 on the other hand barely survived till 40 µg/ml of EtBr. RS05 pulled through 80 µg/ml of EtBr and RS07 made it till 150 µg/ml.

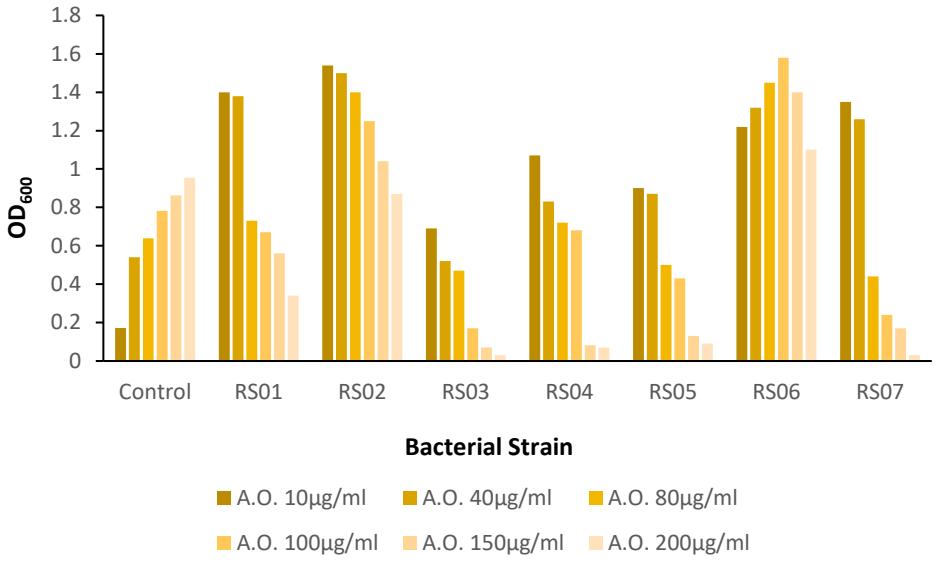

Fig. 8 showing different concentrations of Acridine Orange tolerance by rice seed endophytic bacteria [In the above figure A.O. represents Acridine Orange]

From the above bar graph (Fig. 8) we can predict the enduring capacity of bacterial strains in presence of Acridine Orange by comparing it with the control. It is clearly evident from the graph that bacterial strains RS02 and RS06 were capable of enduring all the A.O. concentrations in the media i.e., up to 200 µg/ml. The strains RS01 and RS04 strived up to 80 µg/ml while RS03, RS05 and RS07 could tolerate up to 40 µg/ml of A.O. concentration in LB media.

6 Conclusion

Endophytes present in seeds improves plant growth, delay flower senescence, help phytoremediation and extend plant tolerance against biotic and abiotic stress. The main motive behind this whole research is to replace the conventional method of using fertilizers with endophytic bacteria to increase plant productivity and yield. Furthermore, in a world where the society frequently faces unpredictable climatic changes and drastic changes in weather, it is necessary to think of sustainable agriculture, particularly in situations like heavy rainfall, drought, intense heat, higher salinity in soil especially in coastal areas. Despite of rice being a staple food crop all over India, especially West Bengal damage of rice crops due to such abiotic stress factors or climatic changes is a serious threat to the mankind. These conditions can decline the rate of plant growth and increase the chances of damage thereby concerning the demand to meet the needs of food. Therefore, if we can implement these potential bacteria in agriculture effectively, the outcome will be propitious- be it in terms of combating plant

pathogens or dealing with abiotic stress or plant growth promotion. It would also be promising towards agroeconomic aspects. By reinstating fertilizers and pesticides with endophytic bacteria, we can also reduce soil pollution, and promote a healthy environment. The potential bacteria obtained from the research work can hence be of great importance, valuable for agriculture and lead towards the betterment of the society.

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