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BIOCHEMICAL AND MORPHOLOGICAL CHARACTERIZATION OF RHIZOSPHERIC *BACILLUS SUBTILIS* ISOLATES

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ABSTRACT

The present investigation was carried out to isolate, characterize, and evaluate the biochemical properties of rhizospheric isolates of *Bacillus subtilis* collected from different cropping systems across Maharashtra. A total of thirty bacterial isolates (RBs-1 to RBs-30) were obtained from rhizosphere soils of crops such as cotton, soybean, pigeon pea, sorghum, green gram, paddy, sugarcane and chickpea using the serial dilution technique on nutrient agar medium. Morphological characterization revealed that all isolates were gram positive and rod shaped, which is a characteristic feature of *Bacillus subtilis*. The isolates exhibited rapid growth within 24 hours at 30°C and showed the ability to grow across a wide temperature range, indicating their adaptability to diverse environmental conditions. Biochemical characterization revealed that all isolates were positive for catalase, oxidase, starch hydrolysis, gelatin liquefaction, hydrogen sulfide production, citrate utilization, nitrate reduction, and voges proskauer test, while negative for KOH, indole production, methyl red, arginine dehydrolase, and urease tests. In the phosphate solubilization test most isolates exhibited clear zone formation, indicating their ability to solubilize insoluble phosphate, whereas a few isolates showed minimal or no activity. Similarly, in the casein hydrolysis test, the majority of isolates showed positive results, while RBs-20 and RBs-26 were negative, indicating the absence of proteolytic activity in these strains. The variability observed among the isolates in biochemical and functional traits highlights their differential potential in plant growth promotion and soil nutrient cycling. The study demonstrated that selected isolates of *Bacillus subtilis* possess significant potential for use as biofertilizers and biocontrol agents. Further evaluation under field conditions is recommended to explore their practical applicability in sustainable agriculture.

Key words : *Bacillus subtilis*, *Bacillus*, Biocontrol agent, Biochemical study, Isolation, collection, Morphology.

Introduction

Bacillus subtilis is also known as hay *Bacillus* or grass *Bacillus*. The *Bacillus* species is classified in the order Eubacteriales and family Bacillaceae. The species is gram positive and produces the endospores, to survive extreme environmental conditions of temperature and desiccation. Endospores in *Bacillus subtilis* are mostly formed in the tips of protuberances (Piggot *et al.*, 2004). *Bacillus subtilis* is peritrichously flagellated, enabling it to move efficiently through liquid environments. *Bacillus* species are widely distributed in soil and the rhizosphere, with recorded concentrations reaching as high as 107 CFU/g of rhizosphere soil (Pandey *et al.*, 1997).

Bacillus serves as a model organism for studying bacterial biology. It is well known for its ability to form endospores and is a facultative anaerobe, capable of surviving in both the presence and absence of oxygen. *Bacillus subtilis* exists in the environment as both an epiphyte and an endophyte and it can protect plants from pathogen attacks through a diverse range of mechanisms. It is the best characterized member of the *Bacillus* genus and has become a model organism for studying gram-positive bacteria. (Kunst *et al.*, 1997). *Bacillus subtilis* is an antagonistic bacterial biocontrol agent capable of

managing numerous airborne, seed borne and soil borne diseases affecting crops such as paddy, wheat, sugarcane, jute, groundnut, cotton, rubber, soybean, tobacco and various vegetables. These bacteria colonize the root and leaf systems of plants, where they compete with suppress the growth of plant pathogenic organisms (Akond *et al.*, 2016).

Among bacterial biocontrol agents *Bacillus* genus encompasses a large genetic biodiversity and present in an extremely large palette of environments ranging from sea water to soil and are even found in extreme environments like hot springs (Hoch *et al.*, 1993). This bacterium could be one of the major sources of potential microbial biopesticides because it retains several valuable traits (Ongena and Jacques, 2008). *Bacillus subtilis* is a well-studied organism which facilitates their rational use. The US Food and Drug Administration (USFDA) has granted the "generally regarded as safe" (GRAS) status to *Bacillus subtilis* which is thus recognized non-pathogenic (Harwood and Wipat, 1996).

Materials and Methods

Materials

A) Chemicals : Following chemicals, reagent, fungicides and antibiotics were obtained from the Department of Plant Pathology, PGI, Dr. PDKV, Akola, Maharashtra. It is used for *in-vitro* studies, undertaken during present investigation.

B) Reagents : (Table 2)

C) Collection of soil samples : Soil samples were collected from the rhizosphere of different crops from different places. The soil samples were collected from rhizosphere of healthy plants by uprooting the plants, 80-100 g of rhizosphere soil was collected for isolation of *Bacillus subtilis* (Table 3).

D) Preparation of media

Nutrient Agar medium (NA)

Peptone	: 5.0 g
Sodium chloride	: 5.0 g
Yeast extract	: 2.0 g
Beef extract	: 3.0 g
Agar agar	: 20 g
Distilled water	: 1000 ml

Nutrient broth

Peptone	: 10 g
NaCl	: 5 g
Beef extract	: 1.5 g
Yeast extracts	: 1.5 g
Distilled water	: 1000 ml
pH was adjusted to	: 7.2

Table 1 : Details of chemicals used in the present investigation.

S. no.	Chemicals	S. no.	Chemicals
1.	Crystal violet	12.	Potassium tetra borate
2.	Lugol's iodine solution	13.	Sodium chloride (NaCl)
3.	Alcohol	14.	Cystine
4.	Saffranin	15.	Skim milk powder
5.	Potassium hydroxide (KOH)	16.	Urea
6.	Soluble starch	17.	Potassium nitrate
7.	Hydrogen peroxide	18.	Meat extract
8.	Gelatine	19.	Glucose
9.	Lead acetate	20.	K_2HPO_4
10.	Peptone	21.	Colloidal chitin
11.	Sodium acetate buffer		

Table 2 : Reagents used for biochemical test.

S. no.	Reagent
1	Kovac's reagent
2	Sulfanillic acid
3	Alpha-Napthylamine reagent
4	Methyl red reagent
5	Barritt reagent A
6	Barritt reagent B
7	P-dimethylaminobenzaldehyde

Methodology

A) Isolation of *Bacillus subtilis*

Bacillus subtilis isolates were obtained from rhizospheric soil samples by serial dilution technique. Rhizosphere soil samples were collected from diseased symptomatic plants of different crops. Test tubes containing 9 ml distilled water were sterilized in autoclave for preparation of water blank.

Procedure : 1 gram of soil sample was mixed with 9 ml of sterilized distilled water in a glass tube. The soil suspension was then heated at 80^0C for 20 minutes to eliminate non-spore-forming organisms. From this heated suspension, 1 ml was taken and added to the first test tube. A serial dilution was carried out by transferring 1 ml from the first test tube to the second test tube containing 9 ml of distilled water. This dilution process was repeated similarly up to the sixth test tube. From the 5^{th} and 6^{th} test tubes, 0.1 ml of the diluted suspension was taken and transferred onto sterile petri plates. Nutrient agar (NA) medium was then poured using the pour plate method. The plates were incubated at 30^0C for 48 hours

Table 3 : Details of rhizospheric samples collected from different locations.

S. no.	District	Location	Crop	Designation of isolate
1	Amaravati	Amarawati	Cotton	RBs-1
2	Akola	Paras	Soybean	RBs-2
3	Akola	Pulses Research Unit, Dr.PDKV, Akola	Pigeon pea	RBs-3
4	Buldhana	Buldhana	Pigeon pea	RBs-4
5	Yavatmal	Darwha	Cotton	RBs-5
6	Washim	Amkheda	Green gram	RBs-6
7	Nagpur	Umarkhed	Cotton	RBs-7
8	Wardha	Hinganghat	Soybean	RBs-8
9	Bhandara	Sakoli	Paddy	RBs-9
10	Gadchiroli	Alapalli	Paddy	RBs-10
11	Chandrapur	Bhadrawati	Cotton	RBs-11
12	Gondia	Amgaon	Paddy	RBs-12
13	Parbhani	Parbhani	Black gram	RBs-13
14	Jalna	Partur	Cotton	RBs-14
15	Bid	Ambejogai	Sorghum	RBs-15
16	Nanded	Ardhapur	Soybean	RBs-16
17	Hingoli	Basmath	Sorghum	RBs-17
18	Latur	Ausa	Soybean	RBs-18
19	Nandurbar	Shahada	Black gram	RBs-19
20	Dhule	Sindkheda	Bajra	RBs-20
21	Jalgaon	Raver	Ginger	RBs-21
22	Nashik	Malegaon	Bajra	RBs-22
23	Pune	Junnar	Sugarcane	RBs-23
24	Satara	Koregaon	Sugarcane	RBs-24
25	Sangli	Tasgaon	Cotton	RBs-25
26	Solapur	Akole	Sorghum	RBs-26
27	Solapur	Tembhurni	Sorghum	RBs-27
28	Kolhapur	Radhanagari (Turambe)	Sugarcane	RBs-28
29	Sindhudurg	Kankavli	Paddy	RBs-29
30	Ratnagiri	Chiplun	Paddy	RBs-30

for bacterial growth observation (as per Kumar *et al.*, 2012).

B) Maintenance of the culture

The cultures of *Bacillus subtilis* isolated were used for studying the biochemical characterization and bioefficacy test. The cultures were sub cultured on nutrient agar (NA) slants and allowed to grow at 25°C for 10 to 15 days. Such slants were preserved in refrigerator at 4°C and sub cultured once in 30 days.

C) Morphological studies of *Bacillus subtilis*

The confirmations of the *Bacillus subtilis* isolates were performed with the following studies. Pure culture of thirty selected isolates, were streaked on nutrient agar medium petriplate separately for colony development. The individual colonies were examined for colony colour and shape.

Shape and colour : The shape and colour was observed on nutrient agar media.

Shape of colonies : Circular and irregular.

Elevation : Flat, raised and convex.

Colour : Yellow, dull white and cream white.

D) Biochemical studies

Biochemical tests as per the Bergey's manual of Determinative Bacteriology some test were carried out the confirmation of bacterial isolates of *Bacillus subtilis viz.*, Gram's reaction, catalase, KOH test, starch hydrolysis, gelatin liquefacation, H_2S production, casein hydrolysis, oxidase test, citrate utilization, nitrate reduction, arginine dehydrolase, methyl red, urease test and vogas proskauer reaction test.

All the isolates of *Bacillus subtilis* were also evaluated for plant growth promoting properties *viz.*, Indole acetic acid (IAA) production and phosphate solubilization.

Gram's reaction

Identification was made by gram staining and by studying the morphological characters of the *Bacillus subtilis* isolates.

Procedure : 1) Bacterial smear was prepared by holding a clean slide at edges.

2) A loopful of bacterial suspension was transferred in the centre of slide, with the help of wire loop.

3) The drop was smeared over slide and air dried.

4) Dried smear was fixed by passing the slide 3 to 4 times rapidly over the flame.

5) The smear was flooded with crystal violet for 30 seconds, washed in the tap water.

6) Then it was immersed in potassium iodide/Lugol's

iodine solution for 30 seconds, washed in tap water then decolorized with 95% alcohol and rinsed with water.

7) Counterstained with saffranin for 10 second, again washed with tap water and air dried.

8) Drop of cedar wood oil was placed on the slide and examined the smear under oil immersion objective.

Potassium hydroxide solubility test (KOH)

Two drops of potassium hydroxide (KOH) solution were placed on a clean glass slide. A portion of the bacterial colony was aseptically picked using a sterile inoculating needle and gently mixed with the KOH drops for approximately 10 seconds. The needle was then slowly lifted to a height of 0.5 to 2 cm. The formation of a visible mucilaginous thread during this process was considered indicative of a positive KOH test.

Starch hydrolysis test

Starch is a polymer of glucose units joined through alpha-glucoside linkages. The amylose (soluble) type of starch is employed in this test. The hydrolysis of starch occurs when the bacterium produces an extracellular enzyme amylase. The enzyme beta-amylase hydrolyses starch completely to maltose units, whereas, alpha-amylase causes partial hydrolysis to erythrodextrin and dextrin. Iodine solution is used to detect the hydrolysis of starch. It gives a blue colour with starch, brown with erythrodextrins and no colour with maltose (Kim *et al.,* 2012).

Medium : Starch Agar : nutrient agar + 0.2% soluble starch

Test reagent : Lugol's iodine

Bacterial culture was inoculated on starch agar plates and incubated for 7 days. After incubation, the plates were flooded with Lugol's iodine solution. Presence of starch hydrolysis indicated, by the appearance of clear zone. Reddish zone indicated that the starch was partially hydrolysed to dextrin.

Catalase test

The production of catalase is evidenced by the fact that catalase enzyme breaks hydrogen peroxide (H_2O_2) into H_2O and O_2. A loopful of single well isolated colony was placed on a clean microscope slide, put a drop of an aqueous solution of H_2O_2 30 per cent (v/v) and mixed. A positive result of catalase production was characterized by the rapid evolution of O_2 which is evidenced by bubble formation (Yunting *et al.,* 2013).

Gelatin liquefaction test

Gelatin is a protein produced by hydrolysis of collagen, a major component of connective tissue and tendons in humans and other animals. Hydrolysis (liquefaction) of gelatin is brought about by bacterium capable of producing a proteolytic exoenzyme known as gelatinase, which acts to hydrolyze this protein to amino acids.

Medium : Nutrient gelatin = Nutrient broth + 1.5% gelatin

Bacterial cultures were inoculated through stab of a nutrient gelatin tube and incubated for 7 days, uninoculated tubes serves as control and observed for liquefaction. Deep gelatin inoculated tubes that remain liquified produce gelatinase and show positive test for gelatin hydrolysis and those tubes that remain solid demonstrate negative reaction for gelatin hydrolysis.

Hydrogen sulfide test (H_2S)

This test reveals the ability of the bacterium to produce H_2S after dissimilation of sulphur containing amino acids like cystine and methionine. The usual bacteriological peptone contains cystine in enough concentration and it is used as a substrate (Kim *et al*., 2012).

The following medium (peptone water) was used for the study.

Peptone	: 10.0 g
NaCl	: 5.0 g

5 ml medium was dispensed into tubes and then autoclaved. To detect H_2S, the lead acetate test strips were prepared as follows: Whatman No.1 filter paper was cut into 5 x 50 mm strips, which were then soaked in warm saturated solution of lead acetate. The strips were then dried, autoclaved and again dried at 60^0C. The medium in each tube was inoculated with a loopful of 48 hour old growth of the bacterium. After the inoculation, a test strip was inserted in between the plug and inner wall of the tube, so that, it hangs just above the broth but does not touch it. The tubes were incubated at 27^0C and observations were recorded at regular intervals up to 14 days.

Indol production test

Tryptophan, an essential amino acid, is oxidised by some bacteria by the enzyme tryptophanase resulting in formation of indole, pyruvic acid and ammonia. The indole test is performed by inoculating a bacterium into tryptone broth, the indole produced during the reaction is detected by adding Kovac's reagent which produces a cherry-red reagent layer.

Medium : Tryptone broth 1% : 10 g of peptone in 1 litre of distilled water.

Test reagent : Kovac's reagent (P- di-methylamino benzaladehyde 50 g, amyl alcohol 750 ml, HCL 250 ml).

Procedure : The medium was distributed in test tubes and autoclaved. The bacterium was inoculated and incubated for 48 hrs. Followed by addition of Kovac's reagent in incubated test tube and were allowed to stand. Development of a cherry (deep) red colour on the top layer of the tube is a positive test for indole production. Absence of red colouration is indole negative.

Phosphate solubilization test

Pikovskaya's agar was modified by Sundara Rao and Sinha (1963). Phosphate solubilization test was performed by spot inoculation of test organism on Pikovskaya's medium. The plates were incubated at 27± 2^0C for 4-5 days. Phosphate solubilizing bacteria will grow on this medium and forms a clear zone around the colony, formed due to phosphate solubilization in the vicinity of the colony.

Oxidase test

Oxidase disc are sterile filter paper disc impregnated with a colourless dye such as N, N-dimethyl-p-phenylenediamine oxalate and α-naphthpol serve as an artificial electron acceptor for the enzyme oxidase. An inoculating loop was taken. A well isolated colony was touched and spread on an oxidase disc. The reaction was observed within two minutes at 25 to 30^{0}C. Deep purplish blue colouration of disc indicated positive reaction.

Arginine test

Arginine decarboxylase media was used for detection of Arginine dehydrolase producing microorganism.

Medium : Peptone 1 g/lit, Sodium chloride 5.00 g/l, Dipotassium hydrogen phosphate 0.3 g/l, L-arginine 10.0 g/l, Bromo cresol purple 0.01 g/l, Agar 3.0 g/l.

Procedure : Inoculated the bacteria on arginine decarboxylase media in test tubes and incubated for 3 days. Purple colour indicated positive reaction and yellow colour or no colour change indicated negative reaction.

Nitrate reduction test

The nitrate broth is recommended for detection of nitrate reduction by bacteria,

Medium: Peptic digest of animal tissue 5 gm/l, Meat extract 3 gm/l, Potassium nitrate 1 gm/l, Sodium chloride 30 gm/l.

Test reagents

a) Sulfanilic acid : Dissolve 8 gm of sulfanilic acid in 1 litre 5 N acetic acid

b) Alpha-Napthylamine reagent : Dissolve 5 gm of alpha-napthylamine in 1 litre 5 N acetic acid

Procedure : Few drops of each reagent were poured into the tube containing culture to be tested. A distinct red or pink colour indicates nitrate reduction. The results should be recorded within 5 to 10 seconds as the colour fades on standing. A control tube should also be tested for comparison.

Citrate utilization test

The citrate test was performed by inoculating the micro organisms into an organic synthetic medium, Simmon's citrate agar, where sodium citrate is the only source of carbon and energy. Bromothymol blue was used as an indicator. When the citrate acid was metabolized, the CO_2 generated and combined with sodium and water to form sodium carbonate an alkaline product, which changed the colour of the indicator from green to blue and this constitutes a positive test.

Casein hydrolysis test

Many bacteria are equipped with enzymes that hydrolyze protein.

Medium: Skim milk agar : Nutrient Agar + 2 % raw skim milk

Procedure : Inoculated the bacteria on skim milk agar plates and incubated for 3 days. Colonies of organism which digest casein need to be surrounded by clear zones. Areas in which the casein was attacked remained slightly opaque.

Methyl red (MR) test

The test was performed to see the cleavage of glucose commonly used in the differentiation of organisms.

Medium: Glucose phosphate broth - Glucose 0.5%, K_2HPO_4 - 0.5%, Peptone 0.5% and distilled water 1000 ml.

Test reagent : 0.1 g Methyl red dissolved in 300ml of 95% ethanol and made up to 500 ml with distilled water.

Procedure : Medium was distributed in the test tubes and incubated for 7 days at 27± 2^0C temperature after inoculation. Five drops of the indicator methyl red were added to culture. A red colour described as a positive test for methyl red (MR). A yellow colouration was recorded as negative test.

Vogas-proskauer reaction (VP) test

The test was performed to see the cleavage of glucose commonly used in the differentiation of organisms.

Medium: Glucose phosphate broth - Glucose 0.5%, K_2HPO_4-0.5%, Peptone 0.5% and distilled water 1000 ml.

Test reagent: a) α-naphthol solution – 5% ethanilic of α-naphthol.

b) KOH solution - 16% aqueous solution of potassium hydroxide.

Procedure : Inoculated test tubes with a pure culture of test organism. Incubate for 24 hours at 27± 2^0C. Added 0.5 ml of 5% α-naphthol, followed by 0.5ml of 16% KOH solution and the tube was shaken. Development of red coloration within 5 minutes constitutes a positive VP reaction.

Urease test

The urease test detects the ability of micro organisms to produce the enzyme urease, which hydrolyzes urea into ammonia and carbon dioxide. Ammonia increases the pH, turning the indicator (phenol red) from yellow/ orange to pink.

Medium

Urea agar base (without urea initially)

40% Urea solution (added after sterilization)

Procedure : The urease test using urea agar base and 40% urea solution is performed by first preparing the basal medium without urea. The urea agar base is dissolved in distilled water and sterilized by autoclaving at 121^{0}C for 15 minutes. Urea is prepared separately as a 40% urea solution and sterilized by membrane filtration. After autoclaving, the basal medium is allowed to cool to about 45–50^{0}C and the sterile 40% urea solution is aseptically added and mixed thoroughly. The medium is then dispensed into sterile tubes and allowed to solidify in a slanting position. The prepared urea agar slants are inoculated with the test organism using a sterile loop and incubated at 35–37^{0}C for 24–48 hours. Urease-producing organisms hydrolyze urea into ammonia, increasing the pH and turning the phenol red indicator from yellow/ orange to pink, indicating a positive result, while no colour change indicates a negative result.

Results

Isolation of *Bacillus subtilis* and collection of rhizospheric samples

A total of thirty bacterial isolates were successfully obtained from the rhizosphere soils of various *kharif* and *rabi* crops, including cotton, soybean, pigeon pea, sorghum, green gram, paddy, sugarcane and chickpea collected across different districts of Maharashtra. The isolation was carried out using the serial dilution technique on nutrient agar medium and the isolates were designated as RBs-1 to RBs-30.

The present results correspond with the findings by

Fig. 1 : Rhizospheric soil samples collected from different location for isolation.

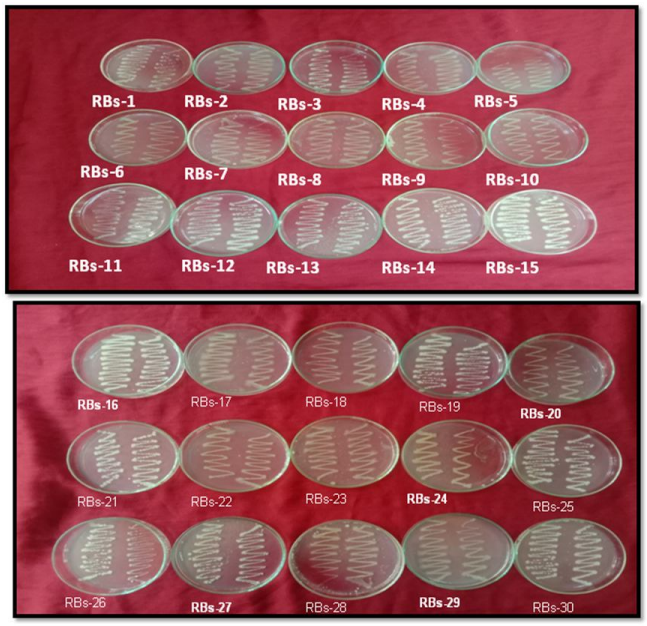

Fig. 2 : Culture of *Bacillus subtilis.*

Amin *et al*. (2015) reported that total of fifty soil samples were collected from various locations and examined for the presence of *Bacillus* species using nutrient broth and nutrient agar media. Karthick *et al*. (2017) a total of five *Bacillus* species. (TB1, TB2, TB3, TB4 and TB5) were isolated from rhizhospheric soil samples of tomato. Smitha *et al*. (2017) reported that *Bacillus* isolate CaB5 and *Bacillus subtilis* strain CcB7 were obtained from chickpea and pigeon pea rhizosphere soils in Tamil Nadu through serial dilution plating. Rajkumar *et al*. (2018) thirty isolates of *Bacillus subtilis* collected from different rhizosphere soil samples of chilli, chickpea, cotton, groundnut, onion, marigold, mustard, niger, pigeon pea, paddy, sorghum, sunflower and wheat of Bagalkot, Ballari, Raichur and Koppal parts of North Eastern Karnataka by serial dilution and plate count technique on nutrient

Table 4 : Morphological and physiological characters of *Bacillus subtilis* isolates.

Isolates	Gram staining	Cell shape	Colony shape	Colony colour
RBs-1	+	Rod	Smooth	Cream white
RBs-2	+	Rod	Smooth	Cream white
RBs-3	+	Rod	Smooth	Cream white
RBs-4	+	Rod	Smooth	Cream white
RBs-5	+	Rod	Rough	Cream white
RBs-6	+	Rod	Smooth	Dirty white
RBs-7	+	Rod	Smooth	Dirty white
RBs-8	+	Rod	Rough	Dirty white
RBs-9	+	Rod	Smooth	Dirty white
RBs-10	+	Rod	Rough	Dirty white
RBs-11	+	Rod	Smooth	Cream white
RBs-12	+	Rod	Rough	Cream white
RBs-13	+	Rod	Smooth	Dirty white
RBs-14	+	Rod	Rough	Dirty white
RBs-15	+	Rod	Smooth	Dirty white
RBs-16	+	Rod	Smooth	Dirty white
RBs-17	+	Rod	Rough	Dirty white
RBs-18	+	Rod	Smooth	Dirty white
RBs-19	+	Rod	Rough	Cream white
RBs-20	+	Rod	Rough	Cream white
RBs-21	+	Rod	Smooth	Dirty white
RBs-22	+	Rod	Rough	Dirty white
RBs-23	+	Rod	Rough	Dirty white
RBs-24	+	Rod	Rough	Dirty white
RBs-25	+	Rod	Smooth	Dirty white
RBs-26	+	Rod	Smooth	Dirty white
RBs-27	+	Rod	Rough	Dirty white
RBs-28	+	Rod	Rough	Dirty white
RBs-29	+	Rod	Smooth	Dirty white
RBs-30	+	Rod	Rough	Dirty white

agar medium.

Morphological characterizations of rhizospheric *Bacillus subtilis* isolates

All the thirty isolates of rhizospheric *Bacillus subtilis* were rod shaped. The isolates of *Bacillus subtilis* that were grown on nutrient agar medium showed typical well separated white colonies with the colour variation from cream white to dirty white.

Data presented in the Table 4, plate revealed that, all the *Bacillus subtilis* isolates from rhizospheric were gram positive and rod shape. The isolate RBs-1, 2, 3, 4, 6, 7, 9, 11, 13, 15, 16, 18, 21, 25, 26 and 29 are smooth texture and the isolate RBs-5, 8, 10, 12, 14, 17, 19, 20, 22, 23, 24, 27, 28, 30 are rough texture.

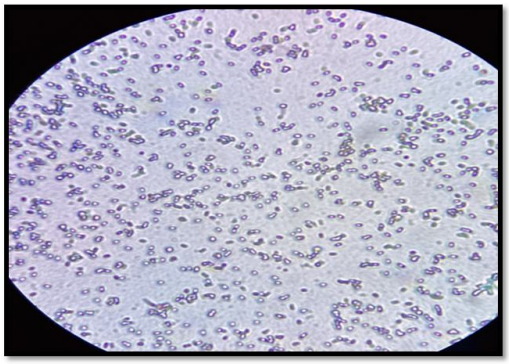

Fig. 3 : Gram reaction (100 X magnification).

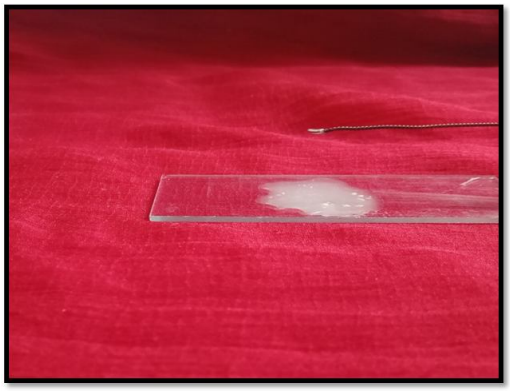

Fig. 4 : KOH test.

Fig. 5 : Catalase test.

Similar results have been reported by Patel *et al*. (2020) confirmed that *Bacillus subtilis* isolates were uniformly gram positive, rod-shaped and motile. Parmar *et al*. (2021) reported that *Bacillus subtilis* consistent gram positive, rod morphology, motility and endospore formation. Similarly, Kumar *et al*. (2020) observed that

Fig. 6 : H_2S Production test.

Fig. 7 : Gelatin Liquifaction test.

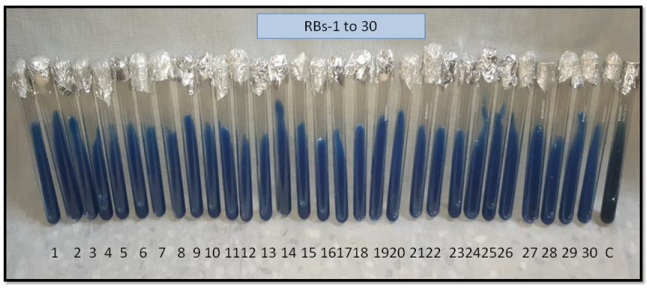

Fig. 8 : Citrate Utilization test.

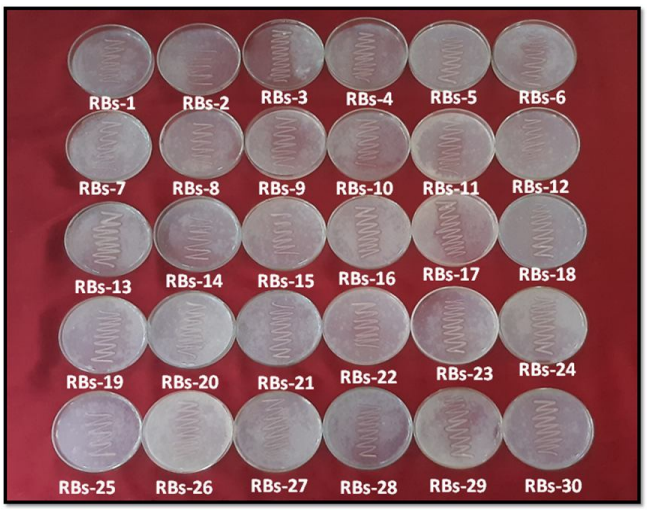

Fig. 9 : Casein hydrolysis test.

colonies of *Bacillus subtilis* on nutrient agar exhibited rough, dry and irregular colony morphology with gram positive rod shaped cells under microscopic examination. These observations align with classical descriptions and validate the use of morphological characterization as a reliable preliminary identification tool.

Biochemical characteristics of *Bacillus subtilis* isolates

Bacillus subtilis is a gram positive, rod shaped, endospore forming bacterium with a broad spectrum of biochemical characteristics that aid in its identification

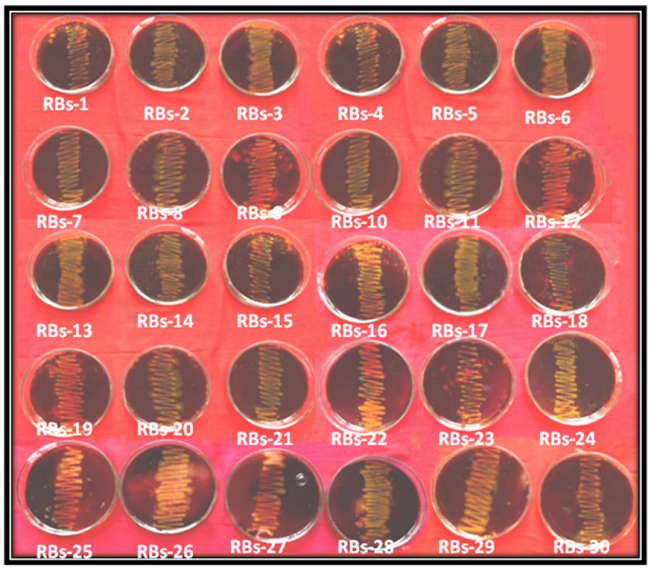

Fig. 10 : Starch Hydrolysis test.

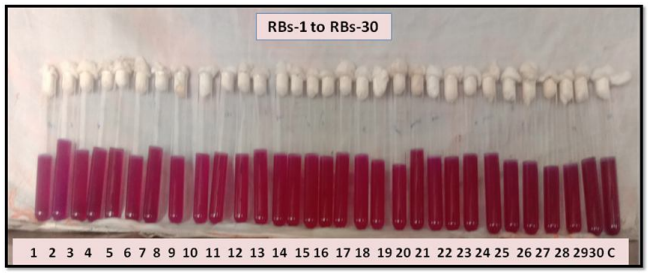

Fig. 11 : Arginine test.

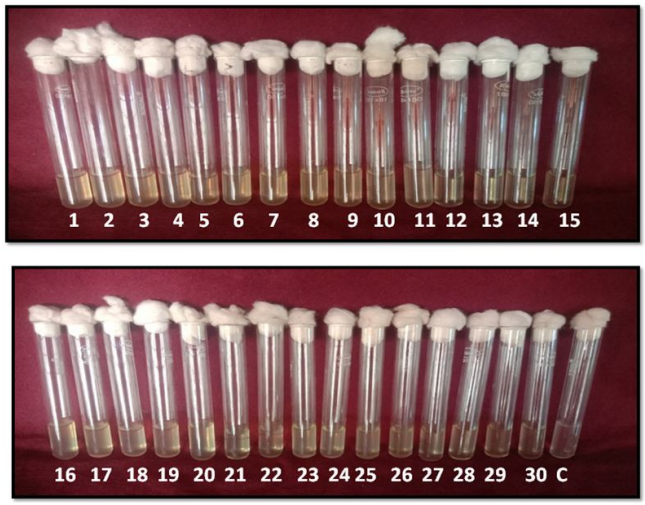

Fig. 12 : Indole test.

and functional classification. The biochemical profile of *Bacillus subtilis* isolates plays a crucial role in distinguishing them from other rhizobacteria and in confirming their plant growth promoting potential.

Data presented in the Table 5 indicated that all the isolates of *Bacillus subtilis* showed positive reaction in respect of starch hydrolysis, gelatin liquefaction, H_2S production, casein hydrolysis, citrate utilization, nitrate reduction, VP, catalase, oxidase test. Negative reaction

Table 5 : Biochemical characteristics of *Bacillus subtilis* isolates.

S. no.	Isolates	Reaction Test																
		Shape	Gram reaction	KOH test	Catalase test	H_2S Production	Gelatin liquefaction	Casein hydrolysis	Starch hydrolysis	Citrate utilization	Arginine Dehydrolase	Indole test	Phosphate solubilization	Oxidase test	MR Test	Urease Test	VP test	Nitrate reduction
1	RBs1	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
2	RBs2	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
3	RBs3	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
4	RBs4	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
5	RBs5	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
6	RBs6	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
7	RBs7	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
8	RBs8	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
9	RBs9	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
10	RBs10	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
11	RBs11	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
12	RBs12	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
13	RBs13	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
14	RBs14	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
15	RBs15	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
16	RBs16	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
17	RBs17	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
18	RBs18	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
19	RBs19	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
20	RBs20	R	Gram +ve	-	+	+	+	-	+	+	-	-	+	+	-	-	+	+
21	RBs21	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
22	RBs22	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
23	RBs23	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
24	RBs24	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
25	RBs25	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
26	RBs26	R	Gram +ve	-	+	+	+	-	+	+	-	-	+	+	-	-	+	+
27	RBs27	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
28	RBs28	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
29	RBs29	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+
30	RBs30	R	Gram +ve	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+

RBs- Rhizosphere *Bacillus subtilis*, + Positive, - Negative reaction and R- Rod shape.

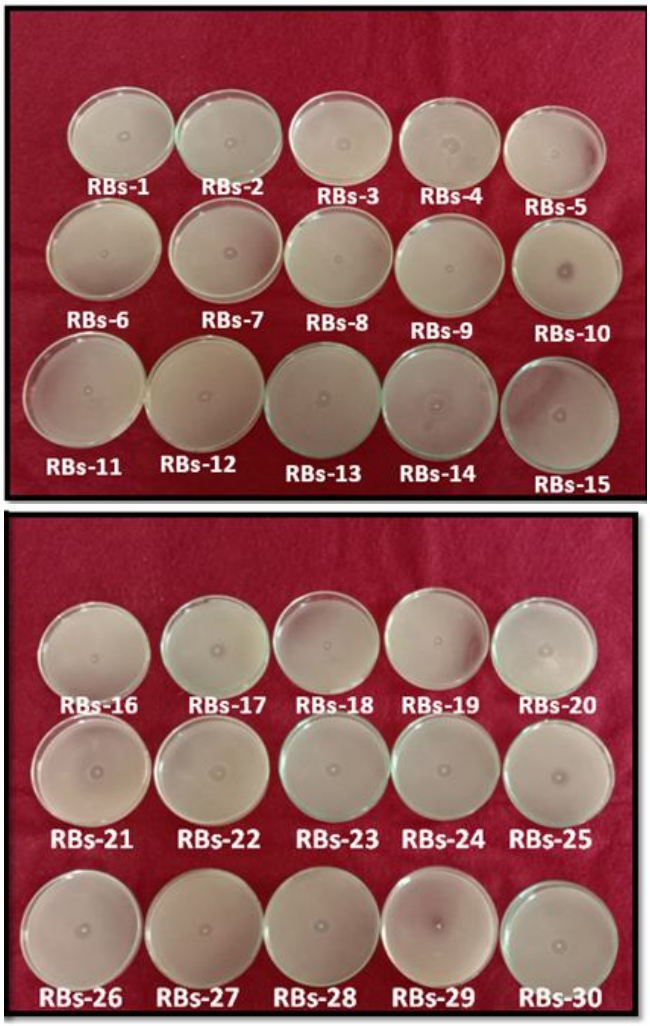

Fig. 13 : Phosphate Solubilization test.

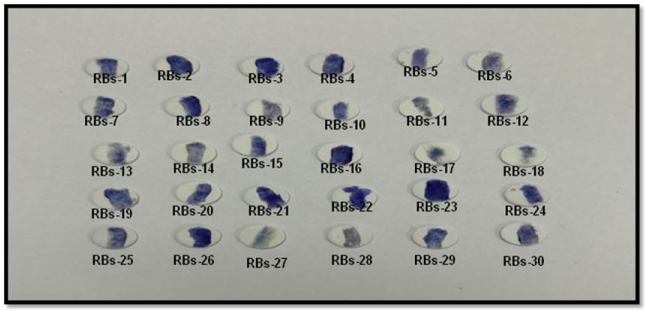

Fig. 14 : Oxidase test.

Fig. 15 : MR test.

regarding with *Bacillus subtilis* for indole production, KOH, MR test, arginine dehydrolase. Among the test

Fig. 16 : Urease test.

Fig. 17 : VP test.

Fig. 18 : Nitrate Reduction.

starch hydrolysis and phosphate solublization showed positive and negative reaction in *Bacillus subtilis* isolates.

Similar findings were Abbo *et al*. (2014) and Jha *et al*. (2016) recorded positive reaction with *Bacillus subtilis* for catalase, H_2S and acid production, starch hydrolysis, phosphate solubilization and gelatin liquefaction. However, they observed negative reaction for KOH test, gas and indol production and starch hydrolysis. Bala *et al*. (2022) reported biochemical characters of *Bacillus* species, S18 isolates showed positive reaction to starch hydrolysis, catalase test, nitrate reduction, indole production, citrate utilization, and negative reaction to urease test.

In the casein hydrolysis test, most of the isolates exhibited a positive reaction, indicating their ability to produce proteolytic enzymes that degrade casein. However, isolates RBs-20 and RBs-26 showed a negative response, suggesting the absence of casein hydrolyzing activity in these strains. The positive isolates formed clear zones around their colonies on skim milk agar, confirming casein degradation. This variation among isolates reflects differences in enzymatic capabilities. Overall, the majority of the isolates demonstrated strong proteolytic potential.

In the phosphate solubilization test, isolates RBs-1,

RBs-2, RBs-3, RBs-4, RBs-7, RBs-10, RBs-13, RBs-14, RBs-15, RBs-17, RBs-21, RBs-22, RBs-23, RBs-24, RBs-25, RBs-26, RBs-28, RBs-29 and RBs-30 produced clear zones around their colonies, indicating positive phosphate solubilizing ability. In contrast, isolates RBs-5, RBs-8, RBs-9, RBs-11, RBs-12, RBs-16, RBs-18, RBs-19, RBs-20, and RBs-27 produced minimal zones, while the remaining isolates did not produce any clear zone. The formation of a clear halo is attributed to the solubilization of insoluble phosphate in the medium. This variation among isolates suggests differences in their plant growth promoting potential.

Discussion

The present investigation included isolation and identification of *Bacillus subtilis* isolates, biochemical characterization of *Bacillus subtilis*. The results obtained in these investigations are summarized below.

Thirty isolates of *Bacillus subtilis* were collected from different rhizosphere soil samples of cotton, soybean, pigeon pea, sorghum, green gram, paddy, sugarcane and chickpea, collected across different districts of Maharashtra by serial dilution technique on nutrient agar medium and designated as RBs-1 to RBs-30.

All the *Bacillus subtilis* isolates from rhizospheric were gram positive and rod shape. The isolate RBs-1, 2, 3, 4, 6, 7, 9, 11, 13, 15, 16, 18, 21, 25, 26 and 29 are smooth texture and the isolate RBs-5, 8, 10, 12, 14, 17, 19, 20, 22, 23, 24, 27, 28, 30 are rough texture. All the isolates grew even at 4 ºC and 45 ºC. All the isolates were generally fast growing within 24 h at 30 ºC ambient temperature.

Biochemical characterization revealed that all isolates were positive for starch hydrolysis, gelatin liquefaction, casein hydrolysis, citrate utilization, nitrate reduction, voges–proskauer (VP), catalase, oxidase, hydrogen sulfide production, and phosphate solubilization. These results indicate strong enzymatic activity and metabolic versatility. The ability to hydrolyze complex substrates such as starch, gelatin and casein reflects the production of extracellular enzymes, which play a significant role in nutrient cycling and soil fertility. Similarly, phosphate solubilization is a key plant growth–promoting trait that enhances phosphorus availability to plants, thereby improving crop productivity.

The casein hydrolysis test demonstrated that most isolates possessed strong proteolytic activity, as evidenced by the formation of clear zones on skim milk agar. This indicates the production of extracellular proteases, which play an important role in nutrient cycling and suppression of plant pathogens. However, isolates RBs-20 and RBs-26 showed negative results, indicating the absence of casein-degrading enzymes in these strains. Such variations highlight the metabolic diversity among the isolates.

The positive response for catalase and oxidase tests confirms the aerobic nature and efficient oxidative metabolism of the isolates. Nitrate reduction and VP positivity further indicate active nitrogen metabolism and fermentative pathways, respectively. Collectively, these biochemical attributes are consistent with previously reported characteristics of *Bacillus subtilis* strains isolated from agricultural soils.

In contrast, all isolates showed negative reactions for indole production, methyl red (MR), KOH test, and arginine dehydrolase activity. These negative results are also in agreement with standard biochemical profiles of *Bacillus subtilis*, thereby validating the identification of the isolates.

The observed differences in phosphate solubilization and casein hydrolysis abilities suggest that not all isolates have equal potential for plant growth promotion and biocontrol. These findings are consistent with earlier reports that emphasized the strain-specific functional diversity of *Bacillus subtilis* in the rhizosphere.

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