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Biochemical and physiological characterizations of *Bacillus subtilis*

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Abstract
An investigation entitled "Biochemical and physiological characterizations of *Bacillus subtilis*" was undertaken at Plant Pathology Section, College of Agriculture, Nagpur during the year s2017-18. Fifteen isolates of *Bacillus subtilis* were isolated from rhizospheric soil of different crops. Out of 15 samples only 7 samples yielded *Bacillus subtilis* on the King's B medium. The isolates were characterized by standard biochemical and physiological characters viz. Gram reaction, cell shape, colony, H_2S production, gelatin liquefaction, starch hydrolysis, catalase test, KOH test, acid and gas production, phosphate solubilization and IAA production test were carried out. The bacteria found to be Gram positive and rod shaped. All the isolates shows maximum growth at 30 and 35ºC temperature and grew well in 6 to 8 pH range. All the isolates were found positive for catalase test (except BS-4), positive for gelatine liquefaction, negative for KOH test, positive for acid and gas production, positive for H_2S test (except BS-1 and BS-6), positive for starch hydrolysis (except BS-2, BS-5, BS-7), negative for IAA production, positive for phosphate solubilization (except BS-4). In this investigation antagonistic study of *Bacillus subtilis* against *Xanthomonas axonopodis* pv. *citri.* Also done and BS-5 recorded maximum inhibition zone which is 16.60 mm followed by BS-2 which is 16.20 mm. Minimum inhibition zone was recorded by BS-6 which is 15.20 mm.

Keywords: *Bacillus subtilis*, *Xanthomonas*, biochemical, antagonism

Introduction
Bacillus subtilis is Gram positive, rod-shaped, 0.7-0.8 X 2.0-3.0µm, variation occurred with 0.6-1.0 X 1.3-6.0µm motile but occasionally non motile. (Reva *et al*., 2004) [9]. The endospore size 0.6-0.9 X 1.0-1.5µm ellipsoidal to cylindrical, central or paracentral, thin walled. It was first identified in 1835 by the German scientist Christian Ehrenberg, who called it *Vibrio subtilis* or 'thin, bent rod'. Ferdinand Cohn later gave this bacterium its current name. *Bacillus subtilis* colonies are dull and may be wrinkled cream to brown in colours, when grow in broth has a coherent pellicle usually single arrangement. *B.subtilis* is a catalase negative bacterium.
Bacillus subtilis also used in control of citrus canker. Studies of biological control of citrus canker is still in preliminary stages using antagonistic bacteria strains such as *Bacillus subtilis*. Therefore, biological control and use of biofertilizer of *Bacillus subtilis* are used to overcome these problems and annual lossess and to get more yield. According to Silo-Suh *et al*., (1994) [11], the metabolites produced by *Bacillus* spp. can also affect the microflora on the rhizosphere, providing an environment antagonistic to the pathogen or they can trigger host defence responses.
However, further studies is to be performed for finding an effective antagonist bacteria or antibiotic compounds produced by its secondary metabolism with an effective ability to control citrus canker.

Materials and Methods
Collection of soil samples
Soil samples were collected from different locations and from different field as given in table 1. One gram soil sample drawn from each sample was used for isolation purpose.

Isolation of *Bacillus subtilis*
The soil samples were collected randomly from rhizosphere of different crops. These samples were used for isolation of *Bacillus subtilis* by serial dilution method using King's B medium.

Morphological characters of *Bacillus subtilis*

Gram reaction was carried out for bacterial cultures to classify them in two groups i.e. Gram positive and Gram negative Salle (1967) [10].

Biochemical characteristics of *Bacillus subtilis*

Biochemical testswas carried out for biochemical confirmation of *Bacillus subtilis* according to Aneja, 2009.

1) KOH test
2) Starch hydrolysis
3) Gelatin liquefaction
4) H_2S production
5) Acid and gas production
6) Catalase test

Also all the isolates of *Bacillus subtilis* were evaluated for plant growth promoting properties viz., IAA production and phosphate solubilization.

Physiological studies

Effect of temperature

The study was initiated to find the optimum temperature requirement for growth of *Bacillus subtilis* using King's B medium. A loop full of 48 hrs. Old bacterial culture was inoculated in 100 ml conical flask containing 30 ml of King's B broth. The inoculated flask were incubated at different temperature level viz., 5, 10, 15, 20, 25, 30, 35 and 40°C respectively for 72 hours. Observations were recorded for the optical density of the broth culture turbidiometrically using spectrophotometer at 600 nm after 72 hr.

pH requirement

Effect of pH on the growth of *Bacillus subtilis* was studied by adjusting pH of the King's B medium to various levels viz., 4, 5, 6, 7, 8, 9 and 10 using appropriate phosphate buffer. A loop full of 48 hour old bacterial culture was mixed in 100 ml conical flask containing 30 ml King's B broth. Inoculated flask were incubated at room temperature for 72 hrs. After the incubation period observations were recorded for the growth of bacterium turbidiometrically using spectrophotometer at 600 nm.

Antagonism of *Bacillus subtilis* against *Xanthomonas axonopodis* pv. *citri*

Xanthomonas axonopodis pv. *Citri* isolate was collected from Plant Pathology Section, COA, Nagpur and antagonistic effect of *Bacillus subtilis* was tested. Different isolates of *Bacillus subtilis* were evaluated for their efficacy against the growth of *Xanthomonas axonopodis* pv. *citri* by inhibition zone assay method.

Result and Discussion

Collection of soil samples and isolates of *Bacillus subtilis*

Soil samples were collected from rhizosphere of citrus, cotton, soyabean, jowar, maize, groundnut, chickpea, pigeonpea, bajra etc. (Table 1).

Table 1: Location and isolates of *Bacillus subtilis*

S. N.	Crop	Location	Isolation code	Rhizosphere population (cfu) × 10^6
1.	Cotton	Plant Pathology Field, COA, Nagpur.	BS -1	13
2.	Soyabean	Botany Field, COA, Nagpur.	BS -2	12
3.	Jowar	Agronomy Field, COA, Nagpur.	BS -3	10
4.	Maize	Botany Field, COA, Nagpur.	BS -4	15
5.	Citrus	NRCC, Nagpur.	BS -5	19
6.	Chickpea	Plant pathology Field, COA, Nagpur.	BS -6	9
7.	Pigeonpea	KVK, Nagpur.	BS -7	11
8.	Bajra	Agronomy Field, COA, Nagpur.	--	--
9.	Groundnut	Plant pathology Field, COA, Nagpur.	--	--
10.	Paddy	Kamthi, Dist. Nagpur.	--	--
11.	Maize	Hingna, Dist. Nagpur	--	--
12.	Cotton	Ramtek, Dist.Nagpur	--	--
13.	Bajra	Kuhi, Dist.Nagpur	--	--
14.	Rice	Mauda, Dist.Nagpur	--	--
15.	Jowar	Hingna,Dist.Nagpur	--	--

Rhizospheric population of *Bacillus subtilis* varied from 9×10^6 to 19×10^6, maximum rhizospheric population was obtained from BS-5 (19×10^6) isolated from citrus rhizosphere, followed by BS-4 (15×10^6) isolated from maize rhizosphere and BS-1 (13×10^6) which is from cotton rhizosphere. Minimum rhizospheric population was of BS-6 (9×10^6) obtained in chickpea rhizosphere.

Apet *et al.*, (2018) [3] tested bioagents viz., *B. subtilis*, *P. fluorescence*, *T. virens* and *T. harzianum* against *Xanthomonas axonopodis* pv. *punicae* by inhibition zone technique. Among all field crops citrus supported maximum number of rhizospheric population.

Characterizations of *Bacillus subtilis* isolates

Colony morphology

The shape, size, margin of the colony were observed in the culture plates with King's B used as a nutrient medium. The observations were noted as under.

Gram's staining

Morphological characterizations of isolate strains were done by Gram's staining and it was found that all the isolates were Gram positive *Bacillus*.

Table 2: Morphology of *Bacillus subtilis* isolates

Strains	Gram staining	Cell shape	Colony shape	Pigmentation (Colony)
BS-1	+	Rod	Circular, wet, smooth, Concave	Coloured
BS-2	+	Rod	Circular, wet, smooth, Concave	Coloured
BS-3	+	Rod	Circular, dry, smooth, flat and irregular with lobate margin	Coloured
BS-4	+	Rod	Circular, dry, smooth, flat and irregular with lobate margin	Coloured
BS-5	+	Rod	Circular, wet, smooth, Concave	Coloured
BS-6	+	Rod	Circular, dry, smooth, flat and irregular with lobate margin	Coloured
BS-7	+	Rod	Circular, wet, smooth, concave	Coloured

'+'positive test. '-'negative test.

It can be seen from table 2, that all the isolates were Gram positive and rod shape. Similar results has been reported by Toppo *et al.*, (2015) [12]. They isolated fifteen isolates and characterized. Among fifteen isolates 6 isolates were found Gram positive, rod shaped. These morphological characters confirms that the bacteria was *Bacillus subtilis*.

Biochemical characterization

For the identification and characterization of isolate bacterial strains. Bergey's manual of determinative bacteriology was used.

Table 3: Biochemical characteristics of *Bacillussubtilis* isolates

SN.	Biochemical tests	BS-1	BS-2	BS-3	BS-4	BS-5	BS-6	BS-7
1	Catalase test	+	+	+	-	+	+	+
2	KOH test	-	-	-	-	-	-	-
3	Gelatin liquefaction	+	+	+	+	+	+	+
4	H_2S production	-	+	+	+	+	-	+
5	Starch hydrolysis	+	-	+	+	-	+	-
6	Acid and gas production	+	+	+	+	+	+	+

'+'positive test. '-'negative test.

Growth promoting characteristics of *Bacillussubtilis*

Table 4: Plant growth promoting characteristics of *Bacillus subtilis*

SN.	Characters	BS-1	BS-2	BS-3	BS-4	BS-5	BS-6	BS-7
1	IAA	-	-	-	-	-	-	-
2	Phosphate solubilization	+	+	+	-	+	+	+

'+' positive test. '-' negative test.

Indole acetic acid (IAA)

Table 4, indicates growth promoting characteristics of *Bacillus subtilis*. Development of pink colour upon addition of Kovac's reagent to culture supernatant of *Bacillus subtilis* strain confirmed IAA production. From the above table it was found that all the isolates were found negative for the production of IAA.

Phosphate solubilization

Phosphate solubilization by bacterial strain was found positive as they formed clear zone on Pikovskaya's agar medium. Only isolate BS-4 don't produce the clear zone (Plate 6). It has been reported that the ability of several isolates to solubilize tricalcium phosphate shows the possible application of the isolates in the crop field and *Bacillus s*pecies were capable of increasing the availability of phosphorus in the soil as per Wahyudi *et al.*, (2011) [13].

Physiological study

Effect of temperature

Table 5: Effect of temperature regimes on the growth of *Bacillus subtilis*

SN.	Temperature	BS 1	BS 2	BS 3	BS 4	BS 5	BS 6	BS 7
1	5ºC	+	+	+	+	+	+	+
2	10 ºC	+	+	+	+	+	+	+
3	15 ºC	++	++	++	++	++	++	++
4	20 ºC	++	++	++	++	++	++	++
5	25 ºC	+++	+++	+++	+++	+++	+++	+++
6	30 ºC	+++	+++	+++	+++	+++	+++	+++
7	35 ºC	+	+	+	+	+	+	+
8	40 ºC	+	+	+	+	+	+	+

+:0.01 to 0.30 ++:0.31 to 0.60 +++:≥0.60

Table 5 indicates the growth of *Bacillus subtilis* on temperature range like 5ºC, 10ºC, 15ºC, 20ºC, 25ºC, 30ºC and 40ºC, mean optical density (72 hrs) T 600 nm. All the isolates shows minimum growth on 5ºC, 10ºC, 35ºC and 40 ºC. All the isolates show the normal temperature from 15ºC to 20ºC. All the isolates shows the maximum growth on 25ºC and 30ºC. The present result are in conformity with the result obtained as per (Ratkowsky *et al*. 1983). The growth of *Bacillus subtilis* is maximum on temperature range between 25 and 30 ºC.

pH requirement

Table 6: Effect of pH on the growth of *Bacillus subtilis*

S.N	Ph	BS 1	BS 2	BS 3	BS 4	BS 5	BS 6	BS 7
1	4	+	+	+	+	+	+	+
2	5	+	++	+	+	+	+	+
3	6	++	++	++	++	++	++	++
4	7	+++	+++	+++	+++	+++	+++	+++
5	8	++ +	+++	+++	+++	+++	++ +	+++
6	9	+	+	+	+	+	+	+
7	10	+	+	+	+	+	+	+

+:0.01 to 0.30 ++:0.31 to 0.60 +++:≥0.60

Table 6, shows the growth of *Bacillus subtilis* on pH range. The mean optical density (72 h) at 600nm. The growth of all the isolates was minimum in 4, 5, 9, 10 pH range while it was normal at 6 pH range. The maximum growth of *Bacillus subtilis* is on 7 and 8 pH range.

Antagonistic studies

Table 7: Antagonistic studies of *Bacillus subtilis* against *Xanthomonas axonopodis* pv. *Citri*

S. N.	Bacterial Strain	Inhibition Zone (mm)
1	*Bacillus subtilis* BS-1	15.60
2	*Bacillus subtilis* BS-2	16.20
3	*Bacillus subtilis* BS-3	15.90
4	*Bacillus subtilis* BS-4	16.10
5	*Bacillus subtilis* BS-5	16.60
6	*Bacillus subtilis* BS-6	15.20
7	*Bacillus subtilis* BS-7	-

The data presented in Table 7 indicates that there were differences in inhibition zone. It was revealed from the data there was minimum inhibition zone recorded by the isolate BS-6 had 15.20 mm of inhibition zone. It was followed by BS-1 had 15.60 mm inhibition zone. Maximum inhibition zone was recorded by BS-5 had 16.60 mm. These results showed the correlation between the reports of Monteiro *et al*., (2005) [7]; Ali *et al*., (2017) [1] which shows the maximum inhibition zone. *Bacillus subtilis* (BS-5) which was isolated from citrus rhizosphere which is followed by BS-2 which was isolated from soyabean rhizosphere. The minimum inhibition zone was recorded from BS-6 which was isolated from chickpea rhizosphere.

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