Characterization of *Bacillus cereus* symbiotic to hemi-parasitic plant *Santalum album* L.

Short title: Characterization of *Bacillus cereus* associated with *Santalum album* Linn.

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Abstract:

Aims:

Santalum album L., known as sandalwood plant (white sandal) belongs to the family Santalaceae, is characteristically a hemi-parasite that requires host plant in the early stages for the better growth and development. Besides its extreme economic importance, significant work has not been done to reveal the relationship of beneficial microorganisms with these plants for their better growth and development. Present investigation is an attempt to isolate and characterize the rhizospheric soil bacteria of *Santalum album* L. occurring in some areas of Bankura district of West Bengal, India.

Methodology:

The microbial colonies in the soils were estimated as colony forming units (cfu/g dr.soil) from plates prepared by different medium. Phenotypic, biochemical and molecular characters of the bacteria were studied following standard methods. The physico-chemical parameters, and microbial population was determined on the rhizospheric soil of the hemiparasitic sandalwood plant *Santalum album* Linn. occurring at four locations of Bankura district in West Bengal, India.

Results:

The population diversity of cultivable heterotrophic, Gram negative, nitrifying, phosphate solubilizing, starch hydrolyzing, spore forming bacteria were higher at Hirbandh with higher organic carbon level than other three locations. Bacterial population was comparatively lower in Basudevpur due to lower water holding capacity. One spore forming bacterium (SW1) was isolated from Hirbandh soil. The isolate (SW1) was characterized by phenotypic properties, scanning electron microscopy, biochemical properties, analysis of fatty acid methyl esters and 16S rRNA gene sequence and identified as *Bacillus cereus* (KT626448) which branched with *Bacillus cereus* BSFN12r (KM405329) with 100% bootstrap support.

Conclusion:

Present investigation is an attempt to isolate and characterize the rhizospheric soil bacteria from economically important plant *Santalum album* L**.** Further studies may find out the positive role of the symbiotic association of *Bacillus cereus* (SW1) with the root of *S. album* as a key factor for the better growth and development of this economically important plant occurring in Bankura district, West Bengal, India.

Key words: *Santalum album* L., Rhizospheric bacteria, *Bacillus cereus* (SW1), Scanning Electron Microscopy, 16S rRNA gene sequence

Introduction

Santalum album L., commonly known as sandalwood plant (white sandal) belongs to the family Santalaceae comprising of more than 15 species and their variants. It is characteristically a hemi-parasite and requires host plant in the early stages for the better growth and development. Rao (1911) reported that sandal seedlings were incapable to grow beyond one year without haustoria, and confirmed its selectivity for host as although almost all plants in its surroundings may be attacked but the better growth was observed in association with *Pongamia pinnata, Albizia lebbeck, Tectona grandis* etc Rao (1911) .Though the plant grows naturally in wide agroclimatic conditions like warm desert of Australia, dry and monsoon climate of India, Vanuatu, eastern Indonesia, subtropical climate of Hawaii and New Caledonia which receives almost uniform rainfall but in India the plant is mostly restricted in southern part only. Presently, there is a few patches in one or two districts of West Bengal with white sandal plants, but it can be introduced in many more areas of West Bengal because it can adapt to various soils although prefers light to medium and well drained soil (Merlin et al., 2006; Rao et al. 2011). Fragrant wood and essential oil obtained from sandal are used for the preparation of incense, perfumes, carving and medicine. In spite of its extreme economic importance, significant work has not been done to reveal the relationship of beneficial microorganisms with this plant for their better growth and development. Present study is an attempt to isolate and characterize the rhizospheric soil bacteria of *Santalum album* L. occurring at some areas of Bankura district of West Bengal, India in relation to its possible role on the growth of the plant.

Materials and methods

Site of soil collection

The rhizospheric soil samples were collected from four different places viz., Hirbundh, Basudevpur, Bagaldhara and Maitybundh of Bankura district of West Bengal, India.

Isolation of the bacteria from soil

100 g of soil samples from the rhizosphere of the plant were collected from different areas of Bankura district of West Bengal, India. Soil samples were mixed thoroughly and the soils were put separately in sterile polythene bags, sealed with rubber bands and analyzed in the laboratory. Viable aerobic bacterial population were assessed from the plates prepared from the soil suspensions after incubating the plates at 30±0 ^{o}C for required days (3-21 d) in a BOD incubator. The microbial colonies in the soils were enumerated as colony forming units (cfu/g dr. soil) from plates prepared with 10 µl soil suspension of (10^{-2}) dilution mixed with 100 ml of different medium. Soil suspension was heated at 60ºC ± 0.1ºC for 30 minutes for enrichment culture of the spore formers. Gram negative bacterial population was determined in Nutrient Agar (NA) (g/l: peptone 5, beef extract 3, agar 2, pH 7.0) medium and crystal violet (peptone 5 g/l, beef extract 3 g/l, lactose 10 g/l, crystal violet 0.0033 g/l, agar 15 g/l, pH 6.8 ± 0.1) solution was added to the

medium before plating (Pelczar et al., 1957; Lacey1997;Dangar et al., 2010;Chatterjee et al,2012 ; Chatterjee et al,2015). To determine gram-negative bacterial population, crystal violet (0.01 g/l) was added to the medium before plating. The nitrifying bacterial population were assessed on Winogradsky's medium containing $(NH_4)_2SO_4$ (1.0 g/l) and the colonies were visualized (pink colour) by flooding the plates with sulphanillic acid reagent (Pelczar et al., 1957). Nitrifying bacterial colonies were recorded from 5-30 d (5 d intervals) but other colonies were counted after 3 day of incubation. The inorganic phosphate solubilizing bacteria were assessed from the halo zone formation around the bacterial colonies on the insoluble phosphate [$Ca_3 (PO_4)_2$] containing medium Pelczar et al. (1957). The asymbiotic nitrogen fixing bacterial populations were determined on the nitrogen free medium Pelczar et al. (1957).

Characterization of the bacterial isolate

The predominant bacterial colonies isolated from the medium were purified and characterized.

The cultural and morphological characters viz. shape, size, elevation, margin, colour, opacity and consistency of the colonies were recorded. Phenotypic and staining properties of the bacteria were studied. Antibiotic sensitivity tests were done with standard antibiotic discs (Brown 2007). The bacterial isolate was identified on the basis of biochemical properties, FAME analysis (MIS,MIDI,Sharlock®USA) and 16S rRNA gene sequence analysis (Janssen 1994). The spore forming bacterial isolates were observed under scanning electron microscope. The smear preparation of bacterial suspension was done on a cover glass, air dried and heat fixed over a flame for one to two seconds followed by 2.5% glutaraldehyde (aquous) for 45 min. Slides were then dehydrated passing through 50%, 70%, 90% and finally with absolute alcohol for 5 min. each. Then the gold coated suspensions were observed under scanning electron microscope (HITACHI S-530). For the fatty acid methyl ester analysis (FAME), whole cell fatty acids were converted to methyl ester and analysed by gas chromatography. The fatty acid methyl ester composition of bacterial isolates was compared to Sherlock library of known bacterial strains in order to find a closest match.

16S rRNA gene sequence analysis

Pure cultured colony of bacterial isolate SW1 was picked up with a sterilized toothpick, suspended in 0.5 ml of sterilized saline in a 1.5 ml centrifuge tube and centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet was suspended in 0.5 ml of Insta Gene Matrix (Bio-Rad, USA), incubated at 56℃ for 30 min and then heated 100℃ for 10 min. After heating, supernatant was used for PCR. The PCR reaction was prepared with 1µl of template DNA in 20 µl of PCR reaction solution using primers and amplified for 35 cycles at 94℃ for 45 sec., 55℃ for 60 sec. and 72℃ for 60 sec. which produced about 1,400 bp DNA fragment. dNTPs from PCR products were purified by using Montage PCR clean up kit (Millipore) and the purified PCR products were sequenced using the forward and reverse primers.

Results and discussion

Population dynamics of microorganisms in rhizospheric soils of *S. album* showed that the total aerobic heterotrophic bacteria ranged from 2.9 $x10^7$ to $3.3x10^7$ cfu/g, the nitrifying bacterial were also higher ranging from 5.2 $x10^6$ cfu/g in Basudevpur to 5.7 $x10^6$ cfu/g in Hirbandh, phosphate solubilising bacteria ranged from 7.9 $x10^2$ cfu/g (Basudevpur) to 8.3 $x10^2$ cfu/g (Hirbandh), starch hydrolyzing bacteria ranged from 8.0 $\times10^4$ to 10.0 $\times10^4$ cfu/g and spore forming bacteria ranged from 7.5×10^4 to 8.0 $\times10^4$ cfu/g (Table 1).

The organic carbon level varied from 0.37 to 0.51%, nitrogen, phosphorus, potassium and soil pH were also recorded (Table 2). The soil physico-chemical parameters, as well as, the soil types are important factors which influence the soil microbial community. Rhizospheric soil is a hot spot of bacterial diversity and harbours those bacterial strains that may have some impact on soil functional status, as well as, the growth of the plant.

The bacterial isolate (SW1) was found in all rhizospheric soil samples of *S. album* of all locations throughout the year. The bacterial colony was off-white with smooth margin and vegetative cell was more than 1 µm and rod shaped. Under SEM study only spores were visualized but no crystal was detected (Fig.1). The isolate was positive for the tests of catalase, methyl red- Vogues-Proskauer, starch, casein and gelatin hydrolysis and negative for citrate utilization test (Table 3). The isolate reduced nitrate to nitrite which reflects its role nitrogen metabolism in the rhizospheric soil (Table 3). The strain was sensitive to nalidixic acid (30 µg/disc), doxycycline (30 µg/disc), bacitracin (10 µg/disc) and tetracycline (30 µg/disc), and resistant to amoxycillin (10 µg/disc), ampicillin (10 µg/disc), polymyxin-B (50 µg/disc) and nystatin (100 µg/disc)(Table 3).On the basis of morpho-physiolological and biochemical properties, the bacterium SW1 was identified as *Bacillus* sp. Through the FAME analysis, 16:1w7c alcohol, 17 isow10c fatty acid supports that the organism belongs to the genus *Bacillus* and the 17:1 iso w5c, 12:0 iso fatty acid supports that the organism belongs to the species *Bacillus cereus* (Fig. 2). Phylogenetic affiliation of the bacterial isolate reveals that *Bacillus* sp. SW1 (KT626448***)*** branched with *Bacillus cereus* BSFN12r (KM405329) with 100% bootstrap support (Fig. 3) which confirmed the identity of SW1 as *Bacillus cereus (*Logan et al, 2009). The nucleotide base composition of 16 rRNA gene sequence of the bacterial isolate SW1 was determined which revealed that the AT and GC content were 47.16% and 52.84%, respectively (Table 3).

It has already been reported that different strains of *Bacillus* act as plant growth promoters for *Saccharum officinerum* sugarcane (Nakade et al., 2013) and *Triticum aestivum* (Rawat et al., 2011 belonging to the family Poaceae. *B. cereus* has been proved to be a growth promoting rhizobacteria of some plants viz..*Brassica juncea* (Aziz et al., 2012), *Arabidopsis thaliana* (Niu et al., 2011), *Sophora alopecuroides* (Zhao 2011) and *Allium ascalonicum* (Aziz et al., 2012), belonging to the families Brassicaceae, Fabaceae and Lilliaaceae respectively. Different strains of *B. cereus* have been established as facultative mosquito pathogens (Krattiger, 1997; Wirth et al., 2004; Teng et al., 2005, Chatterjee et al., 2008) which can colonize in mosquito larval guts in relation to the control of *Aedes aegypti* and *A. subpictus* larvae. But its growth promoting function on sandalwood plant has not known to date. Present study clearly established the strong association of *B. cereus* SW1 with sandalwood plant *Santalum album* L.

Conclusion:

Present study is an attempt to isolate and characterize the rhizospheric soil bacteria from economically important plant *Santalum album* L. It has already been reported that different strains of *Bacillus* act as plant growth promoters for some economically important plants. Further studies may elucidate the positive role of the symbiotic association of *Bacillus cereus* (SW1) with the root of *S. album* as a key factor for the better growth and development of this economically important plant occurring in Bankura district, West Bengal, India.

Table 1. Population density (cfu/g dry soil) of different microbial groups in the rhizosphere of *Santalum album* L occurring at different localities of Bankura district

Sl.no.	Types of organisms	Hirbandh	Basudevpur	Bagaldhara	Maity bandh
1.	Aerobic heterotrophic bacteria ($\times 10^7$)	3.3±0.001	2.9±0.001	3.1±0.001	3.0±0.001
2.	Gram (–) bacteria ($\times 10^6$)	3.9±0.001	3.5±0.002	3.7±0.001	3.3±0.001
3.	Nitrifying bacteria ($\times 10^6$)	5.7±0.003	5.5±0.013	5.2±0.002	5.5±0.013
4.	Phosphate solubilising bacteria ($\times 10^2$)	8.3±0.014	7.9±0.018	8.1±0.011	8.0±0.020
5.	Starch hydrolyzing bacteria ($\times 10^4$)	10±0.02	8±0.02	9.1±0.018	8.5±0.021
6.	Spore forming bacteria ($\times 10^4$)	8±0.012	7.5±0.015	7.8±0.008	7.6±0.018
7.	Fungi ($\times 10^4$)	4.66±0.002	4.1±0.003	4.5±0.001	4.2±0.011

Results are mean of three replication ± SE, cfu: colony-forming unit

Table 2. Physicochemical properties of rhizospheric soil

Sl. no.	Area	N (kg/Acre)	P (kg/Acre)	K (kg/Acre)	pH	Organic carbon(%)	Texture
1	Hirbundh	94.67± 0.89	30±0.03	6.9±0.013	6.5±0.019	0.44±0.001	Lateritic, hard rocky
2	Basudevpur	72.43±0.40	25±0.01	6.35±0.011	6.2±0.001	0.37±0.001	Lateritic, hard rocky
3	Bagaldhara	82±0.71	50±0.06	7.5±0.02	6.4±0.017	0.51±0.002	Lateritic, hard rocky
4	Maity bundh	76± 0.46	40±0.04	7.1±0.017	6.3±0.013	0.47±0.001	Lateritic, hard rocky

Results are mean of three replication ± SE

Table 3. Biochemical properties of the bacterial isolate (SW1)

Name of the tests	**Observations/ Result**
Colony characters	Off white, spherical, elevated
Bacterial properties	rod shaped, >1 µm, motile
Biochemical properties	
Citrate utilization	- ve
Nitrate reduction	+ ve
Catalase	+ ve
Methyl red	+ ve
Voges-Proskauer	+ ve
Antibiotic sensitivity tests:	
amoxycillin (10 µg/disc)	R
ampicillin (10 µg/disc)	R
polymyxin-B(50 µg/disc)	R
pystatin (100 µg/disc)	R
nalidixic acid (30 µg/disc)	S
doxicycline (30 µg/disc)	S
bacitracin (10 µg/disc)	S
tetracycline (30 µg/disc)	S

Where, S = sensitive; R = Resistant

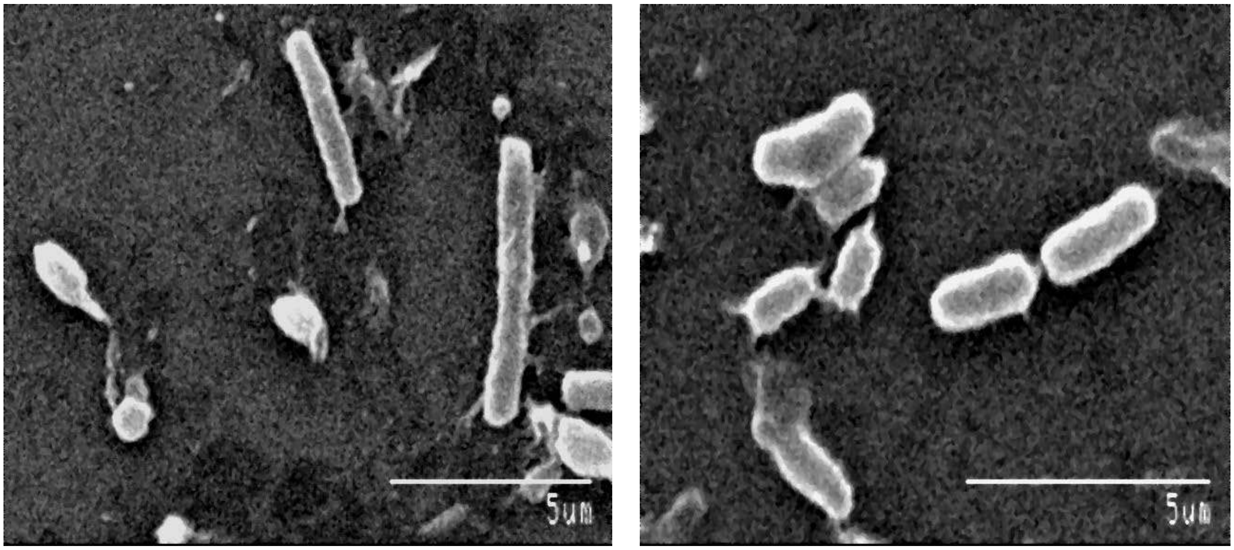

Plate 1 Plate 2

Fig. 1: Scanning electron micrograph of *Bacillus cereus* SW1 (Plate 1 and 2)

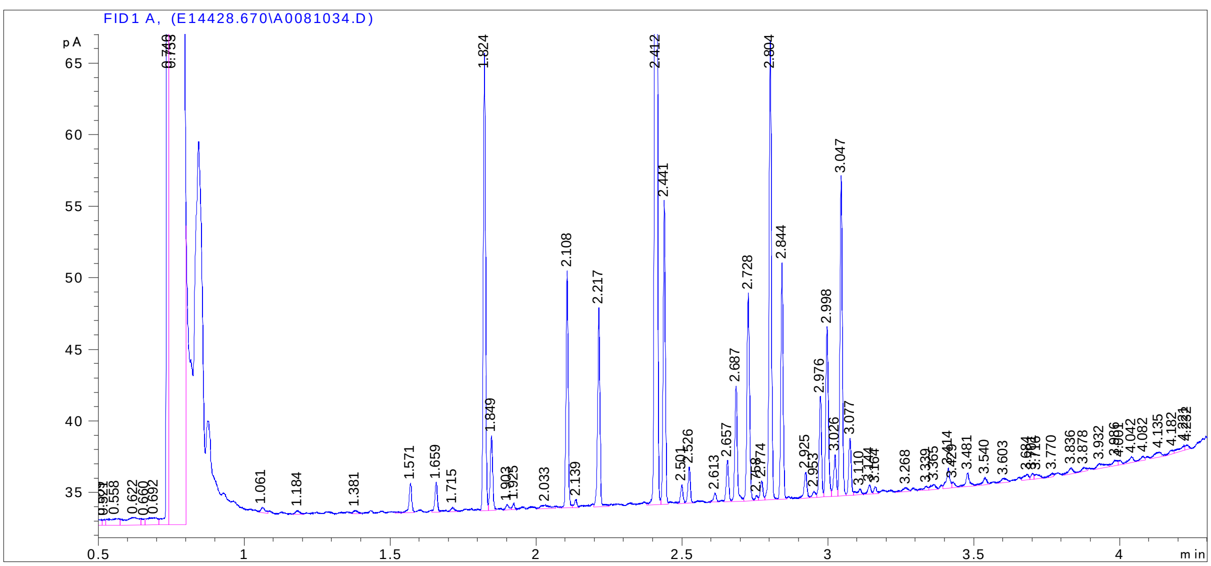

Fig 2. FAME analysis of the bacterial isolate SW1

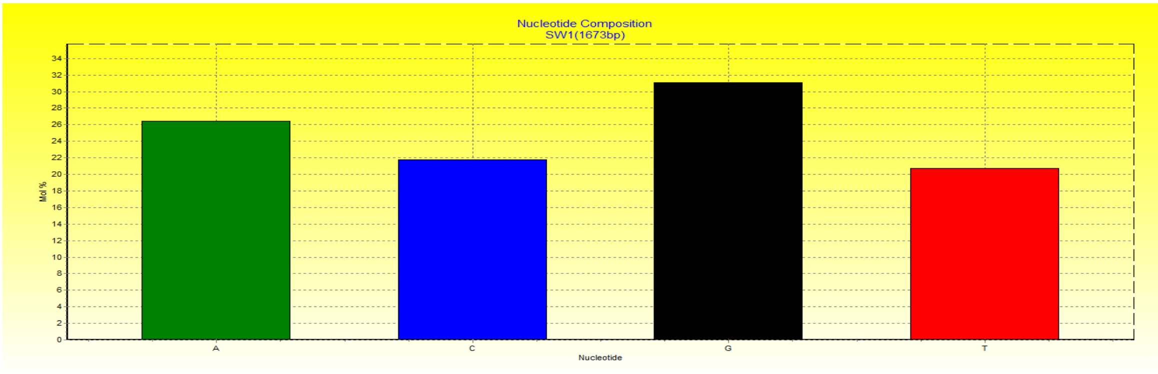

Fig. 3 : A, C, G ,T content (Mol%) of 16S rDNA gene of bacterial isolate SW1

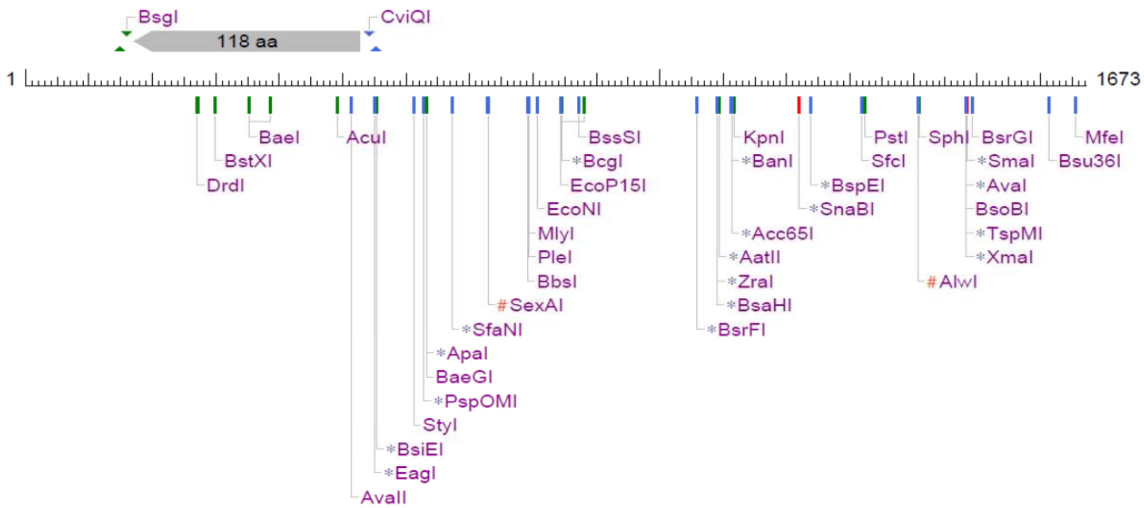

Fig. 4: Restriction map of 16S rDNA gene sequence of bacterial isolate SW1

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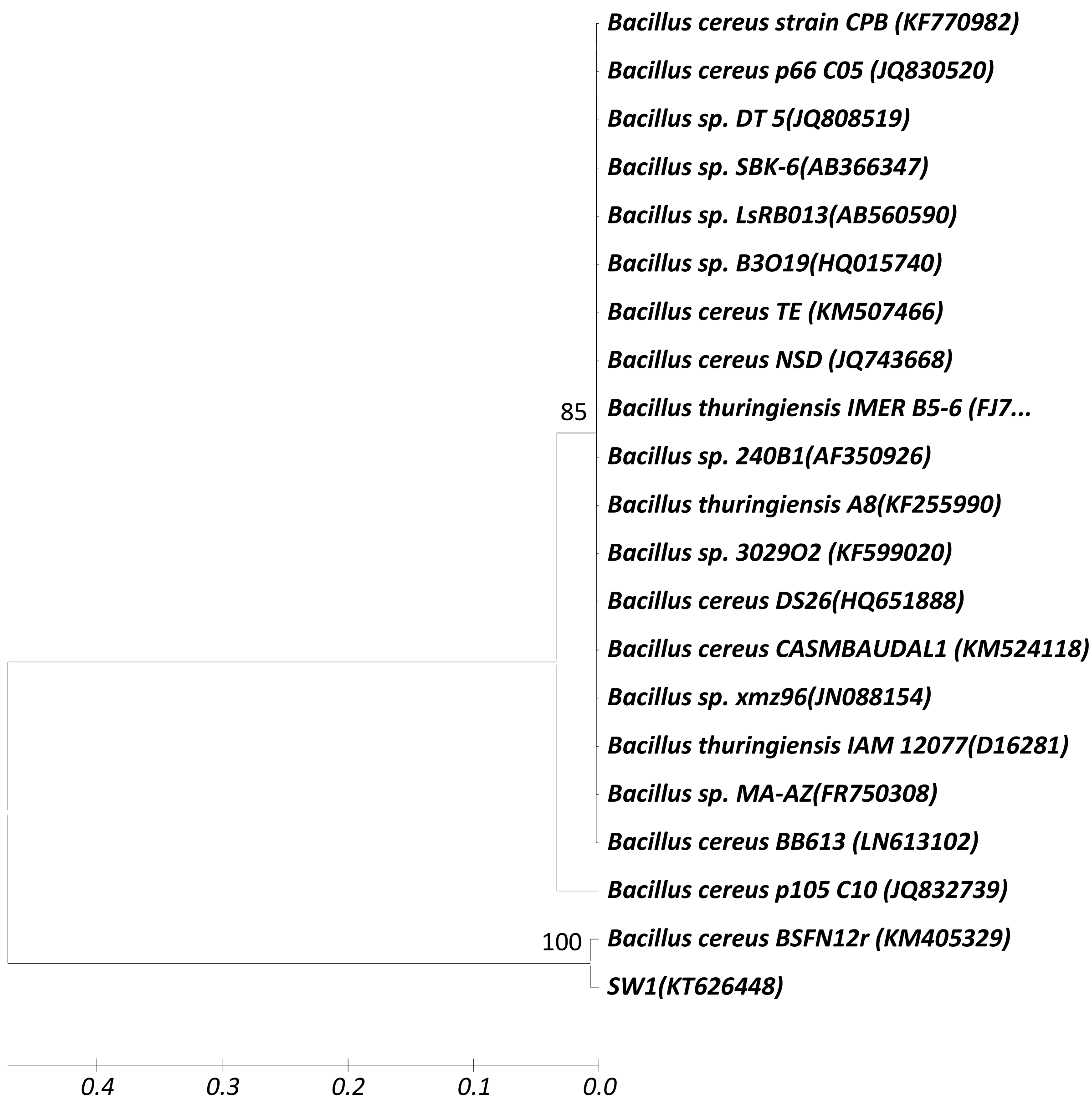

Fig. 5 : Neighbor-joining tree based on 16S rRNA genes sequences of *Bacillus cereus* SW1 (KT626448) strain along with few other 16S rRNA genes retrieved from NCBI.

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