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REGULAR ARTICLE

ANTIBACTERIAL AND CYTOTOXIC ACTIVITY OF *BACILLUS METHYLOTROPHICUS*-SCS2012 ISOLATED FROM SOIL

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ABSTRACT

The strain *Bacillus methylotrophicus*-SCS2012 (*Bacillus* sp. SCS2012) identified by 16S rRNA gene sequencing was isolated from soil. Ethyl acetate extract of *B. methylotrophicus*-SCS2012 showed antibacterial activity against both Gram positive and Gram negative bacteria. The crude metabolite extracted from *B. methylotrophicus*-SCS2012 exhibited strong antibacterial activity against *Streptococcus agalactae*, *Bacillus cereus, Escherichia coli, Shigella sonnei* and *Shigella dysenteriae* with the zone of inhibition range 28.33 to 32.16 mm at 100µg/disc. Minimum inhibitory concentration (MIC) of the extract against *Streptococcus agalactae, Bacillus cereus, Escherichia coli, Shigella sonnei* and *Shigella dysenteriae* was found to be 156, 156, 312, 312 and 625µg/ml respectively. The ethyl acetate extract also exhibited cytotoxic effects on brine shrimp lethality bioassay with LC_{50} value of 10.78 µg/ml. Thus the ethyl acetate extract of *Bacillus methylotrophicus*-SCS2012 has broad spectrum activity with moderate cytotoxicity.

Keywords: *Bacillus methylotrophicus*-SCS2012, antibacterial activity, minimum inhibitory concentration, cytotoxicity

INTRODUCTION

Antibacterial substances are widely produced by a large number of microorganisms. Currently microorganisms of genera *Bacillus, Penicillium, Streptomyces, Cephalosporium* and *Micromonospora* are known to produce more than 5000 different antibiotics **(Tador, 2002)**. *Bacillus* is the largest antibiotic producing genus, producing both antibacterial and antifungal, and also a wide range of other bioactive compounds. *Bacillus* sp and its related genera have been identified as potential biocontrol agent as they produce wide range of cyclic lipopeptides active against various microorganisms **(Kim *et al.,* 2003)**. Most *Bacillus* antibiotics are active against gram positive bacteria but small number have been found to have activity against gram negative bacteria, yeast or fungi **(Katz and Demain, 1977).** Nowadays rapid emergence of antibiotic resistance pathogens causing increase in infectious disease. One of the major reasons for the increase in incidence of infectious disease is the emergence of multidrug resistance pathogens **(Cassell and Mekalanos, 2001).** This scenario enforces researcher to search for new and potent antimicrobial compound.

Therefore the aim of our research is to the screening of potent antibiotic producing microorganism from the natural sources such as soil, sewage, and water. The bacterial strains such as *Bacillus subtilis, Bacillus amyloliquefaciences* are well studied for the production of antibiotic, but there are few scientific reports on the production of antibiotics from *Bacillus methylotrophicus.* Although **Yan *et al.,* (2011)** observed that the strain *Bacillus methylotrophicus* showed antimicrobial activity against some pathogenic bacteria and fungus, but the study on *Bacillus methylotrophicus* for antibiotic production is very early stage at present. So we were interested to the screening and isolation of *Bacillus methylotrophicus* processing antimicrobial properties from soil.

MATERIALS AND METHODS

Sample collection

Soil samples from different places of the campus of Rajshahi University were collected in the sterile small reagent bottles from various depths of the surface and labeled properly and stored at 4ºC until examination.

Isolation of pure colonies

Approximately 1 gram of soil sample was suspended in 9 ml sterile distilled water and vortexed vigorously to make uniform suspension. After that successive serial dilutions were made by transferring 1 ml of aliquots to 2nd test tube containing 9 ml of sterile water and in this way dilution up to 10^{-5} were made. An aliquot of 0.1 ml of each dilution was taken and spread evenly over surface of glucose nutrient agar plates and incubated overnight at 37ºC. After 24 hours plates were examined and few colonies nearly same in appearance showed very thin clean zone around the colony were selected for isolation and purification. Selected colonies were re-cultivated several times for purity. The purified strains were stored on nutrient agar slant at 4ºC for one or two months and at -20ºC as glycerol store for longer period.

Preliminary screening of antibacterial activities

Preliminary screening for antibacterial activity of the isolates was checked by cross streaking technique. Pure isolate was streaked on nutrient agar plate in a single line and then incubated at 37ºC for two days to allow the isolate to secrete antibiotics into the medium. After incubation period the overnight growth culture of test pathogenic bacteria were cross streaked perpendicularly along the line of fully grown isolate. Each streaking was started near the edge of the plate and streaked toward the isolate growth line. The plates were then incubated at 37ºC for 24 hours. Absence of growth adjacent to *Bacillus* growth indicated inhibition of target culture and the zone of inhibition was measured using a millimeter **(Shomura *et al.*, 1980).**

Identification of Bacillus isolates

Bacillus isolate showed highest antibacterial activity in preliminary screening test was identified by using conventional method and by 16 S rRNA gene sequence analysis. Purified isolate was characterized by morphological and biochemical analysis using the tests prescribed in Bergey's Manual of Systematic Bacteriology **(Berkly *et al*., 1984, Claus and Berkly, 1986).** *Bacillus* isolate was identified to species level by 16 S rRNA gene sequence analysis using the taxonomy approach **(Stackebrandt and Goebel, 1994).** The 16S rRNA gene sequence was amplified by using primers F1L5´-GAGTTTGATCCTGGCTCAG-3´ and R1L5´-GTATTACCGCGGCTGCTGG-3´. The nucleotide sequence analysis of the 16 S rRNA of the isolates was done at NCBI server using BIBI (Bioinformatics bacterial identfication) tool or BLASTn (www.ncbi.ncm.gob/blast) by aligning the partial sequences with the 16S rRNA gene sequences of recognized species of the genus *Bacillus* obtained from the GenBank/EMBL/DDBJ database. A phylogenetic tree was constructed using neighbour-joining **(Saitou and Nei, 1987)** method by MEGA4 software **(Tamura *et al.*, 2007).**

Extraction of crude antibiotics

The primarily screened bioactive *Bacillus* isolate was inoculated into 100 ml glucose nutrient broth and incubated for 24 hours at 37ºC. After incubation, 10 ml of culture broth was transferred to another 100 ml of sterile broth in 250 ml conical flask and incubated at 37ºC for 3 days with shaking (120 rpm). Following 3 days incubation the broth culture was centrifuged at 10000 rpm at 4ºC. The culture supernatant was collected and mixed with equal volume of ethyl acetate solvent in a separating funnel and then shake gently for 2 hours. The organic solvent was collected and dried at room temperature. The solvent extraction was then assayed for antibacterial activity by agar disc diffusion method. The residue was termed as crude antimicrobial metabolite.

Test organisms

Five bacteria strains were obtained from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) were used to test the antibiotic activity of the isolates. Two of them were gram positive (*Bacillus cereus*, *Streptococcus agalactiae*) and three were

gram negative (*Escherichia coli*, *Shigella dysenteria*, *Shigella sonnei)* bacteria. All the test strains were maintained in nutrient agar slant at 4ºC.

Antibacterial assay

To test antibacterial activities of the crude extract of isolate *Bacillus methylotrophicus*-SCS2012, the disc diffusion method **(Baure *et al.*, 1966)** was used against pathogenic bacteria. The crude extract was dissolved in respective solvent to get a solution of known concentration (mg/ml). Dried and sterilized filter paper discs (6 mm) were then impregnated with known amounts of the test material were placed on nutrient agar medium evenly seeded with the test microorganisms. Standard antibiotic disc (Kanamycin 30µg/disc) and blank discs (impregnated with solvent) were used as a positive and negative control respectively. The plates were then kept in a refrigerator at 4ºC for about 24 hours in order to provide sufficient time to diffuse the sample and standard antibiotic from the discs to surrounding agar medium. Finally the plates were incubated at 37ºC for 24 hours to allow maximum growth of the organisms. The test materials having antibacterial activity inhibited the growth of the microorganism and a clear, distinct zone of inhibition was visualized surrounding the disc on the medium. The antibacterial activity of the test agent was determined by measuring the diameter of the zone of inhibition in terms of millimeter (mm) with a transparent scale and the experiment was carried out in triplicate.

Minimum inhibitory concentration

Minimum inhibitory concentration of the crude extract from *Bacillus methylotrophicus*-SCS2012 was determined against pathogenic bacteria base on a micro dilution method in 96 multi-well micro titer plates as previously described **(Al-Bayati, 2008)**. Briefly pathogenic bacterial strains were cultured overnight at 37ºC on nutrient broth and adjusted to a final density of 10^8 CFU/ml and used as inoculums. The crude extract was dissolved in 5% DMSO and then in nutrient broth to reach a final concentration of 4 mg/ml. Serial two fold dilutions were made in a concentration range from 1000 to 0.5 µg/ml. A 100 µl volume of the extract sample was transferred to the first well of each row and serial two fold dilutions were performed, the remaining 100µl was discarded. A 100 µl volume of working suspension was added to each well. In each microtiter plate, 20% ethanol was used as positive inhibition control and culture medium containing bacterial suspension, but no drug

was used as positive growth control in additional three wells. Plates were then incubated at 37ºC for 24 hours and the growth was visually examined. As an indicator of bacterial growth, 20µl of 0.2mg/ml thiazolyl blue tetrazolium bromide (MTT) was added to the wells and incubated at 37ºC for 1 hour. The lowest concentration of the extract showing no growth was taken as its MIC value. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red coloured formazan product by biologically active organisms **(Eloff, 1998)**. Where bacterial growth was inhibited, the solution in the well remained clear after incubation with MTT.

Determination of relative percentage inhibition

The relative percentage inhibition with respect to positive control was calculated applying the familiar formula **(Ajay *et al.*, 2002)**. Relative percentage inhibition of the test extract,

$$= \frac{[100 \times (a-b)]}{(c-b)}$$

Where, a = Total inhibition area of the test extract

b = Total inhibition area of the solvent

c = Total inhibition area of the standard drug

Total inhibition area was calculated according to area = πr^2; where, r = radius of the inhibition zone.

Brine Shrimp lethality test

Brine shrimp lethality bioassay was performed by using the procedure of **Meryer *et al.*, (1982)**. For this test, eggs of the brine shrimp (*Artemia salina*) were placed in a glass container containing artificial sea water (3.8 % NaCl in H_2O) and incubated at room temperature for 48 hours under a light source to mature shrimp called nauplii. The test sample was prepared by dissolving 6 mg of the extract into 0.6 ml (600µl) of dimethylsulfoxide (DMSO) to get a concentration of 10 mg/ml used as stock solution. From the stock solution 5, 10, 15, 20, 25, 30, 35 and 40µl were transferred into 5 different vials making the volume up to 5 ml by adding sea water. Thus the final concentration of the sample in the vials became10, 20, 30, 40, 50, 60, 70 and 80µg/ml (ppm) respectively. A vial containing 50µl DMSO diluted to 5 ml was used as a control. Ten matured shrimps were applied to each of all experimental

vials and control vial. The vials were then placed under illumination at room temperature. After 24 hours the vials were inspected using a magnifying glass and the numbers of survived nauplii in each vial were counted. From the resulting data, LC_{50} value for the extract was determined using Probit analysis **(Finney, 1971)**. Three replicas of each concentration were used.

RESULTS

The study was performed with an aim of screening and isolation of antimicrobial substances producing bacteria from soil. Three *Bacillus* isolates were isolated from soil sample as they produced thin clear zone around the colony during crowded plate technique and purified by subculture. But one of them showed best activity against pathogenic bacteria in primary screening by cross streaking method (Figure 1). The antibacterial activity was subsequently confirmed by agar disc diffusion method.

The isolate was identified on the basis of morphological and biochemical characteristic (Table 1) by conventional techniques as per Bergey's manual of Determinative Bacteriology **(Berkley *et al.*, 1984; Claus and Berkely, 1986)** and to be *Bacillus* sp. The partial sequence of the 16 S r RNA gene of the isolated strain obtained using different primers which showed closest homology with some known sequences of *Bacillus* sp. in Gene Bank Database and showed highest sequence similarity (96%) to B. methylotrophicus CBMB205. A phylogenetic tree based on the nucleotide substitution rate (Knuc values) indicated that the strain belongs to the genus *Bacillus*. When the phylagentic position of isolate was compared with closely related species of the genus *Bacillus*, the strain formed a monophylectic clade with Bacillus methylotrophicus (Figure 2). Therefore the strain was tentatively identified as *Bacillus methylotrophicus* SCS2012 and the partial sequence was submitted to NCBI gene bank with accession number JX427405.

The isolated strain exhibited inhibitory effect on the growth of different test bacteria and the strain was selected for further study. The crude extract of *Bacillus methylotrophicus*-SCS2012 obtained from ethyl acetate extraction showed strong antibacterial activity against two gram positive bacteria and three gram negative bacteria (Figure 3). The crude extract at a dose of 100µg/disc showed prominent antibacterial activity against all the tested bacteria with the zone of inhibition range 28.33 to 32.16 mm (Table 2). The highest zone of inhibition was found against *Streptococcus agalactae* followed by *Bacillus cereus*, *Escherichia coli* and

Shigella sonei, whereas the lowest activity was shown against *Shigella dysenteriae* at 100µg/disc.

The minimum inhibition concentration (MIC) values were obtained on the basis of micro-dilution assay. The extract showed significant variations in MIC values, which depend upon the test bacteria (Figure 4). The MIC values of the extract were 156, 156, 312, 312 and 625µg/ml against *Streptococcus agalactae*, *Bacillus cereus*, *Escherichia coli*, *Shigella sonei* and *Shigella dysenteriae* respectively (Table 3).

The crude extract of *Bacillus methylotrophicus*-SCS2012 showed the maximum relative percentage inhibition against *Streptococcus agalactiae* (115%) followed by *Shigella dysenteriae* (94%), *Bacillus cereus* (93%), *Shigella sonnei* (91%) and *Escherichia coli* (90%) at the dose of 100µg/ml (Table 4).

In brine shrimp lethality bioassay, the lethality of the crude extract of ethyl acetate extract of *Bacillus methylotrophicus*-SCS2012 to brine shrimp was determined on *A. salina* after 24 hours of exposure of the sample and probit analysis statistical method was used to determine the LC_{50} value. It was observed that LC_{50} value of the extract was 10.78 (Table 5)

DISCUSSION

Screening for new antibiotics from natural sources is becoming increasingly important for the pharmaceutical industry **(Schmidt, 2004)** as pathogenic bacteria are increasingly becoming resistant to commonly used therapeutic agents **(Coates *et al.*, 2002)**. Scientists are now working to explore alternative drugs from microbial sources to discover new and potent antibacterial principles. Secondary metabolites from microorganisms having a diverse chemical structure and biological activities are produced only by some species of a genus *Bacillus* **(Stachelhaus *et al.*, 1995).**

Table 1 Morphological and Biochemical characteristics of *Bacillus methylotrophicus*-SCS2012

Characteristics	Morphological Test				Biochemical Test											
	Gram staining	Rod shaped	Spore formation	Motility	Indol	Catalase	Urease	H_2S	Methyl red	V-P test	Glucose	Sucrose	Lactose	Starch	Citrate utilization	Nitrate utilization
Result	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+

Table 2 Antibacterial activity of crude extract of *Bacillus methylotrophicus*-SCS2012

Pathogenic Bacteria	Diameter of inhibition zone (in mm)		
	Ethyl acetate extract		Kanamycin (30µg/disc)
	50µg/disc	100µg/disc	
Bacillus cereus	30.16±0.62	31.66±0.47	32.83±0.23
Streptococcus agalactiae	29.66±0.62	32.16±0.23	30.00±0.40
Escherichia coli	25.33±0.57	30.66±0.57	32.33±0.47
Shigella sonnei	28.33±0.47	30.33±0.47	31.66±0.47
Shigella dysenteriae	27.50±0.40	28.33±0.47	29.16±0.62

Table 3 Minimum inhibitory concentration (MIC) of crude extract of *Bacillus methylotrophicus*-SCS2012

Pathogenic Bacteria	MIC (µg/ml)
Bacillus cereus	156
Streptococcus agalactiae	156
Escherichia coli	312
Shigella sonnei	312
Shigella dysenteriae	625

Table 4 Relative percentage inhibition of crude extract of *Bacillus methylotrophicus*-SCS2012

Pathogenic Bacteria	Relative percentage inhibition (%)
Bacillus cereus	93
Streptococcus agalactiae	115
Escherichia coli	90
Shigella sonnei	91
Shigella dysenteriae	94

Table 5 LC_{50} value of crude extract of *Bacillus methylotrophicus*-SCS2012

Sample	Dose (µg/ml)	Log dose	% Mortality	LC_{50} value (µg/ml)	95% confidence limit		Regression Equation	x^2 value (df)
					Upper	Lower		
Ethyl acetate extract	5	0.698	40					
	10	0.999	50					
	20	1.301	60					
	40	1.602	60	10.78	33.914	3.429	Y= 0.830X + 4.141	0.583
	60	1.778	70					
	70	1.845	80					
	80	1.903	80					

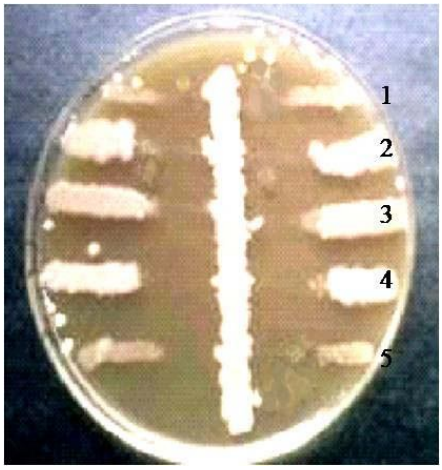

Figure 1 Cross streaking-plate technique to screen the antibacterial activity of *Bacillus methylotrophicus*-SCS2012.The vertical line is the *Bacillus methylotrophicus*-SCS2012 and the lines marked with numerical digits are the test organism- (1) *Shigella dysenteriae*, (2) *Streptococcus agalactiae*, (3) *Shigella sonnei*, (4) *Bacillus cereus* and (5) *Escherichia coli.*

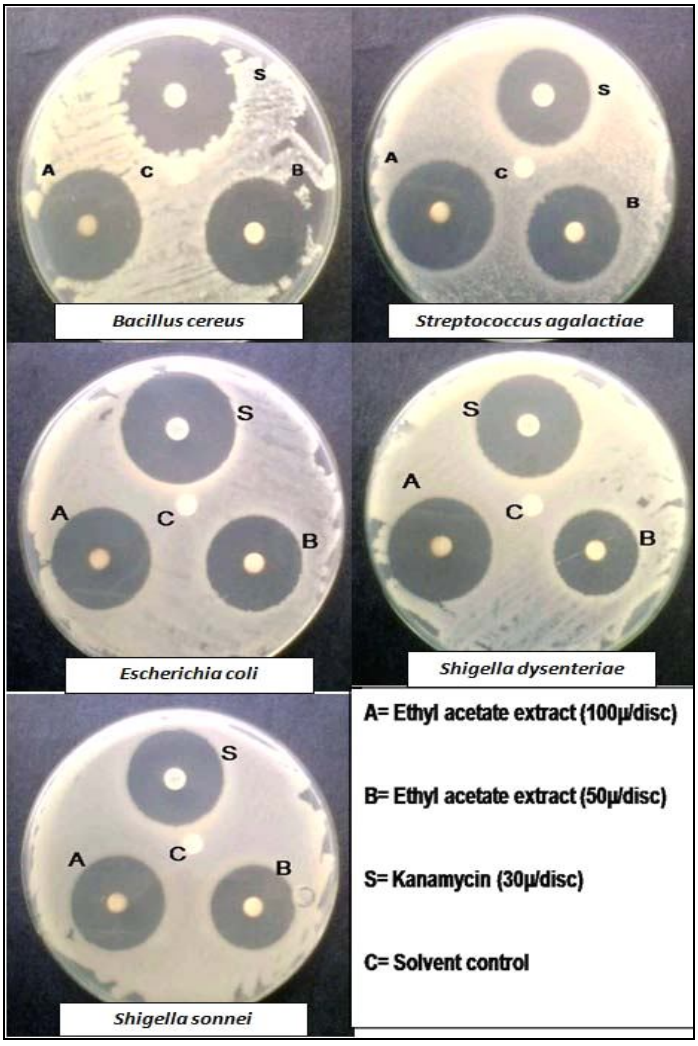

Figure 2 Antibacterial activity of crude extract of *Bacillus methylotrophicus*-SCS2012

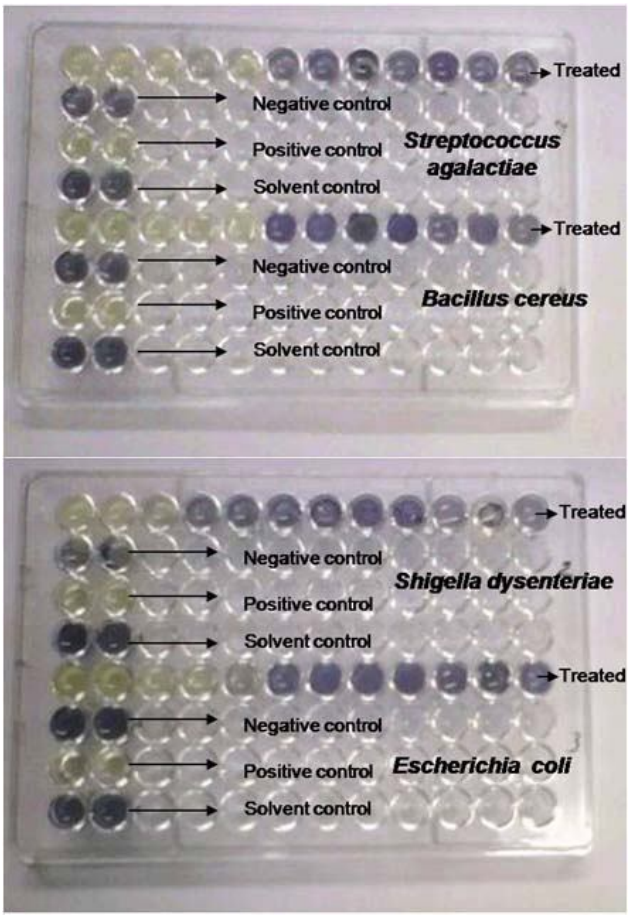

Figure 3 Minimum inhibitory concentration of crude extract of *Bacillus methylotrophicus*-SCS2012

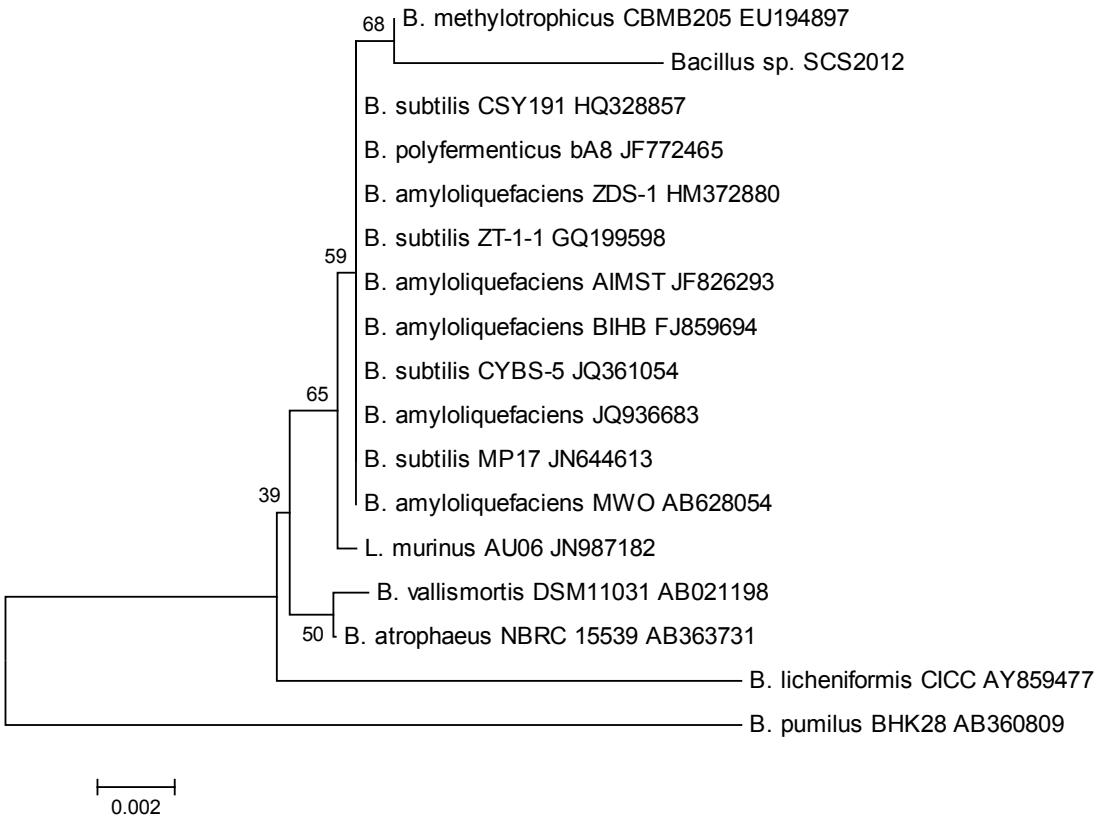

Figure 4 Phylogenetic tree obtained by Neighbor-joining analysis based on 16S rRNA gene sequences showing the phylogenetic position of strain SCS2012 and representatives of some other related taxa. Bootstrap values expressed as percentages of 1000 replications are shown at the branch points. Scale bar, 0.002 estimated substitutions per nucleotide Position

CONCLUSION

The bacterial strain isolated from soil was *Bacillus methylotrophicus*-SCS2012 posessing antibacterial activity. From the above studies we had seen that the crude extract of this strain was active against both gram positive and gram negative pathogenic bacteria with moderate cytotoxicity. So the strain *Bacillus methylotrophicus*-SCS2012 may be considered as a good potential source for the production of new antibiotics in future.

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