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Original Research Article

Isolation and Identification of *Bacillus* Species from Soils and Studies on Their Secondary Metabolites

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Abstract

The study was performed to isolate Bacillus sp. having antibacterial activity isolated from soils of Rajshahi, Bangladesh. The strain *Bacillus amyloliquefaciens* was identified by 16S rDNA gene sequencing. Ethyl acetate extract of *B. amyloliquefaciens* showed antibacterial activity against both Gram positive and Gram negative bacteria. The crude metabolite extracted from *B. amyloliquefaciens* exhibited strong antibacterial activity against Gram positive *Staphylococcus aureus,.Bacillus cereus, Streptococcus agalactiae and* Gram Negative *Escherichia coli, Shigella dysenteriae and Pseudomonas aeruginosa.* Thus the ethyl acetate extract of *Bacillus amyloliquefaciens* has broad spectrum antibacterial activity. Since many species showed inhibitory activity against test bacteria, that suggests Bangladeshi soil could be an interesting source to explore for antibacterial secondary metabolites.

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Introduction

The genus *Bacillus* has been known as an important source of antibiotic producers since the "golden age" of antibiotic discovery (Schaffer, 1969). Just in the organism *Bacillus subtilis*, 12 antibiotic compounds have been isolated from different strains (Stein, 2005). Many of the antibiotics from *Bacillus* are peptide compounds that are synthesized either ribosomally or non-ribosomally (by non-ribosomal peptide synthases) and are resistant to the action of proteases (Schaffer, 1969, Nakano and Zuber, 1990, Stein 2005). Ribosomally synthesized peptides include lantibiotics such as nisin and subtilin, which are active against Gram-positive organisms (Stein, 2005). Bacillomycins and mycosubtilin, which are hemolytic and anti-fungal are examples of non-ribosomal peptides produced by *Bacillus* (Stein, 2005). Many of the peptide antibiotics produced by *Bacillus* contain unusual amino acids and are also resistant to proteases (Schaffer, 1969).

Two peptide antibiotics produced by *Bacillus* have seen widespread use: bacitracin and polymyxin B. Bacitracin is a cyclic dodecapeptide produced by both *Bacillus subtilis* and *Bacillus licheniformis*, and is active mainly against Gram-positive organisms by inhibiting cell wall synthesis (Tay, 2010). Bacitracin is one of the active ingredients in the topical agents neosporin and

polysporin, and is also used in livestock feed. Although this antibiotic has seen 50 years of use, resistance to bacitracin is still rare (Tay, 2010). Polymyxin B is part of the polymyxin class of drugs, cationic peptides that act against Gram-negative organisms by increasing outer membrane permeability (Gales, 2001). In the past polymyxin B was one of the few antibiotics effective against *Pseudomonas aeruginosa*. However, other less toxic compounds have been found, and polymyxin B is now used as a topical agent (Tay, 2010).

Species of *Bacillus* also produce polyketide antibiotics such as difficidin, which has a broad spectrum of activity against Gram-positive and Gram-negative organisms. Many antibiotics produced ribosomally are lantibiotics, which contain the unusual amino acid lanthionine (Stein, 2005). Type A lantibiotics are active by forming voltage-dependent pores in the cell membrane of bacteria (Stein, 2005). Type B lantibiotics inhibit cell wall synthesis by binding to lipid II (Stein, 2005). Non-ribosomally synthesized antibiotics from *Bacillus* are often lipopeptide compounds that exhibit surfactant-like qualities (Stein, 2005).

One of the best well-studied lipopeptides from *Bacillus* is surfactin, which is one of the most powerful surfactants known (Stein, 2005).Surfactin can lower the surface tension of water from 72mN/m to 27mN/m and will also disrupt biological membranes (which is the source of its antimicrobial activity). Polyketide antibiotics are also produced by *Bacillus* such as difficidin, which has broad-spectrum activity against Gram-positives and Gram-negatives. *Bacillus* continues to be a good source of new antibiotics today, such as the recent discovery of a novel antibiotic from *Bacillus licheniformis* (Dischinger, 2009).

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 amyloliquefaciens* are well studied for the production of antibiotic. We observed that the strain *Bacillus amyloliquefaciens* showed antimicrobial activity against some pathogenic bacteria and fungus. Bangladesh is a low-lying riverine country, has a tropical monsoon climate characterized by heavy seasonal rainfall, high temperatures, and high humidity. The alluvial soil of Bangladesh is highly fertile. A few studies have been done so far using Bangladeshi soils to screen for new bioactive compounds.

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 g 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 another test tube containing 9 ml of sterile water and in this way five times dilution were made. An aliquot of 0.1 ml of each dilution was taken and spread evenly over surface of Yeast extract agar plates and incubated overnight at 37ºC. After 24 hrs examination very thin clean zone of few colonies of similar morphology was appeared around the colonies selected for isolation and purification. Selected colonies were re-cultivated several times for purification. The purified strains were stored on glycerol Stock method use for strain preservation (Demain and Davies, 1999) and further identified by detailed conventional biochemical methods and 16S rDNA sequencing.

Morphological studies of the isolated strains

To identify the selected strains, the following morphological characters were studied.

i) Non-microscopic studies like agar colony

ii) Agar slant and nutrient broth culture test were observed and noted.

Microscopic observation

The sizes and shapes of the (vegetative cell) bacteria were determined. The arrangements of the cells whether present singly, in rod shaped, in pairs or chains were also observed. Motility of the organism was observed and gram-staining test was done to characterize the organism. Gram staining tests were done according to the standard method. The presence of crystal Violet color indicated the Gram positive and safranine color indicated the gram-negative organisms (Reynolds et al., 2009).

Biochemical testes used to characterize the isolated strains

Fermentation test: This test is used to differentiate the organisms that ferment carbohydrate (such as glucose, galactose and sucrose). Oxidation fermentation medium is used in the fermentation test of carbohydrate (Table 2).

Citrate utilization test: This test was based on the ability of an organism to use citrate as its only carbon source and ammonia as its only source of nitrogen. The test organism was cultured in a medium, which contains sodium acetate, an ammonium salt and the indicator bromothymol blue, Koser's citrate medium was used in citrate utilization test.

Indole test: This test demonstrates the ability of certain bacteria to decompose the amino acid tryptophan to indole, which accumulates in the medium. Indole is then tested by a calorimetric reaction with p-dimethyl-aminobenzaldehyde. The pH of the media was adjusted to 7.4 and sterilized by autoclaving at 121°C for 15 minutes and stored at 4°C.

Phylogenetic classification and identification of the isolated strains

Molecular identification of bacteria: The identity of the isolate was determined by sequence analysis of the 16S rDNA gene. The overnight cultured bacterial cells were lysed with lysozyme and the DNA was extracted by the phenol: chloroform (1:1) extraction method described by Ausubel (1987). The 16S rDNA was amplified in PCR with the primer pair BcF (TACGGYTACCTTGTTACGACTT) and BtF (AGAGTTTGATCMTGGCTCAG). The amplified region was then sequenced and subject to BLAST analysis for analyzing its phylogeny (Altschul et al., 1990)

PCR: The Polymerase Chain Reaction (PCR) is a powerful and sensitive technique for DNA amplification (Saiki et al., 1985). *Taq*DNA Polymerase is an enzyme widely used in PCR (Powell et al., 1987). The following guidelines are provided to ensure successful PCR using NEB's *Taq*DNA Polymerase. These guidelines cover routine PCR. Amplification of templates with high GC content, high secondary structure, low template concentrations, or amplicons greater than 5 kb may require further optimization.

Agarose gel electrophoresis: Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Most every molecular biology research laboratory routinely uses agarose gel electrophoresis for the preparation and analysis of DNA. Agarose gel electrophoresis was used to determine the presence and size of PCR products.

16S rDNA gene sequencing and Blast

16S rDNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. The amplified region was then sequenced and subject to BLAST analysis for analyzing its phylogeny.

Extraction of secondary metabolites

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 hrs. 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 slant medium at 4ºC.

Antibacterial assay

To test antibacterial activities of the crude extract of isolate *Bacillus amyloliquefaciens*, the disc diffusion method (Bauer 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 5µg/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 hrs 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 hrs 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)

Results and discussion

Characterization of bacteria

The microscopic observation of the isolated strain showed positive for Gram staining, single celled bacillus with motility. Biochemical tests also confirmed Gram positive staining and positive for fermentation test, citrate utilization test and for indole test (Tables 1 and 2). The cultural characteristics of the isolates on agar slant and broth medium showed that the colonies were white in colour, wet circular in nature and grown on the surface of broth medium (Table 3).

Phylogenetic analysis of *B. amyloliquefaciens*

According to BLAST, the 16s rDNA sequence of this strain only shows 99% sequence similarity with complete genome sequence of *Bacillus toyonensis.* The phylogenetic tree was constructed by using Bioinformatics Bacterial Identification Tool (BIBI). The result is shown in the Fig. 2. The phylogenetic tree built by the forward sequence of the strain AIAS-39 showed that the strain belonged to *Bacillus* clade.

The study was performed with an aim of screening and isolation of antimicrobial substances producing bacteria from soil. The antibacterial activity was subsequently confirmed by agar disc diffusion method. The isolate was identified on the basis of morphological and biochemical characteristic (Tables 1, 2 and 3) by conventional techniques as per Bergey's manual of Determinative Bacteriology (Berkley et al., 1984; Claus and Berkely, 1986) and is found to be *Bacillus* sp. The partial sequence of the 16S rRNA 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 similarity to *Bacillus amyloliquefaciens* (Figs. 1, 2 and 3; Table 4) . A phylogenetic tree based on the nucleotide substitution rate indicated that the strain belongs to the genus *Bacillus*. When the phylogentic position of isolate was compared with closely related species of the genus *Bacillus*, the strain formed a monophylectic clade with *Bacillus amyloliquefaciens* (Fig. 2). Therefore the strain was tentatively identified as *Bacillus amyloliquefaciens* and the partial sequence was submitted to NCBI gene bank. 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 amyloliquefaciens* obtained from ethyl acetate extraction (Fig. 4) showed strong antibacterial activity against Gram positive bacteria and gram negative bacteria (Fig. 5). The crude extract at a dose of 100µg/disc showed prominent antibacterial activity against all the tested bacteria with the zone of inhibition range 20 to 25 mm (Table 5).

Table 1. Microscopic observation of isolated strains.

Strain	Gram staining	Cell shape	Arrangement	Motility test	Comment
B. amyloliquefaciens	+	Bacillus	Single	+	May be *Bacillus*

Table 2. Biochemical tests used to characterize the isolated strains.

Strain	Gram staining	Fermentation test	Citrate	utilization test	Indole Test
B. amyloliquefaciens	+	+	+		+

Table 3. Visual observation on agar slant and broth medium.

Strain	Colony color	Colony type	Colony form	Growth in nutrient broth medium
B. amyloliquefaciens	White	Wet	Circular	Surface

Forward sequence of *B. amyloliquefaciens*

The forward sequence (sequence length: 723 bp) of *B. amyloliquefaciens is* given below:

AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCC
GCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTG
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA
ACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTT
CGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCCGTGCCCGGAGATTTTTGGGT
TAAGTCCCCACACCAAGCGCAACCCTTTGATCTTAGTTGGCCAGCTTTCCATTTGGGCCCTC
TANNNCGAGCGGCGTGCTATACATGCAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGT
TAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGA
AACCGGAGCTAATACCGGATAGTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCG
GCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAG
GCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG
CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAA
GAGTAACTGCTTGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGG
CGGTTTC

Fig. 1: Forward sequence of *B. amyloliquefaciens.*

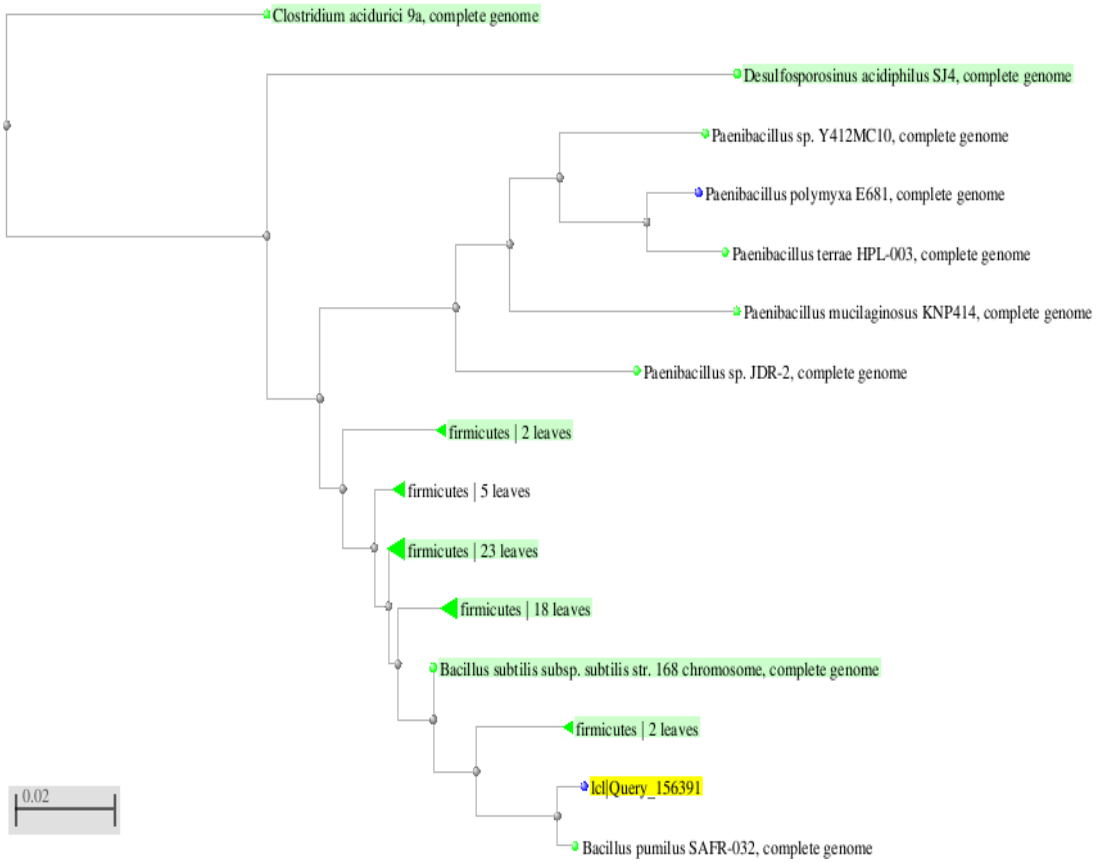

Fig. 2: Phylogenetic tree showing the relationship between isolated strains and representative species of the genus *Bacillus* based on partial 16S rDNA gene sequences (This tree was made from forward sequencing).

Table 4. Sequences producing significant alignments with the complete 16S rDNA of *Bacillus amyloliquefaciens.*

Description	Max score	Total score	Query cover	E value	Identity	Accession
Bacillus amyloliquefaciens subsp. *plantarum* str. FZB42, complete genome	878	13128	100%	0.0	95%	NC_009725.1
Bacillus subtilis subsp. *subtilis* str. 168 chromosome, complete genome	872	14195	100%	0.0	95%	NC_000964.3
Bacillus atrophaeus 1942, complete genome	867	10081	99%	0.0	95%	NC_014639.1
Bacillus infantis NRRL B-14911, complete genome	821	12158	99%	0.0	93%	NC_022524.1
Bacillus licheniformis DSM 13 = ATCC 14580, complete genome	819	9623	99%	0.0	93%	NC_006322.1
Bacillus sp. 1NLA3E, complete genome	806	15991	99%	0.0	93%	NC_021171.1
Bacillus pseudofirmus OF4, complete genome	800	8892	99%	0.0	93%	NC_013791.2
Bacillus megateriumDSM319, complete genome	784	14539	99%	0.0	92%	NC_014103.1
Bacillus halodurans C-125 DNA, complete genome	782	10061	99%	0.0	92%	NC_002570.2
Bacillus toyonensis BCT-7112, complete genome	780	15547	99%	0.0	92%	NC_022781.1

Nucleotide sequence homology data for forward sequence of *B. amyloliquefaciens* alignments

Bacillus: B. amyloliquefaciens subsp. *plantarum* str. FZB42, complete genome

Sequence ID: ref|NC_009725.1|

Length: 3918589

Number of Matches: 19

Genomic Sequence Range 1: 97104 to 97664GenBankGraphics

Alignment statistics for match #1:

Score	Expect	Identities	Gaps	Strand
878 bits(475)	0.0	534/562(95%)	6/562(1%)	Plus/Plus

rRNA-16S ribosomal RNA

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Query  381    GCGGCGTG-CT-ATACATGC-AGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAG  437
              |||||||| || |||||||| ||||||||||||||| |||||||||||||| ||||||||
Sbjct  97104  GCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAG  97163

Query  438    CGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAA  497
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97164  CGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAA  97223

Query  498    ACCGGAGCTAATACCGGATAGTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTC  557
              ||||| |||||||||||||| |||  ||||||||||||||||   || ||||  || |||
Sbjct  97224  ACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTC  97283

Query  558    GGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACC  617
              |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97284  GGCTATCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACC  97343

Query  618    AAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGG  677
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97344  AAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGG  97403

Query  678    CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGG  737
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97404  CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGG  97463

Query  738    AGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAA  797
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97464  AGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAA  97523

Query  798    CAAGTGCAAGAGTAACT-GCTT-GCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAA  855
              |||||||  |   || | |    ||||||||||||||||||||||||||||||||||||
Sbjct  97524  CAAGTGCC-GTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAA  97582

Query  856    CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG  915
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97583  CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG  97642

Query  916    TAAAGGGCTCGCAGGCGGTTTC  937
              |||||||||||||||||||||
Sbjct  97643  TAAAGGGCTCGCAGGCGGTTTC  97664
```

Fig. 3: Genome sequence alignments for the search.

Table 5. Antibacterial assay of ethyl-acetate extract of *B. amyloliquefaciens.*

Test organisms	Zone of inhibition (in mm)		
	***B. amyloliquefaciens* extract (50µg/disc)**	***B. amyloliquefaciens* extract (100µg/disc)**	**Kanamycin (5µg/disc)**
Gram positive bacteria:			
1.*Staphylococcus aureus*	9	20	28
2. *Bacillus cereus*	12	22	31
3. *Steptococcus agalactiae*	13	21	27
Gram negative bacteria:			
4. *Escherichia coli*	15	22	29
5. Shigella dysenteriae	10	23	33
6. *Pseudomonas aeruginosa*	9	24	30

Fig. 4: Ethyl acetate extract of *B. amyloliquefaciens.*

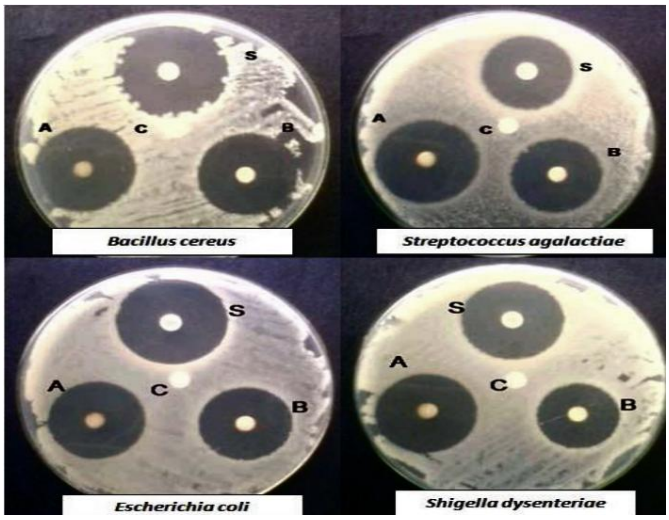

Fig. 5: Antibacterial activity of ethylacetate exrtract of *Bacillus amyloliquefaciens*.
A-Ethyl acetate extract (100µg/disc).
B-Ethylacetate extract (50µg/disc); S-Kanamycin (5µg/disc).
C- Solvent control.

Conclusion

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). The bacterial strain isolated from soil was *Bacillus amyloliquefaciens* possessing 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 amyloliquefaciens* may be considered as a good potential source for the production of new antibiotics in future.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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