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Screening and Characterization of Antimicrobial Substances Produced by *Bacillus* species

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This study isolated two strains of *Bacillus* identified by cultural, morphological and biochemical methods as *Bacillus subtilis* (B1) & *B. coagulans* (B2). Potent antibacterial activity was observed against pathogenic and food-spoilage bacteria *Staphylococcus aureus* MTCC 1144, *Pseudomonas aeruginosa* MTCC 2488, *Shigella flexneri* MTCC 1457, *Listeria monocytogenes* MTCC 1463. MIC values of B1 and B2 were compared to those of standard strain *B.subtilis* MTCC 441 and potent activity was obtained at 640 AU of B1, 160 AU of B2 and 640AU of *B. subtilis* against *S. flexneri MTCC 1457*. Against *S. aureus* MTCC 1144, it was 320 AU of B1 and 640 AU of B2 and *B.subtilis*. Partially purified Anti-Microbial Substances (AMS) obtained from the two strains were low molecular weight polypeptides ranging from 5-25 kDa as detected by SDS-PAGE. Characterization of AMS by HPTLC, LC and GC-MS indicated presence of a mixture of secondary metabolites viz., derivatives of Benzenedicarboxylic acid, 3 OH Fatty acids like Hexadecanoic acid, Heptadecanoic acids and Quinolone compounds. The findings of this study shed light on important aspects of food protection. Also due to the fact that *Bacillus* species have produced antibiotics in the soluble protein structure and that these antibiotics have been found to be cheaper and more effective in studies conducted to date, these microorganisms are preferable for commercial production.

Key words: *Bacillus* spp, Peptide antibiotics, Biocontrol agents, Characterization.

Antimicrobial substances are key among the compounds of microbial origin, providing the basis for treatment of infectious diseases of humans, animals, and plants. Over the past decade there has been an increased development of resistance in organisms that are typical pathogens in humans. With the continuing discovery of new infectious diseases and the development of new disease processes of existing pathogens (i.e., necrotizing fasciitis caused by *Streptococcus pyogenes*), it is important to continue to find anti-infective agents that can be used to treat these infections[12,13]. Development of novel classes of drugs, drugs with fewer side effects, and drugs with shorter lengths of treatment are key in continuing the fight against infectious disease[17]. The confluence of these trends has focused attention on new sources of microbial products and new approaches to synthesize antimicrobial agents.

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Parallel to the screening for new antibiotics, efforts have been focused in finding low molecular weight secondary metabolites with other biological activities. Two main strategies have been used during the screening process of these compounds. First, the screening of known secondary metabolites which had failed as useful antibiotics and second, screening of unknown compounds using new technologies for detecting inhibition of enzymes and biological activities in other targets[17]. Some of the observations regarding secondary metabolites include:

a. They act as an alternative defence mechanism, because only the organisms lacking an immune system are prolific producers of these compounds.
b. They have sophisticated structures, mechanisms of action, and complex and energetically expensive pathways.
c. Act in the competition between microorganisms, plant and animals.
d. They are produced by biosynthetic genes clusters, which would only be selected if the product conferred a selective advantage.
e. The production of secondary metabolites with antibiotic activities is temporarily related with sporulation when the cells are particularly sensitivity to competitors and requiring special protection when a nutrient runs out.

Secondary metabolites are mostly biosynthesised by bacteria, fungi, algae, corals, sponges, plants and lower animals. Actually, filamentous microorganisms are the main source of secondary metabolites with nearly 75 % of all described antibiotics being produced by actinomycetes and 17 % by molds. Moreover, approximately 40 % of the filamentous fungi and actinomycetes produce antibiotics when freshly isolated from nature. Some isolates of several *Streptomyces* species can produce more than 180 different secondary metabolites.

Bacteria that show high natural products production are those belonging to the genus *Bacillus*. Several antibiotics have been isolated from different *Bacillus* strains, moenomycins, difficidins, bacillomycins and bacillaenes are part of this wide variety. *Myxobacterium* is yet another genus of bacteria with interesting antibiotic productivities.

Several *Bacillus* strains have been considered to be natural factories of biologically active compounds such as lipopeptides, bacteriocins, as well as Bacteriocin like substances (BLS) and the significance of their involvement in plant microbial disease control have been demonstrated (Asaka and Shoda 1996; Emmert and Handelsman 1999).

Known antibiotic producers are *Brevibacillus brevis* (e.g. gramicidin, tyrothricin), *Bacillus cereus* (e.g. cerexin, zwittermicin), *Bacillus circulans* (e.g. circulin), *Brevibacillus laterosporus* (e.g. laterosporin), *Bacillus licheniformis* (e.g. bacitracin), *Paenibacillus polymyxa* (e.g. polymyxin, colistin), *Bacillus pumilus* (e.g. pumulin) and *Bacillus subtilis* (e.g. polymyxin, difficidin, subtilin, mycobacillin) (Todar K, PhD: Textbook of Bacteriology).

Bacillus antibiotics share a full range of antimicrobial activity: bacitracin, pumulin, laterosporin, gramicidin and tyrocidin are effective against Gram-positive bacteria; colistin and polymyxin are anti-Gram-negative; difficidin is broad spectrum. Many of these *Bacillus* species produce antifungal compounds like mycobacillin and zwittermicin, fengycins, iturins (Edwards *et al.,* 1994).

Bacillus is generally soil-inhabiting genus or exists as epiphytes and endophytes in the spermosphere (Mundt and Hinckle 1976) and rhizosphere (Zhang *et al.,* 1993). *Bacillus* species are also found in many other environments (Sneath 1986) as their survival is aided by this ability to form endospores. These dormant structures are resistant to desiccation, heat, U.V. irradiation and organic solvents; characteristics which allow for further formulation and commercialization procedures (Rhodes 1990).

The present study aims at isolating *Bacillus* species with potent antimicrobial activity against human pathogenic and food spoilage organisms keeping in view the need for bioactive compounds in treatment of infectious disease and food protection.

MATERIAL AND METHODS

Sample collection:-In order to isolate antagonistic *Bacillus* species, ubiquitous soil bacteria, soil samples were collected from different

areas of Mumbai ie: Andheri, Santacruz (E), Malad (W), Borivali (W). Also manure from Bhavan's College Botanical garden was collected for the same. For crowded plate method, each gram of sample was suspended in 100 ml of sterile distilled water and shaken vigorously for 2 min. Then the soil suspensions were serially diluted in sterile Saline, and the dilutions from 10^{-1} to 10^{-6} were plated on nutrient agar medium. The plates were incubated at 28-37°C for 24-48 hr (Watanabe, Chilkot.C.N, 1993). After incubation the colonies showing antagonism were selected and primary identification of the isolates was done.

Identification and maintenance of test cultures

Several test cultures were used to assess the antimicrobial activity of the *Bacillus* species. The lyophilized vials were reconstituted in Nutrient Broth and revived after 24 hours. *Staphylococcus aureus* MTCC 1144 and *Streptococcus pyogenes* NCIM 2608 specifically were grown in Brain Heart Infusion Broth (BHI) overnight. Purification was done on Nutrient agar and BHI medium and plates were incubated at 37°C for 24 hours. The test cultures were identified using standard biochemicals.

Preliminary screening of antimicrobial activity

Determination of antimicrobial activity of *Bacillus* isolates by Agar streak method

The agar streak method was performed as described by Rosado and Seldin (1993) wherein the isolated *Bacillus* strains were streaked at the centre of media plate and after incubation at RT/ 48 hrs they were killed by exposure to chloroform vapours for 15min. These plates were then inoculated with indicator strains and following incubation at 37°C for 24hrs, inhibition of growth besides the central streak indicated antimicrobial production. Extent of inhibition was reported as follows: (-) No inhibition and (+) clear Inhibition. On the basis of these results further activity studies of isolates were conducted.

Overlay method

A lawn of *Bacillus* isolates was prepared on Nutrient agar plates and incubated for 48 hours for optimal antimicrobial compound production. *Bacillus* plugs of 6mm diameter were made and placed upright on Mueller Hinton agar seeded with standard test cultures (O.D 530nm = 0.1). Results were observed after incubation at 37°C for 24hrs, as diameters of zones of inhibition of test cultures.

On the basis of the above results the selected *Bacillus* isolates were then used for bulk production of AMS in liquid medium. Activity assays were performed using standard disc diffusion and agar cup methods.

Bulk production of selected isolates for AMS

Nutrient broth with 1% glucose, pH 7.0 was inoculated individually with selected species of *Bacillus* (0.4%v/v) and incubated under aerobic, shaker conditions for 72 hours. Cells were harvested by centrifugation at 10,000 rpm for 30 min at 4°C, culture supernatants were filtered using 0·45 *i*m filter membranes and stored at 4°C until utilization (Brandelli *et al*., 2004). This cell free supernatant solution was then divided equally for antibacterial and antifungal studies and subjected to antimicrobial susceptibility testing by agar diffusion methods.

Determination of antibacterial activity of crude Cell Free Supernatant (CFS)

Agar cup method to determine antibacterial activity of Crude Cell free extracts was performed (Tagg and McGiven 1996) on Mueller and Hinton agar medium. The plates were seeded with the test cultures (O.D 530nm = 0.1) prior to the addition of 50 μl CFS into the wells (6mm diameter). Plates were grown overnight at 37°C before examining inhibition zones (Brandelli *et al*. 2004, P.A. Risoen *et al*. 2004). 50µl of Standard Streptomycin solution (15mcg/ ml) was used as control.

Following evaluation of antimicrobial potential of the selected *Bacillus* species, solvent extraction and purification of the active compounds was carried out.

Extraction and purification of AMS

The crude Cell free extracts from selected *Bacillus* species were filter sterilized using 0.45*i*m membrane filters, and proteins precipitated with ammonium sulphate at 65% saturation by constant stirring overnight for 16 h at 4°C (Bizani *et al*., 2005). Precipitated proteins were pelleted by centrifugation (10 000 rpm for 30 min at 4°C), dissolved in 5ml phosphate-buffered saline pH 7·4, and butanol added (1: 4 ratio butanol: sample) for extraction of antimicrobial activity (P.A. Risoen *et al*., 2004). The butanol was evaporated and precipitates were dissolved in 2ml PBS at pH

7.4 for B1 and B2 compounds and for *B.subtilis* the precipitate was dissolved in 3ml of PBS. Following dissolution the extracts were filter sterilized using 0.45*ì*m syringe filter and stored at 4°C before use. This solution was designated as purified antibacterial extract. Disc diffusion assay was performed in duplicates to determine the antibacterial activity of the butanol extracts and results recorded as zones of inhibition. Butanol control was used to eliminate the possibility of butanol inhibiting the test cultures giving a zone.

Determination of Minimum Inhibitory Concentration (MIC)

The Microdilution method using 96 well microtitre plates described by the National Committee for Clinical Laboratory Standards (NCCLS) was performed for the assessment of the minimal inhibitory concentrations (MICs). For this microtitre plate assay, 100µl of butanol extracts were serially two-fold diluted in St MH broth with 0.2% TTC. Then 50 μl of 4 hr young test culture was added to respective wells. The results were observed after 4–5h (and after 24hours) and inhibition of growth was detected visually by colour change to pink when the culture was growing and absence of pink upto concentration that is inhibitory (Modified Hoyle *et al*., method. 2004).

Medium control, extract controls and positive controls were also used for elimination of false positive results.

By definition, MIC is the reciprocal of the highest dilution of the extracts that inhibits growth of the test culture under specified conditions.

Separation of purified AMS by HPTLC method

Separation of active components of the purified antibacterial extracts was done by HPTLC due to its main advantage of enormous flexibility, as follows:

Method

10×10 cm TLC aluminum plates precoated with Kieselgel Silica 60 F_{254} gel (Merck, Darmstadt, Germany) were used. 32µl of individual samples (600ìl of extracts in 2ml St Saline) were applied as 15mm bands on plate 10mm from the bottom edge and at a distance of 14mm by the spray-on technique using the CAMAG ATS autosampler (LINOMAT, CAMAG ISO 9001, Switzerland). Plate was developed in a twin trough saturated tank with a solvent system of Butanol: Acetic acid: Water (3:3:1 v/v/v) to 85mm above the spotting line. The plate was then dried for 1 min, dipped in chamber containing 1% Ninhydrin in butanol solution and heated at 110°C for 2 min. Spots were identified in TLC Scanner-3 attached to CATS software for Chromatogram evaluation enabling high spectral sensitive in-situ spectra recording. Plate was observed and photographed under fluorescence, UV and Visible light in the cabinet.

TLC- Bioautography

The developed chromatographic plates were overlaid with Mueller Hinton agar, inoculated with 0.5ml (O.D530nm = 0.1) of bacterial test culture *S.aureus* MTCC 1144. Results were noted after incubation at 37°C for 16-18hrs as zones/ bands of inhibition in the area in which biologically active compounds diffused. The Rf values of bands showing inhibitory activity on developed plate were compared with that of Rf values of compounds obtained on derivatized plate to identify the location and molecular weight determination of the separated compounds.

Identification and Characterization of the AMS by LC and GC-MS

HPLC was carried out on VARIAN Pro Star/ Dynamax System Model 210 equipped a C-18 column (VARIAN). Pump A was Water and Pump B was Acetonitrile. 20 ìl of the extracts (dissolved in acetonitrile) were injected and run time was 20min with sampling interval of 0.20sec. The Mass Spectra of separated components was detected using VARIAN 500 MS-IT Mass Spectrometer coupled with APCI ionization mode. The active substances were identified using the mass spectra in comparison with the online compound database and library (NIST).

For further identification of active antimicrobial fractions present in the purified extracts, GC-MS was performed on GCD instrument present at SAIF, IIT Powai. The sample was volatilized using 0.03mm column maintained at a temperature of 80°C for a run time of 27min. The temperature and run time were decided depending upon the boiling point of the compounds present in the extracts.

SDS- Polyacrylamide Gel Electrophoresis (SDS- PAGE) for molecular weight determination

Purified antibacterial extracts were separated and analyzed on 12% Polyacrylamide gel containing Sodium Dodecyl Sulphate (SDS) (Techno Source Power Pack and Apparatus) using method described by Laemmli (1970). The gel was electrophoresed at 15mA in Stacking (5%) and 20mA in Resolving (12%) gels respectively for 2 hours. This was followed by staining the gels using Silver staining[21]. The apparent molecular masses of the sample polypeptides were then calculated by comparison with mobility of Bangalore Genei SDS-PAGE standard marker.

RESULTS AND DISCUSSION

Preliminary Antibacterial activity screening

Antagonism is ubiquitous in nature among different species. The genera *Bacillus* produces about 167 biological compounds active against bacteria, fungi, protozoa and viruses (Katz and Demain, 1977; Cordovilla *et al.* 1993, Karuse *et al*. 1990). Many of theses extracellular compounds are indicated as preservatives in food systems and beverages (Wang and Fung 1996), as biological control agents against phytopathogens and as antibiotic producers (Zuber *et al.,* 1993).

By Crowded Plate and Wilkin's overlay method nine *Bacillus* cultures with antimicrobial potential were isolated from soil samples collected from different locations of Mumbai. (Table 2).

Data

1. *Bacillus* isolates from soil and manure B1-B9.

Method

Combined results of a) Agar strip method
b) Overlay method

Antibacterial activity of crude Cell Free extracts

The agar cup assay of Cell Free extracts indicated that most of the selected *Bacillus* strains produced antimicrobial compounds in the late stationary phase ie; within 72 hours of growth. Also the extent of target growth inhibition was found to be good in the extracellular supernatants. Hence further extraction and purification of the active antimicrobial compounds was performed.

From the results of preliminary analysis of antibacterial potency of the *Bacillus* isolates, strains B1, B2, B5, B6 and B7 were found to have

Table 1. Species and sources of standard test cultures

S. No	Test Cultures Human Pathogens	Source
1.	*Staphylococcus aureus*	MTCC 1144
2.	*Streptococcus pyogenes*	NCIM 2608
3.	*Salmonella typhimurium*	NCIM 2501
4.	*Shigella flexneri*	MTCC 1457
5.	*Pseudomonas aeruginosa*	MTCC 2488
6.	*Pseudomonas aeruginosa*	NCIM 2200
7.	*Pseudomonas syringae*	MTCC 1604
8.	*Klebsiella pneumoniae*	MTCC 432
9.	*Erwinia carotovora*	MTCC 1428
10.	*Agrobacterium tumefaciens*	MTCC 431
11.	*Listeria monocytogenes*	MTCC 1143

Table 2. Preliminary activity screening of *Bacillus* isolates

Bacillus strain	STD Test Cultures											Inhibited/11	% Inhibition
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.		
B1	+(11)	+	-	+	-	+	+(11)	-	-	+(11)	+	7	63.6
B2	+(14)	+	-	+	+	+	-	+	+	+	+	9	81.8
B3	+	+	-	+	+	-	+(10)	-	+	-	-	6	54.5
B4	-	+	-	+	+	-	-	-	-	-	-	3	27.2
B5	-		-	+	-	+	+	+	+	-	-	5	45.4
B6	+	+	-	+	+	-	+	-	-	+	-	6	54.5
B7	-	-	-	+	-	+	-	-	-	-	-	2	18.1
B8	+(12)	+	-	-	-	+	-	-	+	-	-	4	36.3
B9	+	-	-	-	-	-	+(12)	-	-	-	-	2	18.1

higher amount of activity against Gram positive and Gram negative test cultures. Hence these strains were selected for further study.

Extraction and Purification of AMS

Antibacterial activity of purified Butanol extracts

The results of agar disc diffusion assay (Table 4) indicate the potent antibacterial activity of the purified antibacterial extracts as compared to whole cell assays used in preliminary studies.

On the basis of above results of pilot studies, *Bacillus* strains B1 and B2 produced most active secondary metabolites under the specified conditions. However strains B3, B6 and B7 showed less inhibition, hence were not considered in further studies. For comparison purposes *B.subtilis MTCC 441* was considered in further studies as the standard strain producing antimicrobial compounds.

AMS produced by strain B1 was detected during sporulation phase, when number of spores had increased as detected by endospore staining. It is possible that sporulation process is required for AMS production or atleast a common regulatory pathway as reported for other antimicrobial compounds (Leifert *et al.,* 1999). Similar results have been reported for antibacterial compounds of *Bacillus subtilis* strains. In case of strain B2 the production of AMS seems to be induced under non-sporulating conditions as a secondary metabolite, as previously reported for other AMS by *Bacillus* species (Galvez *et al.,* 1993). Since in both cases the AMS were produced when the isolates were grown in a rich medium in the late stationary phase, they are considered secondary metabolites.

Bioassay of antibacterial extracts

A bioassay using serial two-fold dilutions of antibacterial extracts of B1, B2 and *B.subtilis* was performed against *S.aureus* MTCC 1144 (Representative test culture for Gram +ve) to confirm the antibacterial activity observed so far.

Table 3. Antibacterial activity of CFS by agar cup method

Bacillus Strain	STD Test Cultures											Total/11
	1.	2.	3.	4.	5	6.	7.	8.	9.	10.	11.	
B1	+	+	-	+	-	+	-	+	-	+	+	7
B2	+	-	-	+	+	-	-	-	-	+	+	5
B3	+	-	-	+	-	+	-	-	+	-	-	4
B4	-	-	-	-	+	-	-	-	-	-	-	1
B5	-	-	-	-	+	-	+	-	-	-	-	2
B6	+	+	-	+	-	-	-	-	-	+	-	4
B7	-	-	-	+	+	-	-	-	-	-	-	2
B8	+	-	-	-	-	-	-	-	-	-	-	1
B9	-	-	-	+	-	-	-	-	-	-	-	1

Key: + Inhibition - No inhibition

Table 4. Disc diffusion assay of purified extracts

Bacillus strains	STD Test Cultures										
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.
B1	+(16)	+	-	+ (14)	-	+	-	+	-	+	+(12)
B2	+(15)	-	+	+	+	-	-	-	-	-	+(15)
B3	+	+	-	-	+	-	-	-	-	-	-
B6	-	-	-	+	+	-	-	-	-	-	-
B7	+(13)	-	-	-	-	-	-	-	-	-	-
Streptomycin	+(20)	-	+(30)	+(20)	-	+(29)	-	+(30)	+(24)	+(28)	-

Key: Inhibition: + No inhibition: - Butanol control: No inhibition

After incubation at 37°C for 24hours the diameters of zones of inhibition were noted in mm. The standard strain *B.subtilis* inhibited the test organism upto 1:16 dilution of the extract whereas B1 and B2 strains had maximum activity upto 1:8 and 1:4 dilutions respectively (Table 5).

MIC determination by Microtitre plate assay

In order to determine the exact concentration of the extracts that inhibits the growth of test cultures a 96 well Microtitre plate assay was performed using serial two-fold dilutions of extracts. Upon incubation at 37°C overnight, colour change to pink was observed in wells where the Test cultures were growing and the rest of the wells showed no colour change indicating the inhibitory action of the antibacterial extracts. In case of B1, the MIC titre for *S.flexnerii MTCC 1457* is 64 units, B2 it is 16 units while *B.subtilis* inhibited the test at 64units concentration. *Staphylococcus aureus* was inhibited at 32 units for B1 and 64 units for B2 and *B.subtilis* (Fig. 2).

Calculation of Arbitrary Units of antibacterial extracts

By definition, 1 Arbitrary Unit per milliliter is the MIC of purified extract x 1000, divided by the volume of extract used for the assay. As compared to the activity of *B. subtilis,* B1 and B2 compounds demonstrated similar and at times even higher in-vitro inhibitory action. The above results thus indicate that B1 is more potent than B2 against Gram negative bacteria, while B2 has better activity than B1 against Gram positive bacteria.

Yield of antibacterial extracts after purification

From initial 100ml broth medium used for antibacterial extraction, about 1gm of

Table 5. Bioassay of purified antibacterial extracts

Dilutions→ *Bacillus* extracts	Und	1:2	1:4	1:8	1:16	1:32
	Staphylococcus aureus MTCC 1144					
B1	18	14	12	11	-	-
B2	15	13	12	-	-	-
B.subtilis	21	18	16	15	12	-
Streptomycin	24	22	20	17	16	-

Table 6. Arbitrary Units / ml determination

Bacillus extracts	Arbitrary Units/ ml against *S. flexneri* MTCC 1457	Arbitrary Units/ml against *S.aureus* MTCC 1144
B1	640	320
B2	160	640
B.subtilis	640	640

Table 7. Bioautography of antibacterial extracts

Bacillus Strains	Bands showing inhibition	Rf values at zone position	Corresponding Rf values at 580nm
B1	Band 1	0.28	*0.2*
	Band 4	0.44	*0.45*
B2	Band 5	0.37	*0.39*
	Band 6	0.49	*0.47*
B.subtilis	Band 2	0.43	*0.41*
	Band 3	0.52	0.53

precipitate of B1 and B2 strains was obtained as against 2.6gm precipitate of *B.subtilis*. The precipitates were dissolved in 2ml PBS (pH 7.4) for B1 and B2 cultures while 3ml for *B.subtilis.*

Separation of antibacterial components by HPTLC

At a wavelength of 254nm the chromatogram for B1, B2 and *B.subtilis* indicated presence of 3, 7 and 5 component bands respectively. At a wavelength of 580nm the chromatogram for B1, B2 and *B.subtilis* indicated presence of 4, 7 and 5 component bands respectively The Rf values of some bands were similar in all three extracts probably suggesting that these compounds belong to the same class of antimicrobial compounds.

TLC- Bioautography for detection of antimicrobial activity

When bioautography with test culture *S.aureus* MTCC1144 was performed, Band 1 and 4 of B1 showed a zone of inhibition. In case of B2 bands 5 and 6 showed zones whereas in *B.subtilis* bands 2 and 3 exhibited zones.

Characterization of separated compounds by LC-MS and GC-MS

For characterization of separated compounds GC-MS was done using a 0.03mm column. Several compound peaks were obtained indicating two possibilities:

1. The samples require further purification and concentration so as to get clear, sharp peks of individual compounds or
2. B1 and B2 produce a mixture of metabolites which may be responsible for the antibacterial activity.

A GCD Library search indicated the presence of 2-chloro-6-nitro-quinoline along with 1, 2-Benzene dicarboxylic acids in B1. Several other phenolic derivatives were identified as also compounds like Estrone, Octadecane, and Docosane etc.

B2 produces compounds like 4-Amino-5-imidazole carboxamide, Hexadecanoic acids and Thiazol ring derivatives. Jorgen *et al*., (2003) have demonstrated the possible role of phenolic compounds and hydroxy fatty acids produced by *Bacillus subtilis, B. amyloliquefaciens* and certain probiotic strains of *Bacillus* like *B. coagulans* against pathogenic bacteria like *S.aureus, Pseudomonas spp* etc

Molecular Mass determination by SDS-PAGE

According to SDS-PAGE analysis, strains B1 and B2 produced low molecular weight polypeptides from 5-25 kDa. In case of *B.subtilis* used as a standard reference strain, the compounds ranged from 8- 28 kDa (Fig. 3). AMS isolated from different *Bacillus* species differ in their relative molecular masses most being in the range

Table 8. GCD-Library search data of B1 antibacterial compounds

Peak No.	Real Time (RT)	Area %	Compounds identified	Quality (%) match with GCD library)
1	11.77	5.95	2-Naphthalenol, 8-amino-Nonanoic acid	*72*
4	13.14	512.30	9-Octadecenoic acid	*76*
10	16.66	25.41	1,2-Benzene-dicarboxylic acid	91

Table 9. Biochemical identification of selected *Bacillus* isolates

No	Sugar fermentation								*TSI*			
	Gluc	Malt	Starch	Ind	Citr	VP	Nitrate	Gelatin	Butt	Slant	H2S	*Gas*
B1	+	-	+	-	+	+	+	-	Acid	Alk	-	-
B2	+	-	+	-	-	+	+	-	Acid	Alk	-	-
B.s	+	-	+	-	+	+	+	+	*Acid*	*Acid*	-	-

of 26 kDa to 32 kDa as stated in literature. Thus the results obtained herein are fairly comparable with the standards indicating that these antimicrobial compounds broadly belong to low molecular weight Peptide antibiotic class.

However confirmation of inhibitory activity on gel is essential to detect which of those bands is the antimicrobial compound. Also a certain discrepancy was seen between the molecular masses as determined by SDS- PAGE and GC-MS analysis. It has been observed that highly hydrophobic proteins behave in an abnormal manner during SDS-PAGE (Kaufmann *et al.,* 1984). Moreover the AMS was transferred from aqueous phase to some of the organic solvents used, indicating a certain degree of hydrophobicity explaining the difference in sizes observed.

Identification of potential soil isolates

The *Bacillus* species isolated from soil samples by designated B1 and B2 were identified by Morphological, cultural and biochemical. The isolates were found to be Gram positive, Catalase positive Lecithinase negative, sporulating rods. On the basis of results of biochemical tests, B1 was identified as *B.subtilis* and B2 as *B. coagulans* (Koneman 1986).

In conclusion, the present study demonstrates the production of wide array of peptide antimicrobial compounds by *Bacillus* strains designated B1 and B2 with possible species identification as *B.subtilis* and *B. coagulans* respectively. B2 was found to have more potent antimicrobial activity against pathogenic cultures than B1. It was observed that Antimicrobial Substances from B2 inhibited potential food pathogens like *S. aureus, S. flexneri, and Listeria monocytogenes* amongst others. Indeed the above findings represent an important aspect of food safety and protection.

Two limiting factors in this study were the small amount of each of the compounds that was obtained and the lack of purity data. The small quantity obtained prevented the performance of an adequate number of repeat tests. It also prevented additional studies that would have allowed further purification and characterization of the compounds. Hence future studies on the *Bacillus* isolates involve optimization of antibiotic production by genetic manipulations and strain improvement techniques, purification by Column chromatography, elucidation of possible mechanism of action and structure analysis of AMS by FT-IR, NMR-MS methods.

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