Bacteria Exhibiting Antimicrobial Activities; Screening for Antibiotics and the Associated Genetic Studies

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Abstract: A total of 118 bacterial strains were isolated from six different soil samples from different parts of Pakistan. 50 out of these strains exhibited antagonistic activities against at least two or more strain from a panel of pathogenic and non-pathogenic microorganisms. Tested strains included three sets; environmental, laboratory and multidrug resistant clinical isolates. Twelve strains exhibited diverse spectrum of activity are focus of further work. The bacterial species identified include *Bacillus subtilis, B. amyloliquefaciens, B. cereus,* and *B. licheniformis.* Identified strains showed interesting biological activities e.g. inhibiting the growth of clinical isolates (*Klebsilla* species), strong antifungal and anti-algal activities and high toxicity against *Artemia* sp., while their TLC and HPLC profile showed an impressive chemical diversity. All the strains were able to produce number of peptides (surfactins, iturins, fengycins, subtilin and subtilosin) in different combinations. Presence of *sboX* gene was not correlated with subtilosin production, however, subtilosin and *sboX* were confirmed in *Bacillus amyloliquefaciens* for the first time.

Keywords: Bacillus, Antimicrobial activities, Antibiotics, *sboX* gene.

INTRODUCTION

Screening for new antibiotics from natural sources is becoming increasingly important for the pharmaceutical industry [1] as pathogenic bacteria are quickly becoming resistant to commonly used therapeutic agents [2]. Secondary metabolites from microorganisms have a diverse chemical structure and biological activities and are produced only by some species of a genus *Bacillus* [3]. Some examples of these antibiotics used in medical treatments are bacitracin, Gramycidin S, polymyxin, and tyrotricidin [4] produced by different *Bacillus spp*. The antibiotics produced by *Bacillus* are more effective against Gram-positive organisms; however, compounds such as polymyxin, colistin, and circulin exhibit activity against Gram negative organisms [5]. Lipopeptides produced by *Bacillus* also demonstrate anti-fungal [6], anti-viral [7], anti-ameobocytic [8] and anti-mycoplasma [9] activities. Lipopeptide antibiotics are members of a particular antibiotic class formed by surfactin [10], iturin [11], fengycin [12] and plipastatin [13] families. The chemical and physical diversity of peptide antibiotics makes them ideal candidates not only for therapeutic applications but also in other areas, especially the agri-food industry [14].

Subtilosin A is one of many antibiotics produced by *Bacillus* strains [15-17]. The spectrum of activity was investigated and proved antagonism against a wide range of bacteria including Gram-positive and Gram-negative bacteria and both aerobes and anaerobes [18]. The production of mature subtilosin is based on the expression of the *sbo-alb* gene cluster encompassing the subtilosin structural gene *sbo* and genes involved in posttranslational modification, processing of presubtilosin and immunity [17, 19].

This study attempts at investigating the antimicrobial activities of *Bacillus* species isolated from 6 different soil samples. The selected strains were identified, their activity spectra were determined against other strains. Further peptide secondary metabolites and presence of *sbo* gene in antimicrobial exhibiting *Bacillus* strains is being reported.

METHODS

Screening and Isolation

Six different soil samples were taken from different localities of Lahore in Pakistan. Each 1 g of the sample was suspended in 9 ml sterile distilled water and shaken vigorously for 2 min. The samples were heated at 80^{o}C for 40 min in a water bath. The soil suspension was serially diluted in sterile distilled water and the dilution from 10^{-6} and 10^{-7} were plated on overlaid Nutrient agar 0.8% [20] with seeded test organisms and incubated at 37°C for 12 to 24 hours, to screen for antagonistic bacteria. Colonies giving zone of inhibition were isolated and re-streaked over a fresh media plate. Eventually selected candidate were collected from the reservoir plate and rechecked for their activity.

Media and Culture Condition

Growth was on Nutrient broth, Nutrient agar and minimal salt medium [21]. In all cases, pre-inoculum was prepared by suspending in 10 ml deionized water colonies taken from minimal salt agar. The suspensions were adjusted to 5×10^{12}cells per ml. Growth temperature and pH values were maintained at 37°C and 7 ± 0.5, respectively. Fermentation was carried out for 24 or 48 hours in 250 ml conical flasks containing 50 ± 5 mL broth on an orbital shaker operating at 120 rev/min.

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Table 1. Samples Collected for the Isolation of Antimicrobial Exhibiting Bacteria (AEB) and Some Physico-Chemical and Microbiological Properties

Sample	Source and Characteristics	No. of Bacterial Isolates	No. of Antagonistic Isolates	Designation of selected strains
A	Punjab, Lahore, Canal bank, P.U. Campus area. 2003. Soil: light brown, loamy, partly humid with root remnants and alkaline (pH 7.8)	21	7	A15
B	Punjab, Lahore, Canal bank, P.U. Campus area. 2004. Soil: brown, loamy, humid with root & leave remnants and alkaline (pH 7.8)	34	15	B3, B4, B5, B7, B8, B29, B30, B34, B71
C	Punjab, Lahore, Botanical garden, P.U. Campus area. 2003. Soil: dark brown, loamy, humid with root & debris and alkaline (pH 7.8)	24	14	C6
D	Punjab, Lahore, Canal bank, P.U. Campus area. 2003. Soil: brown, sandy, dry with some rocks and alkaline (pH 7.9)	29	12	
E	Punjab, Lahore, Canal bank, P.U. Campus area. 2003. Soil: light brown, loamy, partly humid with root remnants and alkaline (pH 8.5)	28	13	
F	Punjab, Lahore, Canal bank, P.U. Campus area. 2004. Soil: black, clayey, highly humid, containing humus and alkaline (pH 8.2)	25	9	

Identification and 16S r DNA Analysis

In the identification of isolated bacteria species, standard taxonomic descriptions were used [22]. Genomic DNA was extracted from overnight incubated bacterial cultures in Luria Bertani LB-broth [20] at 37 ºC with 120 rpm. The extraction was carried out by using gene extraction kit (Biorad, UK). PCR amplification of 16S rDNA was performed successfully following the method described by Hasnain and Thomas [23]. To 0.5-0.1 ng of chromosomal template DNA, 0.25 µM each primer, 200 µM deoxynucleoside triphosphate and 1 unit of Taq polymerase were added. Solution was heated to 94°C for 5 min and passed through 29 cycles as follows: denaturation for 20 s at 94°C, primer annealation for 20 s at 50 °C and extension at 72 °C for 2 min. Final extension was at 72 °C for 5 min. The product was purified using Aqua pure extraction kit (Fermantas, UK) and sequenced using 27f and 1522r as forward and reverse primer respectively [24].

Spectrum of Activity

Spectra evaluation was performed by stabbing method [25] for all selected isolates starting from the indigenous strains found in the same sample (Cross sensitivity) and 13 different Laboratory collected strains (*Escherichia coli* C600, *Escherichia coli* DH5α1, *Bacillus subtilis* PY49, *Bacillus subtilis* 92, *Bacillus subtilis* 29, *Bacillus fusiformis* 5, *Bacillus subtilis* 1A309, *Bacillus subtilis* 5A29, *Bacillus subtilis* 3A22, *Bacillus subtilis* 1G1, *Bacillus subtilis* 22A1, *Bacillus subtilis* 1A321, *Bacillus subtilis* 1A106), 8 clinical isolates of *Klebsiella* spp. (collected from Children Hospital, Lahore, Pakistan), 22 isolates of *Staphylococcus aureus*, 18 isolates of *Streptococcus pyogenes*, and 85 unidentified clinical isolates (Social Security Hospital, Lahore, Pakistan).

Antimicrobial Substances (AMS) Assay

Culture supernatants containing the antibiotic were assayed for activity using an agar-well diffusion assay [26]. 50 µL antibiotic sample was transferred into the well. Sample was allowed to diffuse into the agar, the plate was then inverted and incubated at 37 °C until a lawn of the indicator bacteria appeared on the plate (approximately 8-10 hrs).

Chemical Screening

Chemical screening was performed in parallel to biological screening over crude extracts of antimicrobial exhibiting bacteria (AEB). Preparation of crude extract: broths of grown bacteria with optimum conditions and broth medium were freeze dried and extracted with ethyl acetate three times. Filtered extract was concentrated under vacuum and dried under steam of continuous air.

Thin Layer Chromatography

Crude extracts were run on TLC 30 (F 250) and substances were visualized by UV absorption at 250 and 235 µm, chromogenic reaction with spray reagents e.g. anisaldehyde and Ehrlich. Active bands were visualized using bioautography method in which suspensions of indicator organisms in agar or broth are overlaid on chromatograms to detect bioactive spots/ areas.

HPLC-MS

The application of hyphenated techniques like high-performance liquid chromatography (HPLC) coupled to electrospray ionization tandem mass spectrometry (ESI-MS) has influenced much our approach to explore natural products. HPLC-MS is a sensitive and powerful tool for natural product chemistry permitting a fast screening of the metabolic profiles with a minimum amount of material. The crude extract was dissolved in a HPLC grade methanol injected to a HPLC/MS set up. HPLC was run on a Eurospher C-18 reversed phase column. Every chromatogram HPLC peaks were analyzed directly through ESI-MS to provide the corresponding molecular weights. These results were analyzed with AntiBase [27] and recorded as hits indicating identical or a near match in molecular weights with other known compounds.

Detection and Sequencing of *sbo* Genes

sbo and its flanking region were detected from the environmental *Bacillus* strain.Genomic DNA was extracted from overnight inoculated bacterial culture in L-broth at 37°C with 120 rpm. PCR amplification of ~1375-bp consisted of *sbo* and flanking region was performed successfully with TS13C (GAATTGACACTATCTAG AGAAATGC CG) and TS14 (ATCCGGTGGTGCGGAATT CGATGA) [16]. The optimum PCR conditions were determined as heating 94°C for 5min through 30 cycles as follows: denaturation for 30s at 94 °C primer annealation for 30s at 59°C and extension at 72°C for 1.2 min. Final extension was at 72°C for 3 min. PCR products were analyzed by 1.5% agarose gel electrophoresis. The purified PCR products were sequenced by using an ABI Prism dye terminator cycle sequencing ready reaction kit and an ABI PRISM 377 DNA sequencer (Applied Biosystems, USA). Analyses of DNA sequences were performed by using Prochromas version software (Oxford Molecular, UK).

MALDI-TOF-Mass Spectrometry

Fractions correlated with surfactin from TLC and Reverse phase HPLC were analyzed using MALDI-TOF-MS. 2 µL of samples were mixed on the target plate with 2 µL of matrix solution (2 mg of alpha-hydroxycinnaminic acid per ml in acetonitrile-methanol-water, 1:1:1). MALDI-TOF-MS spectra were recorded by using a 337-nm nitrogen laser for desorption and ionization. The mass spectrometer was operated in the refraction mode at an accelerating voltage of 18 kV with an ion flight path that of 0.7 m. The delay time was 375 ns. Matrix-suppression was also used, and the mass spectra were averaged over 50 to 100 individual laser shots. The laser intensity was set just above the threshold for ion production. External calibration was performed by using the [M+H]+ signals of renin, adenocorticotropic hormone, insulin oxidized B, and bovine insulin (Sigma-Aldrich Co.). Surfactin isomers were anticipated to have an m/z range of 500–1500. The variance of the *m/z* of ± 0.8 Da was considered acceptable.

RESULTS AND DISCUSSION

Isolation and Identification

From six different soil samples a total of 118 morphologically different bacterial strains were isolated among them fifty strains were found to have antimicrobial activities against at least two tested organisms (Table **1**). 42.34% of the strains recovered from all samples could exhibit antimicrobial activities. Selected strains were identified on the basis of 16S rDNA sequence homology compared to their nearest sequence matches. As a result of identification, 12 *Bacillus* spp. strains were identified as *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* (Table **2**).

Evaluation of Spectrum of Activity

The spectra for activity of isolated strains were evaluated against indigenous strains; laboratory collected strains, as well as clinical isolates Fig. (**1**). The lowest activity was exhibited against clinical isolates (19.5%). 36% of the strains were active against laboratory collected strains and 67% were active against environmental isolates. Strains that showed large and clear inhibitory zones against Gram positive or Gram negative test bacterial strains were considered as potential producers of antimicrobial substances (AMS). Twelve out of "118" (10.17%) bacterial strains were observed to be most active and were selected for further study (Table **2**). These strains exhibited a wide spectrum of activity against Gram positive, Gram negative and clinical isolates.

Biological Activity

The inhibitory activity of each of these 76 strains from 3 soil samples (A, B, C as majority of antimicrobial exhibiting isolates pertain to these samples) was detected against all others strains of same sample in cross sensitivity tests and also against at least 14 indicator strains (Table **3**) using stabbing method. Inhibitory activity against endogenous and important pathogenic microbes was observed and marked using symbols; +++ clear and large inhibitory zone >4mm,

Table 2. Bacterial Strains and Assigned Homologous Counterparts Based on 16S rDNA Sequence Analysis

Designation	Highest homology counterpart	Accession No.
Strain A15	*Bacillus licheniformis* strain CICC10093	AY842874
Strain B 7	*Bacillus subtilis* strain 3A25	DQ400916
Strain B 30	*Bacillus subtilis* strain B-FS01	DQ520955
Strain B29	*Bacillus subtilis* strain CGMCC1869	EF159949
Strain B 34	*Bacillus amyloliquefaciens*	AY620954
Strain C6	*Bacillus subtilis* strain BS-S3	AY583216
Strain B 71	*Bacillus* sp. G1DM-10	EU586791
Strain B 3	*Bacillus* sp. cp-h61	EU586784
Strain B 4	*Bacillus* sp.	EU586785
Strain B 5	*Bacillus* sp. cp-h23	EU586783
Strain B 8	*Bacillus* sp.	DQ327713
Strain B 9	*Bacillus* sp.	EU686585

++ clear zone up to 4mm, + presence of zone, ± incomplete zone, - no zone. A total of thirty six out of 76 (47.368%) bacterial isolates showed inhibitory zones on Lauria Bertani agar plates against indicator strains.

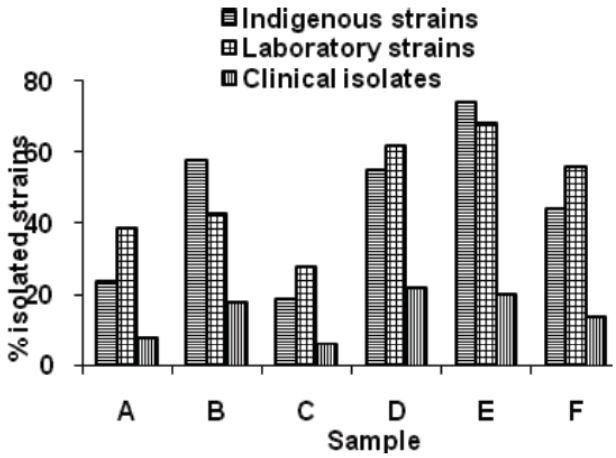

Fig. (1). Antimicrobial activity of bacterial strains isolated from 6 different soil samples against (**a**) indigenous strains, (**b**) laboratory collected strains, and (**c**) clinical isolates.

Each strain that showed large and clear inhibitory zones against gram positive as well as gram negative test bacterial strains was considered as potential producers of antimicrobial substances (AMS). Thirty six out of "76" bacterial strains were observed to be most active and been selected for further study. Twelve out of thirty six strains were observed to be gram positive having potential antimicrobial activity (Table **4**). Three strains were showing large +++ inhibitory zones against *Bacillus subtilis* 5A29. Bacterial strains A15, B3, C6, were showing inhibitory zones ++ against *Escherichia coli* DH5α1. Thirteen out of anti-bacterial isolates were observed to be having +++ activity against B8 endogenous bacterial strains. Final selected strains are A 15, B 7, B 30, B 29, B 34, C 6, B 71, B 3, B 4, B 5, B 8, B 9.

Influence of Temperature, pH and Aeration on the Production of Antimicrobial Substances (AMS)

The influence of temperature, pH and aeration was observed on the production of antimicrobial substances (AMS) in LB medium Cell free supernatants showing inhibitory activity were obtained when the strains were grown in a range of pH 4 - 10, temperature 25 - 45°C and in still or shaking cultures. However, the maximum antimicrobial activity was observed when strains were cultivated as shaking cultures, pH-7.0 and temperature 30°C.

Isolation and Purification of Active Substances

Three different isolation methods were employed for six strains. For strains B 8 and B 5 liquid-liquid extraction in combination with TLC was used. For strains B 9 and B 7 ammonium sulfate precipitation followed by gel filtration

Table 3. Selected Antibiotic Producing Strains and their Spectrum of Activity, (++) Clear Zone >10mm, (+) Clear Zone up to 5mm

Strain	Activity against indicator strains		Activity against indigenous strains		Activity against clinical isolates	
	++	+	++	+	++	+
A15	DH5α1, C600	3A22, 1A321, 29, 92, 5	A7, A18, A19,	A4, A8, A6, A20, A11	*Klebsiella* spp. 3	*Staph. aureus* 12
B7	5A29, 1A309	3A22, DH5α1, 1G1	A10, A14	A3, A16, A15, A21	-	*Staph. aureus* 18
B30	5A29	1A309, 1G1, 22A1, DH5α1	B2, B10	B3, B7, B4, B14, B8	*Staph. aureus* 3	*Strep. pyogenes* 2
B29	1G1	DH5α1, 1A106, 1A309, 1A321	B4, B8, B13	B3, B14, B2, B10	*Staph. aureus* 12	*Staph. aureus* 4
B34	92, 5	1A309, 1A321, 5829, 1G1, 29	C2, C6,	C3, C7, C10, C12	-	*Strep. pyogenes* 5
C6	C600	1A321, 3A22, DH5α1, 29, 92	D2, D8	D3, D5, D6, D10, D13, D15	*Klebsiella* spp. 6	*Klebsiella* spp. 8
B71	1A106, 29, 1A321	3A22, 29, 92, PY79	D10, D15	D2, D3, D7, D16	*Strep. pyogenes* 2	-
B3	1A106, 29	1A309, 1A321, DH5α1, 22A1	E8, E10, E15	E12, E13, E7, E16, E22	*Staph. aureus* 18	*Klebsiella* spp. 3
B4	1A106, 29, 92	1A309, 1A321, 22A1	E10, E15	E11, E28, E9, E6, E23	*Strep. pyogenes* 1	*Strep. pyogenes* 7
B5	1A106, 29	1A309, 1A321, C600, 92, 22A1	E4, E8	E26, E15, E14, E17	*Staph. aureus* 22	*Staph. aureus* 8
B8	1A106, 29, 92 1A309	1A321, PY79, C600, PY79	F10, F7, F14	F4, F24, F21, F16	-	*Strep. pyogenes* 7
B9	1A106, 29, 92 1A309	1A321, PY79, C600, PY79	F5, F7	F4, F9, F12, F15	-	*Staph. aureus* 8

Table 4. Factors Affecting Antimicrobial Production

Strain No.	pH			Temperature			Aeration	
	4	7	10	25°C	37°C	45°C	Still	Shaking
	Zone of Inhibition ZOI (mm)							
A 15	17	37	10	35	35	20	40	35
B 7	10	33	0	37	32	21	34	32
B 30	15.5	18.5	14	28	30	16	25.5	30
B 29	0	18.5	12.5	15	20.5	13	15	20.5
B 34	12.5	34	14	36	36	20.5	36	36
C 6	0	33	36	32	32	21	35	32
B 71	13.5	34	11	34	36	12.5	38.5	36
B 3	14.5	16.5	13	13.5	15	14.5	23.5	15
B 4	12	18	0	27.5	27.5	20.5	32	27.5
B 5	15.5	18.5	14.5	24	16.5	12	24	16.5
B 8	14.5	17	15	18	16	13	25.5	16
B 9	12	15.5	16.5	17	16.5	12.5	25.5	16.5

was employed. For strains B 4 and C 6 acid precipitation followed by solid phase extraction (SPE) was used. Resulting active fractions from all of these methods were further purified using RP-HPLC. The purified product from HPLC was analyzed through MALDI-TOF-MS for molecular mass determination.

Thin Layer Chromatography Analysis

For strain B 8, eight prominent spots were visible under UV having R_f values of 0.08, 0.12, 0.21, 0.37, 0.49, 0.53, and 0.57. However, only one active fraction was observed upon bioassay with R_f value of 0.49. The spot was ninhydrin negative indicating the absence of free amino groups and presence of peptide bonds in the compound. A white spot formed with same R_f value when the plate was sprayed with water indicating that the compound is lipophilic. In addition, spots with R_f values of 0.08 and 0.53 could not be stained with ninhydrin while all other spots were ninhydrin positive. For strain B 5, six prominent spots were visible under UV having R_f values of 0.1, 0.15, 0.26, 0.37, 0.51 and 0.57 with active fraction having R_f value of 0.37. The spot was ninhydrin negative and formed a white spot with same R_f value when the plate was sprayed with water indicating that the compound is lipophilic.

Solid Phase Extraction

Seven fractions were collected by decreasing the polarity with acetonitrile until 100% and tested for activity. For strain B 4, fractions eluted with 5, 15, 25, and 35% acetonitrile did not show any activity However, three active fractions were eluted with 50, 75, and 100% acetonitrile with peak activity at 75% showing maximum product is eluted when the polarity is decreased until 3/4 to that of start. For strain C 6, maximum activity was observed in the fraction when polarity was decreased to 50%. Both of these fractions were further purified by using RP-HPLC.

Gel Filtration Chromatography

Gel filtration chromatography was performed by using EconoPack DG 10 column that was equilibrated with 0.01 M Sodium Phosphate buffer, pH 7.0 for B7 and B9. After the sample was applied, 13 fractions were collected each of 1ml by using same buffer and tested for activity against test strain. The result showed that for both strains sample eluted from the column at two distinct peaks. Initially high molecular weight components were eluted which were above 6 kDa and not of our interest. For strain B7 and B9 maximum activity was found in fraction 9 and 10 respectively which correspond to the molecular weight of about 1.5 kDa. These fractions were selected for purification through RP-HPLC. As initial fractions also showed activity, these may represent the aggregated form of antimicrobial compound present in the sample.

Reverse-Phase HPLC

Fig. (**2**) show the chromatogram of HPLC purification of samples obtained from TLC. Maximum activity was observed in fraction corresponding to retention time 10 min. for both strains B 8 and B 5 with 15% recovery and an increase of specific activity to about 8 folds over that of culture supernatant. There was a fair amount of contamination that eluted early and active peaks were observed at retention time 14 and 16 minutes for SPE and gel filtration chromatography respectively. The results revealed that HPLC provided purified samples for molecular mass determination by mass spectrometry.

Molecular Mass Determination

MALDI-TOF mass spectrometry was used for the accurate determination of molecular masses of purified products from RP-HPLC. The technique has been used as a novel, efficient method for identification and structural

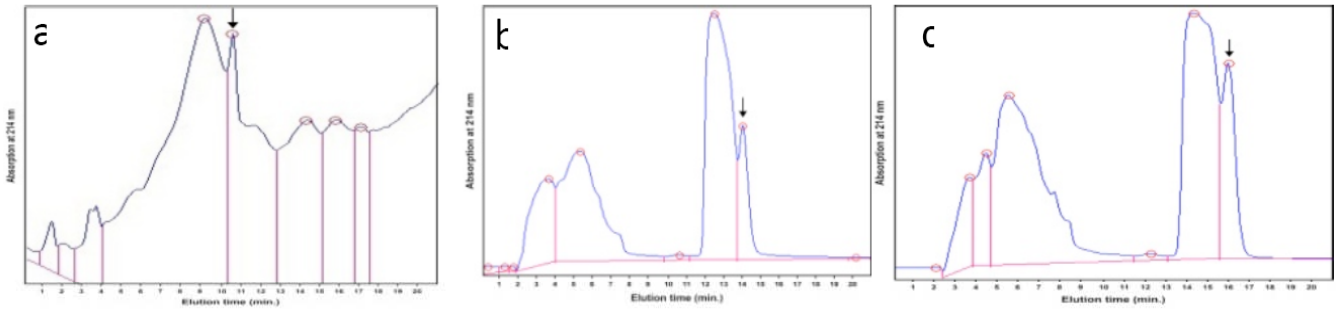

Fig. (2). Reverse phase HPLC chromatogram of antimicrobial substances separated by (**a**) TLC on a Thermo Hypersil-Keystone ODS column, (**b**) SPE on a Thermo Hypersil-Keystone ODS column, (**c**) gel filtration chromatography.

characterization of peptides and proteins with molecular masses ranging from 0.5 to 30 kDa. The results obtained for different strains are presented below:

In strain B 8 a cluster of peaks with mass/charge (m/z) ratios between 1072 and 1098 was observed in linear mode which could be attributed to protonated iturin isoforms and their alkali metal adducts, Fig. (**3a**). The peak at m/z 1084 was the most intense and corresponds to the mass of $[M+H]^+$ ion of iturin with a fatty acid chain length of 17 carbon atoms. It showed that C_{17} iturin like compound is produced by strain B 8.

In strain C 6 a peak cluster was observed in linear mode with m/z ratios between 1012 and 1056 which could be ascribed to protonated surfactin isoforms and their alkali derivatives, Fig. (**3b**). An intense peak at m/z 1022 matches to the mass of $[M+H]^+$ ion of surfactin with 14 carbon atoms fatty acid chain indicating that C_{14} surfactin like compound is produced by strain C 6.

In strain B 5 the linear mode, we observed two clusters of peaks with different mass/charge (m/z) ratios for purified product. One cluster was observed with m/z ratios between 976 and 998 which may be regarded as surfactin isoforms. The peak with a m/z ratio 994 was the most intense and corresponds to the mass of $[M+H]^+$ ion of surfactin with a fatty acid chain length of 13 carbon atoms. Other cluster was detected with m/z ratios between 1088 and 1096, which could be attributed to protonated iturin isoforms. Mass spectrometric analysis revealed that both surfactin and iturin like compounds are produced by strain B 5, Fig. (**3c**).

In strain B 9 a cluster of peaks with m/z ratios between 1022 and 1059 was observed in linear mode which could be attributed to protonated surfactin isoforms and their alkali metal adducts, Fig. (**3d**). The peak at m/z 1036 was the most intense and corresponds to the mass of $[M+H]^+$ ion of surfactin with a fatty acid chain length of 15 carbon atoms.

In strain B 4 in the linear mode, a cluster of peaks with m/z ratios between 1044 and 1112 was observed which could be credited to protonated iturin isoforms and their alkali metal derivatives, Fig. (**3e**). In the peak cluster the peak with m/z of 1070 was most intense and matches to the mass of $[M+H]^+$ ion of iturin with a fatty acid chain length of 16 carbon atoms. This indicated that C_{16} iturin like compound is produced by strain B 4.

In strain B 29 in the linear mode, we observed a cluster of peaks with m/z ratios between 1036 and 1064 which could be ascribed to protonated surfactin isoforms and their alkali metal adducts, Fig. (**3f**). In peak cluster an intense peak at m/z 1036. This peak matches to the mass of $[M+H]^+$ ion of surfactin with 15 carbon atoms fatty acid chain indicating that C_{15} surfactin like compound is produced by strain B 29.

Subtilosin Gene

sbo and its flanking region were detected from the environmental *Bacillus* strain. Results are shown in Fig. (**4**) and summarized in Table **5** in which all of strains screened were secured *sbo* class I. Nucleotide sequences were edited manually using Chromas software (http://www.technelsysiu.com.au/chromas.html). PCR sequences were identified using the basic local alignment search tool and GenBank nucleotide data bank from the National Center for Biotechnology Information, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov). s*boX*, encoded a bacteriocin-like product, a new gene with an unknown function, in strain 168 [19], which resides in an open reading frame overlapping the coding region of *sbo*. While the expression of *sboX* would result in a 22-amino-acid curtailed peptide in W23- like strains compared to the peptide produced by 168-like strains, which makes it unlikely that SboX is produced by W23-like strains. However, a correlation between *sbo* gene and subtilosin production was not established probably due to influence of *sboX* 186 variation. Hence, this study provided additional strains to support the idea of subtilosin gene predominance amongst *Bacillus* strains isolated from environment and to report different species containing homologous genes. This is the first report of the detection of subtilosin production by *B. amyloliquefaciens* based on PCR screening. Further, research must be direct to utilize the *sbo* genes as a means of identifying different *Bacillus* species that produce subtilosin.

Phylogenetic Analysis

Amplified PCR products were sequenced using an ABI Prism dye terminator cycle sequencing ready reaction kit and an ABI PRISM 377 DNA sequencer (Applied Biosystems). Analyses of DNA sequences were performed by using Prochromas version software (Oxford Molecular, UK). The sequences obtained were submitted to GenBank at National Centre of Biotechnology and Information to obtain accession numbers (Table **5**). Sequences of *sbo* for both *Bacillus subtilis* 168 and *Bacillus subtilis* ATCC 6633 were used as positive control representing the two classes/ subsp. *subtilis* (class I) and spizizenii (class II) respectively. These

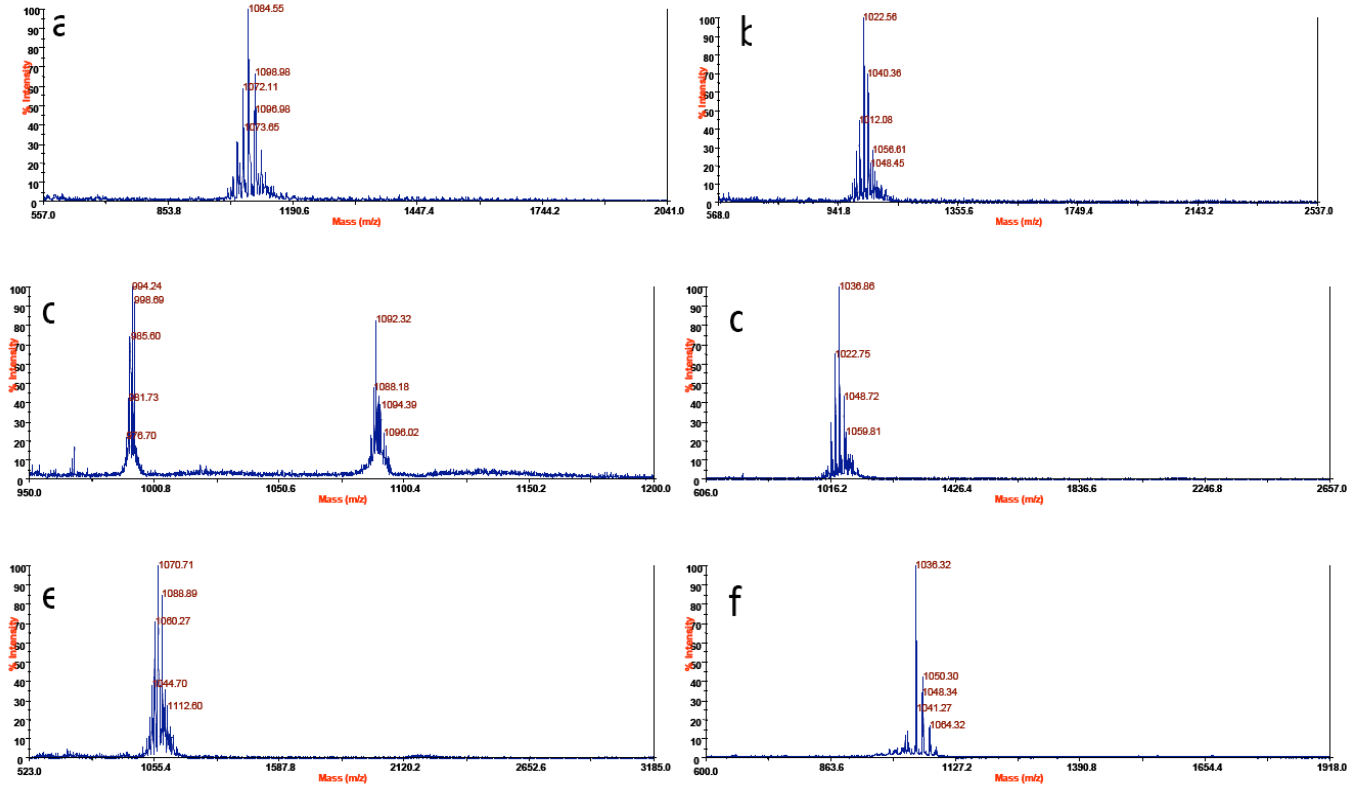

Fig. (3). MALDI-TOF mass spectrometric analysis of (**a**) iturin like compounds produced by strain B 8 detected in the range from m/z 1072 and 1098, (**b**) surfactin like compounds produced by strain C 6 detected in the range from m/z 1012 and 1056, (**c**) surfactin and iturin like compounds produced by strain B 5 detected in the range from m/z 976-998 and 1088-1096 respectively, (**d**) surfactin like compounds produced by strain B 9 detected in the range from m/z 1022 and 1059, (**e**) iturin like compounds produced by strain B4 detected in the range from m/z 1044 and 1112, (**f**) surfactin like compounds produced by strain B 29 detected in the range from m/z 1036 and 1064.

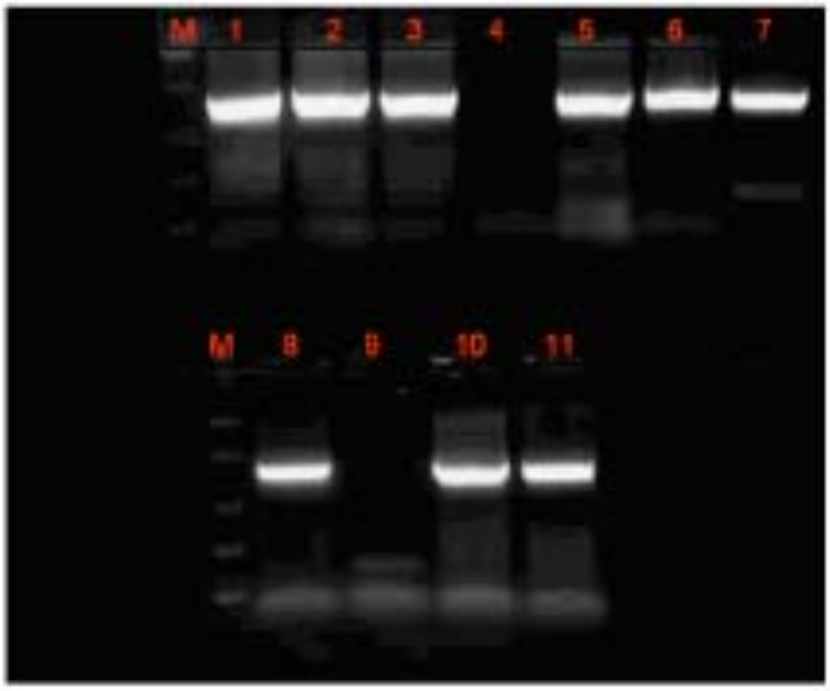

Fig. (4). Positive and negative amplification of *sbo-alb* region. M: Marker, 1: B4 2: A15, 3: B8, 4: B30, 5: C6, 6: B7, 7: B29, M: Marker, 8: B71, 9: B34, 10: B9.

Table 5. Summary of the *sbo* Screened Strains for and Type of Class

S.No.	PCR	Sbo X class (I/II)	Subtilosin production	Strain	GenBank Accession No. of *sbo*
1	+	I	+	*Bacillus subtilis* B4	FJ1 51503
2	+	I	+	*Bacillus subtilis* B7	FJ1 51504
3	+	I	+	*Bacillus* B9	FJ15 1505
4	-	I	-	*Bacillus licheniformis* A15	-
5	+	I	+	*Bacillus subtilis* B30	-
6	+	I	+	*Bacillus subtilis* C6	FJ15 1506
7	+	I	+	*Bacillus subtilis* B29	-
8	+	I	+	*Bacillus subtilis* B71	-
9	-	I	-	*Bacillus* sp. B5	-
10	+	I	+	*Bacillus amyloliquefaciens* B34	FJ1 51507
11	+	I	+	*Bacillus* sp. B9	-

sequences are available from NCBI web site and can be retrieve using search tools or accession numbers. Sequences were analyzed and phylogenetic analyses revealed both classes. All the environmental isolates that produced subtilosin belong to class I, Fig. (**5**). Multiple sequence alignment of *sbo* and its flanking region for the strains is given at this thesis in supplemental material.

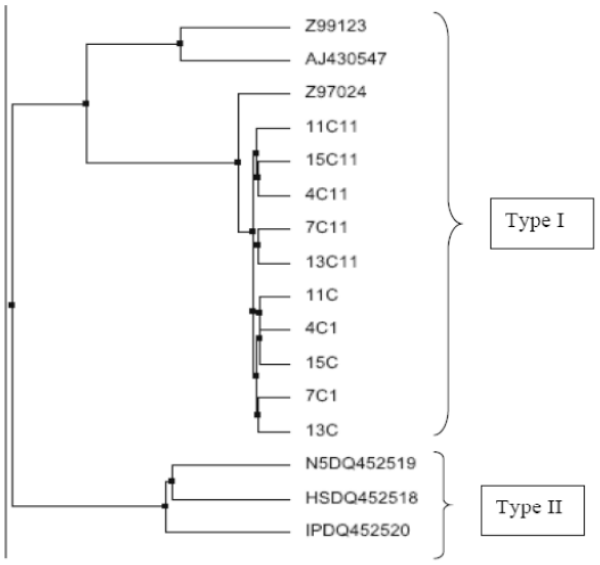

Fig. (5). Phylogenetic diversity of isolated strains using multiple sequence alignments of sequences *sbo* and flanking region. GenBank accession no. Z99123, AJ430547, Z96024, DQ452519, DQ452518 and DQ452520 belong to strains of corresponding class. 11C11:(B9), 15C11:(B34), 4C11:(B71), 7C11:(B29), 13C11:(B9), 11C:(B5), 4C1:(B30), 15C1:(A15), 7C1:(B7), 13C:(B4).

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ABBREVIATIONS

AEB = Antimicrobial Exhibiting Bacteria

BHI = Brain-Heart Infusion

HPLC = High Performance Liquid Chromatogrophy

RP-HPLC = Reverse Phase-High Performance Liquid Chromatogrophy

SPE = Solid Phase Extraction

TLC = Thin Layer Chromatogrophy

ZOI = Zone Of Inhibition

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