Identification and growth optimization of a Marine *Bacillus* DK1-SA11 having potential of producing broad spectrum antimicrobial compounds

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Abstract: Control of harmful bacteria in food, aquaculture, pharmaceuticals, agriculture, hospitals and recreation water pools are of great global concern. Marine bacteria are an enormous source of bio-controlling agents. The aim of this study was to identify and optimize the growth conditions including effect of different biotic and abiotic factors on antimicrobial activity of strain DK1-SA11 isolated from Qingdao Bay of China Yellow Sea. Microscopic characterization, API® 20E and 50 CHB kit base carbohydrates utilization, 16S rDNA and DNA *gyrB* gene sequencing studies identified the bacterium as *Bacillus subtilis* subsp. *spizizenii* DK1-SA11. Antimicrobial spectrum of cell free supernatant (CFS) has shown antimicrobial activities against all test strains including methicillin-resistant *Staphylococcus aureus*, *E. coli* O157:H7, *Candida albicans*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *E. coli*, *Pseudomonas fluorescens*, *Salmonella typhimurium* and *Vibrio cholerae*. Among all the media tested, Marine Broth 2216 was found to be the best medium for bacterial growth and production of antibacterial compounds. The other optimum conditions for growth were pH:7 and incubation temperature: 25°C with ≥ 180 rpm for 60-72 h. Out of 49 different carbohydrates tested, D-mannose increases the antibacterial activity by 33.3% while D-arabitol decreases it by 44.4%. Crude CFS showed activity even after three months of storage below -20°C and boiling for 10 min, whereas it loses 100% of its antimicrobial activity after enzymatic treatments of lipase, trypsin and papain. The production of antimicrobial compounds and broad spectrum of antimicrobial activity against all tested pathogens suggested that the strain DK1-SA11 can be used as a source for probiotics, synbiotics and antibiotics.

Keywords: Marine bacteria; *Bacillus subtilis* subsp. *Spizizenni*, antibacterial agent, broad spectrum, bio-autography.

INTRODUCTION

Control of harmful bacteria in food, aquaculture, pharmaceuticals, agriculture, hospitals and recreation water pools are of great global concerned. Recently, outbreaks and resistance to available treatment options, stimulated researchers to discover new solutions. Hazards associated with synthetic antimicrobial chemicals also demand for natural antimicrobial products. Many natural compounds have been obtained from various microbial sources, and are being successfully used in different fields. To overcome the constantly increasing numbers of multiple drug resistant (MDR) pathogens, discovery of new natural antimicrobial compounds is imperative (Fickers, 2012).

Marine environment is an enormous source of natural compounds, though our knowledge is still very limited. The diversity of aquatic environment enforces microorganisms to adopt unique physiological and structural characteristics and to produce novel compounds (Radajewski *et al.*, 2002). During the past fifty years, more than 10,000 marine metabolites were sieved, searched, separated and studied by researchers. Among these, 18% are from bacterial origin (Bhatnagar and Kim, 2010). Up till now, statistics show that only a little proportion of antimicrobial compounds produced by Gram positive bacteria has been studied (Fickers, 2012).

Genus *Bacillus* is an outstanding source of antagonistically active natural compounds (Stein, 2005, Westers *et al.*, 2004). Production of antibiotic with ecological stress resistant endospores enhances their survival in natural competitive conditions. Gene sequencing has revealed that in *B. subitilis*, about > 4% of total genome has potential genes for production of bacteriocins, BLIS (bacteriocin like inhibitory substance), polyketides, nonribosomal peptide, as well as other unusual antibacterial compounds (Abriouel *et al.*, 2011, Arguelles-Arias *et al.*, 2009, Chen *et al.*, 2009, Deng *et al.*, 2011, Kunst *et al.*, 1997).

Current study reports the identification and cultural conditions for growth and antimicrobial compounds production by the marine isolate DK1-SA11 a bacterium isolated from Qingdao Bay of China Yellow Sea (Khan *et al.*, 2015).

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MATERIALS AND METHODS

Sampling and isolation of bacteria

Antibacterial compound producing bacteria were isolated from surface attached marine invertebrate samples obtained from coast of Qingdao Bay in China (N 36° 03' 35.5", E 120° 18' 34.4"). Broad spectrum antibacterial activities of isolates were screened by modified double agar layer method against four pathogenic indicator strains including *S. aureus*, *Escherichia coli*, *L. monocytogenes* and *V. cholerae* (Dopazo *et al.*, 1988). The isolate DK1-SA11 was selected for further studies due to its antagonistic activity against all four strains. The purified isolate was preserved in BHI broth and Marine broth 2216 with 30% glycerol at -80°C (Khan *et al.*, 2015).

Identification of the isolate

Morphology of bacterium DK1-SA11 was studied by colonial characteristics on growth media and Gram staining was observed under compound microscope (Olympus). Biochemical characteristics were studied by API® 20E and 50 CHB kit (bioMerieux, France) followed by database analysis on api*web*™ identification software (database V 4.0). Genotypic characteristics were studied by 16S rDNA (Acinas *et al.*, 1999) and *gyrB* gene of subunit B protein of DNA gyrase as described previously (Yamamoto and Harayama, 1995). BLAST analysis and phylogeny trees were constructed via Mega 5.1 software by maximum likely hood method and tree topology were reproduced by bootstrap analysis of 1000 replicates (Hall, 2013). Genetic sequences were submitted to NCBI databank [GenBank:JX391980, GenBank:KF767445].

Growth and production of CFS

Single colony of DK1-SA11 was inoculated in 3 ml Marine Broth 2216, incubated at 28°C in shaker incubator (180 rpm) for 24 h and 1 ml of this culture was transferred into 100 ml Marine Broth and incubated for 72h. Cell free supernatant (CFS) was obtained by removing cells by centrifugation (9000 g for 15 min at 4°C) followed by 0.22 µm filtration, then stored at -20°C .

Antimicrobial activity of CFS

Antimicrobial activity against test organisms by CFS were analyzed by using Oxford Cup (8-mm outer diameter stainless steel cylinder) method (Zhou *et al.*, 2006). Overnight cultures of test organisms were adjusted to 10^8 cfu/ml (0.2 optical density @ 600 nm) in Brain Heart Infusion (BHI) broth for bacteria and Yeast extract Peptone Dextrose (YPD) broth for yeast strain, and were spread on pre-poured plates of 0.7% BHI agar and 0.7% YPD agar from their respective broths. After 30 min of absorption at room temperature, sterile Oxford cups were placed on agar surface, then 100 µL CFS and 100 µL of un-inoculated broth (negative control) were poured into the cups and placed at 4°C for 2 h for diffusion of antibacterial compounds in agar. Plates were then incubated at the respective optimum temperature of test organisms. After 24 h, activity of CFS was evaluated by measuring zone of inhibition around the cups.

Selection of growth media

The growth and antibacterial compound production in different commercially available microbial growth media (Marine broth 2216, Alkaline peptone water, Brain heart infusion broth, Luria Bertani broth, Mueller Hinton broth, Nutrient broth, Tryptic soya broth, Tryptic soya yeast extract broth and Yeast Extract peptone dextrose broth) were tested up to 160 h at 28°C with 180 rpm rotation. Optical densities at 600 nm (OD_{600}) were measured by Spectrophotometer (Shimadzu) and pH of media were observed by pH meter (Jenway) after every 12 h. Antimicrobial activities were tested against *S. aureus* by Oxford cup agar diffusion method from CFS of growth media. The best bacteriological media was selected on the basis of maximum antibacterial activity, shorter time of production, and consistency of growth with antimicrobial activity.

Optimization of growth conditions

Effect of incubation temperatures

Incubation temperature for growth and antimicrobial compounds production were tested to find out the optimum growth temperature. Marine broth 2216 were inoculated by 1% (v/v) of 24 h old culture of strain DK1-SA11 bacterial suspension in 100 ml medium, followed by incubation at 15, 20, 25, 30, 35, 40, 45 and 50°C. OD_{600}, pH and antibacterial activity in terms of zone of inhibitions were measured after 12 h interval, as described before, up to 96 h of incubation with rotation speed of 180 rpm.

Effect of rotation speed

Effect of agitation on growth and antimicrobial activity was tested at optimized temperature of 25°C without rotation and with rotation speed of 60, 120, 140, 160, 180, 200 and 220 rpm. Growth of bacteria and antibacterial activities were recorded as described above.

Effect of NaCl

Marine broth 2216 with additional 0.5%, 1%, 2%, 4%, 6% and 8% salt were used to check the effect of NaCl on growth and antibacterial activity of DK1-SA11. Bacterial growth OD_{600}, pH of medium and zone of inhibition were measured after 12 h interval up to 96 h with incubation temperature of 25°C and 180 rpm. Salt containing media without bacteria were used as negative control.

Effect of pH

Effect of pH for bacterial growth and antimicrobial activity were tested by adjusting pH of medium with 1M HCl and 1M NaOH from pH 1-13. OD_{600} and antibacterial activity were measured after 72 h of incubation at 25°C with 180 rpm.

Table 1: Differential characteristics of strain DK1-SA11 and closely related species of the Bacillus Taxa: 1, Marine isolate DK1-SA11; 2, *B. axarquiensis* LMG 22476^T; 3, *Brevibacterium halotolerans* DSM 8802^T; 4, *B. mojavensis* BCRC 17124^T; 5, *B. mojavensis* BCRC 17531; 6, *B. subtilis* subsp. *subtilis* BCRC 10255^T; 7, *B. amyloliquefaciens* BCRC 11601^T; 8, *B. atrophaeus* BCRC 17123^T; 9, *B. vallismortis* BCRC 17183^T; 10, *B. licheniformis* BCRC 11702^T; 11. *Bacillus tequilensis* strain 10b^T. Data are from this study and Wang *et al.* (2007b), Delaporte and Sasson (1967), Wink (2009), and Gatson *et al.* (2006).

Characteristics	1	2	3	4	5	6	7	8	9	10	11
Colony pigmentation	Opaque	White	Ivory	White	White	White	White	Brown	White	White	Yellow
Oxidase	+	–	ND	+	+	+	+	–	+	+	+
Urea hydrolysis	–	–	+	–	–	–	–	–	–	–	–
Citrate utilization	–	+	–	+	+	+	+	+	+	+	+
liquefaction	+	+	+	+	+	+	+	+	+	–	+
β-Galactosidase	+	+	+	+	+	+	–	+	+	+	+
Arginine dihydrolase	–	–	–	–	–	+	–	–	–	+	+
Acid production from:											
L-Arabinose	+	+	–	+	+	+	+	–	+	+	+
Ribose	+	+	–	+	+	+	+	–	+	+	+
D-Xylose	+	+	–	+	–	–	–	–	–	–	+
Inositol	+	+	–	+	+	+	–	+	–	W	+
D-Sorbitol	+	+	–	+	+	+	+	+	–	+	+
Methyl α-D-glucoside	+	+	–	+	+	+	+	–	+	+	+
Amygdalin	–	+	–	+	+	+	+	+	+	+	+
Salicin	+	+	ND	+	+	+	–	–	+	+	+
D-Cellobiose	+	+	–	+	+	+	+	+	+	–	+
D-Lactose	–	–	–	–	–	–	+	–	–	–	+
D-Melibiose	–	–	–	–	–	+	–	–	–	–	+
Inulin	+	+	ND	–	W	+	–	–	–	–	+
D-Raffinose	+	+	–	+	–	+	–	–	–	–	+
Starch	+	+	ND	+	+	+	–	–	–	+	–
Glycogen	+	+	–	+	+	+	–	–	–	–	+
Gentiobiose	+	+	ND	–	–	–	–	–	–	–	+
D-Turanose	+	–	ND	–	–	+	–	–	–	–	+
D-glucose	+	+	–	+	+	+	+	+	+	+	+
D-fructose	+	+	–	+	+	+	+	+	+	+	+
D-fructose	+	+	–	+	+	+	+	+	+	+	+
Sucrose	+	+	–	+	+	+	+	+	+	+	+
Esculin	+	+	–	+	+	+	+	+	+	+	+
N-acetylglucosamine	+	–	–	–	–	–	–	–	–	–	+

+: Positive; –: Negative; ND: not determined.

Table 2: Antimicrobial profiles of Marine isolate DK1-SA11 cell free supernatant and EtOAc extract against pathogens

Microorganism	Source	Zone of inhibition (diameter /mm)*			
		CFS	*EtOAc Extract*	*Streptomycin*	*Chloramphenicol*
MRSA	ATCC 43300	22 ± 0.34^{c}	32 ± 0.58^{ab}	R	R
E. coli O 157 H7	Clinical Isolate	20 ± 0.34^{b}	40 ± 0.89^{c}	17 ± 0.34^{a}	22 ± 0.58^{a}
Candida albicans	ATCC 10231	19 ± 0.34^{b}	39 ± 0.58^{c}	-	-
Klebsiella pneumoniae	NTCC 9133	18 ± 0.34^{ab}	32 ± 0.34^{b}	R	22 ± 0.34^{a}
Staphylococcus aureus	ATCC 6538	17 ± 0.58^{a}	30 ± 0.89^{a}	R	22 ± 0.34^{a}
Listeria monocytogenes	NCMCC 54001	17 ± 0.89^{a}	33 ± 0.58^{b}	24 ± 0.89^{c}	32 ± 0.89^{b}
Vibrio parahaemolyticus	ATCC 17802	17 ± 0.89^{a}	30 ± 0.89^{a}	22 ± 0.58^{b}	35 ± 0.58^{bc}
E. coli	ATCC 8739	17 ± 0.58^{a}	31 ± 0.89^{ab}	19 ± 0.34^{a}	20 ± 0.34^{a}
Pseudomonas fluorescens	CCTCC B2010226	18 ± 0.67^{ab}	30 ± 0.89^{ab}	20 ± 0.34^{b}	41 ± 0.67^{c}
Salmonella typhimurium	ATCC 14028	16 ± 0.89^{a}	29 ± 0.89^{a}	16 ± 0.58^{a}	21 ± 0.67^{a}
Vibrio cholerae	ATCC 14035	15 ± 0.34^{a}	39 ± 0.89^{c}	28 ± 0.89^{c}	33 ± 0.34^{b}

CFS: Cell free supernatant MRSA: Methicillin resistant *Staphylococcus aureus* R: Resistant-: Not tested*: Mean of triplicates $\pm$ SEM Different letter superscripts within column are significantly different ($p<0.05$)

Table 3: Effect of Carbohydrates on antibacterial activity by Marine isolate DK1-SA11

Carbohydrates	*ZOI (mm)**	*Increase %*	*Carbohydrates*	*ZOI (mm)**	*Decrease %*
D-Mannose	24±0.34	33.33%	Glycogen	17±1.16	-5.56%
Gentibiose	23±0.34	27.78%	D-Sacchrose(Sucrose)	17±0.34	-5.56%
Inulin	22±0.58	22.22%	L- Rhimnose	17±0.34	-5.56%
D-Raffinose	22±0.58	22.22%	D-Fructose	17±1.16	-5.56%
Methyl-αD-Glucopyranoside	22±1.16	22.22%	Methyl-β D-Xylopyranoside	17±1.46	-5.56%
D-Melibiose	21±0.58	16.67%	D-Adonitol	17±0.34	-5.56%
D-Xylose	21±0.89	16.67%	Glycerol	17±0.34	-5.56%
D-Ribose	21±0.58	16.67%	Potassium 2-Ketogluconate	16±0.34	-11.11%
D-Turanose	20±0.89	11.11%	Potassium Gluconate	16±0.89	-11.11%
Amidon (Starch)	20±0.34	11.11%	Potassium 5-Ketogluconate	15±0.89	-16.67%
D-Maltose	19±0.89	5.56%	Dulcitol	15±0.34	-16.67%
Inositol	19±0.34	5.56%	D-Galactose	15±0.58	-16.67%
D-Glucose	19±0.89	5.56%	L-Xylose	15±0.34	-16.67%
Erythritol	19±0.89	5.56%	L-Arabitol	14±0.34	-22.22%
Xylitol	18±0.89	0.00%	L-Fucose	14±0.89	-22.22%
D-Trehalose	18±0.34	0.00%	D-Lyxose	14±0.89	-22.22%
N-Acetyl Glucosamine	18±0.34	0.00%	D-Melezitose	14±0.58	-22.22%
D-Sorbitol	18±0.89	0.00%	D-Lactose (Bovine origin)	14±0.34	-22.22%
D-Mannitol	18±1.16	0.00%	D- Celiobiose	14±0.34	-22.22%
L-Arabinose	18±0.89	0.00%	Methyl-αD-Mannopyranoside	14±0.34	-22.22%
D-Arabinose	18±0.34	0.00%	D-Fucose	13±0.34	-27.78%
Medium only	18±0.89	0.00%	Arbutin	13±0.89	-27.78%
			Amygdalin	13±0.34	-27.78%
			D-Tagatose	12±0.34	-33.33%
			Salcilin	12±0.34	-33.33%
			Esculin ferric citrate	11±0.34	-38.89%
			L-Sarbose	11±0.34	-38.89%
			D-Arabitol	10±0.34	-44.44%

*Zone of Inhibition, Mean of triplicate ± SEM, by Cell free supernatant of *Bacillus sp*. DK1-SA11 Positive and negative values are percentage increases and decrease in antibacterial activity, respectively. *Medium only* considered as base line.

Effect of carbohydrates on antibacterial activity

Bacterial suspension were prepared in api® 50 CHB/E medium (bioMerieux, France) according to manufacturer's instructions and inoculated in 50 cups (1 blank and 49 carbohydrates containing cups) in API 50 CH strips. After 72 h of incubation, 60 µL of CFS from each cup were tested for antibacterial activity against *S. aureus*. Cup number 1, which has medium only, was considered as base line for zone of inhibition.

Effect of heat shock, cold storage and enzymes treatment on CFS

CFS was placed in water baths set at different temperatures and was autoclaved at 121°C for 15 min at 15 psi to identify residual activity of CFS of DK1-SA11 after heat treatment.

To determine the effect of storage temperature, CFS were stored at 25, 10, 4, -20, -40 and -80°C. Activities were measured after 0, 7, 30 and 90 days.

The effects of α-amylase, lipase, proteinase K, trypsin, pepsin and papain on antibacterial activity of DK1-SA11 were investigated by incubating CFS with equal volume of each enzyme (4 mg/mL) at 37°C for 2.5 h followed by inactivation of enzyme at 100°C for 5 min. The residual activities of CFS were measured as described before. Untreated CFS was used as control.

Table 4: Residual activity of CFS after enzymatic treatment

Enzymes	Residual Activity
Trypsin	0 %
Proteinase K	0 %
Lipase	0 %
Pepsin	75 %
Amylase	100%
Papain	100 %
CFS (positive Control)	100%

Ethyl acetate extract activity

CFS from 1000 ml Marine Broth 2216 was extracted three times with ethyl acetate (EtOAc) (1000 ml x 3). Solvents were concentrated by rotary evaporator under reduced pressure at 37°C (Zheng *et al.*, 2005) and

antimicrobial activity were evaluated by 20 µL of EtOAc extract (10 mg/ml) by Oxford cup agar diffusion method described above. 20 µL of EtOAc was used as negative control and 10 µL of 1 mg/ml streptomycin and chloramphenicol were used as positive control against the test organisms.

Table 5: TLC-bioautography R_f values of active spots against *S. aureus*

Active spots	R_f values		
	48 h	60 h	96 h
1	0.90	0.95	0.35
2	0.76	0.90	0.21
3	0.28	0.84	0
4	0.14	0.71	-
5	0	0.46	-
6	-	0.42	-
7	-	0.35	-
8	-	0.21	-

TLC-Bioautography

To find out R_f value of antimicrobial compound(s) in extract, strain DK1-SA11 was grown in Marine broth 2216 and extracted by EtOAc after 48 h, 60 h and 96 h as described above. Extensive TLC on 0.44 mm silica GF254 plates was developed with different solvents combination to achieve maximum separation. TLC-bioautography was performed with Chloroform: MeOH: H_2O (30:15:2) and visualize under 254 and 365 nm. After 30 min UV sterilization, the TLC plates were aseptically placed in petri-dish and overlaid with overnight culture of *S. aureus* seeded in BHI agar (0.7 %) maintained at 44°C. After solidification at room temperature and diffusion at 4°C for 2 h petri-dish was incubated at 37°C for 12 h. Clear zones of inhibition were observed after spraying aqueous solution of 2 mg/mL MTT on agar surface (Nostro *et al.*, 2000).

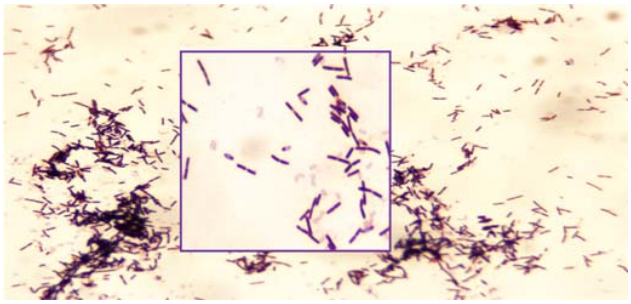
STATISTICAL ANALYSIS

All experiments were performed in triplicate and statistical mean, residual percentage, statistical graph and tables were constructed by software GraphPad® Prism 5.01. Analysis of variance was performed by statistical software SPSS (v 16.0).

RESULTS

Identification of isolate

Morphologically, strain DK1-SA11 cells are Gram positive, rods with sub-terminal endospores (fig. 1). Similarity studies on biochemical utilization with api*web*™ database revealed 98.8~99.2 % similarity with *B. subtilis/amyloliquefaciens*. Genotypic identification based on 16S rDNA sequencing and BLAST analysis showed 97.52% similarity with *Brevibacterium halotolerans* DSM 8802^T, 97.42% with *B. methylotrophicus* CBMB205^T and 97.35% with four type strains, of *B. subtilis* group, including *B. mojavensis* RO-H-1^T, *B. subtilis* subsp. *inaquosorum* KCTC 13429^T, *B. subtilis* subsp. *spizizenii* NRRL B-23049^T and *B. tequilensis* 10b^T. Moreover, there is 97.07% similarity with *B. subtilis* subsp. *subtilis* NCIB 3610^T and *B. vallismortis* DV1-F-3^T. However, *gyrB* gene similarity analysis with existing DNA gyrase subunit-B DNA sequence database revealed that strain DK1-SA11 have 100% similarity with *B. subtilus* subsp. *spizizenii*^{T}, 94.7% with *B. subtilis* subsp. *inaquosorum*^{T}, 93.2% with *B. subtilis* subsp. *subtilis*^{T}, 91.1% with *B. mojavensis*^{T} and 82.5% with *B. amyloliquefaciens* subsp. *plantarum*^{T}. Phylogeny studies confirmed that strain DK1-SA11 belongs to genus *Bacillus*, species *subtilis*, subspecies *spizizenii* (figs. 2 and 3). On the basis of microscopy, API 20E & 50 CHB biochemical characteristics, 16S rDNA and *gyrB* sequences analysis, marine isolate DK1-SA11 was identified as *B. subtilis* subsp. *spizizenii* DK1-SA11. Moreover, biochemical characteristic of strain DK1-SA11 revealed that strain is somewhat distinct from other neighbors of genus *Bacillus* (table 1).



Showing Gram positive rods, mostly in chain with sub terminal endospores as un-stain area in cells (focus in zoom insert)

Fig. 1: Gram Staining of Strain DK1-SA11

Antimicrobial activity of cell free suspension

Cell free supernatant has shown antimicrobial activities against all test strains including MRSA (ATCC 43300), *E. coli* O157:H7 (clinical isolate), *C. albicans* (ATCC 10231), *K. pneumoniae* (NTCC 9133), *L. monocytogenes* (NCMCC 54001), *V. parahaemolyticus* (ATCC 17802), *E. coli* (ATCC 8739), *P. fluorescens* (CCTCC AB2010226), *S. typhimurium* (ATCC 14028) *and V. cholerae* (ATCC 14035). Results are presented in table 2.

Optimum growth conditions

Marine broth 2216 was selected as the best medium for production of antimicrobial compounds (fig. 4), due to its fast and maximum production of antimicrobial activity among all other media. Incubation temperature 25°C (fig. 5), rotation speed > 180 rpm (fig. 6) and pH 7 (fig. 8) were the optimum conditions for best production of antimicrobial compounds. Moreover, strain DK1-SA11 showed the best antibacterial activities in 1% sodium

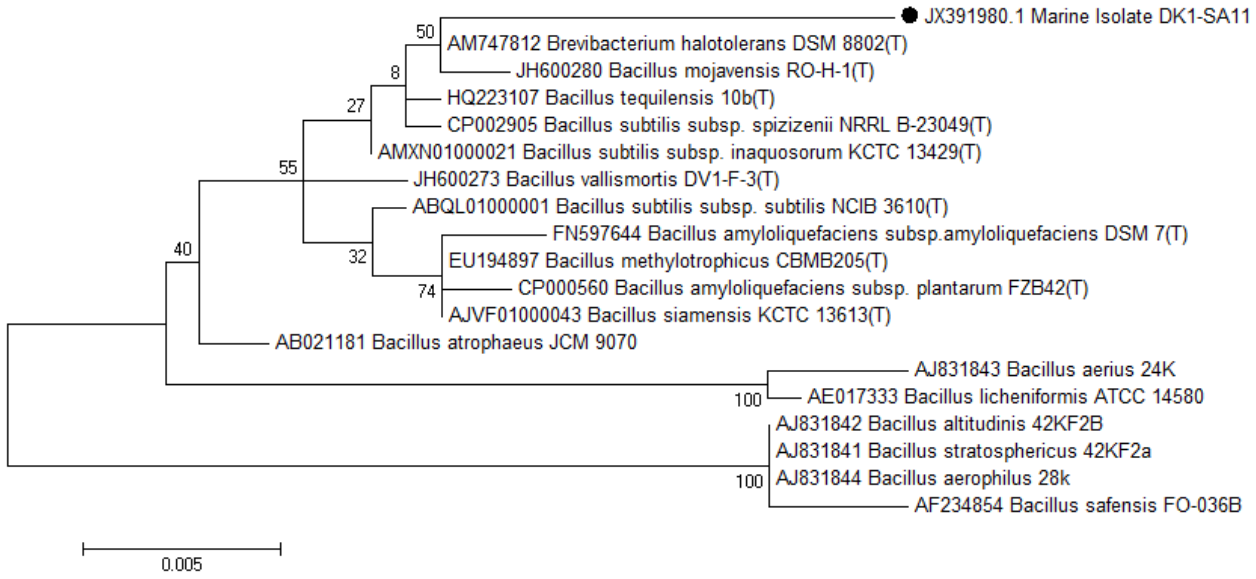
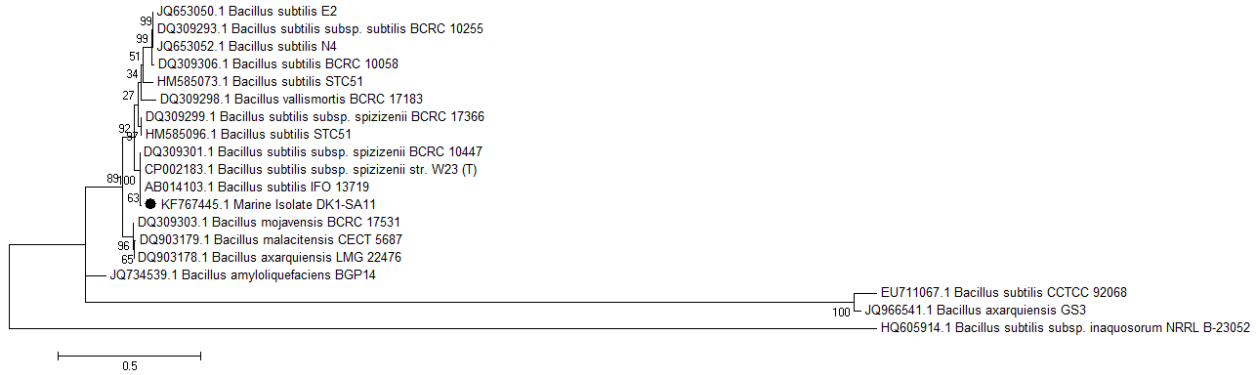


Fig. 2: 16S rDNA base Phylogenetic tree



Phylogeny analysis of Marine isolate DK1-SA11 DNA *gyrB* sequence via MEGA 5.1 software by maximum likelihood method. Percentage bootstrap values with 1,000 resembling are indicated at nodes. Scale bar 0.5 represents sequence divergence

Fig. 3: DNA *gyrB* gene base Phylogenetic tree

chloride, although the rapid log phase was achieved at higher concentration of salt (>4% NaCl) (fig. 7)

Effect of carbohydrates on antimicrobial activity

Among 49 carbohydrates tested D-mannose, gentibiose, inulin, D-raffinose and Methyl-αD-glucopyranoside were the top most inducers for antimicrobial activity in descending order. Sugars xylitol, D-trehalose, N-acetyl glucosamine, D-sorbitol, D-mannitol, L-arabinose and D-arabinose have no effect on increase or decrease in antimicrobial activity. However, D-arabitol, L-sarbose, esculin, salcilin and D-tagatose were leading carbohydrates to reduce the zones of inhibition of CFS against *S. aureus* (table 3).

Effect of heat shock, cold storage and enzymatic treatments

Cell free suspension exhibit 100% residual activity after 10 min heat shock at 100°C, 40 min heat shock up to 60°C. However, 30% reduction in residual activity was observed after 40 min at 100°C. Moreover, after 60 min of incubation, 48%, 16%, 5% and 5% reduction in antimicrobial activity were observed at 100°C, 60°C, 50°C and 40°C, respectively (fig. 9). CFS loses 100% of its activity after autoclaving. After cold storage, CFS has lost its activity after 7 days at 25°C, while at temperature ≤ 10°C it retained 100% activity. After one month, 95% residual activities were observed in CFS stored at ≤ 4°C and 58% at 10°C. About 95% residual activities were observed after 90 days at storage temperature ≤ (-40)°C. Moreover, 90 days storage at (-20)°C, 4°C and 10°C have residual activity of 89%, 63% and 0%, respectively (fig. 10). CFS has lost 100% of its activity after trypsin, proteinase-K and lipase treatments, while there is no effect of amylase and papain on antimicrobial activity against *S. aureus*. A 25% activity loss was observed after pepsin enzymatic treatment (table 4).

Ethyl acetate extraction and TLC –bioautography

EtOAc extract of CFS revealed significantly ($p<0.05$) improved antimicrobial activity against all test strains used for CFS activity (table 2). TLC-bioautography has

revealed five active spots after 48 h growth extraction, eight active spots after 60 h and three active spots after 96 h growth extraction with variable strength of activities (fig. 11). Corresponding R_f values of active spots are listed in table 5.

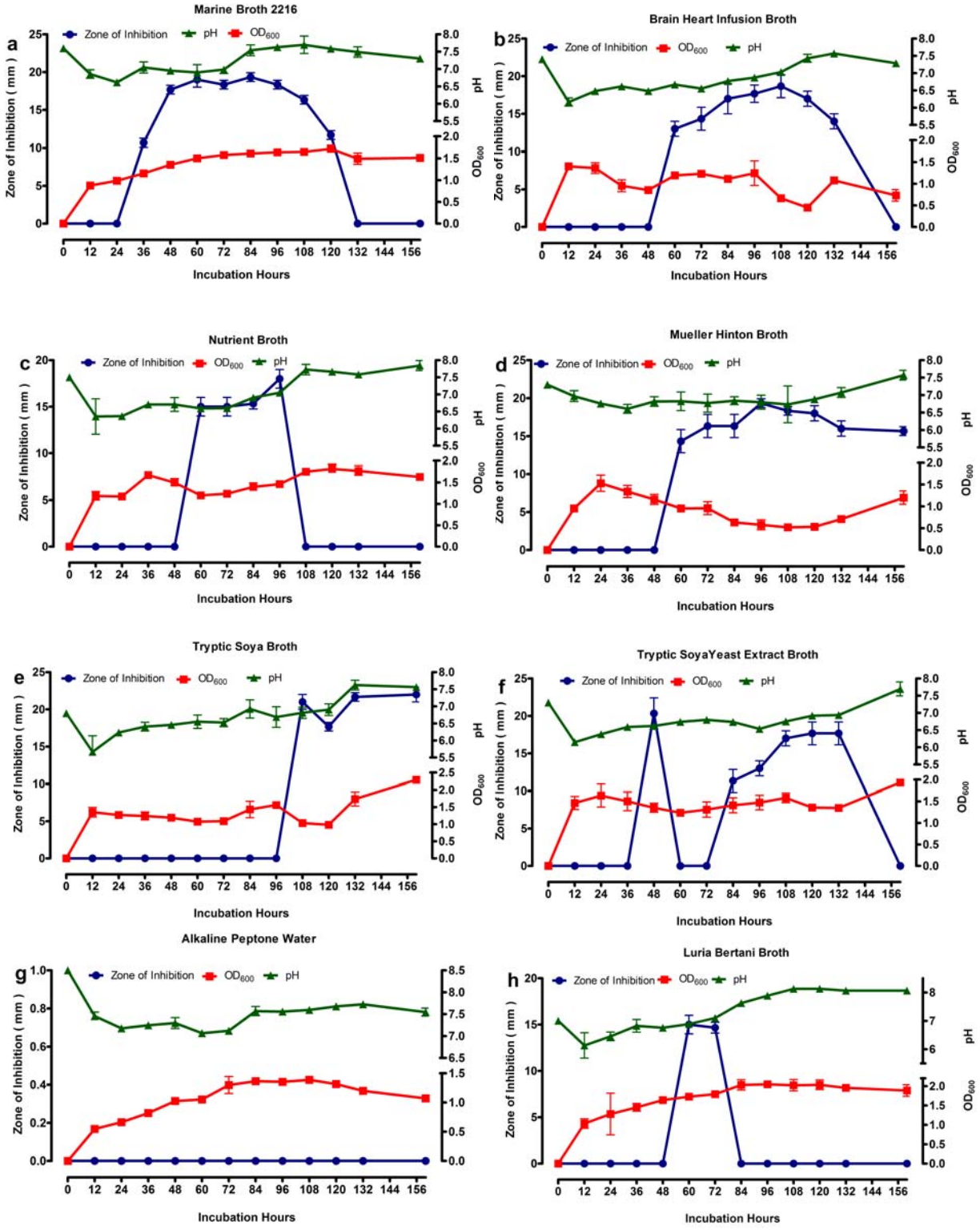
DISCUSSION

Dwelling of microorganisms under extreme and unique habitats result in production of new and unusual products. Most of these metabolites are produced to survive and compete for the available resources (Gallo *et al.*, 2004). Emergence of antimicrobial resistance and potential side effects of the synthetic antibiotics enforced scientists to find out natural solution from herbal, terrestrial and aquatic microorganisms to discover new metabolites (Mehrgan *et al.*, 2008). Marine is the most versatile, variable and natural environment for growth of microorganisms, virtually an unlimited source of chemically novel compounds with potential antibiotic properties (Behal, 2000). Marine inhabitants are supposed to have about 3.67×10^{30} microorganisms (Whitman *et al.*, 1998).

Identification of a microorganism by morphological and biochemical characteristics is not enough to distinguish it from the related species. API system, API 20E and 50CHB, results identified the marine isolate DK1-SA11 as *B. subtilis/amyloliquefaciens,* however strain DK1-SA11 has some different biochemical characteristics when compared with related taxa (table 1). Ribosomal RNA gene sequencing of 16S rDNA was considered as a gold standard to identify and differentiate the bacteria (Joung and Cote, 2002). Strain DK1-SA11 genetic similarity based on 16S rDNA database (NCBI, EzTaxon, EMBL) revealed that it has highest similarity, in descending order, with *Brevibacterium halotolerans* DSM 8802^T and other member of *B. subtilis* group including *B. methylotrophicus* CBMB205^T, *B. mojavensis* RO-H-1^T, *B. subtilis* subsp. *inaquosorum* KCTC 13429^T, *B. subtilis* subsp. *spizizenii* NRRL B-23049^T, *B. tequilensis* 10b^T, *B. subtilis* subsp. *subtilis* NCIB 3610^T and *B. vallismortis* DV1-F-3^T. Phylogeny tree based on 16S rDNA (fig. 2) revealed that strain DK1-SA11 cluster with *B. halotolerans* DSM 8802^T and other member of *B. subtilis* group. This seems that strain DK1-SA11 was *B. halotolerans*. However, in the first edition of Bergey's Manual of Systematic Bacteriology, *B. halotolerans* was classified as incertae sedis (Jones and Keddie, 1986). Studies revealed that *B. subtilis* group is indistinguishable on the basis of 16S rDNA phylogeny. Other phylogenetic markers such as *gyrB* gene, which encodes the subunit B protein of DNA gyrase, is compulsory to identify and distinguish *B. subtilis* group (Wang *et al.*, 2007a). DNA *gyrB* gene alignment and similarity analysis with NCBI, Ez-Taxone, EMBL database confirmed that strain DK1-SA11 has 100% conserved sequence with type and reference strains of *B. subtilus* subsp. *spizizenii*^{T} and phylogenic tree also revealed that strain is cluster with *B. subtilus* subsp. *spizizenii* with higher percentage of bootstrap value (fig. 3). In a recent studies on identification, based on *recA* gene marker, strain DK1-SA11 was identified as *B. subtilus* subsp. *spizizenii* (Khan *et al.*, 2014). Therefore, marine isolate DK1-SA11 was identified as *B. subtilus* subsp. *spizizenii* DK1-SA11. The results of 16S rDNA and *gyrB* phylogenetic studies on strain DK1-SA11 suggest that the strain *B. halotolerance* DSM 8802^T might have been misclassified with the genus *Brevibacterium*, and that it may be considered as a strain of *B. subtilis* group. Our findings are in accordance with the recent volume of Bergey's Manual of Systematic Bacteriology in which *B. halotolerance* DSM 8802^T has been considered as a misclassified strain and it appears to be a representative strain of genus *Bacillus* (Boone *et al*., 2012).

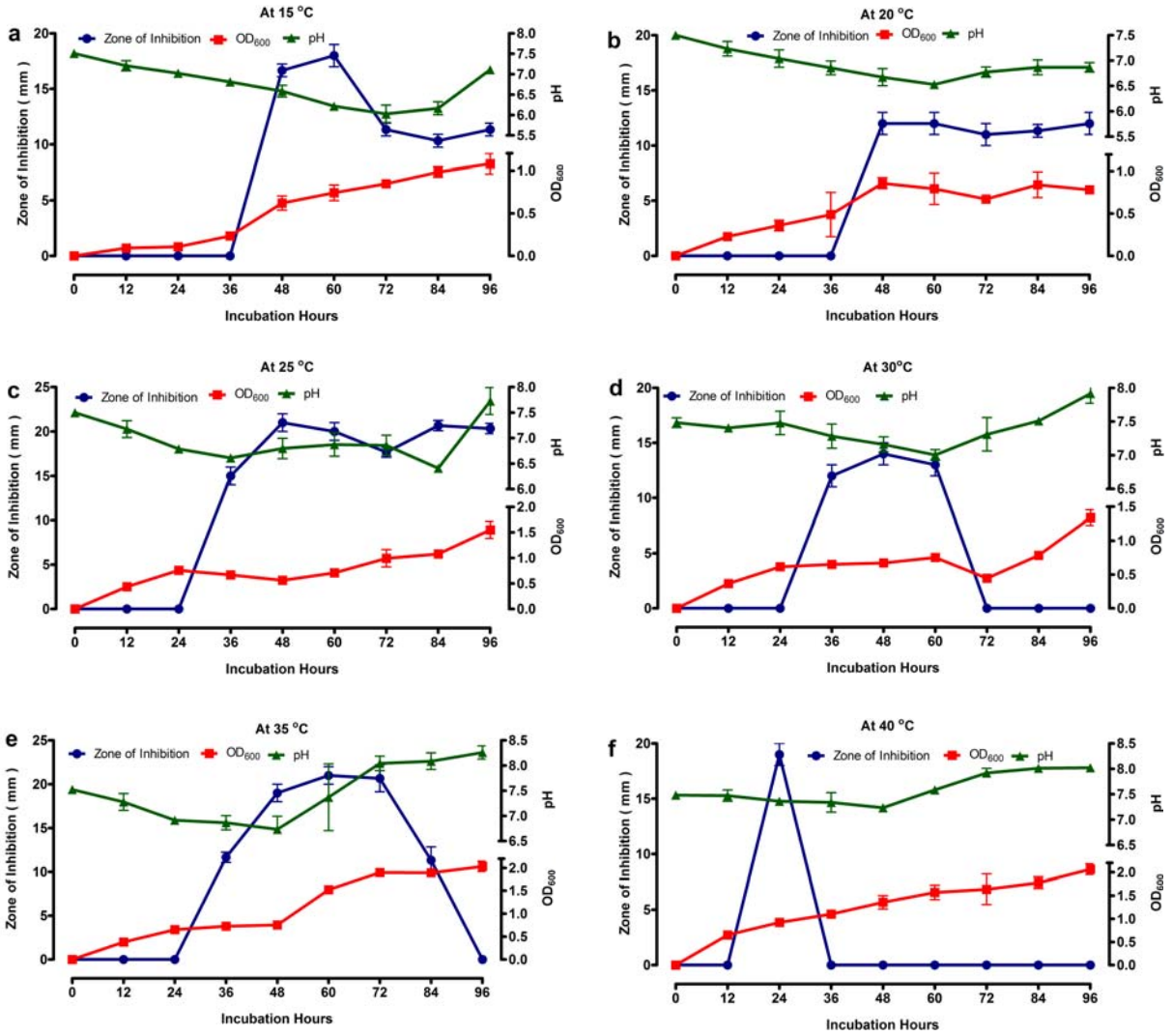
Among the diverse microbial species, isolates of marine *Bacillus* belong to phylogenetically and phenogenetically heterogeneous groups of bacteria. They are ubiquitous in the marine environment and can tolerate adverse conditions such as high temperature, pressure, salinity, and pH (Rampelotto, 2010). The incredible properties of compounds produced by *Bacillus* species, especially from marine *Bacillus*, may be one of the solutions for superbugs and multiple drug resistant (MDR) pathogens (Fickers, 2012). Marine isolate DK1-SA11 has proven antimicrobial activity against Gram positive, Gram negative and pathogenic yeast *Candida albicans* (table 2). Selection of broader spectrum of activity of isolate DK1-SA11 was likely due to extensive contest in oceanic haunt.

Antibiotics are mostly produced in stationary phase i.e. they are secondary metabolites. The presence of genetic sequences does not guarantee the production of particular antibiotics, because environmental conditions play major role in the expression of these genes. Therefore, production of antimicrobial compounds was mostly brought on by elimination of food, addition of an inducer and sometime decrease in the growth rate (Bibb, 2005; Liu and Li, 2011). Leifert *et al.* (1995) also reported that synthesis of antimicrobials was due to substrate medium composition, pH, incubation temperature and concentration of growth components in the medium. To analyze the effects of these factors, strain DK1-SA11 was grown in Marine broth 2216, Alkaline peptone water, Brain heart infusion broth, Luria Bertani broth, Mueller Hinton broth, Nutrient broth, Tryptic soya broth, Tryptic soya yeast extract broth and Yeast Extract peptone dextrose broth. Marine broth 2216 was found to be the best medium for growth and production of antimicrobial compounds by strain DK-SA11, due to maximum antibacterial activity, shorter time, and consistency of growth and antagonistic activity (fig. 4).



Growth and antibacterial activity of Marine isolate DK1-SA11 in different media (a) Marine broth 2216 (b) Brain heart infusion broth (c) Nutrient broth (d) Mueller Hinton broth (e) Tryptic soya broth (f) Tryptic soya yeast extract broth (g) Alkaline peptone water (h) Luria Bertani broth. Each data point of optical density OD_{600}, pH and zone of inhibition was mean value of triplicate experiments

Fig. 4: Growth Medium Optimization

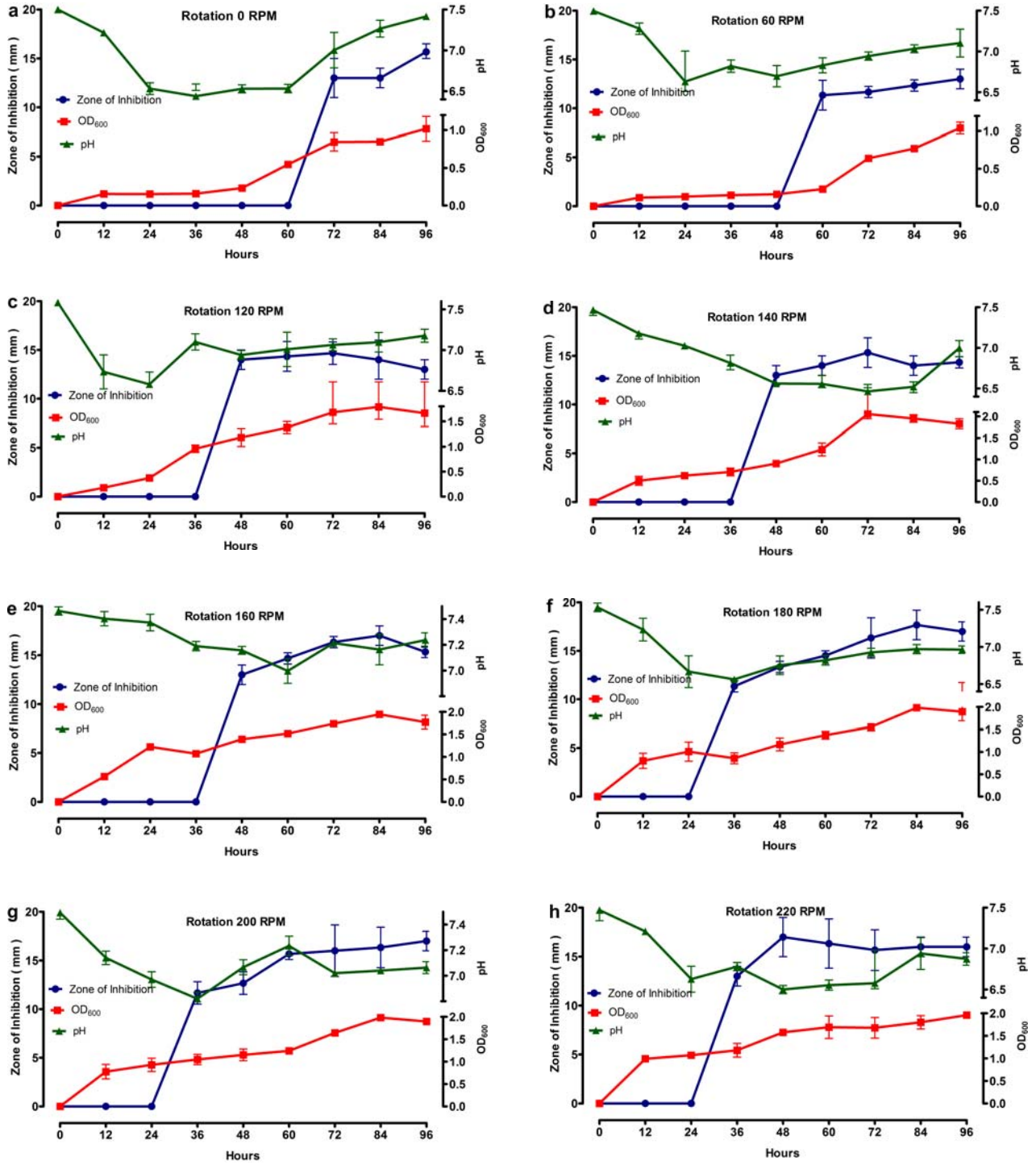


Effect of temperature on growth of Marine isolate DK1-SA11 and its antibacterial activity tested against *S. aureus* after every 12 h in Marine broth 2216 at different incubation temperature: (a) at 15°C (b) at 20°C (c) at 25°C (d) at 30°C (e) at 35°C (f) at 40°C . Each data point of optical density OD_{600}, pH and zone of inhibition was mean value of triplicate experiments

Fig. 5: Growth Temperature Optimization

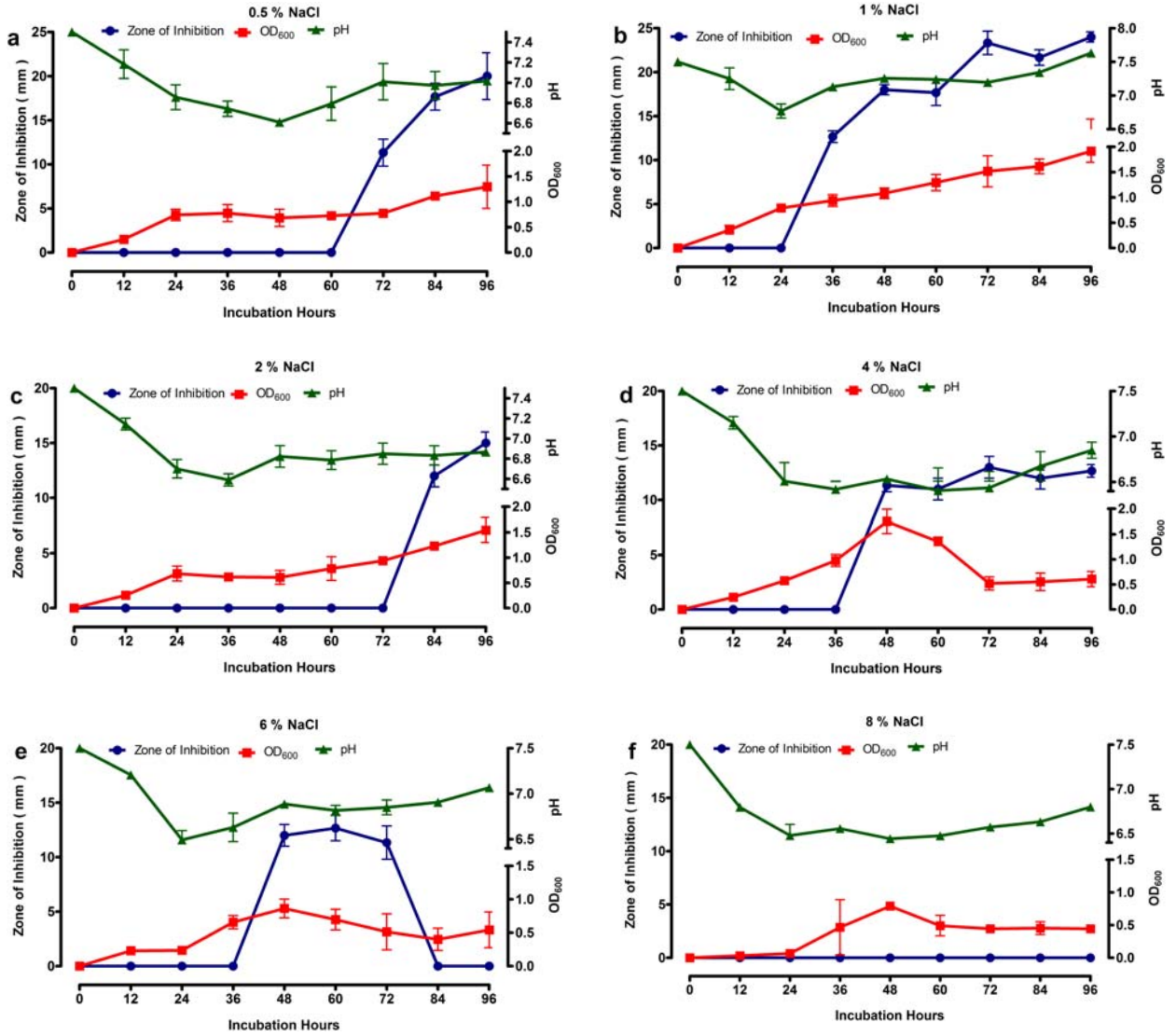
Among all the ingredients in growth medium, carbon has the major role in bacterial growth and antimicrobial production (Nishanth *et al.*, 2012). Antimicrobial substances produced by bacterial species were greatly influenced by variation of carbon sources. Our results revealed that D-mannose, gentibiose, inulin, D-raffinose, Methyl-α-D-glucopyranoside, D-melibiose, D-xylose, D-ribose, D-turanose, amidon, D-maltose, inositol, erythritol and D-glucose were the top inducers for antagonistic activity by *Bacillus* sp. DK1-SA11 (table 2). Mukherjee and Das (2005) reported enhancement of antimicrobial activity by *B. subtilis* in the presence of glucose. Decrease in inhibition activity by glycerol was reported in *B. subtilis* subsp. *subtilis* C9 (Islam *et al.*, 2012). These reports were in accordance with our findings.

Abiotic factors including incubation temperature, pH and rotation speed beyond the optimum growth conditions also influence the antibacterial compounds production (Raaijmakers *et al.*, 2002). The temperature range for production of antibacterial compounds by strain DK1-SA11 was 15-40°C and that for growth was from 15-45°C, where 25°C was found to be the best temperature for antimicrobials production, as highest and consistent production for longer period of time was observed at this temperature (fig. 5). Our results are in accordance withFickers *et al.* (2008), who reported 30 fold increase in lipopeptide production by *B. subtilis* ATCC 6633 when temperature was decreased from 37°C to 25°C. Antimicrobial activity was increased with increase in rotation speeds 0-220 rpm, and ≥ 180 rpm was best for maximum antibiotic production (fig. 6). Our results are similar to Jafarzade *et al.* (2013), who observed an increase in antimicrobial activity with the increase in aeration speed, against *S. aureus* and MRSA by a marine isolate.



Effect of rotation on growth of Marine isolate DK1-SA11 and its antibacterial activity tested against *S. aureus* after every 12 h in Marine broth 2216 at 25°C with different rotation speed : (a) 0 rpm (b) 60 rpm (c) 120 rpm (d) 140 rpm (e) 160 rpm (f) 180 rpm (g) 200 rpm (h) 220 rpm. Each data point of optical density OD_{600}, pH and zone of inhibition was mean value of triplicate experiments

Fig. 6: Growth Agitation Optimization



Effect of NaCl concentration on growth of Marine isolate DK1-SA11 and its antibacterial activity tested against *S. aureus* after every 12 h in Marine broth 2216 at 25 ^{o}C and 180 rpm with different salt concentrations: (a) 0.5 % NaCl (b) 1% NaCl (c) 2% NaCl (d) 4% NaCl (e) 6% NaCl (f) 8% NaCl . Each data point of optical density OD_{600}, pH and zone of inhibition was mean value of triplicate experiments

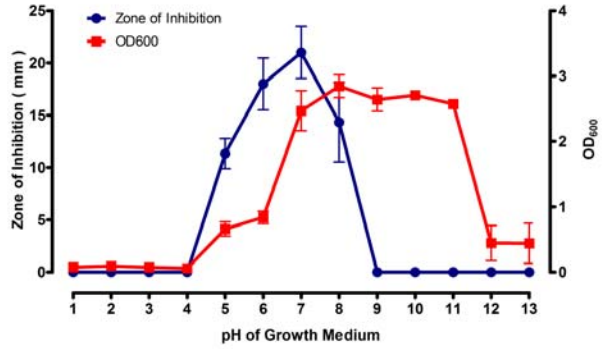
Fig. 7: Growth Salt Conc. Optimization

Growth of vegetative *Bacillus* was mostly rapid with increasing salt concentration, depending on species and source of isolation (Okanlawon *et al.*, 2010). Strain DK1-SA11 has shown higher growth rate at higher salt concentration, although the antimicrobial compounds production in ≥ 2% additional NaCl was not consistent (fig. 7). At higher concentration of NaCl, reduction in antimicrobial compounds production by marine bacteria was also reported by Jafarzade, Yahya *et al.* (2013).

Intracellular pH of microorganisms is mostly consistent near neutral, irrespective of the pH of outside medium. Although, as the proton gradient around the cell membrane changes, the organisms assign most of their assets to balance the desired intracellular pH, thus alteration in the pH of outside medium influence growth and production of secondary metabolites (Chang *et al.*, 1991). Antibacterial activity by our isolate was seen from pH 5-8 while pH 7 was best for antimicrobial production. Moreover, growth was observed from pH 4-11 (fig. 8). These results indicate that Strain DK1-SA11 was able to grow under extreme pH conditions.

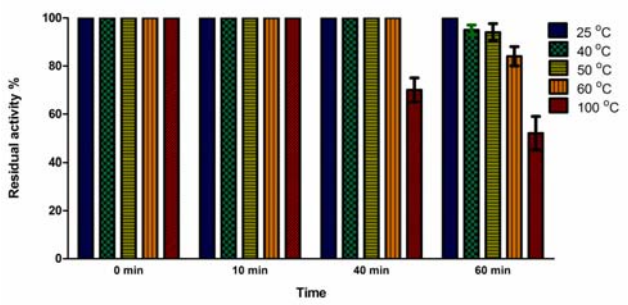
Characterization of CFS revealed that compounds produced by strain DK1-SA11 might be lipoamides, lipopeptides or lipoproteins as destroyed by lipase and some proteolyses by enzymatic treatments (table 3). Recent genomic characterization of a marine *B. subtilis*

subsp. *spizizenii* strain gtP20b, isolated from the Indian Ocean, indicated the presence of huge number of genes for biosynthesis of secondary metabolites including subtilisin A, surfactin, β-lactamase precursor, and replicative DNA helicase (Fan *et al.*, 2011). CFS was stable at lower temperatures for 90 days (fig. 10) while heat shock treatments revealed that compounds are thermo-stable even after boiling temperature (fig. 9). Heat resistant antibacterial compound with broad spectrum of activity, against both Gram positive and Gram negative bacteria, and pH tolerance ability were also reported by Paik *et al.* (1998) from *B. subtilis* B38.



Effect of pH on growth of Marine isolate DK1-SA11 and its antibacterial activity tested against *S. aureus* after, 72 h of incubation at 25 °C with 180 rpm, adjusting pH of Marine broth 2216 to pH-7 from pH 1-13. Each data point of optical density OD_{600} and zone of inhibition was mean value of triplicate experiments

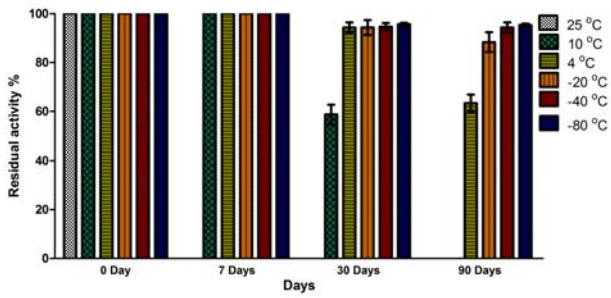
Fig. 8: Growth medium pH Optimization



Residual activity of Cell free supernatant (CFS) after heat treatment at 25, 40, 50, 60, and 100 °C for 0, 10, 40 and 60 min. Each bar shows mean value of triplicate experiments

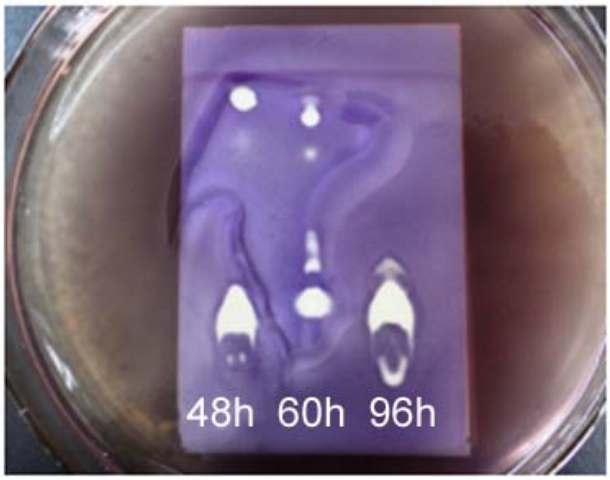
Fig. 9: Heat Shock Stability

It has been reported in many studies that composition of medium, with differences in source of carbon and nitrogen, play major role in production of antibacterial compounds or enzymes responsible for synthesis of antagonistically active components (Ansari *et al.*, 2012, Lisboa *et al.*, 2006, Moshafi *et al.*, 2011, Sihem *et al.*, 2011). Bioautography results after solvent extraction revealed more than one antimicrobial component in CFS, which was dependent on extraction time (fig. 11). These findings suggest that the deviations in antimicrobial activity in different media and other abiotic factors were due to more than one antimicrobial compounds produced by the isolate DK1-SA11 in response to variation in growth medium and abiotic factors. After 48 h, five active components, after 60 h, eight active components, and after 96 h, three active components with variable R_f values (table 4) and intensity of antibacterial activities were observed. Antibacterial activity of filtrates and concentrates after ultra-filtration of CFS via Amicon® Ultra-15 centrifugal filter device with 3K, 10K, 30K and 100K (data not shown) also suggest more than one active compounds with different molecular weights. Four active spots on TLC –bioautography were also reported by Moshafi, Forootanfar *et al.* (2011) from soil *Bacillus sp*. FAS1. Although only two were active against each test organism as compared to our finding where eight spots active against *S. aureus* were observed. Similarly, three active spots by TLC bio-autography of EtOAc extract against *S. aureus* by marine *Bacillus sp.* were reported by Zheng, Han *et al.* (2005) and four different R_f values of antibacterial compounds were reported by Sihem, Rafik *et al.* (2011).



Residual activity of Cell free supernatant (CFS) after Cold storage at 25, 10, 4, -20, -40 and -80 for 0, 7, 30 and 90 days. Each bar shows mean value of triplicate experiments

Fig. 10: Cold Storage Stability



After 48 h, 60 h and 96 h showing variable active components against *S. aureus*

Fig. 11: TLC-Bioautography of CFS extract

CONCLUSION

The purpose of this study was to identify and optimize the broad spectrum antimicrobial compounds producing strain DK1-SA11. Morphological, biochemical and phylogeny analysis based on 16S rDNA and *gyrB* gene identified the isolate as *B. subtilis* subsp. *spizizenii* DK1-SA11. Identification experiments revealed that strain *B. halotolerance* DSM 8802^T might have been misclassified with the genus *Brevibacterium*, and it may be considered as a strain of *B. subtilis* group. Moreover, among all the media tested, Marine Broth 2216 was found to be the best medium for bacterial growth and production of antibacterial compounds. The other optimum conditions for growth were pH:7 and incubation temperature: 25°C with ≥ 180 rpm for 60-72 h. Out of 49 different carbohydrates tested, D-mannose increases the antibacterial activity by 33.3% while D-arabitol decreases it by 44.4%. Crude CFS showed activity even after three months of storage below -20°C and boiling for 10 min, whereas it loses 100% of its antimicrobial activity after enzymatic treatments of lipase, trypsin and papain. Antimicrobial compounds, produced by strain DK1-SA11, individually or in combination might prove to be the solution for treatment against MDR and superbugs and may be a source for new probiotics, synbiotics and antibiotics.

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