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Bioactivity of Marine *Bacillus licheniformis* Ksawd3 Isolated from Arabian Gulf, Saudi Arabia

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***Bacillus licheniformis KSAWD3*, isolated from sea water of Arabian Gulf, Tarut Island of east Coast of Saudi Arabia, was selected as a potential strain that showed considerable bioactivity from amongst 300 isolates. The isolate was identified based on morphological, biochemical and gene sequence of 16sRNA. This bacterium showed inhibitory activity against ATCC reference strains and clinical pathogens obtained from Military Hospital in Riyadh. The tested pathogens included *Salmonella enterica subsp enterica serovar Typhimurium* (ATCC 13311), *Shigella sonnei* (ATCC 11060), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 15442*), Staphylococcus aureus subsp. aureus* (ATCC 6538P, 25923, & 33591), *Streptococcus pneumoniae, Haemophilus influenzae*, *Salmonella sp. Campylobacter jejuni* and *Streptococcus pyogens*. Bioactivity was tested employing cross streaking, agar well diffusion method, agar overlay method, and assay of growth inhibition in broth. This isolate showed broad spectrum of growth inhibition by recording inhibition of growth against more than one pathogen. It was observed that the culture filtrate of *B. licheniformis KSAWD3* showed considerable inhibition against the pathogens which showed resistance against chloremphenicol, gentamycin, tetracycline, penicillin, neomycin, and ampicillin. Results indicate scope for deriving potential bioactive principles from this marine *B. lichenifirmis* of Arabian Gulf for use against the well known human pathogens.**

Key words: Bioactivity, Antibiotics, Marine biodiversity, Pathogens, Drugs.

Enormous efforts are being invested in the effective management and eradication of microbial pathogens, from environment ever since they were recognized. In fact microbes are turning out to be intelligent organisms which have evolved rapidly in developing resistance to antibiotics, and there is not only recurrence of pathogens but also have developed stronger resistance to conventional antibiotics which are being used in therapy. Some resistant, mutant pathogenic bacteria strains have developed as a consequence of inappropriate use of antibiotics and over prescription in the treatment of various diseases, and as a result of the widespread use of antibiotics in food products and livestock. In spite of the fact that the ocean covers 71% of the earth surface and contains approximately half of the total global biodiversity, the marine microbes and the versatility of their bioactive metabolites have not been fully explored till date. Around 2500 new metabolites (MNPs) were reported from marine organisms ranging from microbes to fish, during the period from 1977 to 1987, which accounts for less than

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1.0% of the total marine organisms[1,2]. Nevertheless, the search for new metabolites from marine organisms has resulted in the isolation of more or less 10,000 metabolites many of which are endowed with pharmacodynamic properties[3]. To date approximately 16,000 marine natural products have been isolated from marine organisms and several bioactive compounds had antiviral, antibacterial, antimalarial, anti-inflammatory, antioxidant, and anticancer potentials. The marine biodiversity of Kingdom of Saudi Arabia have not been explored for biomedical molecules. In this context the present study was initiated towards screening of microorganisms associated with marine environments, including mangroves, around the Kingdom of Saudi Arabia for bioactive molecules. In this study, we present the preliminary observations made on the prospective bioactive substances isolated from the bacteria isolated from the decomposing leaf litter samples associated with the mangrove environments of Arabian Gulf of Saudi Arabian Eastern Coast which showed inhibitory activity against well known human pathogens.

MATERIAL AND METHODS

Isolation of microorganisms from samples

Samples of water, sediment and leaf litter (leaves decayed and remain as a suspension in the water) were collected from the mangrove area of Tarot Island and Qatif near Dammam on the Arabian Gulf coast. Samples were transported to laboratory in iced conditions and plated on ZoBell's Marine Agar employing pour plate techniques. Inoculated plates were incubated at 28°C for 5-10 days. A total of 320 single cell cultures were registered.

Screening bioactive strains from all marine isolates

Screening of bioactive cultures was performed in three phases against standard reference strains (ATCC) And Clinical pathogens which were obtained from the Military Hospital in Riyadh. The tested pathogens included *Salmonella enterica* subsp *enterica* serovar *Typhimurium* (ATCC 13311), *Shigella sonnei* (ATCC 11060), *E. coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* subsp. *aureus* (ATCC 6538P, 25923, & 33591), *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Salmonella* sp. *Campylobacter jejuni* and *Streptococcus pyogens*. During the first phase all those presumptive cultures which showed halo zones around them on Zobell's agar and actinomycetes agar media were tested for bioactivity against the selected pathogens by cross streaking the cultures against one another on Brain Heart Infusion (BHI) Agar medium as well as in Zobells agar medium at 37°C for 3 days.

In the second phase standard agar well diffusion assay was performed against the pathogens. This test was done on Brain Heart Infusion agar (BHI) agar plates. A well was made in the agar plate after inoculating the pathogen on the plate by spread plate and adding 100 micro liter of the cell free extract obtained from the test isolates grown in Zobells Broth for 48 hours was used. The plates were incubated at 37°C for 48hours and the halo zones formed around the agar well were measured and the positive cultures were selected. During the third phase inhibition of growth of pathogens in BHI broth added with cell free extract of the test cultures at 37°C for 48 hours, was determined by turbidity assay. Growth was measured in terms of turbidity in the flasks in a UV-Visible spectrophotometer at 600nm.

Identification of the selected KSADW3 Isolate.

The marine bacteria which showed promising activity against the teased bacterial pathogens was identified based on their morphological, Biochemical, physiological properties and gene sequence of 16sRNA.

RESULTS

Isolation of strains with bioactivity

Water, sediment and leaf litter samples from mangrove environments and sea water from Tarot Island, Al-Qatif and Dammam were screened for microorganisms. Unfortunately no fungi were detected among the cultures that developed on the agar medium. Only bacteria and actinomycete cultures appeared on the plates and focus was given only to bacteria that showed visible halo zones around the colonies developed on the agar medium. Among the 320 marine bacterial isolates that showed halo zones around their colonies, when subjected for further screening process by testing against well known human pathogens, only 47 strains showed activity against the test

organisms, *Campylobacter jejuni*, *Bacillus cereus*, *Pseudomonas aeruginosa, Salmonella enterica* and *Haemophilus influenza*. Subsequent screening through bioassays was performed to select a potential strain that showed relatively maximal bioactivity against the human pathogens. Out of the 47 strains which showed activity, isolate *B. licheniformis* KSAWD3 was selected as potential strain for further screening and identification.

Identification of the strain

Based on the morphological, and biochemical characteristics recorded the marine bacteria *KSAWD3* was tentatively placed under the genera *Bacillus*. The selected isolate was grown in Marine Zobells Broth for three days and growth curve was recorded based on the optical density readings. (Fig. 5). Further, the identity of the selected strain was confirmed based on the 16sRNA sequences and nucleotide homology and phylogenetic analysis using the known sequences available in GENBANK. The amplicon obtained for the PCR amplification for 16SrDNA is presented in (Fig. 3). The BLAST HIT results and homology search results are presented. Present the results obtained for the CLUSTAL W (1.81) multiple sequence alignment. Based on the results presented in Figures the strain *KSAWD3* was identified as *B. licheniformis*.

Forward Sequence

CATTCGGGCCGCCTATAGTGCAGTCTAGCGG
AGAGATG GGAGCTTGCTCCCTGA TGTCA
GCGGCGGACGGGTGAGTAACACGTGGG
TAACCTGCCTGTAAGACTGGGATAACTCCG
G G A A A C C G G G G C T A A T A C C G G A
TGCTTGATTGAACCGCATGGTTCAATTA
TAAAGGTGGCTTTTACCTACCACTTTTGATGG
ACCCGCGGCGCATTGCCTAGTTGGTGAGGT
AACGGCTCACCAAGGCAACAATGCGTAGCGA
A C C T G A A A G G G T G G C C G C C C C A
CTTGGAACTGACACAGGGCCAAGAC
TCCTACGGGAGGCAGCTGTAGGGGATCTT
CCCCAATGTATACAAGTCGGACGGAAC
A C C G C A T C T G G A G G G A G T A A G G
TTTTCAGATCTAAAAT ATCT GTT GTT AGG
AAAGCAAATTTAC GGG TCCC AAAAAGT
TGGACC TTGAGGGTGAATACAC TGTCCCC
CAGGTGTTAACACGCGT GCCTATACCAGAGG
TTATTACTA ATGCGGGAGCTGTT ACCGG
TACTTTATAAGTTTG ATCATCGCCGATTC

Reverse sequence

CACGGTCACCTTCAGCGGCTGGCTCGC
AAAGT TACCTCACCG ACTTCC GGT GTTTC
AAAC TCTCGTGGTGGGATGGGCGGTG TGTA
CAATGCCCGGGAAGT ATTCACCGTCCCG
TGCTGATCCGCTATTACTA GCGAATCCAGCTT

Table 1. Turbidity Assay performed for evaluating growth of Reference ATCC strains in the presence of cell free extracts of marine test strains for assessing bioactivity. (Inhibition% at 600nm)

Pathogen strain	Control (cm.)	At 100µl of *KSAWD3* Extract	Inhibition %
C. jejuni	1.504	0.642	42.69
S. enterica	0.800	0.164	20.50
H. influenzae	0.901	0.083	9.21
P. aeruginosa	1.658	1.130	68.15

Table 2. Comparative evaluation of Bioactivity of substances isolated from *Bacillus licheniformis KSADW3* (100µl) against known pathogens with known antibiotics (ready discs), (Zone of inhibition expressed in millimeter diameter)

Antibiotic	*C. jejuni*	*S. enteric*	*H. influenzae*	*P. aeruginosa*
B. licheniformis	9	10	8	10
Ampicillin	0	14	6	8
Penicillin	0	4	3	6
Tetracycline	5	13	7	6
Neomycin	0	4	10	0
Gentamycin	0	3	4	7
Chloromphenicol	14	14	14	14

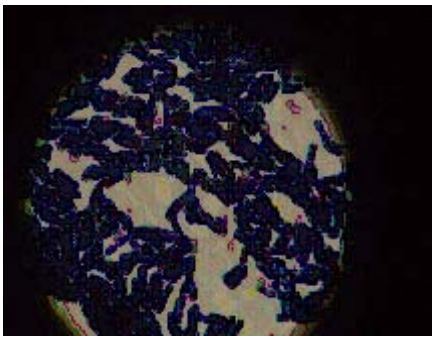


Fig. 1. *B. licheniformis KSADW3* (LM), magnification (100x)

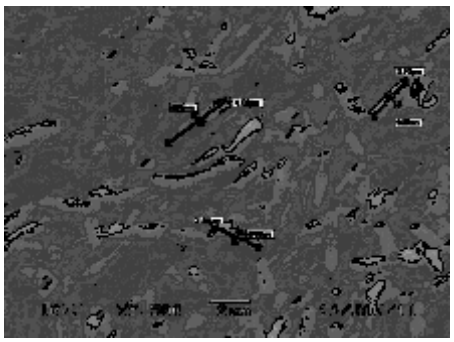
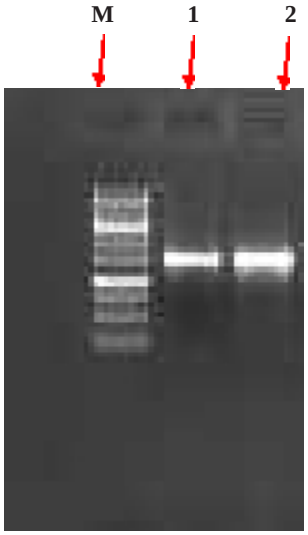


Fig. 2. *B. licheniformis KSAWD3* (SEM)



Lane M. Markers; Lane 1: Sample -1; Lane 2: Sample-2

Fig. 3. Amplicon obtained after PCR amplication of 16SrDNA of *B. licheniformis* KSAWD3, (Molecular weight of the product is 1500bp)

CACGCATTGGTCTTGCA GACTG CGATC
TTTCTGAA AACAGAT TTGTG GGA TTG
GCTTAACCTCG CAGATTTGGGCCCTTTGTTCT
GATAATGTGGTA ATCTGAAGTCCTGCCCATA
CGGGGCCGCTGCTTG AAATC GAACCAC
CTAAAGTGGG GGTGGGACCG GGACTA
AAGGTTTACCTGCC TTTGC ATAAGG ATTC
CCAAGGCAA AACC TTCCTTGTTT AGAA
TTTTTTCTCGTGATTTCG GGGAA TCGC
AATTATTTGGACCCCAGACACTT AGAA
CCAAAAGTTACGCC ACCTC ACCG TATAT ATT
A G ATGGA AAAACGC TATGG GAG ATGGT
TTAAAATTAAGGG CGCCTATTATTTAAAT
CTCTCCAAGC GCGAGAATTTA ATAGATTTAG
GATCAAAGAGAT CCCAAATACTCTTT
TTAGCTCGACAAG AAGGAGAAAAGATTT
T A G A C A A A A T T C A T A T A T T T T G
TACAACGAAGGACTAACAAG

Contig

CATTCGGGCCGCCTATAGTGCAGTCTAG
CGGAGAGATGGG AGCTT GCTCCCTG
ATGTCAGCGGCG GACG GGTGAGTAA CAC
GTGG GT AACCTGCCTGTA AGAC TGGG
ATAACTCCGG GAAACCG GGGCTA ATACCG
GATGC TTGATTGAACCGCATG GTTCA
ATTATAAAGGTGGC TTTTACCT ACCAC
TTTTGATGGAC CCGCGG CGCATTGC
CTAGTTGG TGAGGTAACGG CTCACCA
A G G C A A C A A T G C G T A G C G A A C
CTGAAAGGGTGGC CGCCCCA CTTGG AA
CTGACACAGGGCC AAGACTCCTACGGGAG
GCAGCTGTAGGGGATCT TCCCCAAT GTAT
ACAAGTCGGACGGAACACC GC ATCTGGAGG
GAGTA AGGTTTT CAGAT CTAAAAT
ATCTGTTGTTAG GAA AGCAA ATTTACG
G G T C C C A A A A A G T T G G A C C T T G A
GGGTGAATACACTGTCCC CCAG GTGTT
AACACGCGTG CCTATAC CAGA GGTTA
TTACTAATGCGG GAGCTGTTACCGG TACTTT
ATAAGT TTGATCATCGCCGATTC

Reconfirmation of anti-bacterial activity by *B. licheneformis*

B. licheniformis KSAWD3 which was selected as potential strain with bioactivity was reconfirmed for its potential for production of bioactive substance. The culture was grown as broth and ultra-sonicated to check for presence of bioactive substance as intracellular fraction. After ultra-sonication the cell soup was centrifuged and filtered and using the filtrate bioassays were

performed to reconfirm bioactivity following the same methods mentioned above.

From the results obtained it was observed that the supernatant could demonstrate inhibitory activity against *C. jejuni, B. cereus, P. aeruginosa, S. enterica* and *H. influenza,* when tested by agar well diffusion assay (Fig. 4). Further, the pathogenic cultures were also tested for growth inhibition by addition of *B. licheniformis* culture extract containing prospective bioactive substances in BHI broth (liquid Assay) and measuring growth in terms of absorbance using a UV Spectrophotometer at 600nm. Results obtained for growth is presented in (Table 1) and (Fig. 6). From the results it was inferred that there was significant growth inhibition by *B.licheniformis* culture extract indicating potential bioactivity against human pathogens. However other methods of bioassays cross streaking against the pathogens as well as the agar over lay method did not show any satisfactory results for bioactivity against the tested pathogens.

Antibiotic comparison with *B. licheniformis* KSWD3 supernatant

The antibiotic activities of *B.licheniformis* KSAWD3 against *C. jejuni, S. enterica, H. influenza*e and *P. aeruginosa* were compared with the activity of well known antibiotics against the same strains, antibiotic disks were tested for halo zone around them on the BHI Agar medium plates cultured with the target pathogens, results are presented in (Table 2).

DISCUSSION

In the past, it was presumed that the marine environment was a "desert" with scarcity of life forms[4] However, it is now clear that the oceans are thriving with tremendous diversity of living microorganisms, with cell counts of 10^6–10^9 cells per milliliter[5, 6] and levels of species diversity and richness predicted to exceed many of the Earths rainforests[7-10]. This microbial diversity is presumed to translate into metabolic diversity resulting in the potential for new bioactive molecules to be discovered. In the present study thus a large number of isolates with prospective bioactivity was isolated from marine sediments, sea water and leaf litter samples collected from mangrove areas of marine environments of Arabian coastal environments in Tarut Island, Al-Qatif and Dammam, Saudi Arabia. All of these isolates were subjected to cross streak assay against known pathogens obtained from ATCC and from Military Hospital in Riyadh. Cross streaking assay did not give satisfactory results since all the cultures

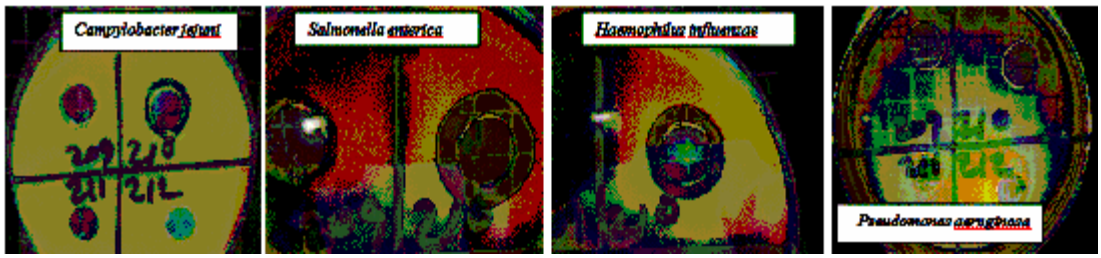


Fig.4. Bioactivity of substance extracted from *B. licheniformis KSADW3* (code No.210) against the pathogens

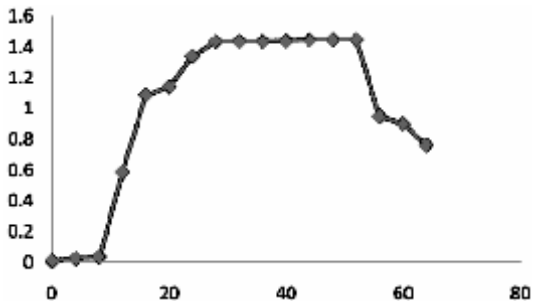


Fig. 5. Growth Curve of *B. licheniformis* KSADW3 recorded during growth in broth

recorded inhibitory zones in the range of 2-3 mm. All the isolates showed very small halo zones around the cross point and the results were not satisfactory. During the process of bioactivity evaluation of the extracts it was noted that cross streaking of standard strains against test isolates obtained from environment showed poor inhibition of growth by standard strains probably due to the inadequacy of the nutrients in the cultivation medium and the differential incubation temperature for the respective isolates of marine origin which grew well at 28°C and the pathogens of human

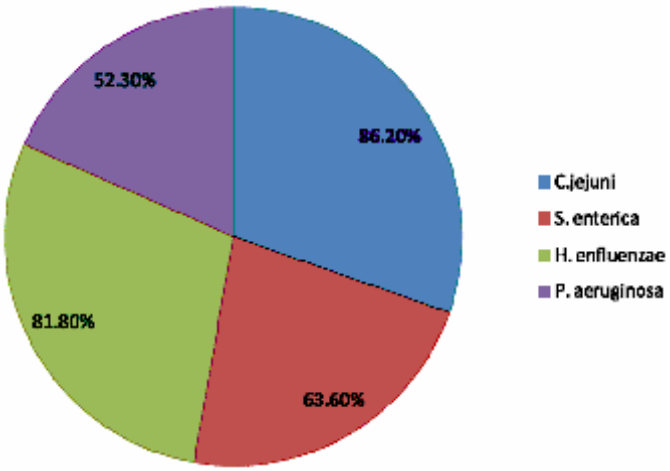


Fig. 6. Inhibition of growth of the pathogens with the bioactive substance extracted from *B. licheniformis KSAWD3* in broth (expressed as percentage)

origin which required 37°C. Likewise during the process of testing bioactivity of the culture extract by agar well diffusion technique there was very clear inhibition zone around the agar well. Hence the agar well diffusion assay was performed to evaluate the potential of the cell free extract to inhibit the pathogens during growth. The agar well diffusion assay yielded satisfactory results and 47 isolates could show inhibition zones around the wells indicating strong bioactivity. The bioactivity test was repeated to confirm their activity. All of them showed inhibition activity (inhibition zones varying from 4mm to 10 mm in diameter around the tested colonies) confirming their potential for production of bioactive molecules.

The current classification of species within the genus *Bacillus* and related genera is well established and is based on a combination of numerous experimental approaches[11]. Systematic studies of the *Bacillus* group have typically focused on terrestrial isolates, even though marine Bacilli are noted for their ability to produce different biologically active compounds[12] although the vast majority of bacterial diversity inhabiting marine sediments appears to be Gram-negative, there is evidence to suggest that Gram-positive bacteria comprise a relatively large proportion of these communities[13]. Most of the bacilli of marine origin belonged to the species *B. subtilis*, according to their phenotypic characteristics, antibiotic susceptibility profiles, and fatty acids patterns[12]. While phenotypic characteristics used for their identification are affected by physiological factors or are not sufficiently sensitive to distinguish between species, fatty acid composition, genetic transformation, DNA–DNA hybridization data and molecular methods based on polymerase chain-reaction (PCR) have been increasingly used in microbial taxonomy[14]. This has been particularly true for the genus *Bacillus*, which has undergone extensive taxonomic restructuring in recent years[15]. Comparisons of 16S rDNA sequences are one of the most powerful tools for the classification of microorganisms[16]. Although the presence of highly conserved sequences in this gene sometimes does not permit the discrimination among closely related species[17] several *Bacillus* species were reclassified based on the alignment of these sequences. Several species resembling *B. subtilis* have been described over the last 15 years, *B. amyloliquefaciens*, *B. atrophaeus*, *B. mojavensis*, *B. vallismortis*, *B. subtilis subsp. subtilis* and *B. subtilis subsp. spizizeni*, *B. tequilensis* and *B. velezensis*[14, 18], Recent studies on marine bacilli showed that strains of *B. marinus*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. cereus* and *B. myicoides* are common inhabitants of the marine habitat[12]. In this study, conventional biochemical tests and partial 16S rRNA gene sequencing were applied in the identification of marine isolates. The strain was identified as *B. licheniformis* based on its morphological, biochemical, physiological, characteristics and gene sequence of 16sRNA.

CONCLUSION

The strain (*KSADW3*) selected as potential strain, and identified as *B. licheniformis* showed strong activity against common well known antibiotics as well as the pathogens *C. jejuni, S. enterica,* and *H. influenzae* and *P. aeruginosa*. These observations suggest that marine *B. licheniformis KSAWD3* has potential for isolation and use of antibiotic molecule and subsequent use as drug. The results obtained during the course of the present study strongly indicated the potentials of marine bacteria for deriving potential antibiotic biomolecules active against clinical human pathogens at comparable and at enhanced levels when compared with commercially available antibiotics.

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