Saudi Journal of Biological Sciences 25 (2018) 1743–1754

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Phylogenetic affiliation and determination of bioactive compounds of bacterial population associated with organs of mud crab, *Scylla olivacea*

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ARTICLE INFO

Article history:
Received 24 July 2018
Revised 25 August 2018
Accepted 25 August 2018
Available online 27 August 2018

Keywords:
Scylla olivacea
16S rRNA gene
Modular polyketide synthase (PKSI)
Cytochrome P450 hydroxylase(CYP)
Non-ribosomal peptide synthetase (NRPS)

ABSTRACT

Mud crab belongs to the genus *Scylla* is an economically valuable and preferred species for costal aquaculture in Asian countries, including India. In recent years, there has been a tremendous expansion of *Scylla* farming, which has led to increasing research on its habit and habitats. However, there has been no study undertaken to understand the role of the bacterial population associated with the different organs of the mud crab, *Scylla olivacea*. In total, 43 isolates were recovered from four selected parts of the crab (carapace, n = 18; abdomen n = 11; leg, n = 8; and hand, n = 6), and the 16S rRNA gene was used to identify the bacterial isolates. The antimicrobial potential along with the detection of modular polyketide synthase (PKSI), cytochrome P450 hydroxylase (CYP) and non-ribosomal peptide synthetase (NRPS) gene clusters were investigated to show a relationship among the biosynthetic genes with their useful aspects. Additionally, the potential three strains (BPS_CRB12, BPS_CRB14 and BPS_CRB41), which showed significant antimicrobial activities, also showed the presence of twenty volatile compounds (VOCs) using GC–MS analysis. We conclude that the strain *Aneurinibacillus aneurinilyticus* BPS_CRB41 could be source for the production of bioactive compounds.
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1. Introduction

Scylla olivacea (Crustacea; Brachyura) is an economically important species, prevalent in the Southeast Asian region. It belongs to the genus *Scylla*, comprised of three species, which are also predominant on the Indian coasts and one sub-species (*Scylla serrata* var. *paramamosain*) (Estampador, 1949). *Scylla* species are commercially valuable and preferred species for coastal aquaculture (Keenan et al., 1998). Crabs are a rich source of vitamin B_2 and have high amounts of calcium and phosphorus that can play vital role in the formation of red blood cells and remain the bones, blood vessels, nerves and the immune system healthy. In crabs, a high amount of selenium has been reported, which act as vital role in the human's antioxidant defense system. At the same time, crab meat has low fat and contains quantifiable amounts of Omega-3 polyunsaturated acids, which provides protection from heart disease and aids brain development (Kim et al., 2012; Chun et al., 2009).

Aquatic animals, as demonstrated by several researchers, are commercially useful in different ways. Research on the antimicrobial activity of the hemolymph of fresh water crab *Oziotelphusa senex senex* showed a strong response against clinical pathogens, which confirms the immune mechanisms of fresh water crabs and would be a potential place for the improvement of novel antimicrobials (Sumalatha et al., 2016). At the same time, the intestinal bacteria based on antimicrobial potential was obtained from freshwater fish also showed significant antimicrobial activity against eighteen targeted pathogens, which further suggests that the intestinal bacteria can fight against pathogenic bacteria to protect the host fish at some extent (Miranda et al., 2013). The study of

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2018.08.025
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hemolymph obtained from *Charybdis lucifera* has shown its antimicrobial potential against bacteria and pathogenic fungal strains, and this serves as a baseline source of data that may confirm that crab hemolymph is indeed a promising source of compounds with biological activities (Rameshkumar et al.,2009). The speedy growth of antibiotic resistance and the emergence of new infectious diseases lead to the identification of unique sources for the discovery of novel antimicrobials. Boman (1995) reported that antimicrobial peptides having ability to protect a many animal species.

Several antimicrobial peptides were obtained from crab species over the past several years. Among them, proline peptide of 6.5 KDa was the first antimicrobial peptide obtained from hemocytes of the shore crab *Carinus maenas* (Ai et al., 2009), followed by the 3.7KDa cationic antimicrobial peptide, Callinecin, which was recovered from *Callinectes sapidus* (Ma et al., 2010). Recently, an anionic antimicrobial peptide, scygonadin, was obtained from a mud crab *Scylla serrata* (Spersad et al., 2009). Hence, this study was planned to explore the locally available edible mud crab, *Scylla olivacea,* for the isolation of the bacterial population associated with different parts of the crab and to check for their biosynthetic potential, which might be considered an alternative natural resource for antimicrobial compounds production.

2. Materials and methods

2.1. Collection of crabs

Fresh crabs were collected randomly from Lengte, Mamit district of Mizoram (23°46′19.7″N 92°35′56.6″E), Northeast India. The samples were kept in sterile Hi-media bags, carried into the Molecular Microbiology and Systematic laboratory and immediately processed to estimate the bacterial population.

2.2. Isolation of bacteria from different parts of crab

The collected crabs were cleaned in continuous tap water to remove unwanted debris and organic material. The crab was dissected into defined parts (i.e., abdomen, carpace, leg and hand), and the individual parts were put together and ground with a mortar and pestle. Two grams of each part were grinded with 10 ml of 10 mM potassium phosphate buffer. One hundred microlitre (μl) of homogenate was spread on different nutritional media. The plates were incubated at 37 °C for 24 h to observe the bacterial colonies. Clearly distinguished isolated single colonies were picked up and were re-streaked on respective media to obtain pure isolates. The pure culture was stored on agar test tube slants at 4 °C.

2.2.1. Isolation media

Five specific nutritional media were used to obtain the high number of bacteria. The used media were as follows: (1). Nutrient agar (peptone, beef extract/yeast extract, NaCl, agar); (2). Actinomycetes isolation agar (sodium caseinate, L-asparagine, sodium propionate, dipotassium phosphate, magnesium sulfate, ferrous sulfate, agar); (3). Tryptic soya agar (casein peptone, sodium chloride, soy peptone, agar); (4). Tap water yeast extracts agar (tap water, dipotassium phosphate, yeast extract, agar); and (5). Starch casein agar (casein peptone, starch, sea water, agar).

2.3. Molecular identification of obtained bacterial isolates

2.3.1. DNA isolation, amplification of 16S rRNA gene and phylogenetic analysis

Total genomic DNA was extracted using the bacterial DNA Purification Kit (In-vitrogen, Life technologies KT-110052) as per the manufacturer's instructions. Isolated bacterial DNA was used to amplify the16S rRNA gene as per Cui et al. (2001). The amplified products of PCR were cleaned using a QIA quick gel extraction kit (Qiagen, Hilden, Germany) and sequencing was done commercially.

The obtained sequences were compared with the NCBI database and showing similarity percentage of 97–100% with their closest strains. The sequences were deposited to NCBI GenBank and phylogenetic tree was constructed using lowest BOC and AIC values using MEGA6, and the phylogenetic tree was evaluated based on bootstrap data with 1000 replicates (Saitou and Nei, 1987; Felsenstein, 1985).

2.4. Antimicrobial activity using agar well diffusion method

The antimicrobial screening was carried out against the bacterial pathogens *Staphylococcus aureus* (MTCC-96), *Pseudomonas aeruginosa* (MTCC-2453), *Escherichia coli* (MTCC-739), *Micrococcus luteus* (MTCC-7950), *Bacillus subtilis* (MTCC-2057) and *Candida albicans* (MTCC-3017) using agar well diffusion procedure as per Saadoun and Muhana (2008) & Zothanpuia et al. (2016).

2.5. Biosynthetic gene amplification (PKS type II; NRPS and CYP)

PKS typeII:

Polyketide synthase (PKS) type II gene was amplified using KS∞ Forward 5/-TSGCSTGCTTGGAYGCSATC-3′ and KSβ Reverse 5′-TGG AANCCGCCGAABCCTCT-3′ primers according to Ayuso-Sacido and Genilloud (2005).

NRPS gene:

Non-ribosomal peptide synthase (NRPS) gene was amplified using NRPS forward (5′-GCSTACSYSATSTACACSTCSGG-3′) and NRPS reverse (5′-SASGTCVCCSGTSCGGTAS-3′) primers as per Meng Yuan et al. (2014).

CYP gene:

Cytochrome p450 (CYP) gene was amplified using CYP forward (5′-TGGATCGGCGACGACCGSVYCGT-3′) and CYP reverse (5′-CCGW ASAGSAYSCCGTCGTACTT-3′) primers as described by Meng Yuan et al. (2014).

2.5.1. Phylogenetic analysis of biosynthetic genes (PKS type II; NRPS and CYP)

The biosynthetic genes sequences were compared with similar sequence found from NCBI database using the protein BLAST (Tamura et al., 2011), and a multiple sequence alignment was carried out on all the gene sequences with the Clustal W software packaged in MEGA 5.05 (Thompson et al., 1997). For PKS type II, the evolutionary model was selected based on the lowest BIC value (4942.558) and highest AIC value (4839.212) using MEGA 5.05 (Saitou and Nei, 1987). The transition and transversion bias ratio was 1.54. The maximum log likelihood for the substitution computation was −2404.648. The phylogenetic tree was constructed by the maximum likelihood method using MEGA 5.05 software with Kimura 2-parameters model (Tamura et al., 2011; Saitou and Nei, 1987). Similarly, for the NRPS gene, the evolutionary model was selected based on the lowest BIC value (4443.679) and highest AIC value (4336.327) using MEGA 5.05 (Saitou and Nei, 1987). The transition and transversion bias ratio was 1.24. The maximum log likelihood for substitution computation was −2170.530. The phylogenetic tree was constructed by maximum likelihood method using MEGA 5.05 software with the Hasegawa Kishino Yano model (Tamura et al., 2011; Saitou and Nei, 1987). Further, for the CYP gene, the evolutionary model was again selected based on the lowest BIC value (2381.152) and highest AIC value (2248.220) using MEGA 5.05 (Saitou and Nei, 1987). The transition and transversion bias ratio was 1.58. The maximum log likelihood for the substitution computation was −1101.948. The phylogenetic tree was

constructed by the maximum likelihood method using MEGA 5.05 software with Kimura 2-parameters model (Tamura et al., 2011; Saitou and Nei, 1987).

2.6. Detection of volatile compounds (VOCs) using GC–MS analysis

The potential isolates based on antimicrobial activity was performed to detect and identified volatile compounds using GC–MS according to Ser et al. (2015) and Sharma et al. (2016). The peaks were matched with NIST, library.

3. Results and discussion

Totally, 43 bacterial isolates were obtained and were identified from different parts of *S. olivacea*. The crab was collected from Lengte, Mamit district of Mizoram, North East India, and its morphological characteristics were recorded and documented (Fig. S1). Different parts of the Crab- Carpace, Abdomen, Leg and Hand were taken for study. The evenness value and Berger-Parker index were similar in all parts of the crab (1.0). The range of Fisher's alpha and the Menhinick index in all the parts of the crab was 0.2357–0.4082 and 0.2263–0.3426, respectively. From the total isolates, 41.8% of the bacterial population was obtained from carapace, followed by abdomen (26.8%), leg (19.5%) and hand (14.6%). Similar statement reported by Kim et al. (2013), who described that 67% of *Bacillus* sp. was available in the heart, although 33% was obtained in the gill and carapace in *C. japonicas*.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.sjbs.2018.08.025.

Bacteria were isolated from all five selected isolation media, although 21 strains were obtained from TSA, 11 from AIA, 5 from TH_2O, 4 from SCA and 2 strains by using NA media. These results showed that TSA was the most suitable medium for the recovery of the bacterial population, followed by AIA and TH_2O. This result was likewise accounted by Kim et al. (2013), who stated that TSA and NA has the utmost media for the isolation of bacteria from the *C. opilio* snow crab. Moreover, Saha and Santra (2014) reported that 66.6% of the bacterial population was recovered from NA media.

To assign the taxonomic position, the obtained 16S rRNA gene sequences were clustered by nucleotide BLAST. All the strains were compared with the type strains obtained from the NCBI GenBank. The results revealed that all the microbes were grouped into six families and seven taxonomic groups: *Bacillus* sp. (n = 17; 39.5%), followed by *Staphylococcus* sp. (n = 8; 18.6%), *Pseudomonas* sp. (n = 6; 13.9%), *Enterobacter* sp. (n = 4; 9.3%), *Aeromonas* sp. (n = 3; 6.9%), *Alcaligenes* sp. (n = 3; 6.9%) and *Acinetobacter* sp. (n = 2; 4.6%) (Table 1). The evolutionary tree exhibited 98–100% identical

Table 1
Identification of bacterial strains based on 16S rRNA gene sequences similarity with closest type strains from Eztaxon database.

Sl. No	Strain name	Isolate name	Accession No	Similarity strain	Identity	Biosynthetic Genes		
						PKSII	NRPS	CYP
1	CRB 1	*Acinetobacter* sp.	MF421767	*Acinetobacterdispersus* ANC 4105	99.51%	–	–	–
2	CRB 2	*Acinetobacter* sp.	MF421768	*Acinetobacterseifertii* NIPH 973	100%	–	–	–
3	CRB 3	*Enterobacter* sp.	MF 421769	*Enterobacter aerogenes* KCTC 2190	99.80%	–	–	–
4	CRB 4	*Enterobacter* sp.	MF421770	*Enterobacter aerogenes*KCTC2190	99.77%	–	–	–
5	CRB 5	*Enterobacter* sp.	MF421771	*Enterobacter xiangfangensis* 10–17	99.77%	–		–
6	CRB 6	*Bacillus thuringiensis*	KX369561	*Bacillus thuringiensis* ATCC 10792	98.81%	+	–	–
7	CRB7	*Enterobacter cloacae* subsp. *cloacae*	MF 421772	*Enterobacter cloacae* subsp. *cloacae* ATCC 13047	99.54%	–	–	–
8	CRB 8	*Alcaligenes* sp.	MF 421773	*Alcaligenes faecalis* subsp. *faecalis* NBRC 13111	99.24%	–	–	–
9	CRB 9	*Alcaligenes faecalis*	MF421774	*Alcaligenes faecalis* subsp. *faecalis* NBRC 13,111	100%	–	–	–
10	CRB 10	*Alcaligenes* sp.	MF 421775	*Alcaligenes faecalis* subsp. *faecalis* NBRC 13111	100%	–	–	–
11	CRB 11	*Lysinibacillus sphaericus*	KX369562	*Lysinibacillus sphaericus* KCTC 3346	98.50%	–	+	+
12	CRB 12	*Bacillus* sp.	KX369563	*Bacillus cereus* ATCC 14579	99.03%	+	+	+
13	CRB 13	*Bacillus* sp.	KX369564	*Bacillus thuringiensis* ATCC 10792	99.11%	+	–	–
14	CRB 14	*Bacillus anthracis*	KX369565	*Bacillus anthracis* ATCC 14578	98.47%	+	+	+
15	CRB 15	*Pseudomonas stutzeri*	MF 421776	*Pseudomonas stutzeri* ATCC 17588	94.27%	–	–	–
16	CRB 16	*Pseudomonas alcaligenes*	MF 421777	*Pseudomonas mendocina* NBRC 14162	99.62%	–	–	+
17	CRB 17	*Bacillus cereus*	KX369566	*Bacillus cereus* ATCC 14579	99.86%	+	–	+
18	CRB 18	*Bacillus cereus*	KX369567	*Bacillus thuringiensis* ATCC 10792	99.27%	+	+	–
19	CRB 19	*Pseudomonas* sp.	MF 421778	*Pseudomonas baetica* a390	99.54%	–	–	+
20	CRB 20	*Pseudomonas aeruginosa*	MF 421779	*Pseudomonas aeruginosa* JCM 5962	99.73%	–	–	–
21	CRB 21	*Pseudomonas fulva*	MF 421780	*Pseudomonas parafulva* NBRC 16636	99.89%	–	–	–
22	CRB 22	*Pseudomonas* sp.	MF 421781	*Pseudomonas koreensis* Ps 9–14	99.83%	–	+	+
23	CRB 23	*Lysinibacillus* sp.	KX369568	*Lysinibacillus fusiformis* NBRC 15717	99.67%	–	–	–
24	CRB 24	*Bacillus cereus*	KX369569	*Bacillus cereus* ATCC 14579	100%	–	–	–
25	CRB 25	*Lysinibacillus sphaericus*	KX369570	*Lysinibacillus sphaericus* KCTC 3346	98.39%	–	–	–
26	CRB 26	*Aeromonas hydrophila*	MF 421782	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	100%	–	–	–
27	CRB 27	*Aeromonas* sp.	MF 421783	*Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658	99.50%	–	–	–
28	CRB 28	*Aeromonas* sp.	MF 421784	*Aeromonas sobria* ACC 43979	99.09%	–	–	–
29	CRB 29	*Staphylococcus* sp.	MF 421785	*Staphylococcus pasteuri* ATCC 51129	94.29%	–	–	–
30	CRB 30	*Staphylococcus* sp.	MF 421786	*Staphylococcus hominis* subsp. *hominis* DSM 20328	99.46%	–	+	–
31	CRB 31	*Bacillus anthracis*	KX369571	*Bacillus anthracis* ATCC 14578	99.89%	+	–	–
32	CRB 32	*Bacillus anthracis*	KX369572	*Bacillus anthracis* ATCC 14578	100%	+	–	–
33	CRB 34	*Staphylococcus* sp.	MF 421787	*Staphylococcus hominis* subsp. *hominis* DSM20328	99.54%	–	–	–
34	CRB 35	*Bacillus* sp.	KX369574	*Bacillus anthracis* ATCC 14,578	100%	–	–	–
35	CRB 36	*Staphylococcus aureus* subsp.*aureus*	MF 421788	*Staphylococcus aureus* subsp. *aureus* DSM20231	99.81%	–	–	–
36	CRB 37	*Staphylococcus condimenti*	MF 421789	*Staphylococcus condimenti* DSM 11674	100%	–	–	–
37	CRB 38	*Staphylococcus* sp.	MF 421790	*Staphylococcus haemolyticus* MTCC 3383	99.87%	–	–	–
38	CRB 39	*Staphylococcus carnosus*	MF 421791	*Staphylococcus carnosus* subsp. *carnosus* ATCC 51365	100%	–	–	–
39	CRB 40	*Staphylococcus capitis*	MF 421792	*Staphylococcus capitis* subsp. *capitis* ATCC 27840	99.89%	–	–	–
40	CRB 41	*Aneurinibacillus aneurinilyticus*	KX369575	*Aneurinibacillus aneurinilyticus* ATCC 12856	97.05%	+	+	+
41	CRB 42	*Aneurinibacillus* sp.	KX369576	*Aneurinibacillus aneurinilyticus* ATCC 12,856	99.88%	–	–	–
42	CRB 43	*Lactobacillus* sp.	MF 421793	*Lactobacillus paraplantarum* DSM 10667	100%	–	–	–
43	CRB 44	*Lactobacillus* sp.	MF 421794	*Lactobacillus nagelii* DSM 13675	99.75%	–	–	–

similarity with their strain types. The NCBI accession numbers of obtained sequences were as follows: KX369561-KX369572 (12), KX369574-KX369576 (03) and MF421767-MF421794 (28) respectively. The phylogenetic tree was build using the neighbor-joining method with the Kimura 2-model (K2 + G) (Fig. 1). The maximum log likelihood for the substitution matrix computation estimate was −2987.162.

The phylogenetic tree exhibited that gram-positive bacterial strains were divided into different clades with a bootstrap value of 82%. All the *Staphylococcus* species were closed with their reference types obtained from the EzTaxon databases with a bootstrap of 100%. Similarly, all the *Bacillus* species were closed with their reference strains under a bootstrap value of 100%, whereas a few genera, such as *Lactobacillus*, *Lysinibacillus* and *Aneurinibacillus,* were closely clustered separately from their reference strains. In the gram-negative bacteria, the phylogenetic tree was build based on the neighbor-joining method with the Kimura 2-model (K2 + G) (Fig. 2). The maximum log likelihood for the substitution matrix computation estimate was −1449.670. The phylogenetic tree exhibited gram-negative bacterial strains divided into two clades (clade I and clade II) with a bootstrap value of 71%. In clade I, all the strains were divided again into two clades (clade IA and clade IIB). In clade IA, all the *Pseudomonas* sp. and *Acinetobacter* sp. were clustered together with their strain types with a bootstrap supported value of 63%, whereas in clade IB, all the *Aeromonas* sp. and *Enterobacter* sp. were clustered together with their strain types under a bootstrap supported value of 50%. In clade II, *Alcaligenes* sp. strain BPS CRB8, *Alcaligenes faecalis* strain BPS CRB9 and *Alcaligenes*sp. Strain BPS CRB10 was clustered together with the strain type *Alcaligenesfaecalis*subsp. *Faecalis* NBRC-13111 under a bootstrap supported value of 98%. Here, *Escherichia coli* ATCC-11775 and *Nocardiopsis dassonvillei* subsp. *albirubida* DSM-40465 were used as the out group in the phylogenetic trees.

All the bacterial microbes were grouped into six families and seven genera, which showed a good relationship between the bacterial populations associated with crab. In our study, *Bacillus* was the dominant genus, followed by *Staphylococcus*, *Pseudomonas*, *Enterobacter*, *Aeromonas*, *Alcaligenes* and *Acinetobacter.* Similarly, Sivasubramanian et al. (2017) reported that *Bacillus* was the most familiar and dominant genus, which was found in all observed crabs, whereas some other genera, such as *Bacteroides*, *Acinetobacter*, *Flavobacterium*, *Chryseobacterium* and *Porphyrobacter,* were available in crab guts. Moreover, few other bacterial genera, such as *Pseudomonas*, *Aeromonas*, *Alcaligenes*, *Photobacterium*, *Vibrio*, *Enterobacter* and *Staphylococcus,* were found in crab gut (Sivasubramanian et al., 2017). The crab gut microbial belongs to gram-positive bacteria, such as *Bacillus*, *Micrococcus*, *Corynebacterium,* and gram-negative bacteria, such as *Pseudomonas*, *Vibrio*, *Flavobacterium* and few under the family *Enterobacteriaceae*, which were reported by Soundarapandian and Sowmiya (2013). Many previous researchers state that Proteobacteria and Firmicutes are greatest phyla in the gastro-intestines of insect gut (Harkin et al., 2014; Sathiyamurthy et al., 1990; Sivasubramanian et al., 2017). In our study, Firmicutes was abundant phylum (n = 25; 58.2%),

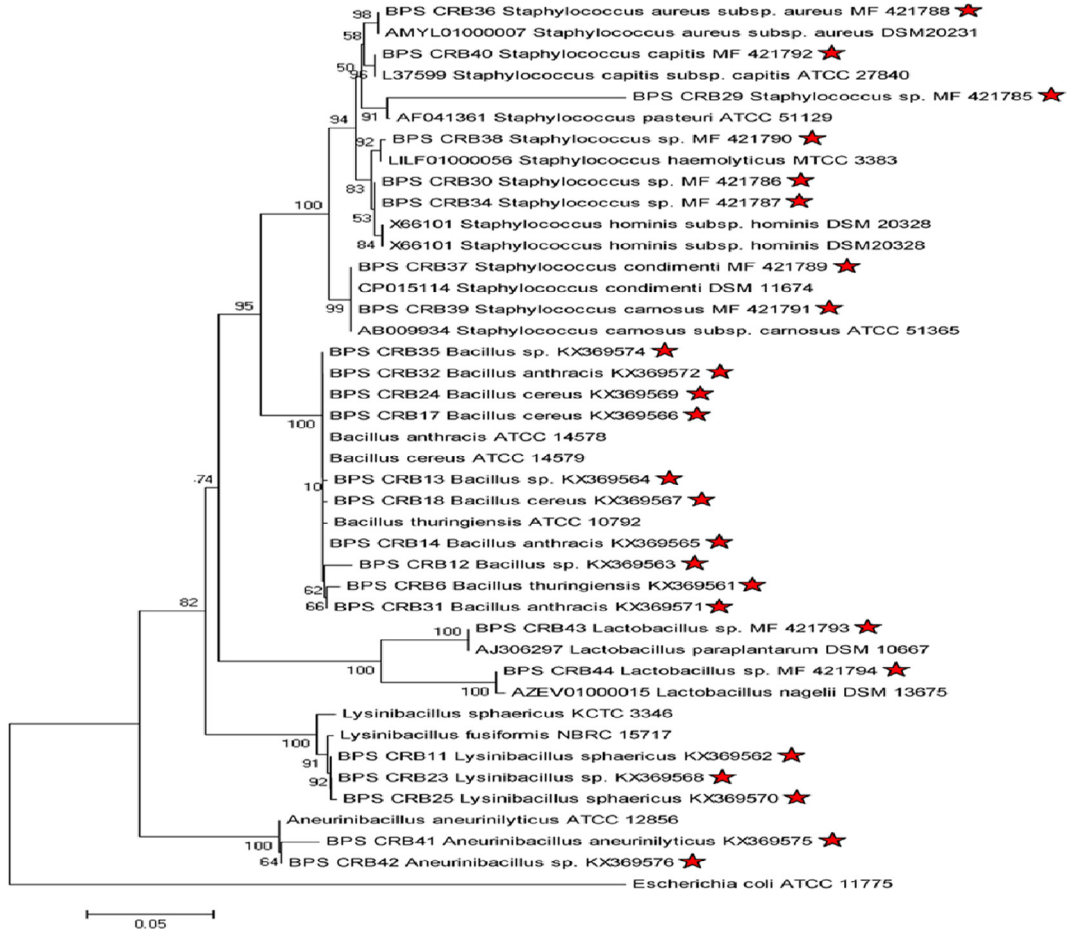


Fig. 1. Phylogenetic relationship based on partial 16S rRNA gene sequence of the crab bacterial isolates with strain types retrieved from the EZ-Taxon database. The phylogenetic tree was constructed using the neighbor-joining method with the Kimura 2-parameter model (K2 + G) in gram-positive bacteria with a bootstrap supported value based on 1000 replicates.

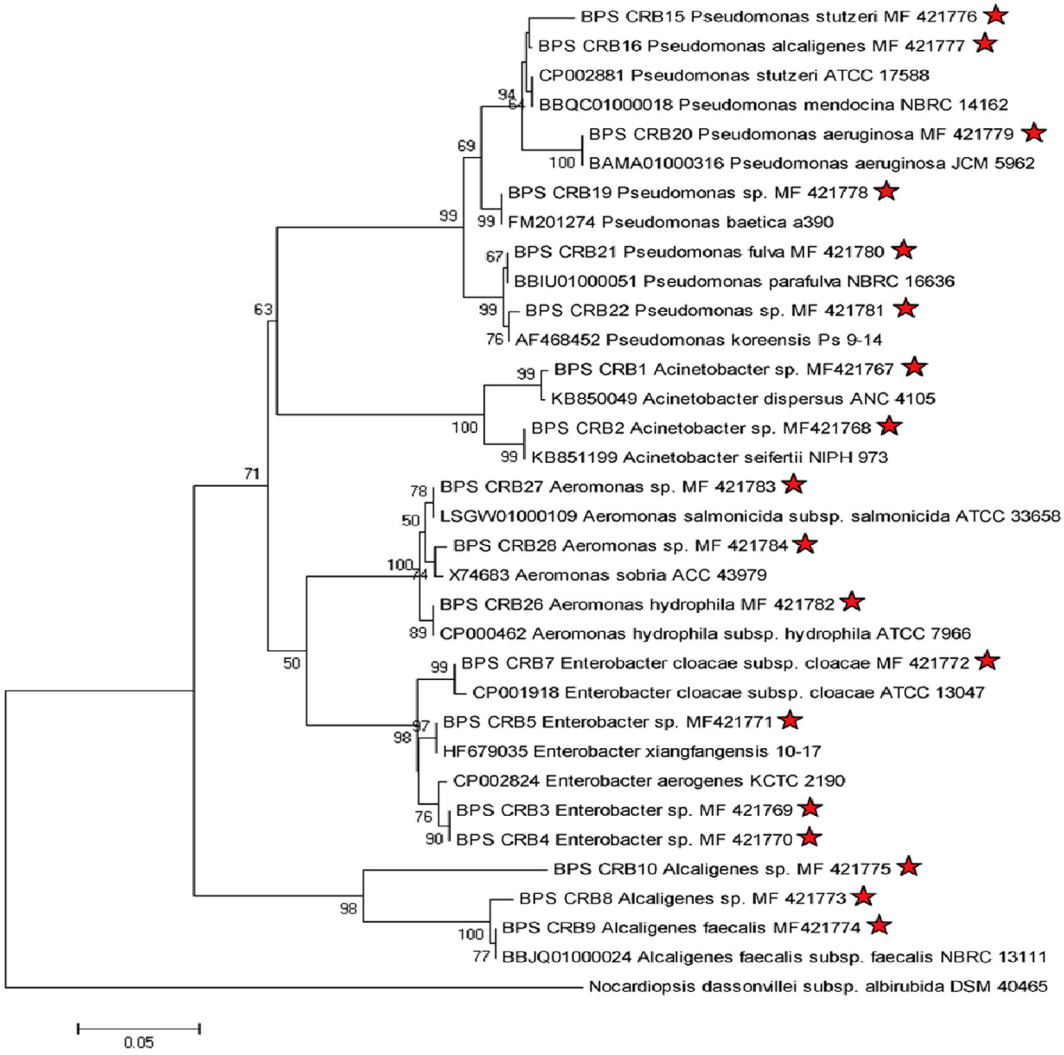


Fig. 2. Phylogenetic relationship based on the partial 16S rRNA gene sequence of crab bacterial isolates with type strains retrieved from the EZ-Taxon database. The phylogenetic tree was constructed using the neighbor-joining method with the Kimura 2-parameter model (K2 + G) in gram-negative bacteria with a bootstrap supported value based on 1000 replicates.

followed by Proteobacteria (n = 18; 41.8%). Similarly, Firmicutes was rich phylum, subsequently Proteobacteria and Bacteroidetes from the gut samples of crab (Liu et al., 2011; Venkateswaran et al., 1981; Harkin et al., 2014).

Approximately 60% of the strains exhibited antimicrobial potential against two or more pathogens. Out of the 43 isolates, 72.1% of the strains exhibited antimicrobial action against *P. aeruginosa*, while 55.8%, 51.2% and 41.6% of the strains showed activity against *B. subtilis*, *E. coli* and *S. aureus,* respectively. *Bacillus* sp. strain BPS_CRB12 showed maximum activity against *S. aureus* (9.5 mm) and *E. coli* (12.0 mm), whereas the *Bacillus anthracis* strain BPS_CRB14 was found to have maximum antimicrobial activity against *C. albicans* (11.0 mm) and *B. subtilis* (10.0 mm). At the same time, the *Aneurinibacillus aneurinilyticus* strain BPS_CRB41 had the highest antimicrobial activity against *P. aeruginosa* (10.5 mm) and *M. luteus* (8.5 mm) (Table2). The findings of the zone of inhibition were similarly reported by Perez et al. (1992). Further, *Bacillus* sp. strain BPS_CRB 12 revealed the maximum antimicrobial activity beside *S. aureus* and *E. coli,* which was supported by Munoz-Atienza et al. (2013), who stated that 20% of the bacteria have antimicrobial activity against three of the eight tested bacterial pathogens. *Bacillus anthracis* strain BPS_CRB14 exhibited significant antimicrobial activity (Haber and Ilan, 2013; Crawford et al., 2009; Kim et al., 2015; Addae et al., 2014; Athamna et al., 2004).

To develop new treatment methods and find new bioactive compounds that can fight against antibiotic-resistant bacteria, it is suggested that the target-based screening approach was not as useful as supposed. Hence, the "classical" bioactivity-based approach is considered the best technique to isolate and identify new bioactive compounds from unique sources, which might be useful in fighting against multi-drug-resistant pathogens. Newman and Cragg (2012) proved that 80% of all the antimicrobial drugs that are useful in the pharmaceutical industry are natural products obtained from microbial sources. A PCR-based technique for the presence of biosynthetic genes producing bioactive secondary metabolites can be easily screened from potential isolates that can produce bioactive compounds (Yuan et al., 2014). Based on the antimicrobial activity, a PCR-based method was used to detect the existence of three biosynthetic genes: Polyketide syntheses type II (PKSII), Non-ribosomal peptide synthetase (NRPS) and Cytochrome P450 hydroxylase (CYP) genes. Three isolates, BPS_CRB12, BPS_CRB 14 and BPS_CRB 41, indicated positively amplified products of PKS type II, NRPS and CYP genes (Fig. S2). The accession numbers were deposited in NCBI GenBank: PKSII gene (MF871609 to MF871611), NRPS gene (MF871612 to

Table 2
Antimicrobial activity of the obtained bacterial isolates from different parts of crab.

Isolate	*B. subtilis*	*E. coli*	*C. albicans*	*P. aeruginosa*	*S. aureus*	*M. luteus*
BPS_CRB1	–	8.4 ± 0.05	–	8.4 ± 0.05	–	–
BPS_CRB2	–	10.0 ± 0.10	10.0 ± 0.10	9.0 ± 0.10	7.4 ± 0.05	10.4 ± 0.05
BPS_CRB3	–	9.0 ± 0.10	–	8.0 ± 0.05	–	–
BPS_CRB4	–	8.0 ± 0.05	–	9.0 ± 0.10	–	–
BPS_CRB5	7.4 ± 0.05	8.4 ± 0.05	–	9.0 ± 0.10	–	–
BPS_CRB6	8.4 ± 0.1	–	–	10.0 ± 0.10	8.0 ± 0.10	–
BPS_CRB7	*8.0 ± 0.11*	9.0 ± 0.10	–	8.5 ± 0.05	9.0 ± 0.10	–
BPS_CRB8	8.4 ± 0.05	–	–	9.0 ± 0.10	8.0 ± 0.05	–
BPS_CRB9	9.0 ± 0.11	9.0 ± 0.05	–	8.4 ± 0.05	9.0 ± 0.10	–
BPS_CRB10	7.4 ± 0.05	–	–	7.0 ± 0.10	9.4 ± 0.05	–
BPS_CRB11	–	–	–	8.0 ± 0.05	9.0 ± 0.10	7.4 ± 0.05
BPS_CRB12	–	8.0 ± 0.10	–	8.0 ± 0.10	9.0 ± 0.10	–
BPS_CRB13	–	–	–	7.4 ± 0.05	9.0 ± 0.10	7.4 ± 0.05
BPS_CRB14	–	8.4 ± 0.05	–	9.4 ± 0.05	9.0 ± 0.05	7.4 ± 0.05
BPS_CRB15	–	–	–	8.4 ± 0.05	9.0 ± 0.10	7.4 ± 0.05
BPS_CRB16	–	–	–	6.0 ± 0.05	–	8.0 ± 0.10
BPS_CRB17	–	–	7.4 ± 0.05	–	–	8.3 ± 0.01
BPS_CRB18	–	–	9.0 ± 0.1	9.0 ± 0.10	–	7.4 ± 0.05
BPS_CRB19	–	–	–	9.0 ± 0.05	–	8.0 ± 0.05
BPS_CRB20	–	–	7.4 ± 0.05	9.0 ± 0.05	–	–
BPS_CRB21	9.0 ± 0.11	–	8.4 ± 0.05	8.0 ± 0.05	–	–
BPS_CRB22	8.4 ± 0.05	–	–	9.0 ± 0.05	–	–
BPS_CRB23	8.0 ± 0.10	8.4 ± 0.05	7.4 ± 0.05	9.0 ± 0.05	–	–
BPS_CRB24	9.0 ± 0.11	8.0 ± 0.05	–	9.0 ± 0.05	–	–
BPS_CRB25	10.0 ± 0.10	–	8.0 ± 0.05	8.4 ± 0.05	–	–
BPS_CRB26	–	8.4 ± 0.05	–	9.0 ± 0.49	–	–
BPS_CRB27	–	9.0 ± 0.05	–	7.4 ± 0.05	–	–
BPS_CRB28	–	8.0 ± 0.05	–	9.0 ± 0.05	–	–
BPS_CRB29	–	8.0 ± 0.05	–	9.0 ± 0.05	–	–
BPS_CRB30	–	8.0 ± 0.05	–	8.4 ± 0.05	–	–
BPS_CRB31	7.4 ± 0.05	5.0 ± 0.05	–	–	8.4 ± 0.05	–
BPS_CRB32	7.4 ± 0.05	9.0 ± 0.05	–	–	9.0 ± 0.10	–
BPS_CRB34	7.4 ± 0.05	8.0 ± 0.10	–	–	9.0 ± 0.10	–
BPS_CRB35	8.4 ± 0.05	8.0 ± 0.80	–	–	9.0 ± 0.11	–
BPS_CRB36	7.4 ± 0.05	–	–	–	–	–
BPS_CRB37	7.0 ± 0.10	–	–	–	–	–
BPS_CRB38	7.0 ± 0.10	–	–	–	–	–
BPS_CRB39	7.4 ± 0.05	–	–	–	–	–
BPS_CRB40	–	–	–	–	–	–
BPS_CRB41	9.4 ± 0.05	10.0 ± 0.05	10.0 ± 0.11	8.4 ± 0.05	6.0 ± 0.11	7.4 ± 0.05
BPS_CRB42	6.4 ± 0.06		9.3.0 ± 0.5			4.5 ± 0.05
BPS_CRB43	–	8.0 ± 0.05	–	6.5 ± 0.05	–	
BPS_CRB44	7.0 ± 0.05	–	8.0 ± 0.11	–	9.0 ± 0.11	–

MF871614) and CYP gene (MF871615 to MF871617). Nucleotide sequences of the biosynthetic genes (PKS type II, NRPS and CYP) showed 92–100% similarity with the strain types obtained from the NCBI-BLASTn database. The tree was constructed using the nucleotide sequences of the PKS type II gene, which showed the *Bacillus* sp. strain BPS_CRB12, *Bacillus anthracis* strain BPS_CRB14 and *Aneurinibacillus* sp. BPS_CRB41 were closely related to the *Bacillus* sp. strain 9A clone 9A-5, *Bacillus anthracis* strain FDAARGOS_341 and *Aneurinibacillus* sp. strain XH2 with bootstrap supported values of 93%, 100% and 100%, respectively (Fig. 3A). Moreover, the NRPS gene sequences of *Bacillus* sp. strain BPS_CRB12, *Bacillus anthracis* strain BPS_CRB14 and *Aneurinibacillus* sp. BPS_CRB41, was clustered with *Bacillus* sp. strain 1s-1, *Bacillus anthracis* strain Tyrol 4675 and *Aneurinibacillus* sp. strain XH2 with bootstrap supported values of 100%, 78% and 100%, respectively (Fig. 3B). Similarly, the CYP gene sequences of the *Bacillus* sp. strain BPS_CRB12, *Bacillus anthracis* strain BPS_CRB14 and *Aneurinibacillus* sp. BPS_CRB41 were 98–100% similar with the *Bacillus* sp. strain 1s-1, *Bacillus anthracis* strain 14RA5914 and *Bacillus licheniformis* strain SRCM100027 with a bootstrap value of 90%, 59% and 49%, respectively (Fig. 3C).

Our findings state that PKS type II, NRPS and the CYP pathway may be extensive in the various crab gut intestine bacteria. Similarly Jami et al. (2015) also investigated the number of genes closely relation with secondary-metabolite biosynthesis, including PKS type II, NRPS and CYP genes, among the bacteria. Moreover, NRPS genes are occupied in the development of bioactive compounds and may help in quorum sensing (Passari et al., 2015). Additionally, the CYP gene can represent cytochrome P450 hydroxylase enzyme that can help for the synthesis of polyene antibiotics (Lee et al., 2006), also detected in the genera *Bacillus* and *Pseudomonas*. However, this is the first attempt antimicrobial biosynthetic (PKS type II, NRPS and CYP) genes have been detected in crab-associated bacteria. The potential strains could be useful for the production of novel compounds.

GC–MS is considered as the suitable methods to recognize the components of volatile compounds used by researchers (Jog et al., 2014; Sharma et al., 2016). Bacteria produced many volatile compounds, including alcohols, ketones, esters and derivatives (Kai et al., 2009). In the present study, a total of 20 volatile compounds were found in three methanolic extracts of the strains BPS_CRB12, BPS_CRB14 and BPS_CRB41 using GC–MS analysis. The methanolic extract of BPS-CRB12 detected two volatile compounds i.e. Di-n-octyl phthalate and 1-Bromo-3,7-Dimethyloctane. On the other hand, the strain BPS_CRB14 detected 11 volatile compounds (Benzaldehyde; 1H-Benzimidazole,1-Ethyl; Benzeneacetic Acid,4-(1 h-1,2,3,4-Tetrazol-1-Yl); Benzyl 2-(3-Aminopropionyloxy)Acetate; Benzene, (3-Chloro-3-Methylbutyl); Benzaldehyde, 4-Methyl-,

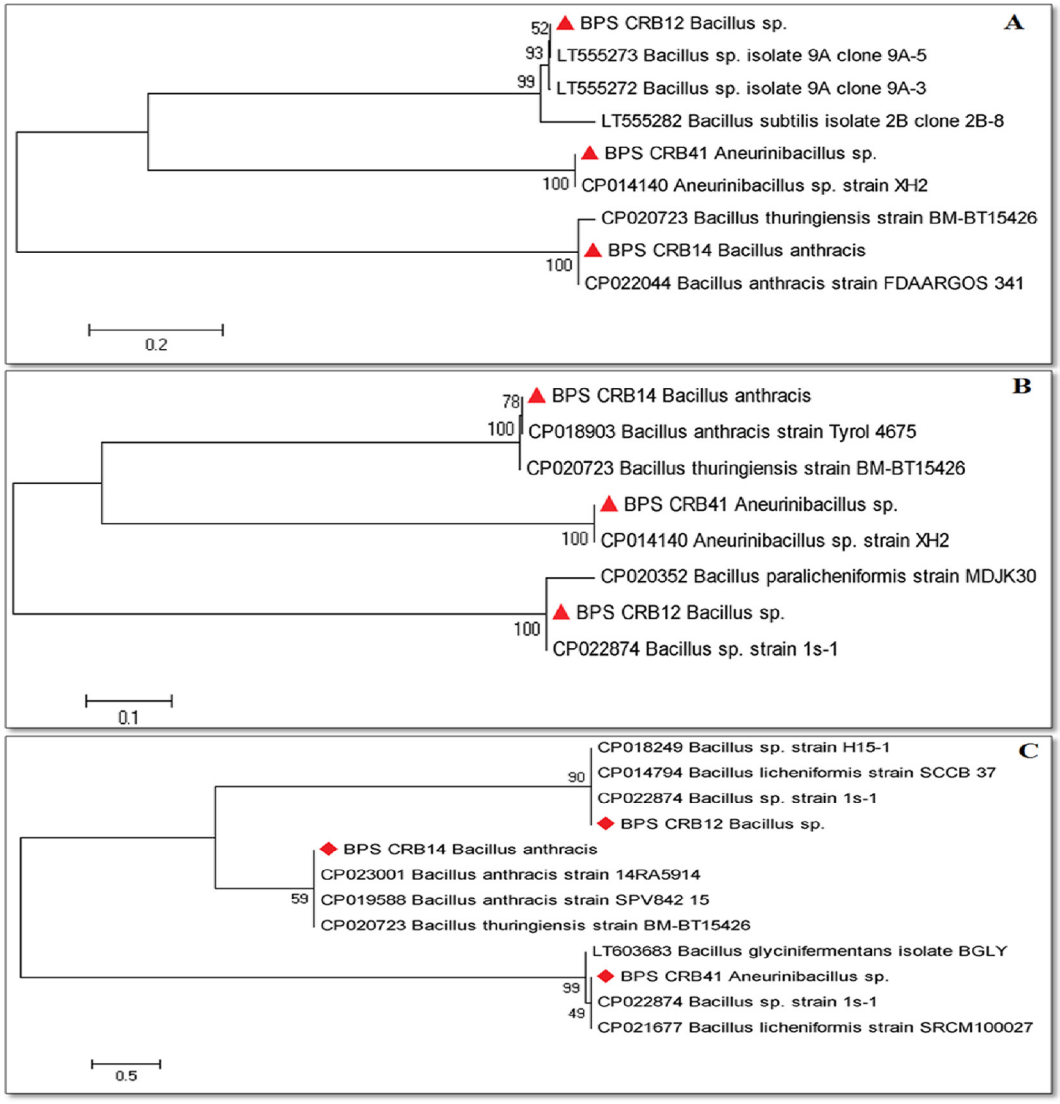
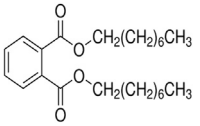
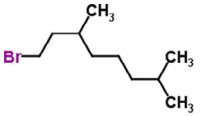
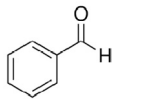
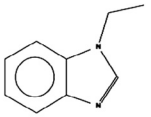
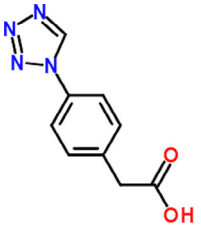
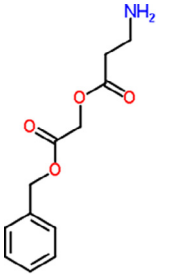
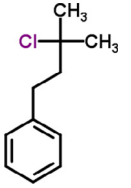


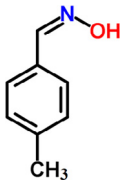
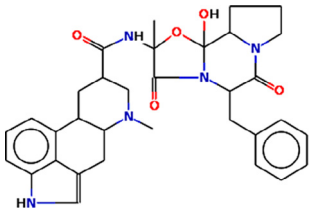
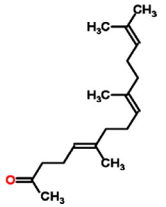
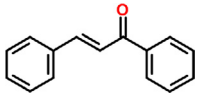
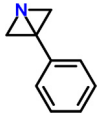
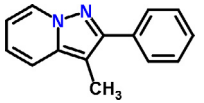
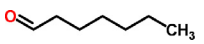
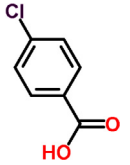
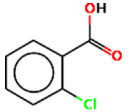
Fig. 3. Maximum likelihood tree based on: A, PKS type II gene sequences, B, NRPS gene sequences and C, CYP gene sequences, illustrating affiliations between *Bacillus* sp. strain BPS_CRB12, *Bacillus anthracis* strain BPS_CRB14 and *Aneurinibacillus* sp. strain BPS_CRB41, with strain types retrieved from the NCBI-BLASTn database. The bootstrap supported values were based on 1000 replicates.

Oxime; Ergotaman-3′,6′,18-Trione, 9,10-Dihydro-12′-Hydroxy-2′-Methyl-5′-(Phenyl Methyl)(5′-α, 10-α); 5,9,13-Pentadecatrien-2-One,6,10,14-Trimethyl-, (E,E); 2-Propen-1-One,1,3-Diphenyl-,(E); 3-Phenyl-1-Aza-Bicyclo[1.1.0]Butane and Pyrazolo[1,5-A]Pyridine, 3-Methyl-2-Phenyl. Moreover, the methanolic extract of BPS-CRB41 detected seven volatile compounds (Heptanal; Benzoic Acid, 4-Chloro; Benzoic Acid, 2-Chloro; 4-Chlorobenzoic Acid, 4-Hexadecyl Ester; 2-Chlorobenzoic Acid, 3-Methylbutyl Ester; Anthranilic Acid, N-Methyl-, Butyl Ester and Benzeneacetic acid, α.-Oxo-, Trimethylsilyl Ester (Table3).

Di-n-octyl phthalate constituted 71% of the total amount present in the *Bacillus* sp. strain BPS_CRB12, which was also reported by Sarkar et al. (2012) and Chen et al. (2013). Benzaldehyde, which is known to have very effective cytotoxic potential, is major volatile compounds found in the strain BPS_CRB14. This compound was accounted by Ulker et al. (2013) and Gupta et al. (2015), who stated that benzaldehyde has the capacity to destroy cancer cells at a minimum concentration. Moreover, Ergotaman-3′, 6′, 18-Trione, 9, 10-Dihydro-12′-Hydroxy-2′-Methyl-5′-(Phenyl Methyl) (5′-α, 10-α) was found in isolate BPS_CRB14, that have antimicrobial potential reported by Shanthakumar and Kumar (2015), who stated that the compounds Ergotaman-3′, 6′, 18-Trione, 9, 10-Dihydro-12′-Hydroxy-2′-Methyl-5′-(Phenyl Methyl) (5′-α, 10-α) exhibited antimicrobial activity against several tested pathogens: *S. enteric*, *S. flexneri*, *E. coli* and *K. pneumoniae*, respectively. Pyrazolo [1,5-A] Pyridine, 3-Methyl-2-Phenyl was also detected in the methanolic extract of BPS_CRB14 and was reported to have anticancer activity (Kamal et al., 2016). Additionally, Heptanal was identified in methanolic extract of BPS_CRB41 and was reported to have lung anticancer activity (Chen et al., 2017; Xu and Wang, 2012). This is the first attempt of eight compounds, (1) 1 h-Benzimidazole, 1-Ethyl; (2) Benzeneacetic Acid, 4-(1 h-1,2,3,4-Tetrazol-1-Yl); (3) Benzyl 2-(3-Aminopropionyloxy) Acetate; (4) Benzene, (3-Chloro-3-Methylbutyl); (5) Benzaldehyde, 4-Methyl-, Oxime; (6) 5,9,13-Pentadecatrien-2-One, 6,10,14-Trimethyl-, (E,E); (7) 2-Propen-1-One, 1,3-Diphenyl-, (E) and (8) 3-Phenyl-1-Aza-Bicyclo[1.1.0] Butane, were reported from the methanolic extracts of *Bacillus anthracis* strain BPS_CRB14. Furthermore, six volatile compounds, (1) Benzoic Acid, 4-Chloro; (2) Benzoic Acid, 2-Chloro; (3) 4-Chlorobenzoic Acid, 4-Hexadecyl Ester; (4) 2-Chlorobenzoic Acid, 3-Methylbutyl Ester; (5) Anthranilic Acid, N-Methyl-, Butyl Ester; and (6) Benzeneacetic Acid, α.-Oxo,

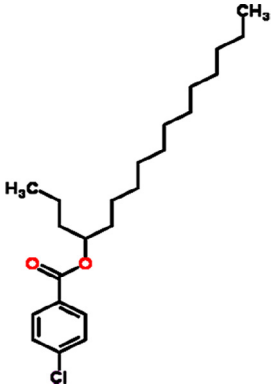
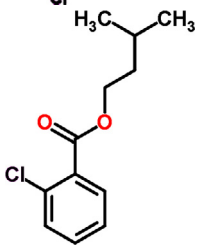
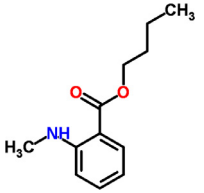
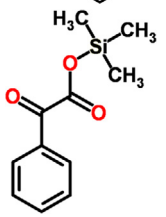
Table 3
GC–MS Chromatogram detected volatile compounds from methanolic extract of potential three bacterial strains compared with NIST library.

Sl.N0	Compound name	Formula	MW	RT	Height	Area %	Norm %	Structure
BPS_CRB12								
1	Di-n-octyl phthalate	C24H38O4	390	25.283	61,828,900	77.671	71.31	
2	1-Bromo-3, 7-Dimethyloctane	C10H21Br	220	28.474	17,773,776	22.324	28.69	
BPS_CRB14								
1	Benzaldehyde	C7H6O	106	13.95	5,106,615	10.521	77.08	
2	1H-Benzimidazole, 1-ethyl-	C9H10N2	146	20.074	6,852,816	4.982	14.61	
3	Benzeneacetic Acid, 4-(1 h-1,2,3,4-Tetrazol-1-Yl)	C9H8O2N4	204	20.185	6,811,223	4.164	12.21	
4	BENZYL 2-(3-Aminopropionyloxy)ACETATE	C12H15O4N	237	21.355	22,046,354	34.099	100.00	
5	Benzene, (3-Chloro-3-Methylbutyl)	C11H15Cl	182	22.05	13,929,964	24.592	72.12	

6	Benzaldehyde, 4-methyl-, oxime	C8H9ON	135	24.336	14,152,776	15.034	44.09	
7	Ergotaman-3′,6′,18-trione, 9,10-dihydro-12′-hydroxy-2′-methyl-5′-(phenyl METHYL)	C33H37O5N5	583	26.427	5,505,286	3.507	10.28	
8	5,9,13-Pentadecatrien-2-one, 6,10,14-trimethyl-, (E,E)-	C18H30O	262	26.912	29,037,002	2.201	6.46	
9	2-Propen-1-one, 1,3-diphenyl-, (E)-	C15H12O	208	27.502	6,753,267	2.523	7.40	
10	3-Phenyl-1-AZA-bicyclo[1.1.0]butane	C9H9N	131	27.663	7,003,659	3.534	10.36	
11	Pyrazolo[1,5-A]pyridine, 3-methyl-2-phenyl-	C14H12N2	208	28.118	6,951,140	5.365	15.73	
BPS_CRB41								
1	Heptanal	C7H14O	114	16.098	9,468,074	4.267	3.08	
2	Benzoic acid, 4-chloro-	C7H5O2Cl	156	18.229	34,652,576	78.814	100.00	
3	Benzoic Acid, 2-Chloro	C7H5O2Cl	156	21.570	9,142,963	4.593	5.83	

(*continued on next page*)

Table 3 (*continued*)

Sl.N0	Compound name	Formula	MW	RT	Height	Area %	Norm %	Structure
4	4-Chlorobenzoic acid, 4-hexadecyl ESTER	C23H37O2Cl	380	22.265	9,899,289	5.559	7.05	
5	2-Chlorobenzoic Acid, 3-Methylbutyl Ester	C12H15O2Cl	226	22.270	9,563,491	32.711	6.21	
6	Anthranilic acid, N-methyl-, butyl ester	C12H17O2N	207	27.412	10,908,732	6.277	7.96	
7	Benzeneacetic acid, ALPHA.-oxo-, trimethylsilyl ESTER	C11H14O3Si	222	27.698	9,487,375	4.756	6.03	

Trimethylsilyl Ester, were reported for the first time in *Aneurinibacillusaneurinilyticus* strain BPS_CRB41 using GC–MS analysis.

4. Conclusion

From the present study, we conclude that the bacterial population associated with mud crabs showed significant antimicrobial potential and which can be exploited for the production of bioactive antimicrobial compounds. The selected strains were also showed the presence of biosynthetic genes and volatile compounds which further proves their ability to use as a potential source for the discovery of antimicrobial agents.

Authors contributions

JZ, AKP, BPS: designed the experiments. JZ, AKP, Z, PC: performed the experiments. JZ, AKP, BPS, NSK: wrote and finalize the draft of the paper. JAM, AH, EFA, AAA: Manuscript editing and helping in analyzed the data statically. CN: analyzed GC–MS data. BPS, NSK finally approves the manuscript.

Competing interests

The authors declared that there are no competing interests.

Acknowledgements

This study was completed by funds received from the Department of Science and Technology (DST), Government of India, New Delhi, as a young scientist project to BPS (No: SERB/F/2501/2013-14). The authors are also given special thanks to the Department of Biotechnology for the establishment of the DBT-BIF center and DBT-Advanced Level State Biotech Hub in the Department, which has been used for the present study. The authors are thankful to the VIT-SIF Lab, SAS, Chemistry Division for GC-MS analysis. The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding to the research group number (RG-1435-014).

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