# DISTRIBUTION OF BACTERIAL POPULATION ASSOCIATED WITH DIFFERENT ORGANS OF EDIBLE MUD CRAB (*Scylla olivacea* Herbst, 1896) AND THEIR ANTIMICROBIAL POTENTIAL

**Dissertation submitted in fulfilment of the**

**Requirements for the Degree of**

**Master of Philosophy**

**In Biotechnology**

By
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**Registration No. MZU/M.Phil/358 of 26.5.2017**

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# Declaration of the Candidate

I, **Joanne Zote**, hereby declare that the subject matter of this dissertation entitled "**Distribution of bacterial population associated with different organs of edible mud crab (*Scylla olivacea* Herbst, 1896) and their antimicrobial potential"** is a record of work done by me, that the contents of this dissertation did not form basis of the award of any previous degree by me or to the best of my knowledge to anybody else, and the dissertation had not been submitted by me for any research degree in any other University/ Institute.

This is being submitted to Mizoram University for the award of the degree of Master of Philosophy in Biotechnology.

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# CERTIFICATE

This is to certify that the dissertation entitled **"Distribution of bacterial population associated with different organs of edible mud crab (*Scylla olivacea* Herbst, 1896) and their antimicrobial potential"** submitted to Mizoram University for the award of Master of Philosophy in Biotechnology by **Joanne Zote Registration No. MZU/M.Phil/ 358 of 26.5.2017**, Research scholar in the Department of Biotechnology, is a record of research, based on the result of the experiments and investigations carried out independently by her during the period from 2016- 2017 of study, under my guidance and supervision and has not been previously submitted for the award of any Indian or foreign University.

It is further certified that the scholar fulfilled all the requirements as laid down by the University for the purpose of submission of M.Phil dissertation.

Place : Prof. N. SENTHIL KUMAR

Date : (Signature of the Supervisor)

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**DATE:** **Joanne Zote**

## ABBREVIATIONS

- AIC – Akaike Information Criterion
- AIA – Actinomycetes Isolation Agar
- BIC – Bayesian Information Criterion
- BLASTN – Basic Local Alignment Tool nucleotide
- BS- Bacillus Subtilis
- CYP – Cytochrome peroxidise P450
- CA – Candida albicans
- E.Coli – Escherichia coli
- GC- MS – Gas Chromatogarphy Mass Spectrometry
- LOD – Linearity, limits of detection
- LAQ – Linearity, limits of quantification
- NRPS – Non – Peptide Polyketide Synthase
- NA – Nutrient Agar
- NCBI – National Centre for Biotechnology Information
- MIC- Minimum Inhibitory Concentration
- MA – Micrococcus luteus
- MEGA – Molecular Evolutionary Genetic Analysis
- PCR – Polymerase Chain Reaction
- P.A – Pseudomona aeruginosa
- PKS II – Polyketide Synthase Type II
- S.A – Staphylococcus aureus
- SCA – Starch Casein Agar
- TSA – Tryptic Soya Agar
- $TH_20$ – Tap Water Yeast Extract Agar
- UPLC – Ultra Performance Liquid Chromatography
- VOC's – Volatile Organic Compounds

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# CHAPTER – I

## INTRODUCTION

Mud crab (Scylla *olivacea* ) is an important commercial species of crab, they are mostly large portunids as well and they are a fast growing species(MacNae.,1968). They are majorly found in the mangroves of Africa, Australia and Asia. They belong to genus *Scylla*, Phylum- Arthropoda, subphylum- Crustacea, Order- Decapoda, Family- Portunidae and species-Serrata. Scylla genus is the most fast growing species which are widely used in aquaculture farming in some tropical and sub- tropical Southeast Asian countries, and also fetch a significant high price in international sea food market (BOBP 1992) . Among the genus, *S. serrata* is a large crab widespread throughout the Indo- West Pacific (IWP) region (Macao, 1968). Genus *Scylla*, comprises of three species (*S. serrata, S. olivacea and S. tranquebarica*) and one sub- species (*Scylla Serrata var. Paramamosain*) among which *S. serrata* is the most widespread (Estampador, 1949). Taxonomic classification of mud crabs along Indian coasts, including the Bay of Bengal, reported the occurrence of three species *i.e. S. serrata*, *S. olivacea*, and *S. tranquebarica* (Radhakrishna & Smuel, 1982).*Scylla olivacea* was the species which was identified by some researches as *Scylla.serrata* because some biological studies o mud crab suggested only the presence of one single species of the mud crab. later mow, the genus of the mud crab has been identified and revised where it states the occurrence of three additional species which was proposed *Scylla Serrata var. Paramamosain* (Estampador), *S. olivacea* (Herbst) and *S. tranquebarica* (Fabricius) and beside *S. serrata* (Kennel et al., 1998). These four species of the crab follow the same life cycle, and their distribution along the coastal plains as well as having similarities in their morphological features, especially juveniles. The studies of individual species of *Scylla* were also confirmed. The majority abundance of the species, recruitment and mortality of the mangrove population was studied with the help of allozyme electrophoresis and morphometric analysis, thus come to conclusion that the species was recognized as '*Scylla olivacea'.* (Overton et al., 2000)

The species *Scylla* are the most preferred species for the coastal aquaculture in Bangladesh (Keenan et al., 1998), as it has been mostly reported by major scientific literatures from Bangladesh.

Crab holds for a good nutritional value as they are a rich source of Vitamin $B_2$ and contains a high quantity of calcium and phosphorous involving in the development of red blood cells, blood vessels and maintain the bones and immune system healthy. Apart from this, it also contain high amount of selenium playing a vital role as an important antioxidant in humans. It also contains Omega -3 polyunsaturated acids, which is helpful for brain development as well as protection from heart diseses (Kim et al., 2012; Chun et al., 2009; Yong, 2003).

The aquaculture has been well known and important sector with many potential products, they have been an important contributors in pharmaceutical industries as well as for food industry. Recently, the production of crab in 2005 has reached 660,000 ton globally, with nearly 100% contribution from the Asian countries (Paterson, 2009).

Research done on the antimicrobial potential of Hemolymph of fresh water crab (*Oziotelphusa senex senex)* reported that there is a strong evidence when tested against clinical pathogens, where this results confirm that they have a strong immune mechanism that might be an efficient and important antimicrobial agents that might be helpful for the synthesis of novel compounds for future use (Sumalatha et al., 2016). Similarly, research done on the intestinal bacteria isolated from the freshwater cultured fish gut also reported that when tested against eighteen pathogenic bacteria including human pathogen, they confirmed that the 7 strains containing the antimicrobial compounds has the capacity to inhibit some pathogenic bacteria and has the capacity to protect the fish gut from some pathogenic bacteria (Miranda *et al.,* 2002). The study of hemolymph obtained from *Charybdis lucifera* has also revealed that they produced antimicrobial activity in both Gram positive, Gram negative bacteria and in some pathogenic fungal strains, this gives a great importance and indeed a potential source of data for finding a novel compounds with biological potential.

Study of antimicrobial activity from haemolymph *Ocypode macrocera*, Ravichandran et al., (2010) when treated against eleven bacterial stains namely, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi, Shigella flexineri, Klebsiella sp., Vibrio cholera, Streptococcus pyogenes, Actinobacter sp., Sallmonella typhi, Staphylococcus and* five fungal strains namely*; Aspergillus fumigates, Rhodotorula sp., Candida albicans, Cryptococcus neoformans, Aspergillus niger* states that out of all the sixteen strains tested against the extract of haemolymph, maximum zone of inhibition (2 mm) was obtained against the tested *Salmonella typhi* and *Klebsiella sp.* and shows significant activity against the microbial strains. However, this suggests that the hemolymph of *Ocypode macrocera*, contain good antimicrobial activity could be further useful for the discovery of useful antibiotics.

Aquatic biodiversity provides a great potential to enable the aquaculture sector to further enhance its contribution to food security and meet future challenges in feeding a growing human population. There is no systematic study done so far on the distribution and biosynthetic potential of bacterial population isolated from different parts of edible mud crab in Mizoram.

# CHAPTER – II

## REVIEW OF LITERATURE

The genus *Scylla* also called as the mud crab as they used mostly a muddy substrate as their major habitat. They are a large portunids and are widely found along the coastal regions along the shorelines, rivers, ponds and they are ordinary inhabitant of the mangrove areas (Hoq, 2008). Most crabs live in the sea, even the land crabs, which are abundant in tropical countries. Mud crabs hide in burrow to make themselves cold during low tide as well as protecting themselves against predators (Macintosh, 1988). Scylla species is a fast growing species and they are economically and commercially important species worldwide (Acharya and Kamal, 1994). Scylla species are the most preferable species for aquaculture along the coastal regions including Bangladesh. The taxonomic classification was quite controversial. But lately, Kennel et al. (1998) classification was accepted worldwide.

The Mud Crab has a high nutritional significance and is consumed by humans as well as for the marine lives. Researchers have also concluded that mud crab have a high prospective exhibiting antimicrobial properties as well as a good antioxidant agent. In some places like Malaysia, crabs are used for some traditional medicinal purpose, for reducing the symptoms of dengue fever. (Yan et al., 2011). Eighty percent of the world's population is covered by marine life; they are the main source for discovery of bioactive compounds, pharmaceuticals and industrial purposes. A total of more than hundred compounds have been detected and characterized from marine environment (Kambooj, 1999). The mud crabs have a strong innate immunity system (in particular the defend mechanism involves the humoral and cellular responses) they are the only marine invertebrates which can protect themselves from pathogens. The circulating hemolymph present in marine invertebrates contains biologically active compounds such as complements, lectin, clotting factors and antimicrobial peptides which are responsible for the productionmand discovery of some bioactive molecules and novel compounds (Miyata, 1989). In addition to this, antimicrobial peptides present acts as a great role in humoral immunity and antioxidant to fight against infectious pathogens. In particular, Antimicrobial peptides (AMP's) has drawn attention since 1988 due to their properties in production of antibacterial agents. The first antimicrobial peptides was recovered from crab (Nakamura et al.,1988) which includes tachyplesin, a peptide derived from combination of 18 animo acids isolated from the hemocytes of Japanese horseshoe

crab, *Tachypleus tridentatus*. Their high immunity level plays immense role when they are exposed to pollution and high stress level. Due to the changes in environmental temperature and salinity, the immune system of the crab modulate to the changes. Antimicrobial potential has been detected in some decapods, cryfish, shrimps and crabs, and this potential could be very useful in some biomedical area.

Bioactive compounds of nearly 6,500 have been isolated from the marine organisms from the year 1960's (Kamboj, 1999). Antimicrobial peptides are very important as they play a role in host defence mechanism in animal species (Boman, 1991; Zasloff, 1992). Antimicrobial peptides (AMP,s) performed the base line for many host defence mechanism against invading microorganisms. Antimicrobial processing bacteria have been well- known in some nest materials of different arthropods lineages. The phylum actinomycetes (actenbacteria) is the best study phylum that produces wide variety of antimicrobial chemicals (Lam, 2006).

Arthropods bacteria relates with derived symbioses, whereas actinomycetes help out in nest or colony hygiene by creating antimicrobial compounds (Seipke et al., 2011; Kaltenpoth, 2009). Intensive farming which has resulted in immense use of antibacterial agents for therapeutic use for the treatment of bacterial fish diseases (Smith et al., 1994). The use of antimicrobial has a massive influence which led to the increase frequency of resistant in bacterial microflora (DePaola et al., 1988; Sugita et al., 1998). There is an increasingly use of antibacterial chemotherapy in fish farming, as it helps in compromising the development of resistance in fish pathogens (Aoki, 1992; Smith et al., 1994). Due to this factor, the salmon industry requires the use of more expensive antibacterial with much higher efficiencies, as resistance develop much less, thus significantly increased the production costs of this activity.

The presence of Antibiotic resistance influenced the microorganisms in many different ways. Some microorganisms like *Streptococcus pyogenes* have always revealed sensitive to Penincillin, even on contact to this drugs for a long time (Denyer et al., 2004). The occurrence of resistance genes can also spread antibiotic resistance in bacteria, the resistance genes might have some capacity which have developed from mutation or the bacteria may possibly received it from others bacteria with resistance genes. Resistance genes multiply by plasmid, transposes or integrands (Witte, 2000). In Chilean salmon farming, regular use therapeutic concentrations of antibiotics has become widely used, as this practise induces the fast development of antibiotic- resistant bacteria (Lebek and egger, 1983). The most common used antibiotic against various diseases caused by both Gram- ngative and Gram- positive bacteria is Oxytetracycline, and is widely used in Chilean fish farming. The

used of this antibiotic (Oxytetracycline) resistance has been followed widely in many fish cultures (Sugita et al., 1998). Some reports showed a realationship between the intestinal and aquatic microflora in the use of Oxytetracycline resistance, and led to the administration of Oxytetracycline – medicated supply to the catfish as well (DePaola et al., 1988).

The study of bacterial phylogeny and taxonomy is done by 16S rDNA and is the most common housekeeping genetic markers for a number of reasons. It includes (i) they are present in all almost all bacteria, exist as a multi – gene family. (ii) the function of 16s rDNA has not changed but there is a change in random sequences but precise measures in time. (iii) 16S rDNA is 1500 bp long and vast enough for information purposes (Patel, 2001). The 16S rDNA gene is universal in bacteria, and used for measuring relations among bacteria (Woo, 2003). It allows separation among organism at the genus level all across the major phylum of bacteria, also helps in classifications of stains at different levels, as well as the name of the species and sub-species level. 16S rDNA gene sequencing helps in relating more than one well known species having the identical or related sequences.

In the genomic era, there is easy access for the production of bioactive molecules by means of molecular methods. Various natural products have been approved to be the best treatment and use for theraupetic uses. Genetic approach is a promising tool for the detection of bioactive compounds for the biosynthetically capable microorganisms. This natural products are known as secondary metabolites - secondary metabolites such as non- ribosomal peptide synthase (NRPS's) and ployketide synthases (PKS,s) which act as antibiotic, immunosuppresents, antitumour agents and toxin. They are large multi- modular and multi- domain enzymes. Ketosynthase (KS), acyltransferace (AT)and acyl carrier proteins are the miimal set of domains that has to be present in the biosynthetic pathway in PKS's (Khan et al., 2014). Adenylation(A), Condensation (C) and peptidyl carrier proteins (PCP's) for peptide elongation responsible for the biosynthetic pathway in NRPSs (Jenke- Kodama and Dittmann, 2009). Most of the current research on polyketide biosynthesis is determined by; first, the biological activities and massive industrial value of these natural products, remain the most successful candidates for new drug discovery; secondly, due to the extraordinary structure , mechanism and catalytic reactivity of PKSs that supply an extraordinary chance to explore the molecular mechanisms of enzyme catalysis, molecular identification and protein- protein interaction; and thirdly, the remarkable versatility and amenability of PKSs that provid the generation with novel compounds, not easy to access by combinatorial biosynthesis process. The variability and the presence of

these genes results in the production of bioactive compounds. Some antibiotic molecules like erythromycin, tetracycline and glycopeptides of the vancomycin family have been found originated from PKS and NRPS gene production (Fischbach and Walsh, 2006).

Cytochrome P450 monooxygenases involves in the oxidation of various cellular metabolites and xenobiotic compounds, and comes from the family of microsomal enzymes. They are classified into 32 gene families according to their amino acid sequences (Nelson et al., 1993). Types of P450 have been recognized in insects and they are studied mainly in relation to insecticide resistance and detoxification of plant metabolites (Hodgson, 1985). For the reason that P450 can matabolize xenobiotic compounds including insecticides, implanted in insecticide resistance. This further support the observations that resistant insect strain contain higher level of more than one CYP gene compared to their suspectible counterparts (Carino et al., 1992). The large biodiversity of CYPs is not just found in wood- rot fungus, but also in saprophytic organisms via *Aspergillus oryzae* (Nazmul et al.,2010) as well as in soil actionomycetes such as 18 CYPs in *S. Coelicolor* A3 (Lamb et al., 2002) and roughly 40 in *Mycobacterium smegmatis* (Jackson et al., 2003; Brodhun et al.,2009). The capability of these organisms to biotransform xenobiotics is well identified, its occurrence and its capacity of aspergilla in industrial steroid hydroxylation. In several human CYPs have been useful for detoxification of natural products in the diet which are further useful for drug metabolism, some microbial CYPs have the ability to metabolize drugs and been used widely for mammalian drug metabolites. Further, these metabolites which will be helpful for pharmacological and toxicological evaluation in the drug processing mostly in the days before heterologous human cytochrome P450 were commercially available. Few microbial CYPs might engaged in detoxification as well as synthesis of natural products in both microbial system of fungus and bacteria. They play a great role in bioremediation, as for example *Rhodococcus* and *Gordonia* strains have the capacity to degrade petrol additives methyl tert-butyl ether, ethyl tert-butyl ether and tert-amyl butyl ether and found an association to CYP249. (Malandain et al., 2010).

Fungal CYPs have an important role in cycling nutrients which includes CYP55 of fungi, de-nutrification enzyme of fungal responsible for the nitrogen cycle (Kobayashi et al., 1996). De-nitrification is a method where nitrate or nitrite is condensed to a gaseous form of nitrogen (N2 or N2O) and nitrous oxide reductase absent in fungi produce nitrous oxide, a greenhouse gas, as a final product (Ma et al., 2008). Studies have exposed that fungi the major provider to de-nitrification in grasslands, semi-arid and forest soils by implementing soil fertility (Hayatsu et al., 2008). Microbial CYPs have been publicized to be an important

food security because of the fungal pathogens of crops are inhibited by inhibitors of CYP51 that lump egosterol biosynthesis (Parber et al., 2011) and were first introduced in the early 1970s. Regarding the fungicides, more than 40 percent are reserved for the agrochemical market as it increases yields and will meet the requirement for continued use in the future.

# CHAPTER – III

## MATERIALS AND METHODS

*Sample Collection*

The fresh crab samples were collected from Lengte market, Mamit district, Mizoram, Northeast India during September 2016. The samples taken were free from disease. They were collected in sterile polythene bags, bought into the laboratory and processed for further isolation.

*Isolation of Bacteria from different parts of the crab*

Before processing the isolation, surface sterilization was done by washing the collected crab sample thoroughly with running water to remove organic materials and impurities. The isolation of bacteria was carried out by spread plate technique. The different parts of the crab were taken namely- abdomen, carpace, leg and hand. Each part was taken individually and grind them with motor and pestle. Two gram of each part was homogenized with 10 ml of 10 mM potassium phosphate buffer. 100 µl of each homogenate parts was taken and was spread on five different nutritional media. The plates were incubated at 37 $^{o}$C for 24 h to observe the colonies of bacteria. Isolated single colonies were picked and re-streaked on respective media to obtained pure isolates. The pure isolates were maintained on agar slants at 4 $^{o}$C.

*Isolation Media*

Five specific nutritional media were used to obtain the population of bacteria. The used media are: 1. Nutrient agar (peptone, beef extract/yeast extract, Nacl, agar); 2. Actinomycetes isolation agar (Sodium caseinate, L-asparagine, sodium propionate, dipotassium phosphate, magnesium sulphate, ferrous sulphate, 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).

Note- See the Appendix for the Media composition.

*Screening of Antimicrobial activity using the agar well diffusion method*

The antimicrobial screening was performed against the bacterial pathogens *Staphylococcus aureus* (MTCC-96), *Pseudomonas aeruginosa* (MTCC-2453), *Escherichia coli* (MTCC-739), *Micrococcus luteus* (MTCC-5262), *Bacillus subtilis* (MTCC-2097) and *Candida albicans* (MTCC-3017). The tested organisms were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India, and maintained in the Molecular Microbiology and Systematic Laboratory in the Department of Biotechnology at Mizoram University. The antimicrobial activity was performed using agar well diffusion method (Saadoun and Muhana, 2008 & Zothanpuia et al., 2016).

*Minimum Inhibitory Concentration (MIC) of the Potential Isolates*

The potential isolates showing the best antimicrobial activity were selected for broth micro dilution technique of 96 wells microtiter plate as described by Eloff (1998). The bacterial culture suspension was prepared to make the final concentration of $1.0 \times 10^4$ CFU/mL (OD= 0.403). Crude extract of the potential isolates was prepared at different concentration (1-10 mg/ml) with 10% DMSO solution. The prepared crude extract of different concentration was added in 96 wells microtiter plate, along with test bacterial culture suspension ($1.0 \times 10^4$ CFU/mL)**.** Standard antibiotic (ampicillin, 0.01µg/ml) along with bacterial cultures which will be used as positive control whereas; DMSO containing bacterial cultures was used as negative control. The plates were incubated at 37 ºC for 36 h and absorbance was taken at 620 nm in spectrophotometer UV-VIS (MultiscanTM GO, Thermo Scientific, MA, USA). Results were documented as $IC_{50}$ values which indicate 50% reduction of bacterial growth. The $IC_{50}$ values were calculated by using calibration curve drawn by using linear regression.

*Molecular Characterization of bacterial isolates*

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

Total genomic DNA was extracted using the Bacterial DNA Purification Kit (Invitrogen, Life technologies KT-110052) as per the manufacturer's instructions. All the isolates were subjected to amplification of the 16S rRNA gene using the PA universal primers (forward 16S rRNA 5'-AGAGTTTGATCCTGGCTCA-3' and PH reverse 16S rRNA primer 5'-ACGGCTACCTTGTTACGACT-3') as described by Cui et al. (2008). The amplified products of PCR were cleaned using a QIA quick gel extraction kit (Qiagen, Hilden, Germany) and sequencing was performed using Sci-Genome Pvt. Ltd, Cochin, India.

The 16S rDNA gene sequences were contrast with the NCBI database and showing a high level of identity (97-100%) were selected as the closest match. All the sequences were submitted to NCBI GenBank, and accession numbers were obtained. The evolutionary models were selected based on the lowest BIC ("Bayesian Information Criterion") and AIC ("Akaike Information Criterion") values using MEGA 6.0, and the phylogenetic tree was evaluated by bootstrap analysis with 1,000 resamplings using a *p*-distance model (Saitou and Nei, 1987; Felsenstein, 1985).

*Biosynthetic gene amplification (PKS type II; NRPS, CYP gene )*

*Polyketide Synthase type II (PKS type II)gene*

Polyketide synthase (PKS) type II gene fragments were amplified using degenerate primers, as described by Ayuso-Sacido and Genilloud (2005).

PKS II forward (KS∞-5′-TSGCSTGCTTGGAYGCSATC-3′

PKS II reverse ( KSβ-5′-TGGAANCCGCCGAABCCTCT-3′)

*Non-Ribosomal Peptide Synthase (NRPS) gene*

Non-ribosomal peptide synthase (NRPS) fragments were amplified using degenerate primers, as per Meng Yuan *et al.* (2014).

NRPS forward (5′-GCSTACSYSATSTACACSTCSGG-3′)

NRPS reverse (5′-SASGTCVCCSGTSCGGTAS-3′)

*Cytochrome P450(CYP) gene:*

Cytochrome p450 (CYP) fragments were amplified using degenerate primers as per Meng Yuan *et al.* (2014)

CYP forward (5′-TGGATCGGCGACGACCGSVYCGT-3′)

CYP reverse (5′-CCGWASAGSAYSCCGTCGTACTT-3′)

*Phylogenetic analysis of Biosynthetic genes (PKS type II; NRPS,CYP genes)*

Biosynthetic gene sequences (PKS type II, NRPS, CYP) were varying with the sequences obtained from the NCBI database using the BLASTn search tool (Tamura *et al.,* 2011), and a multiple sequence alignment was performed on all the gene sequences using the Clustal W software package in MEGA 6.0 (Thompson *et al.,* 1997). The phylogenetic tree was constructed by the maximum likelihood method using MEGA 6.0 software with Kimura 2-parameters model (Tamura *et al.*, 2011; Saitou and Nei, 1987).

*Detection of Antibiotics using Ultra-Performance Liquid Chromatography (UPLC-ESI MS/MS)*

*Preparation of Standard Solution*

Standard antibiotics (trimethoprim, fluconazole, ketoconazole, nalidixic acid, and rifampicin) were prepared in methanol. The standard solution was prepared as per Passari et al. (2017).

*Instrumentation and analytical conditions*

An Acquity ultra-performance liquid chromatography (UPLC$^{TM}$) system consisting of an autosampler and binary pump (Waters, Milford, MA) was used to carry out the analysis as per Passari et al. (2016) and Zothanpuia et al. (2017). MRM parameters were optimized to achieve the most abundant, specific and stable MRM transition for each compound, as shown in Table 4.

*Method Validation*

*Analytical Method Validation*

The proposed UPLC-MRM method for quantitative analysis was validated according to the Passari et al. (2016) & Mishra et al. (2017).

*Linearity, limits of detection (LOD) and quantification (LOQ)*

LOD and LOQ were determined by the calibration curve method as per Passari et al., (2016) & Mishra et al., (2016).

*Precision, Stability and Recovery*

The precision, s*tability and recovery* was measured as per Passari et al., (2106) & Mishra et al., (2017). Three replicates were performed at each level.

*Gas Chromatography Mass Spectroscopy (GC-MS) Analysis*

The volatile compounds present in the selected potential isolates were examined and identified using GC-MS, as described by Ser *et al.* (2015) and Sharma *et al.* (2016), with minor modifications. The peaks were identified by matching the mass spectra with the library from the National Institute of Standards and Technology (NIST, USA).

# CHAPTER – IV

## RESULTS

*Bacterial population from different parts of the crab*

Totally, 43 bacterial isolates were obtained and identified from different parts of *S. olivacea* based on their morphological characteristics. The crab was collected from Lengte, Mamit district of Mizoram, North East India, and its morphological characteristics were recorded and documented Fig.1(a). The sample was dissected from different parts (abdomen, carpace, leg and hand). Among the selected parts, the highest bacterial population was recovered from carapace (n=18; 41.8%) followed by the abdomen (n=11; 26.8%), leg (n=8; 19.5%) and hand (n=6; 14.6%). Trypticase soya agar (TSA) media (n=21; 48.8%) was the best nutritional media for the recovery of bacteria, followed by AIA (n= 11; 26.8%), $TH_2O$ media (n= 5; 12.1%), SCA (n=4; 9.7%) and NA (n= 2; 4.8%). The obtained bacterial isolates shows rough texture, colony color, which could be yellow, white, red, pink, or brownish white, while some isolates formed pigment in the media. 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 to 0.4082 and 0.2263 to 0.3426, respectively.


**Fig:1(a) Morphology of the mud crab '*Scylla olivacea*' (A) Frontal view (B) Dorsal view**

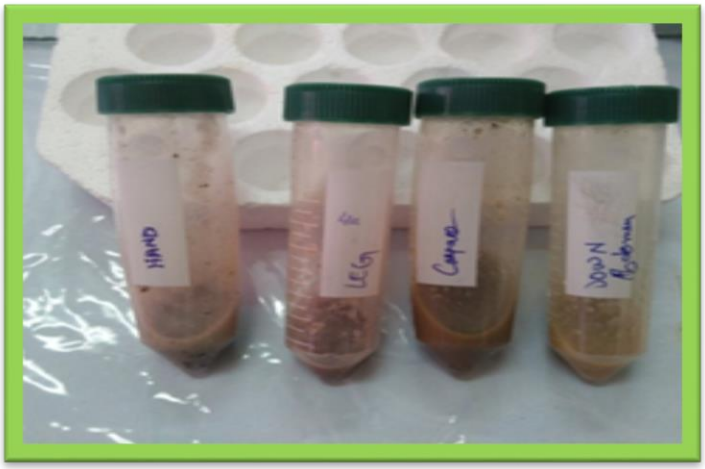



**Fig:1(b)Different parts of the crab taken for study (ie Carpace, abdomen, leg and hand)**

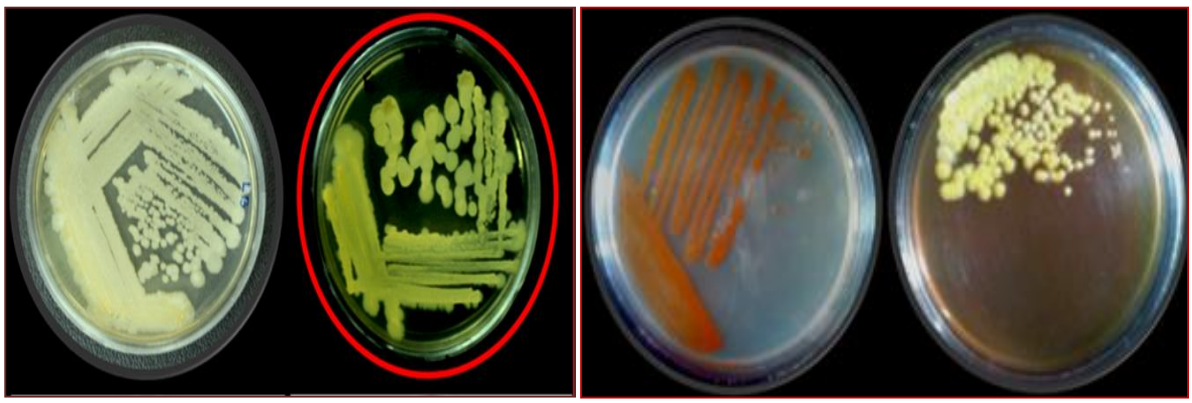


**Fig 1 (c) Morphological characteristics of the isolated bacterial isolates**

*Screening for antimicrobial potential of bacterial Isolates*

All 43 strains were tested for their antimicrobial potential against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus*, *Bacillus subtilis* and yeast *Candida albicans*. 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 beside to *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). (Table.1). Three isolates, i.e., *Bacillus* sp. strain BPS_CRB12, *B. anthracis* strain BPS_CRB14 and *A. aneurinilyticus* strain BPS_CRB41, showed a broad spectrum of antimicrobial activities and are considered the most promising isolates for further analysis.

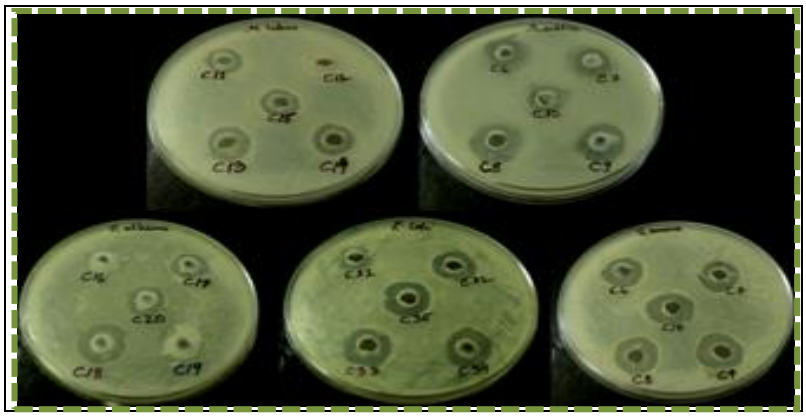


**Fig 2: Antimicrobial actiivity of the bacterial isolates**

**Table 1**: **Antimicrobial activity of the obtained bacterial isolates from different parts of the 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 ± 0.5 | 6.0±0.10 | - | 6.0±0.10 | | 9.4±0.10 |
| **BPS_CRB13** | - | - | - | 7.4±0.05 | 9.0±0.10 | 7.4±0.05 |
| **BPS_CRB14** | 8.4 ± 0.05 | | - | 6.0±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_CRB33** | 5.4±0.05 | 9.0±0.10 | - | - | 9.0±0.05 | - |
| **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 | 4.0±0.11 | 9.0±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 | - | |

Antimicrobial activity ( % inhibition ± SD*)

*Minimum Inhibitory Concentration of the potential isolates*

The three best isolates (BPS_CRB 12, BPS_CRB14 and BPS_CRB41) showing antimicrobial activity were tested for broth micro dilution assay against the 6 bacteria pathogens viz. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus*, *Bacillus subtilis* and yeast *Candida albicans*. Out of the three tested isolates, methanolic extract of BPS_CRB 41 showed maximum 50% inhibition against *M. luteus* (70.71%) and *E. coli* (85.11%) whereas; isolate BPS_CRB14 exhibited highest inhibition against *S. aureus* (71.76%). Moreover, isolate BPS_CRB12 was found to possess maximum 50% inhibition against *B. subtilis* (92.82%). *P. aeruginosa* was the most susceptible pathogen against all three strains. All the strains showed very less inhibition against *P. aeruginosa* pathogen. All the bacterial pathogen was compared with standard antibiotics ampicillin (30 µg/mL) whereas, yeast pathogen *C. albicans* was compared with Fluconazole (30 µg/mL). The list of value is shown in Table.2

**Table 2**: **MIC for Broth- micro dilution Assay**

| Sample | ***B. subtilis*** **$IC_{50}$µg/ml** | ***E. coli*** **$IC_{50}$µg/ml** | ***C.albicans*** **$IC_{50}$µg/ml** | ***P.aeuroginosa*** **$IC_{50}$µg/ml** | ***S .aureus*** **$IC_{50}$µg/ml** | ***M. luteus*** **$IC_{50}$µg/ml** |
|---|---|---|---|---|---|---|
| BPS_CRB12 | 92.82* | 257.8 | - | 288.8 | - | 98.34* |
| BPS_CRB14 | 381.8 | - | 227.4 | 414.9 | 71.76* | 96.76* |
| BPS_CRB41 | 218.9 | 85.11* | 135.0* | 190.2* | 706.5 | 70.71* |

***- Isolates shows significant values**

*Molecular Characterization of bacterial isolates*

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

Genomic DNA of all the strains was extracted using the Pure-Link Bacterial Purification Kit (Life Technologies, Invitrogen, USA). The taxonomic position of the strains was predicted by sequencing the 16S rRNA gene. The amplified product was purified using the Pure-Link PCR Purification Kit (Life Technologies, Invitrogen) and was sequenced at the Department of Biotechnology, Mizoram University. To assign the taxonomic position, the obtained 16S rRNA gene sequences were aligned using BLASTN analysis. All the strains were compared with the type strains obtained from the NCBI GenBank databases. 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%). The phylogenetic tree showed 98-100% identical similarity with their strain types. All the nucleotide sequences were deposited in the NCBI GenBank database under the accession numbers KX369561-KX369572 (12), KX369574-KX369576 (03) and MF421767-MF421794 (28).

For the obtained positive bacteria, the phylogenetic tree was constructed using the neighbor-joining method with the Kimura 2-parameter model (K2+G) according to lowest BIC values (6822.441) using Mega 6.0 (Fig. 1). Gaps were removed by pairwise deletion, and the estimated transition/transversion bias (R) was 1.68. 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 clustered together with their reference types obtained from the EzTaxon databases with a bootstrap support value of 100%. Similarly, all the *Bacillus* species were clustered together 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 under a bootstrap value of 100% each. In the gram-negative bacteria, the phylogenetic tree was constructed based on the neighbor-joining method with the Kimura 2-parameter model (K2+G) according to the lowest BIC values (3507.569) using Mega 6.0, and the estimated transition/transversion bias (R) was 1.90 (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 were clustered together with the strain type *Alcaligenes faecalis* 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 tree.

**Table.3 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 | *Acinetobacter dispersus* ANC 4105 | 99.51% | - | - | - |
| 2 | CRB 2 | *Acinetobacter sp.* | MF421768 | *Acinetobacter seifertii* 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 13111 | 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 14578 | 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 12856 | 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 % | - | - | - |

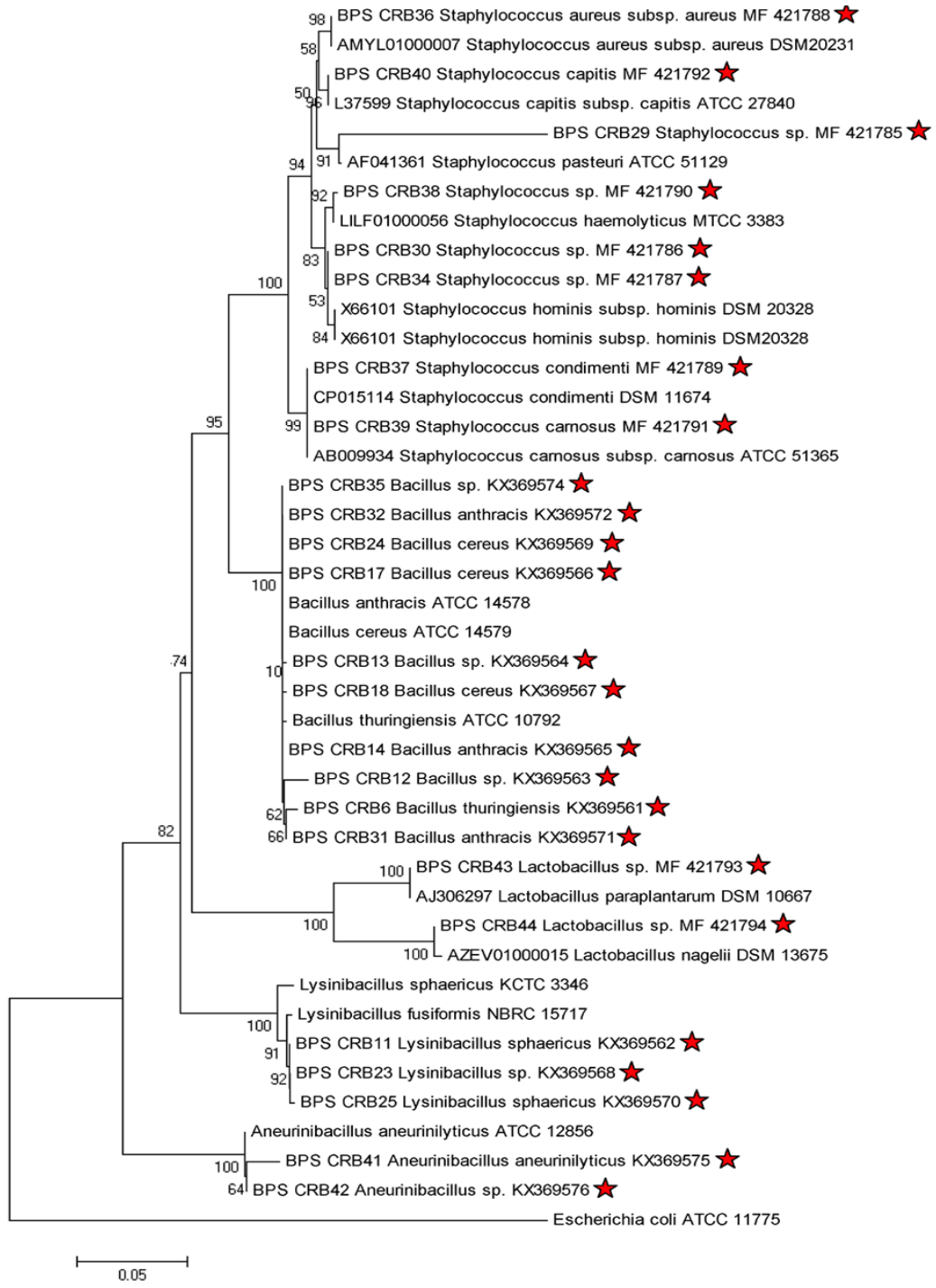



**Fig 3: Phylogenetic relationship based on partial 16S rRNA gene sequence of crab bacterial isolates with type strains retrieved from EZ-Taxon database. The phylogenetic tree was constructed using neighbour-joining method with Kimura 2-parameter model (K2+G) in gram positive bacteria with bootstrap supported value based on 1000 replicates.**

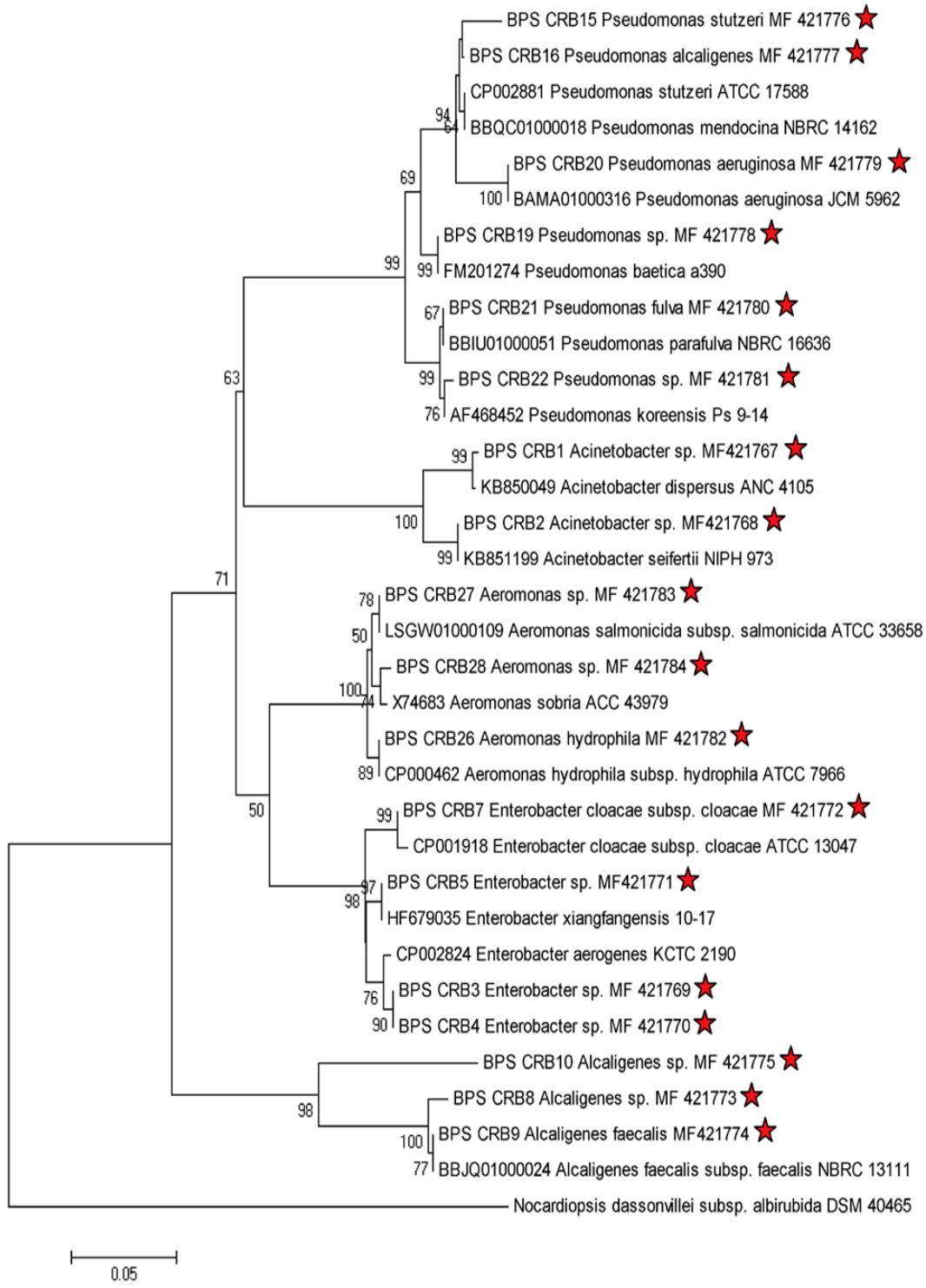



**Fig 4: Phylogenetic relationship based on partial 16S rRNA gene sequence of crab bacterial isolates with type strains retrieved from EZ-Taxon database. The phylogenetic tree was constructed using neighbour-joining method with Kimura 2-parameter model (K2+G) in gram negative bacteria with bootstrap supported value based on 1000 replicates.**

*Detection of biosynthetic genes: Polyketide synthases (PKS type II), Non-ribosomal peptide synthetase (NRPS),Cytochrome P450 hydroxylase (CYP) gene clusters*

Based on the antimicrobial activity, a PCR-based method was used to detect the presence of three biosynthetic genes: Polyketide syntheses type II (PKSII), Non-ribosomal peptide synthetase (NRPS). Cytochrome P450 hydroxylase (CYP) genes .The results showed that all 4 genes were detected in three strains (BPS_CRB12, BPS_CRB14 and BPS_CRB41). The gene sequences were submitted in the NCBI database and accession numbers were obtained: PKSII gene (MF871609 to MF871611), NRPS gene (MF871612 to MF871614), CYP gene (MF871615 to MF871617).

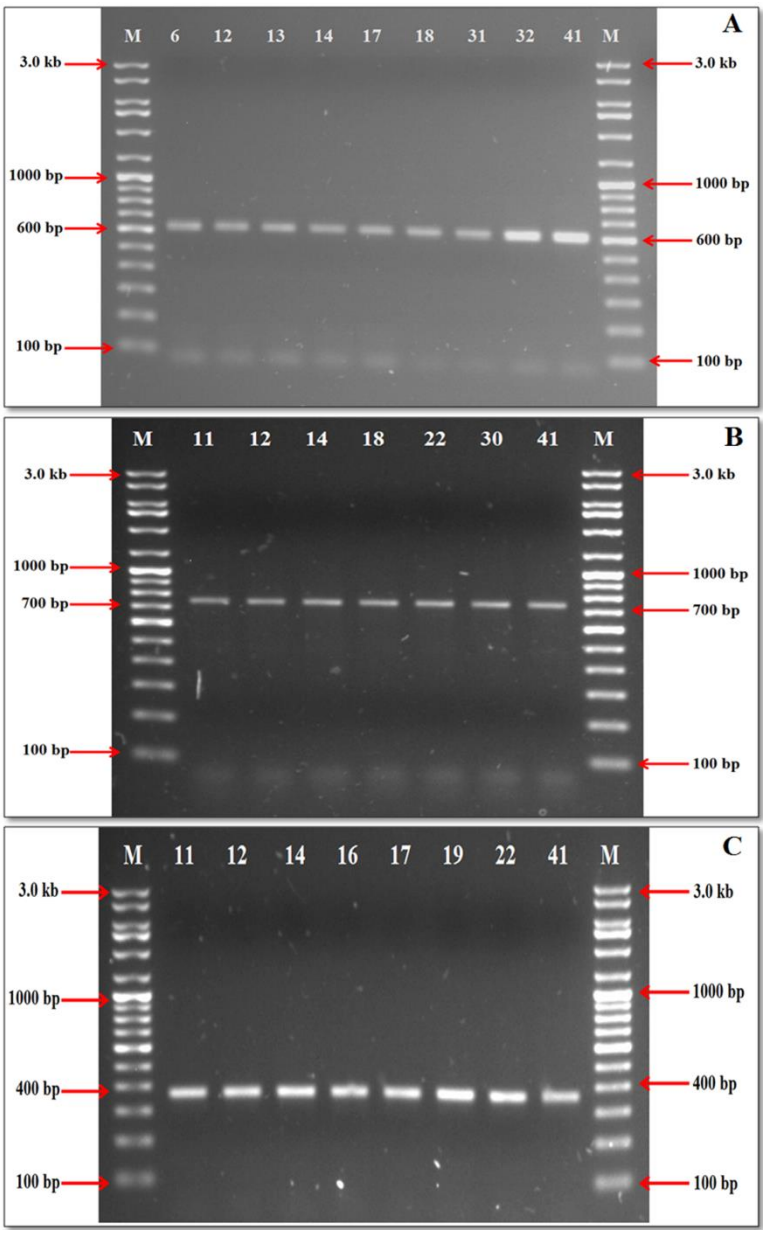



**Fig 5: Detection of Biosynthetic genes (A) detection of PKS II gene in 9 strains (B) detection of NRPS gene in 7 strauns (C) detection of CYP gene in 8 strains**

*Phylogenetic analysis of biosynthetic genes*

Nucleotide sequences of the biosynthetic genes (PKS type II, NRPS and CYP) showed 92% to 100% similarity with the strain types obtained from the NCBI-BLASTn database. The tree was build 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.6(A). 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. 6 (B) . 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. 6 (C).

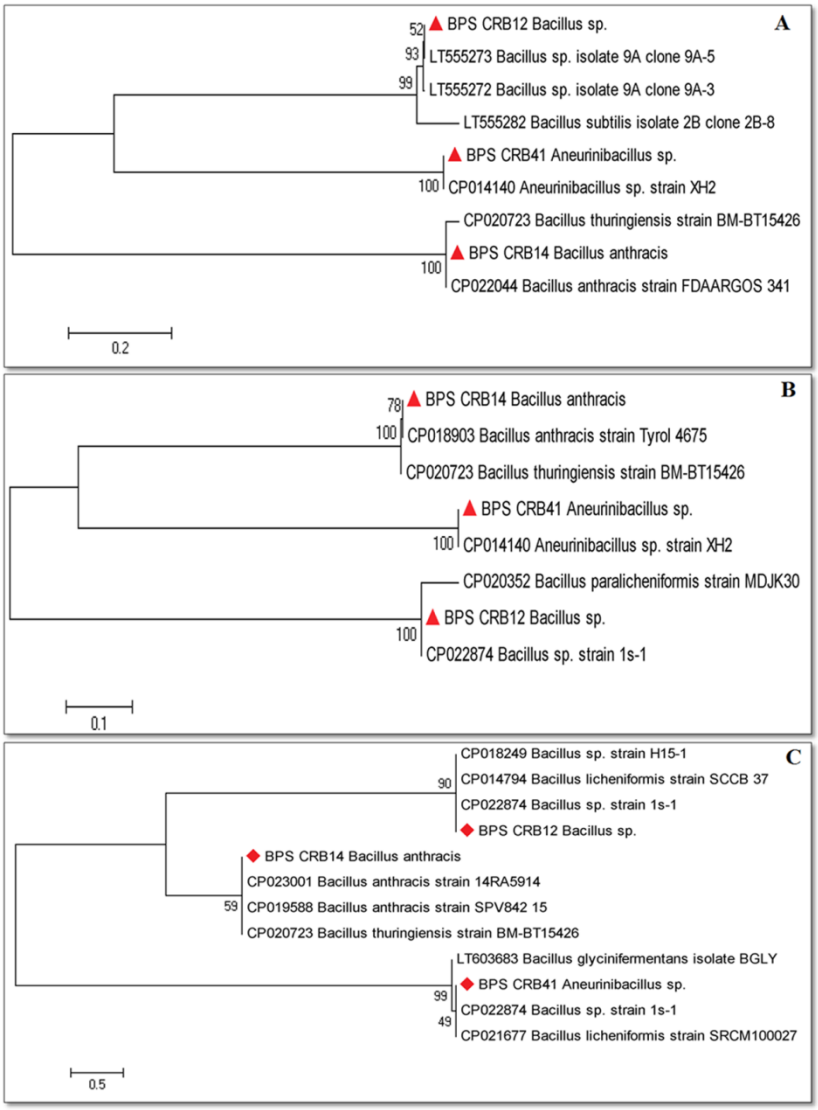



**Fig 6: Maximun Likelihood tree based on (A) PKS type II gene sequences; (B) NRPS gene sequences and (C) CYP gene sequences illustrating affiliations between *Bacillus* sp. strain BPS_CRB12, *Bacillus anthracis* strain BPS_CRB14 and *Aneurinibacillus* sp. strain BPS_CRB41 with type strains retrieved from NCBI-BLASTn database. The bootstrap supported value based on 1000 replicates**

*Detection and Quantification of Antibiotics using UPLC-ESI-MA/MS method*

*Analytical Method Validation*

The UPLC-ESI-*MA/MS* method for quantitative analysis was validated as described earlier in Passari et al., 2016

The calibration curve method was used to determine the LOD and LOQ. A series of concentrations of standard solutions was prepared for establishing the calibration curves. The LOD and LOQ were calculated as described in Passari et al., (2016). The results are listed in Table 6. The precision, stability and recovery were performed according to Passari et al., (2016). Three replicates were used to complete the experiment (Table 5).

The UPLC-ESI-MS/MS technique was performed to detect and quantify six antibiotics (fluconazole, chloramphenicol, ampicillin, ketoconazole, rifampicin and miconazole) (Table3). All the antibiotics were used to detect the methanolic extract of the *Bacillus* sp. strain BPS_CRB12, *Bacillus anthracis* strain BPS_CRB14 and *Aneurinibacillus aneurinilyticus* strain BPS_CRB41. The *Aneurinibacillus aneurinilyticus* strain BPS_CRB41 isolate had the highest concentration of the antibacterial antibiotic chloramphenicol (386.7 µg/g), antifungal antibiotic ketoconazole (221.3 µg/g) and miconazole (46.0 µg/g), whereas the maximum amount of antibacterial antibiotic ampicillin (14.8 µg/g) and rifampicin (33.5 µg/g) was detected in the isolate *Bacillus* sp. strain BPS_CRB12. Meanwhile, Miconazole (0.11 µg/g) was detected least in the *Bacillus anthracis* strain BPS_CRB14 among all the isolates. This is the first stab to *Aneurinibacillus aneurinilyticus* strain BPS_CRB41 isolate, which was used to discover and quantify the standard antibiotics chloramphenicol, ketoconazole and miconazole using the UPLC-ESI-MS/MS method. The MS spectra and MRM extracted ion chromatogram of the six mixed standards and the selected three strains are shown in Fig.8 respectively.

**Table 4:. LC-MS/MS optimized parameters**

| Analytes | *rt* (min) | Q1 | Q3 | Ion species | DP | EP | CE | CXP |
|---|---|---|---|---|---|---|---|---|
| Ampicillin | 1.51 | 348 | 207 | $[M-H]^-$ | -50 | -9 | -16 | -19 |
| Fluconazole | 1.74 | 307.1 | 220.1 | $[M+H]^+$ | 59 | 10 | 27 | 8 |
| Chloramphenicol | 2.07 | 322.1 | 153.1 | $[M-H]^-$ | -56 | -7 | -24 | -27 |
| Ketoconazole | 2.30 | 532.1 | 82.0 | $[M+H]^+$ | 106 | 10 | 68 | 10 |
| Nalidixic acid | 2.31 | 233.1 | 215.1 | $[M+H]^+$ | 49 | 8 | 21 | 8 |
| Rifampicin | 2.66 | 823.5 | 791.4 | $[M+H]^+$ | 53 | 9 | 24 | 19 |
| Miconazole | 2.80 | 417.1 | 159.1 | $[M+H]^+$ | 168 | 10 | 42 | 24 |

**Table : 5 Valiation Parametres**

| Analytes | Regression Equation | $r^2$ | Linear range ng/ml | LOD ng/ml | LOQ ng/ml | Precision RSD (%) | | Stability | Recovery RSD (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Intra-day (n=6) | Inter-day (n=6) | RSD (n = 5) | |
| Ampicillin | y = 2144.8x - 5235 | 0.9997 | 10-250 | 2.16 | 6.54 | 0.92 | 1.29 | 1.46 | 1.65 |
| Fluconazole | y = 11305x + 3944.4 | 0.9996 | 0.5-100 | 0.13 | 0.38 | 1.22 | 0.74 | 1.72 | 2.33 |
| Chloramphenicol | y = 202.22x - 758.72 | 0.9999 | 10-250 | 3.44 | 7.35 | 1.64 | 1.52 | 0.99 | 1.19 |
| Ketoconazole | y = 303.92x + 873.6 | 0.9998 | 1-100 | 0.28 | 0.79 | 0.67 | 0.85 | 2.42 | 0.92 |
| Nalidixic acid | y = 8127.3x - 7517.5 | 0.9997 | 0.5-100 | 0.12 | 0.40 | 0.88 | 1.03 | 2.01 | 1.34 |
| Rifampicin | y = 8237.7x - 18833 | 0.9992 | 1-100 | 0.31 | 0.82 | 2.05 | 1.61 | 1.15 | 1.84 |
| Miconazole | y = 551.37x + 1508.5 | 0.9996 | 0.5-100 | 0.17 | 0.43 | 1.83 | 0.94 | 1.28 | 1.69 |

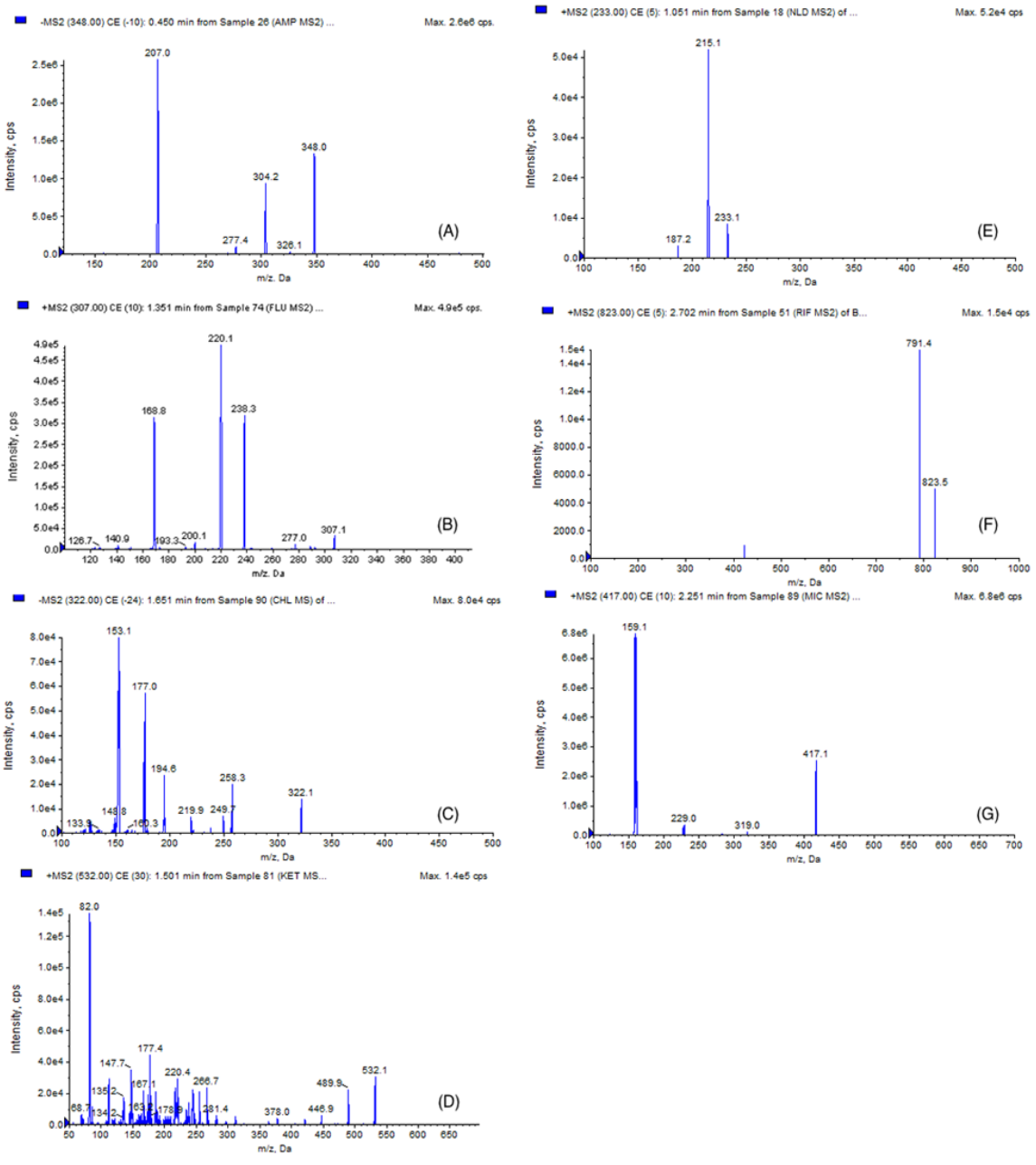



**Fig 7: MS/MS Spectra of reference analytes; (A) ampicillin, (B) fluconazole, (C) chloramphenicol, (D) ketoconazole, (E) nalidixic acid, (F) rifampicin, (G) Miconazole**

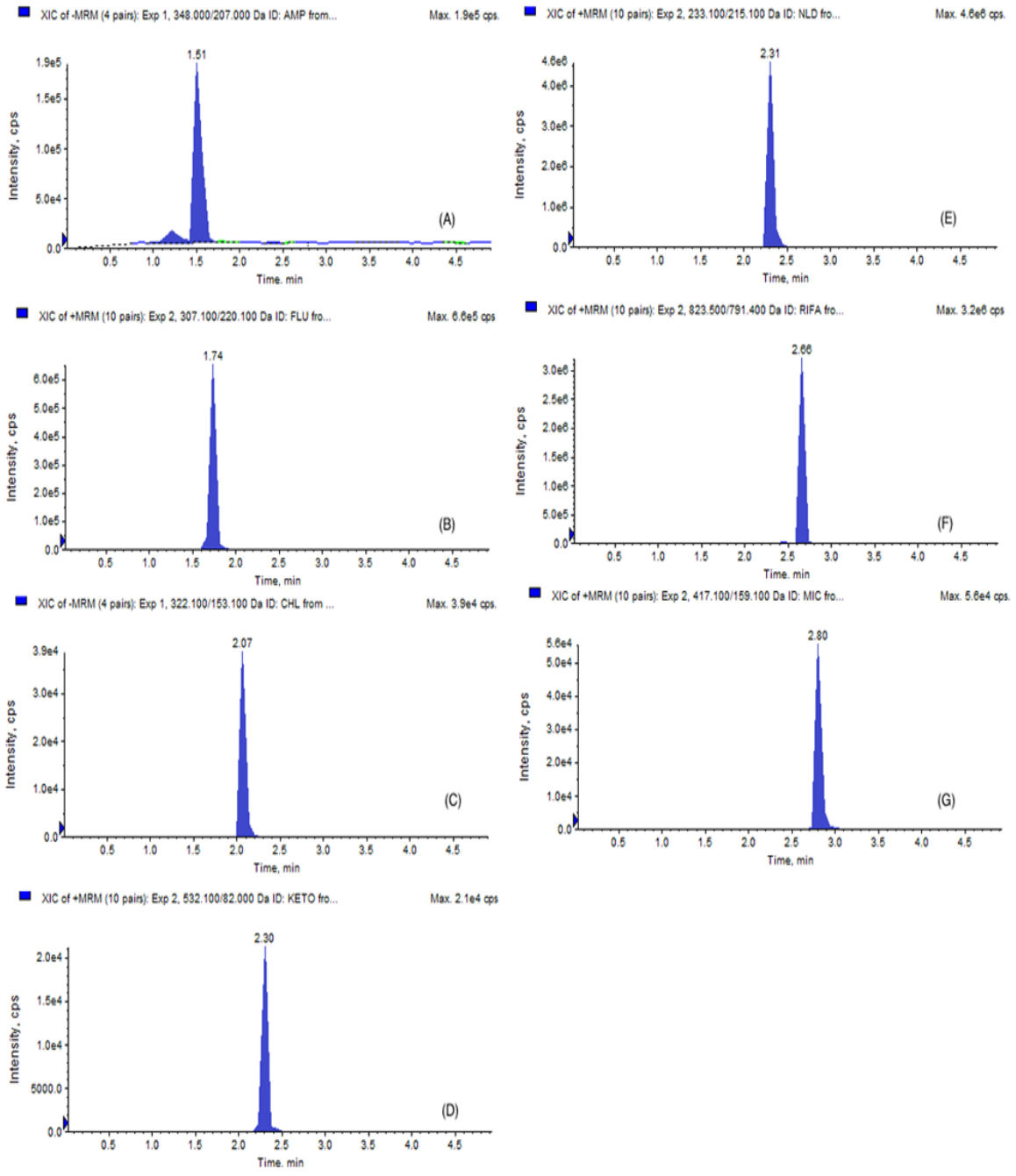



**Fig 8: MRM extracted ion chromatogram of reference analytes; (A) ampicillin, (B) fluconazole, (C) chloramphenicol, (D) ketoconazole, (E) nalidixic acid, (F) rifampicin, (G) Miconazole**

**Table 6: Quantification of potential three isolates using UPLC-ESI/MS/MS (µg/g)**

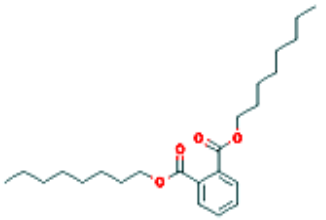
| | Ampicillin | Chloramphenicol | Fluconazole | Ketoconazole | Miconazole | Nalidixic acid | Rifampicin |
|---|---|---|---|---|---|---|---|
| BPS_CRB12 | 14.80 | 99.3 | 42.1 | 163 | 5.0 | 44.2 | 33.5 |
| BPS_CRB14 | 0.26 | 122 | 43 | 164 | 0.11 | 40.5 | 26.6 |
| BPS_CRB41 | 1.56 | 386.7 | 22.5 | 221.3 | 46 | 83.7 | 17.0 |

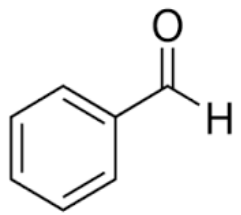
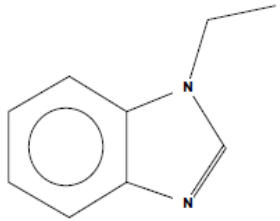
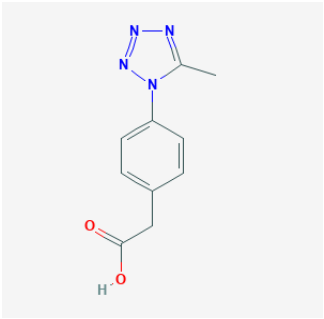
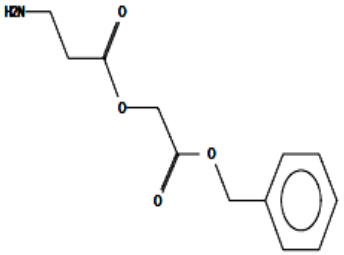
*Gas Chromatography Mass Spectroscopy (GC-MS) Analysis*

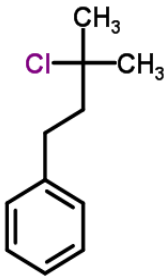
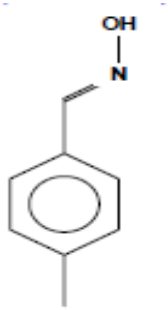
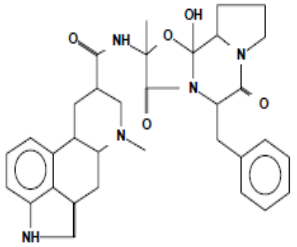
Methanolic extract of the three strains, BPS-CRB12, BPS-CRB14 and BPS-CRB41, based on the antimicrobial activity was estimated using GC-MS, and 20 volatile compounds were identified which is compared with the NIST library based on their peak area, molecular weight, percentage of the area, molecular formula, and retention time (Table 7). The GC –MS chromatogram of the crab isolate are given in (Table 8).

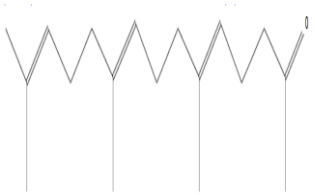
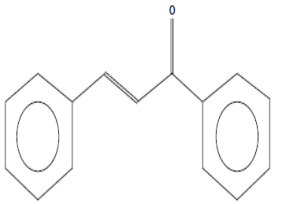
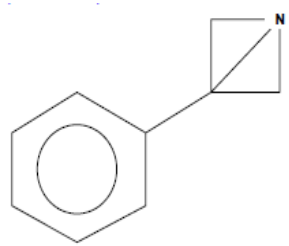
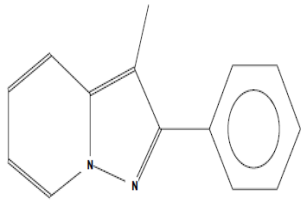
The methanolic extract of BPS-CRB12 detected two volatile compounds: (1) Di-n-octyl phthalate and (2) 1-Bromo-3,7-Dimethyloctane. Conversely, the strain BPS_CRB14 detected 11 volatile compounds: (1) Benzaldehyde; (2) 1h-Benzimidazole, 1-Ethyl; (3) Benzeneacetic Acid, 4-(1h-1,2,3,4-Tetrazol-1-Yl); (4) Benzyl 2-(3-Aminopropionyloxy)Acetate; (5) Benzene, (3-Chloro-3-Methylbutyl); (6) Benzaldehyde, 4-Methyl-, Oxime; (7) Ergotaman-3',6',18-Trione, 9,10-Dihydro-12'-Hydroxy-2'-Methyl-5'-(Phenyl Methyl) (5'-Alpha, 10-Alpha); (8) 5,9,13-Pentadecatrien-2-One, 6,10,14-Trimethyl-, (E,E); (9) 2-Propen-1-One, 1,3-Diphenyl-, (E); (10) 3-Phenyl-1-Aza-Bicyclo[1.1.0]Butane; and (11) Pyrazolo[1,5-A]Pyridine, 3-Methyl-2-Phenyl. Moreover, the methanolic extract of BPS-CRB41 detected seven volatile compounds: (1) Heptanal; (2) Benzoic Acid, 4-Chloro; (3) Benzoic Acid, 2-Chloro; (4) 4-Chlorobenzoic Acid, 4-Hexadecyl Ester; (5) 2-Chlorobenzoic Acid, 3-Methylbutyl Ester; (6) Anthranilic Acid, N-Methyl-, Butyl Ester; and (7) Benzeneacetic acid, .Alpha.-Oxo-, Trimethylsilyl Ester. The highest peak area (%) of 71.31 was found in the Di-n-octyl phthalate compound with a retention time of 25.28, and the lowest peak area (%) of 28.69 was detected in 1-Bromo-3,7-Dimethyloctane with a retention time of 28.47 in the BPS_CRB12 strain. Similarly, a maximum peak area (%) of 34.099 was found in the Benzyl 2-(3-Aminopropionyloxy) acetate compound with a retention-time 21.355 in the BPS_CRB14 strain, whereas the greatest peak area (%), 78.814, was found in the Benzoic acid, 4-Chloro compound with a retention time of 18.229 in the methanolic extract of the BPS_CRB41 strain. The chemical structures are shown in Table 7.

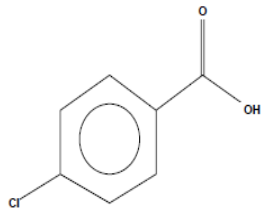
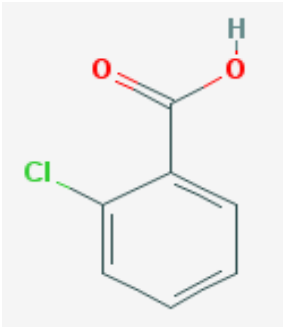
**Table 7: GC-MS Chromatogram detected volatile compounds from methanolic extract of potential three bacterial strains compared with NIST library**

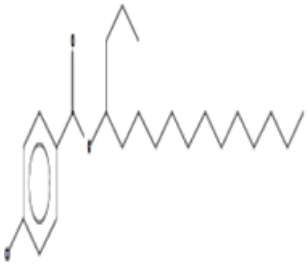
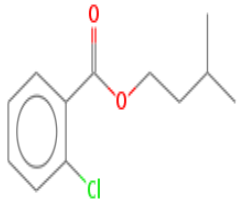
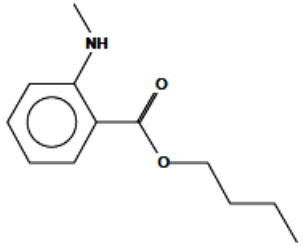
| Sl.N0 | Compound Name | Formula | MW | RT | Height | Area % | Norm % | Structure |
|---|---|---|---|---|---|---|---|---|
| **BPS_CRB12** | | | | | | | | |
| 1 | Di-n-octyl phthalate | C24H38O4 | 390 | 25.283 | 61,828,900 | 77.671 | 71.31 |  |
| 2 | 1-Bromo-3, 7-Dimethyloctane | C10H21Br | 220 | 28.474 | 17,773,776 | 22.324 | 28.69 |  |
| **BPS_CRB14** | | | | | | | | |

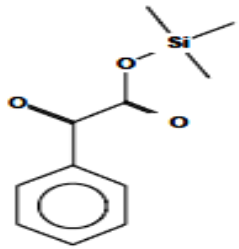
| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1 | Benzaldehyde | C7H6O | 106 | 13.95 | 5,106,615 | 10.521 | 77.08 |  |
| 2 | 1H-BENZIMIDAZOLE, 1-ETHYL- | C9H10N2 | 146 | 20.074 | 6,852,816 | 4.982 | 14.61 |  |
| 3 | Benzeneacetic Acid, 4-(1h-1,2,3,4-Tetrazol-1-Yl) | C9H8O2N4 | 204 | 20.185 | 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 | 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 |  |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 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 |  |

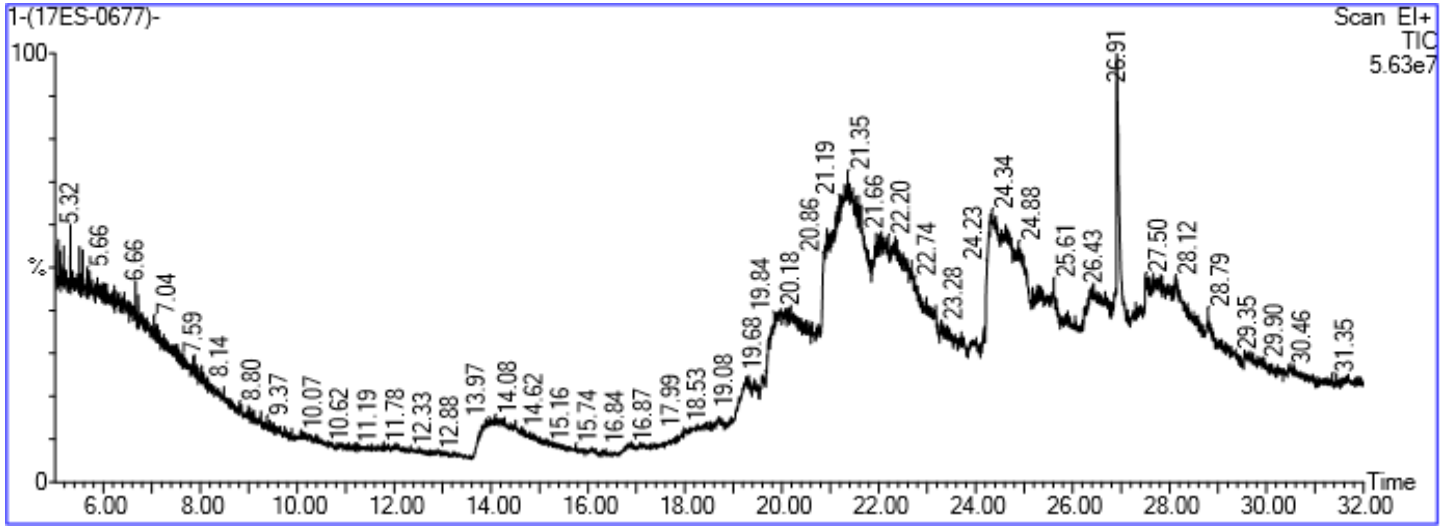



**Fig 9. GC –MS chromatogram of the crab sample from the isolate BPS_CRB 14.**

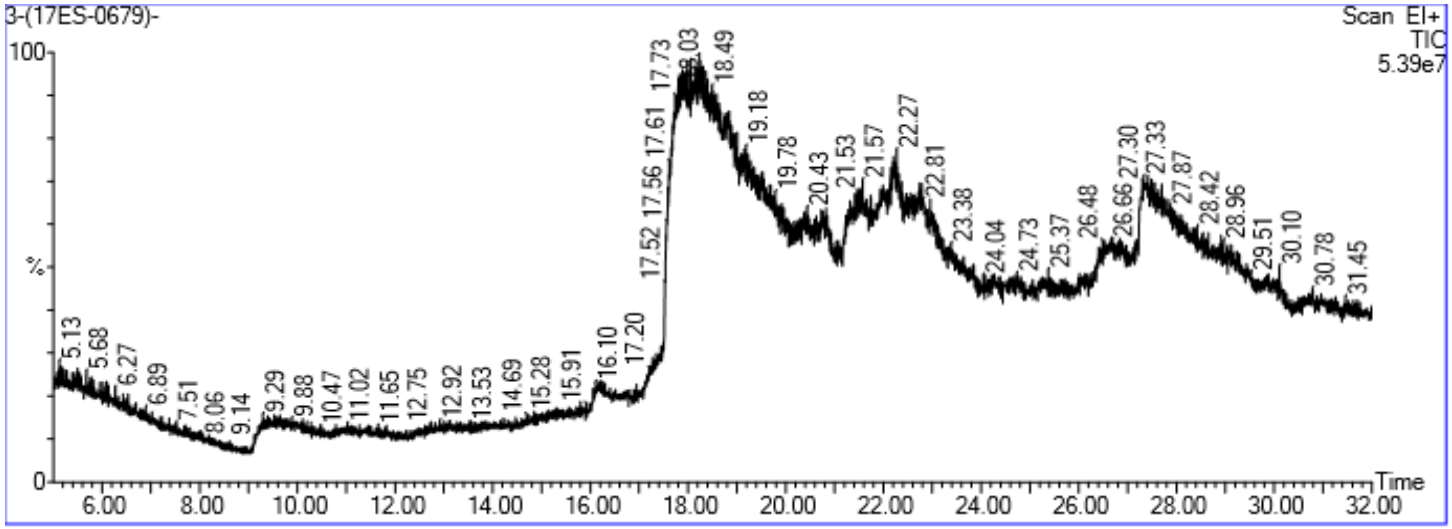



**Fig. 10. GC- MS chromatogram of the crab sample from the isolate BPS_CRB 41**

# CHAPTER- V

## DISCUSSION

Marine life provides useful bioactive compounds helpful in developing potential pharmacological utilization. Now a day there is a great emergence of infectious diseases and resistance to antibiotics by the present ones lead to the improvement of new drug discovery. Antimicrobial peptides serve as the most potential and important host defence system of many animal species. Bacterial population isolated from the freshwater marine life serves as an alternate source for some antimicrobial agents (Mayer et al., 2011; Olicard et al., 2005; Roch et al., 2008; Zothanpuia et al., 2016), even phylum Arthropoda strongly contribute to contain antimicrobial agents. Some Brachyuran crabs have shown evident activities which may be helpful in many biomedical areas. So, research in finding the potential of crabs is still rarely unexplored, for the discovery of many useful bioactive compounds. Hence, it is very important to isolate the mud crab and analyze their potential importance and understand their biosynthetic potential.

In the present study, a total of 43 isolates were recovered from the different parts of the crab, where 41.8% of the bacterial population was obtained from carapace, followed by abdomen (26.8%), leg (19.5%) and hand (14.6%). These findings were consistent with the conclusion of Kim *et al.* (2013), who stated that 67% of *Bacillus* sp. was localized in the heart, while 33% was found in the gill and carapace in *C. japonicas*. Five selected isolation media was used for isolation process, From TSA media 21 strains were obtained, 11 from AIA, 5 from $TH_2O$, 4 from SCA and 2 strains by using Nutrient Agar media. Out of the 5 nutritional media, TSA serves to be the best nutritional media for recovering the bacterial population, followed by AIA and $TH_2O$. These findings were in accordance with Kim *et al.* (2013), who affirmed that TSA and NA would be the best 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.

*Bacillus* sp. strain BPS_CRB12, *Bacillus anthracis* strain BPS_CRB14 and *Aneurinibacillus aneurinilyticus* strain BPS_CRB41 showed antimicrobial activities against most of the tested bacterial and fungal pathogens. The highest activity against *P. aeruginosa* and *M. luteus* was shown by isolate *Aneurinibacillus aneurinilyticus* strain BPS_CRB41. The result of the zone

of inhibition were similarly reported by Perez et al. (1992), who confirmed that the *B. subtilis* MIR 15 strain also displayed antimicrobial activity against *P. aeruginosa*, *E. coli* and *M. luteus*. Further, The maximum antimicrobial activity beside *S. aureus* and *E. coli* was revealed by *Bacillus* sp. strain BPS_CRB 12 which was supported by Munoz-Atienza et al. (2013), who reported that 20% of the bacteria have antimicrobial activity against three of the eight tested bacterial pathogens. *Bacillus anthracis* strain BPS_CRB14 exhibit the most significant antimicrobial activity (Haber and Ilan, 2013; Crawford et al., 2009; Kim et al., 2015; Addae et al., 2014 and Athamna et al., 2004). Isolate BPS_CRB 12, BPS_CRB 14 and BPS_CRB 41, showed a good $IC_{50}$ concentration against the tested bacterial and fungal pathogens. From our findings, isolate BPS_CRB 41 showed maximum 50% inhibition against *M.leuteus* (70.71%)and *E.coli* (85.11%), our findings is controversial with the findings of Lee et al., 2007 who states that the maximum inhibition is maximum against *P. aeruginosa* and *S. aureus*, which was isolated from marine polychaete *Arenicola marina.*

All the bacterial isolates were recognized using PCR amplification of the 16S rDNA gene. They were grouped into six families and seven genera, and shows superior relationship between the bacterial populations associated with crab. In our study, *Bacillus* the dominant genus, followed by *Staphylococcus*, *Pseudomonas*, *Enterobacter*, *Aeromonas*, *Alcaligenes* and *Acinetobacter*. likewise, Sivasubramanian et al. (2017) reported that most common and dominant genus was *Bacillus* which was currently found in all the examined crabs, while some other genera, such as *Bacteroide*s, *Acinetobacter*, *Flavobacterium*, *Chryseobacterium* and *Porphyrobacter*, were known in crab digestive gut. Furthermore, minority of other bacterial genera, such as *Pseudomonas*, *Aeromonas*, *Alcaligenes*, *Photobacterium*, *Vibrio*, *Enterobacter* and *Staphylococcus*, were also found in crab gut (Sivasubramanian et al., 2017). Gram-positive bacteria belongs to the gut microflora in crabs, 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 recommend that Proteobacteria and Firmicutes are the major phyla present in gastro-intestines of insect gut (Harkin et al., 2014; Sathiyamurthy et al., 1990; Sivasubramanian et al., 2017). From our findings, the dominant phylum was found to be Firmicutes (n=25; 58.2%), followed by Proteobacteria (n=18; 41.8%). Same way, Liu et al.(2011); Venkateswaran et al. (1981); Harkin et al. (2014) also reported that Firmicutes the most dominant phylum, followed by Proteobacteria and Bacteroidetes from the gut samples of crab.

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 6.0 (Saitou and Nei, 1987). The transition and transversion bias ratio was 1.54. The maximum log likelihood for the substitution computation was -2404.648. 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 6.0 (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 6.0 software with the Hasegawa Kishino Yano model (Tamura et al., 2011; Saitou and Nei, 1987). Further, for the CYP gene, the evolutionary model was selected based on the lowest BIC value (2381.152) and highest AIC value (2248.220) using MEGA 6.0 (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 6.0 software with Kimura 2-parameters model (Tamura *et al.*, 2011; Saitou and Nei, 1987).

In our study, three isolates, BPS_CRB12, BPS_CRB 14 and BPS_CRB 41, indicate positively amplified products of PKS type II, NRPS, CYP and PhzE genes. These results recommend that PKS type II, NRPS,CYP gene pathway may be widespread in the various crab gut intestine-derived bacteria. This finding was reliable with the finding of Jami et al. (2015), stated that the diversity of thees genes are associated with secondary-metabolite biosynthesis, including PKS type II, NRPS, CYP genes, among the bacteria. In addition to this, NRPS genes are engaged in the expansion of bioactive compounds and perform a role in iron metabolism system or quorum sensing (Finking and Marahiel, 2004; Passari et al., 2015). Moreover the CYP gene encodes the cytochrome P450 hydroxylase enzyme in polyene antibiotics biosynthesis (Lee et al., 2006), which might be detected in the genera *Bacillus* and *Pseudomonas*. However, this is the first effort made for screening antimicrobial biosynthetic (PKS type II, NRPS, CYP) genes detected in crab-associated bacteria. The potential strains could be useful for genetic manipulation and combinatorial biosynthesis, which may help the production of novel pharmaceutical compounds.

Standard antibiotics, such as fluconazole, chloramphenicol, ampicillin, ketoconazole, rifampicin and Miconazole, were detected and identified from bacterial strains BPS_CRB12, BPS_CRB14 and BPS_CRB41, for the first time from the crab which exhibited relevant antimicrobial activity. The highest concentration of the antibacterial antibiotic chloramphenicol (386.7 µg/g) was detected from *Aneurinibacillus aneurinilyticus* strain

BPS_CRB41 and antifungals ketoconazole (221.3 µg/g) and miconazole (46.0 µg/g), whereas in *Bacillus* sp. strain BPS_CRB12, maximum amount of the antibacterial antibiotics ampicillin (14.8 µg/g) and rifampicin (33.5 µg/g) was detected. This finding was reliable with the finding of Passari et al. (2017), who stated that the endophytic *Streptomyces olivaceu*s strain BPSAC77, *Streptomyces thermocarboxydus* strain BPSAC147, *Streptomyces* sp. strain BPSAC101 and *Streptomyces* sp. strain BPSAC121 showed the existence of several antibiotics, such as erythromycin, ketoconazole, fluconazole, chloramphenicol, rifampicin and miconazole, using the UPLC-ESI-MS/MS method. The standard antibiotics, chloramphenicol (Moore et al., 2013) and ampicillin (Lloyd-Williams and Robinson, 1980), which distinguished to inhibit the growth of *S. aureus* and *E. coli*, were reported for the initial time from *Bacillus* sp. strain BPS_CRB12, *Bacillus anthracis* strain BPS_CRB14 and *Aneurinibacillus aneurinilyticus* strain BPS_CRB41 using the UPLC-ESI-MS/MS method and might be thoughtful to be responsible for its antimicrobial activities. At the same time, the *Bacillus* sp. strain BPS_CRB12, *Bacillus anthracis* strain BPS_CRB14 and *Aneurinibacillus aneurinilyticus* strain BPS_CRB41 contained the antibiotics ketoconazole and fluconazole, which are helpful in fighting against the fungi *Candida albicans* (Martin, 1999; Finkel et al., 2009).

GC-MS is an appropriate method to identified the components of volatile compounds, that are used by researchers (Teng hern et al., 2015; Jog et al., 2014; Sharma et al., 2016, Zothanpuia et al., 2017). Volatile compounds which are produced by bacteria, includes alcohols, ketones, esters, small alkenes, monoterpenes, sesquiterpenes, and derivatives (Kai et al., 2009). In this study, totally 20 volatile compounds were detected in three methanolic extracts of the strains BPS_CRB12, BPS_CRB14 and BPS_CRB41 using GC-MS analysis. In *Bacillus* sp. strain BPS_CRB12, Di-n-octyl phthalate constitute 71% of the total amount present which was also reported by Sarkar et al. (2012) and Chen et al. (2013). Benzaldehyde,well known to be very effective cytotoxic potential, major volatile compounds found in the strain BPS_CRB14. This compound was similarly reported by Ulker et al. (2013) and Gupta et al. (2015), stated that benzaldehyde has the ability to devastate cancer cells at a minimum concentration. Furthermore, Ergotaman-3', 6', 18-Trione, 9, 10-Dihydro-12'-Hydroxy-2'-Methyl-5'-(Phenyl Methyl) (5'-Alpha, 10-Alpha) was detected in strain BPS_CRB14, which showed antimicrobial activity according to Shanthakumar et al. (2015), who declared that these volatile compounds Ergotaman-3', 6', 18-Trione, 9, 10-Dihydro-12'-Hydroxy-2'-Methyl-5'-(Phenyl Methyl) (5'-Alpha, 10-Alpha) exhibit antimicrobial activity against some tested pathogens: *S. enteric, S. flexneri, E. coli* and *K. pneumoniae,* respectively. Pyrazolo [1,5-A] Pyridine, 3-Methyl-2-

Phenyl was detected in the methanolic extract of BPS_CRB14 and reported to have anticancer activity (Kamal et al., 2016). Moreover, Heptanal was recognized in methanolic extract of BPS_CRB41 and was
reported to have lung anticancer activity (Chen et al., 2017; Xu & Wang, 2012). This is the first effort to analyze eight volatile compounds, (1) 1h-Benzimidazole, 1-Ethyl; (2) Benzeneacetic Acid, 4-(1h-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]. From the methanolic extracts of *Bacillus anthracis* strain BPS_CRB14, Butane was identified and reported. 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, Alpha.-Oxo-, Trimethylsilyl Ester, were known and detected for the first time in *Aneurinibacillus aneurinilyticus* strain BPS_CRB41 using GC-MS analysis.

From our findings, the bacterial isolates producing good secondary metabolites can be further analyse and checked for their antimicrobial compound. The volatile compounds detected can be further screened for their antimicrobial activity whereas for anti- cancer activity which will be beneficial for pharmaceuticals industries. Identification and characterization of bacterial isolates can be useful in medicinal production and can be further analyse for mass production of bioactive noval compounds and antioxidant compounds which will be further beneficial for human.

## SUMMARY and CONCLUSION

- A total of A total of 43 isolates were obtained from the different parts of the Crab
- The isolate *Bacillus sp* (BPS_CRB 12), *Bacillus anthracis* (BPS_CRB 14) and *Aneurinibacillus aneurinilyticus* (BPS_CRB 41) showed best positive results for antimicrobial activity.
- Among the three best potential isolates, *Aneurinibacillus aneurinilyticus* (BPS_CRB 41) showed the maximum50% inhibition of pathogens activity against *M. luteus* and *E. coli.*
- Methanolic extract of the selected extract detected 20 volatile compounds in *Bacillus sp. , Bacillus anthracis , ,Aneurinibacillus aneurinilyticus.*
- Antibiotic detection using UPLC MS/MS method, revealed that these three isolates showed best result out of which *Aneurinibacillus aneurinilyticus* (BPS_CRB 41) have the best antibiotic content.
- Overall, among the 43 isolates *Aneurinibacillus aneurinilyticus* (BPS_CRB 41) was the best isolate showing both antimicrobial potential and antibiotic content.
- The present study described the genetic diversity of bacterial isolates along with their biosynthetic potential against bacterial pathogens.
- The isolates that produced good secondary metabolites can be further studied in details for specific antimicrobial compound extraction.
- Thus in conclusion the identification and characterization of potential bacterial strains isolated from different organs of crab; can be useful as medicinal as well as production of bioactive compounds and antioxidant compound which are beneficial for human.

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## APPENDIX

MEDIA COMPOSITION OF FIVE RESPECTIVE MEDIA:

1. Nutrient agar (10g peptone, 5g beef extract/yeast extract, 10g NaCl, 20g of agar in 1000 ml of distilled water, ph 7.2)

2. Actinomycetes isolation agar (2g sodium caseinate,0.1g L-asparagine, 4g sodium propionate, 0.5g dipotassium phosphate, 0.1g magnesium sulfate, 0.001g ferrous sulfate,15g agar in 1000 ml of distilled water, ph 8.1);

3. Soyabean Casein Digest Medium (tryptone 17g, soya peptone 3g, sodium chloride 5g, dipotassium hydrogen phosphate 2.5g, Dextrose 2.5 g, agar 20g in 1000ml of distilled water ph 7.3)

4. Tap Water Yeast Extracts Agar (2g dipotassium phosphate, 5g yeast extract, 20g agar in 1L of tap water, ph 7.2).

5. Starch Casein Agar (10 g of starch, 1 g of M- protein powder, 37g of sea water, 15 g of agar in 1 L of distilled water, ph 7.2).

## Conference and Workshop attended:

- **Phylogenetic Affiliation and Biosynthetic Potential of Bacteria Isolated from Different Parts of Fresh Water Edible Crab**

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- National Level Workshop on Biostatistics and Bioinformatics, held during 1st September – 7th September 2016 organized by the Department of Biotechnology, Mizoram University Sponsored by Bioinformatics Infrastructure Facility, Department of Biotechnology (DBT), New Delhi.

- Understanding Basic Principles in Human Molecular Genetics held during 7th September – 11th September organized by the Department of Biotechnology, Mizoram University Sponsored by Bioinformatics Infrastructure Facility, Department of Biotechnology (DBT), New Delhi.

- Antibiotic Awareness and Infection Control Program held during 14th November,2017 organized by the Department of Biotechnology, Mizoram University Sponsored by Bioinformatics Infrastructure Facility, Department of Biotechnology (DBT), New Delhi.

Paper Communicated –

- Prediction of bacterial population associated with organs of mud crab, *Scylla olivacea* and their biosynthetic potential in **Saudi Journal of Biological Sciences.**

# DISTRIBUTION OF BACTERIAL POPULATION ASSOCIATED WITH DIFFERENT ORGANS OF EDIBLE MUD CRAB (*Scylla olivacea* Herbst, 1896) AND THEIR ANTIMICROBIAL POTENTIAL

## ABSTRACT

**Dissertation submitted in fulfilment of the**

**Requirements for the Degree of**

**Master of Philosophy**

**In Biotechnology**

By
**Joanne Zote**

**Registration No. MZU/M.Phil/358 of 26.5.2017**

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# CONTENTS

# INTRODUCTION

*Scylla olivacea* one of the species of mud crab is one preferable and an important species among the crab. They belong to the following classification- genus *Scylla*, Phylum- Arthropoda, subphylum- Crustacea, Order- Decapoda, Family- Portunidae and species- *Serrata*. They are found mostly in Asian countries and, Australia including India. They are commercially preferable species for aquaculture. The shell covers varies from molten green, dark brown and black colour. In recent years, the taxonomic classification of the species was controversial, identification was done based on their morphological characters, and various researches have classified the species into different types of species and sub- species. The classification of (Estampador, 1949) was widely accepted- which consists of 3 species (*S. serrata, S. olivacea and S. tranquebarica*) and one sub- species (*Scylla Serrata var. Paramamosain*). Crabs are rich in vitamin $B_{12}$,calcium, phosphorus in keeping the bones and blood vessels strong, and contain Omega 3-fatty acids for brain development and protection against heart disease ( Kim et al., 2012; Chun et al., 2009).

Several researches have proved that marine biodiversity are a rich source of many bioactive compounds. Research on Hemolymph of fresh water crab (*Oziotelphusa senex senex)* concludes that when tested against clinical pathogens, the result confirms that they have a strong immune mechanism that might be an efficient and important antimicrobial agents which might be helpful for synthesis of novel compounds for future use (Sumalatha et al., 2016). Crabs contain antimicrobial peptides play a great role in humoral immunity and they are the aquatic life that can defence themselves against infectious diseases Miyata, 1989. Intensive farming has resulted in massive use of antibacterial agents for therapeutic use for the treatment of bacterial fish diseases (Smith et al., 1994). The use of antimicrobial has a massive influence which led to the increase frequency of resistant in bacterial microflora Exploring these bioactive compounds can really develop the pharmaceutical as well as food industry.

However, as there is no systematic study done so far on bacterial population associated with the different organs related with the genus *Scylla olivacea*, so this study is taken up to understand the bacteria population and the role they play in aquaculture farming as well as in many fields like biomedical fields and food industry.

The aims and objectives set for carrying out the proposed work

- Isolation and identification of bacterial population associated with different organs of mud crab
- Screening for their antimicrobial potential and detection of antimicrobial biosynthetic genes.
- Determination of antimicrobial compounds using UPLC ESI MS/MS and GC-MS.

## Materials and Methods

*Sample Collection*

The fresh crab samples were collected from Lengte market, Mamit district, Mizoram, Northeast India during September 2016. The samples taken were free from disease. They were collected in sterile polythene bags, bought into the laboratory and processed for further isolation.

*Isolation of Bacteria from different parts of the crab*

Before processing the isolation, surface sterilization was done by washing the collected crab sample thoroughly with running water to remove organic materials and impurities. The isolation of bacteria was carried out by spread plate technique. The different parts of the crab were taken namely- abdomen, carpace, leg and hand. Each part was taken individually and grind them with motor and pestle. Two gram of each part was homogenized with 10 ml of 10 mM potassium phosphate buffer. 100 µl of each homogenate parts was taken and was spread on five different nutritional media. The plates were incubated at 37 $^{o}$C for 24 h to observe the colonies of bacteria. Isolated single colonies were picked and re-streaked on respective media to obtained pure isolates. The pure isolates were maintained on agar slants at 4 $^{o}$C.

*Isolation Media*

Five specific nutritional media were used to obtain the population of bacteria. The used media are: 1. Nutrient agar (peptone, beef extract/yeast extract, Nacl, agar); 2. Actinomycetes

isolation agar (Sodium caseinate, L-asparagine, sodium propionate, dipotassium phosphate, magnesium sulphate, ferrous sulphate, 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) .

*Screening of Antimicrobial activity using the agar well diffusion method*

The antimicrobial screening was performed against the bacterial pathogens *Staphylococcus aureus* (MTCC-96), *Pseudomonas aeruginosa* (MTCC-2453), *Escherichia coli* (MTCC-739), *Micrococcus luteus* (MTCC-5262), *Bacillus subtilis* (MTCC-2097) and *Candida albicans* (MTCC-3017). The tested organisms were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India, and maintained in the Molecular Microbiology and Systematic Laboratory in the Department of Biotechnology at Mizoram University. The antimicrobial activity was performed using agar well diffusion method (Saadoun and Muhana, 2008 & Zothanpuia et al., 2016).

*Minimum Inhibitory Concentration (MIC) of the Potential Isolates*

The potential isolates showing the best antimicrobial activity were selected for broth micro dilution technique of 96 wells microtiter plate as described by Eloff (1998). The bacterial culture suspension was prepared to make the final concentration of $1.0 \times 10^4$ CFU/mL (OD= 0.403). Crude extract of the potential isolates was prepared at different concentration (1-10 mg/ml) with 10% DMSO solution. The prepared crude extract of different concentration was added in 96 wells microtiter plate, along with test bacterial culture suspension ($1.0 \times 10^4$ CFU/mL)**.** Standard antibiotic (ampicillin, 0.01µg/ml) along with bacterial cultures which will be used as positive control whereas; DMSO containing bacterial cultures was used as negative control. The plates were incubated at 37 ºC for 36 h and absorbance was taken at 620 nm in spectrophotometer UV-VIS (MultiscanTM GO, Thermo Scientific, MA, USA). Results were documented as $IC_{50}$ values which indicate 50% reduction of bacterial growth. The $IC_{50}$ values were calculated by using calibration curve drawn by using linear regression.

*Molecular Characterization of bacterial isolates*

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

Total genomic DNA was extracted using the Bacterial DNA Purification Kit (Invitrogen, Life technologies KT-110052) as per the manufacturer's instructions. All the isolates were subjected to amplification of the 16S rRNA gene using the PA universal primers (forward 16S rRNA 5'-AGAGTTTGATCCTGGCTCA-3' and PH reverse 16S rRNA primer 5'-ACGGCTACCTTGTTACGACT-3') as described by Cui et al.(2008). The amplified products of PCR were cleaned using a QIA quick gel extraction kit (Qiagen, Hilden, Germany) and sequencing was performed using Sci-Genome Pvt. Ltd, Cochin, In The 16S rDNA gene sequences were contrast with the NCBI database and showing a high level of identity (97-100%) were selected as the closest match. All the sequences were submitted to NCBI GenBank, and accession numbers were obtained. The evolutionary models were selected based on the lowest BIC ("Bayesian Information Criterion") and AIC ("Akaike Information Criterion") values using MEGA 6.0, and the phylogenetic tree was evaluated by bootstrap analysis with 1,000 resamplings using a *p*-distance model (Saitou and Nei, 1987; Felsenstein, 1985).

*Biosynthetic gene amplification (PKS type II; NRPS, CYP gene )*

*PKS type II:*

Polyketide synthase (PKS) type II gene fragments were amplified using degenerate primers, as described by Ayuso-Sacido and Genilloud (2005).

PKS II forward (KS∞-5′-TSGCSTGCTTGGAYGCSATC-3′

PKS II reverse ( KSβ-5′-TGGAANCCGCCGAABCCTCT-3′)

*NRPS gene:*

Non-ribosomal peptide synthase (NRPS) fragments were amplified using degenerate primers, as per Meng Yuan *et al.* (2014).

NRPS forward (5′-GCSTACSYSATSTACACSTCSGG-3′)

NRPS reverse (5′-SASGTCVCCSGTSCGGTAS-3′)

*CYP gene:*

Cytochrome p450 (CYP) fragments were amplified using degenerate primers as per Meng Yuan *et al.* (2014)

CYP forward (5′-TGGATCGGCGACGACCGSVYCGT-3′)

CYP reverse (5′-CCGWASAGSAYSCCGTCGTACTT-3′)

*Phylogenetic analysis of Biosynthetic genes (PKS type II; NRPS,CYP genes)*

Biosynthetic gene sequences (PKS type II, NRPS, CYP) were varying with the sequences obtained from the NCBI database using the BLASTn search tool (Tamura *et al.,* 2011), and a multiple sequence alignment was performed on all the gene sequences using the Clustal W software package in MEGA 6.0 (Thompson *et al.,* 1997). The phylogenetic tree was constructed by the maximum likelihood method using MEGA 6.0 software with Kimura 2-parameters model (Tamura *et al.,* 2011; Saitou and Nei, 1987).

*Detection of Antibiotics using Ultra-Performance Liquid Chromatography (UPLC-ESI MS/MS)*

*Preparation of Standard Solution*

Standard antibiotics (trimethoprim, fluconazole, ketoconazole, nalidixic acid, and rifampicin) were prepared in methanol. The standard solution was prepared as per Passari et al. (2017).

*Instrumentation and analytical conditions*

An Acquity ultra-performance liquid chromatography (UPLC$^{TM}$) system consisting of an autosampler and binary pump (Waters, Milford, MA) was used to carry out the analysis as per Passari et al. (2016) and Zothanpuia et al. (2017). MRM parameters were optimized to achieve the most abundant, specific and stable MRM transition for each compound.

*Method Validation*

*Analytical Method Validation*

The proposed UPLC-MRM method for quantitative analysis was validated according to the Passari et al. (2016) & Mishra et al. (2017).

*Linearity, limits of detection (LOD) and quantification (LOQ)*

LOD and LOQ were determined by the calibration curve method as per Passari et al., (2016) & Mishra et al., (2016).

*Precision, Stability and Recovery*

The precision, s*tability and recovery* was measured as per Passari et al., (2106) & Mishra et al., (2017). Three replicates were performed at each level.

*Gas Chromatography Mass Spectroscopy (GC-MS) Analysis*

The volatile compounds present in the selected potential isolates were examined and identified using GC-MS, as described by Ser *et al.* (2015) and Sharma *et al.* (2016), with minor modifications. The peaks were identified by matching the mass spectra with the library from the National Institute of Standards and Technology (NIST, USA).

## Summary of the findings

The highest bacterial population was recovered from Carpace (41.8%) followed by Abdomen (26.8%), Leg (19.5%) and Hand (14.6%). From this findings, we can conclude that TSA media was the best nutritional media where most of the bacterial population was recovered, which is followed by AIA , $TH_2O$, SCA, NA media. The morphology of the bacteria were smooth and rough texture, forming white, yellowish, brown colour colonies. Out of the 43 isolates tested for antimicrobial activity, 60% of the isolates were showing good activity against the tested one or two pathogens. From our findings, 72.1% of the strains exhibited antimicrobial activity against *P. aeruginosa*, while only approximate 55%,of the strains showed to have activity against *B. subtilis, E. coli and S. aureus* respectively. Besides, BPS_CRB41 revealed highest antimicrobial activity against *P.aeruginosa* and *M. luteus.* Isolates (BPS_CRB 12, BPS_CRB14 and BPS_CRB41) the three best isolates were tested for broth micro dilution assay against the 6 bacteria pathogen viz. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus*, *Bacillus subtilis* and yeast *Candida albicans*. Out of these three tested isolates, maximum 50% inhibition against *M. luteus* and *E. coli* was shown by methanolic extract of BPS_CRB 41 whereas highest inhibition against *S. aureus* (71.76%) was inhibited by isolate BPS_CRB14. Maximum 50% inhibition against *B. subtilis* (92.82%) was obtained from isolate BPS_CRB12. 16S rRNA was used for identifying the bacteria. Detection of modular synthase PKS II gene detected (20.9%) strain, (16.2%) strains in NRPS gene and (18.6%) strains in CYP gene. Furthermore, the three best isolates (BPS_CRB 12, BPS_CRB14 and BPS_CRB41) showing best antimicrobial activity also showed the presence of six antibiotics which is detected using Ultra Performance Liquid Chromatography(UPLC EMS MS/MS) method, and also detected twenty volatile compounds (VOC's) using the method of Gas chromatography Mass-spectrometry(GC- MS). Thus, we can conclude our findings that the strain Aneurinibacillus aneurinilyticus BPS_CRB41 potentially produce bioactive natural products and can be used in pharmaceutical industries.

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