Evaluation of antibiotic producing genes in *Streptomyces* isolated from a desert environment of Saudi Arabia

Mahera M. Shinwari [1], Sulaiman Ali Alharbi*[1], Ismet Ara [1] and Milton Wainwright[1,2]

[1] Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh-114 51, Saudi Arabia.
[1,2] Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, S102TN, UK
[3]King Abdulaziz City for Science and Technology, P.O. Box 6086, Riyadh 11442, Saudi Arabia
sharbi@ksu.edu.sa*

Abstract: In this research 20 actinobacteria isolates species were obtained from a desert extreme environment of the Kingdom of Saudi Arabia. The isolates were identified as species of *Streptomyces* using the diaminopimelic acid method and were evaluated for their ability to produce genes relating to polyketide synthase type I (PKSI), polyketide synthase type II (PKS II) and non-ribosomal peptide (NRPS). In all 20 isolates high frequencies of positive PCR amplification were obtained for PKS-I (15%), PKS-II (50%) and NRPS (50%). High detection levels of biosynthetic systems were for PKSII and NRPS were found and observed in most isolates. However, some strains did not express any of the three genes, although they did exhibit antimicrobial activity.
[Mahera M. Shinwari, Sulaiman Ali Alharbi, Ismet Ara and Milton Wainwright. **Evaluation of antibiotic producing genes in *Streptomyces* isolated from a desert environment of Saudi Arabia**. *Life Sci J* 2013;10(4):974-980]. (ISSN:1097-8135). http://www.lifesciencesite.com. 125

Keywords: Actinobacteria, *Streptomyces*, diaminopimelic acid, polyketide, non-ribosomal peptide, gel electrophoresis.

1. Introduction

Streptomyces species which produce a large number and variety of antibiotics can be readily isolated from soil and cultivated in the laboratory. They are aerobic gram-positive soil bacteria that grow vegetatively as a branching and usually non-fragmenting mycelium. Aerial hyphae arise from the vegetative substrate mycelium of surface grown colonies which produce spores, generally in chains. When growing on laboratory media these bacteria produce colonies which often exhibit alternating surface color which results from spore formation allied with the formation of white powdery aerial mycelium and multiple rounds of germination and sporulation (Dowding, 1973). Germinated spores, vegetative hyphal fragments and aerial hyphal fragments are all capable of initiating a new colony a process which can be blocked by the formation of mutants.

Most antibiotics are products of the secondary metabolism of three main groups of microorganisms namely, eubacteria, actinobacteria and the filamentous fungi. The actinobacteria group produces the largest number and greatest variety of antibiotics (Waksman, 1950). This group of bacteria consists of a wide range of branching unicellular gram-positive bacteria, with DNA rich in guanine and cytosine (70%). They occur extensively in nature, and can be readily isolated from soil, composts, and aquatic habitats. Most species are free-living and saprotrophic, some form symbiotic associations, while others are pathogenic in man, animals and plants.

Polyketides represent a large group of natural products (secondary metabolites) that are produced by several microorganisms including bacteria, fungi, and also by plants. Many of these compounds are clinically important drugs, such as tetracycline, daunorubicin, erythromycin, rapamycin and lovastatin are polyketide antibiotics (Austin, 2003; Staunton & Weissman, 2001), synthesized by repetitive condensations of small carbon precursors; typically acetate or propionate acyl groups which are derived from malonyl or methylmalonyl coenzyme A thioesters, respectively; i.e. polyketides are polymers of ketide units connected together. These compounds fall into the following classes:

Aromatic and complex polyketides

Aromatic polyketides are built mainly from the condensation of acetate acyl units and the β− carbonyl group after each condensation step is left largely unreduced. The polyketide chain is rearranged directly after synthesis to from an aromatic product. Examples of such products include polycyclic aromatic compounds such as oxytetracycline, actinorhodin and anthracycline compounds like daunorubicin. The enzymes responsible for the biosynthesis of aromatic polyketides are encoded by genes called aromatic polyketide synthases or are otherwise known as PKSII (polyketide synthase type II).

Complex polyketides can be built by condensation from acetate, propionate and butyrate acyl units. The level of the β− carbonyl reduction in complex polyketide synthesis can differ from one

condensation round to the next. The polyketide chain continues to grow until the desired length is reached, when the chain is cyclized to form the end product. The enzymes responsible for the biosynthesis of the complex polyketides are encoded by genes termed modular polyketide synthases, or are otherwise referred to as PKSI (polyketide synthase type I).

Nonribosomal peptides (NRPS)

NRPs (non-ribosomal peptides) are a class of peptide secondary metabolites, produced by bacteria and fungi. Non-ribosomal peptide synthetases (NRPS) are large enzymes that are ordered in modules holding specific domains which incorporate amino acid building blocks in a sequence into a growing peptide chain (Mootz et al., 2001, Schwarzer et al., 2003). NRPS gene clusters encode for a wide range of non-ribosomal peptides, including antibiotics (Cane and Walsh, 1999), toxins (Mansson et al., 2011), siderophores (Crosa et al., 2002) to anti-inflammatorials and immunosuppressants (Cane and Walsh, 1999). The importance of these compounds has motivated extensive searches for novel NRPS genes in microbial isolates and environmental samples. Degenerate oligonucleotide primers have been designed and used to unravel the diversity of NRPS genes in actinobacterial and fungal endophytes of plants (Janso and Carter, 2010; Johnson et al., 2007) in actinobacterial (Schneemann et al., 2010; Jiang et al., 2007; Zhang et al., 2009) and fungal (Zhou et al., 2011) isolates of marine sponges, free-living freshwater Cyanobacteria (Ehrenreich et al., 2005), and finally, marine actinobacteria (Gontang et al., 2010; Hodges et al., 2012). Mixed PKS-NRPS gene clusters from the marine sponge *Discodermia dissolute* have also been detected using a metagenomic approach (Schirmer et al., 2005), and a bimodular NRPS gene cluster has been cloned from a *Chloroflexi*-symbiont of the marine sponge *Aplysina aerophoba* using phi29-mediated whole genome amplification (Siegl and Hentschel, 2010).

2. Material and Methods

Cell wall analysis

Diaminopimilic acid (DAP) isomers are important components of the cell wall peptidoglycan of Gram-positive bacteria, including actinobacteria. If a Gram positive bacterium has a peptidoglycan containing one of the DAP isomers, the DAP is mostly located in the cell wall. Therefore DAP isomers of peptidoglycan can be determined by whole-cell analysis.

In order to achieve bacterial cell hydrolysis approximately 3 mg of dried cells was hydrolyzed with 50 µl of 6N HCl (Winlab, UK) in an Eppendorf tube, autoclaved at 121^{o}C for 15 minutes and then centrifuged. The supernatant was then analyzed by thin layer chromatography (TLC) plates (Hasegawa, et al., 1983). Each sample was applied as 3 µl to the base line of a cellulose TLC plate (20 cm X 20 cm) (Merck No.126827) using a capillary tube. A standard of 1 µl of 10mg (0.01g) DL-2, 6-diaminopimelic acid (DL-DAP) (Sigma Chemical Co., St. Louis Mo., USA) was also applied; this authentic material is a mixture of DAP isomers. TLC was developed with a solvent system comprising methanol-water-6N HCl-pyridine (80:26:4:10 v/v) contained in a covered tank. Chromatograph-development took approximately 4 h. The spots were visualized by spraying with 0.2% ninhydrin solution containing (0.2g ninhydrin (Merck, GERMANY), 100ml acetone (Winlab, UK) followed by heating at 100^{o}C for 5 min; DAP isomers appeared as violet color spots with RF values of 0.29 (LL-isomer) and 0.24 (meso- and DD-isomer) (Staneck and Roberts 1974; Ara and Kudo, 2006).

Molecular analysis

In order to extract or extract bacterial DNA, an enzymatic lysis buffer was prepared containing [20mM Tris.HCl, 2mM sodium EDTA and 1.2% Triton X-100; 180µl of the pre-prepared enzymatic lysis buffer was the added to each sample and incubated at 37^{o}C for 30 minutes. Proteinase K (25µl) was added to the samples followed by 200µl of AL buffer (without ethanol) followed by vortex mixing for 20 seconds and the incubation at 56^{o}C for 30 minutes. Ethanol (200µl of a 70% solution) was added to the samples and mixed by vortex for 20 seconds. This mixture was pipetted into mini column filters provided with the kit and centrifuged at 8000 rpm for 1 minute. The filtrate was discarded and a new column was fitted into the filter. 500µl of AW1 (Washing solution) was added to the samples and centrifuged at 8000 rpm for 1 minute. The liquid was discarded and column was fitted again to the filters. 500µl of AW2 (Washing solution) was added to the samples then centrifuged at 14000 rpm for 3 minutes. The filtrate was discarded and the mini filters were then placed in clean Eppendorf tubes followed by the addition of 200µl of AE buffer to the samples. The samples were then incubated at room temperature for 1 minute and then centrifuged at 8000 rpm for 1 minute.

PCR technology

The Master Mix (Table 1) contained all of the components necessary to make new strands of DNA in the PCR process. The prepared DNA was used as template DNA for Taq polymerase. Reactions were performed in a final volume of 25µl containing 2.5µl of each primer, 2.5µM of all four dNTPs (Roche), 0.2µl Taq polymerase (Appligene) with its recommended reaction buffer.

Primers and PCR conditions

The following sets of degenerated primers were used for PCR amplification of the PKS gen type 1, PKS gen type 2 and NRPS gene respectively: set 1:

(K1F: 5` – TSA AGT CSA ACA TCG GBC A - 3`) and (M6R: 5` – CGC AGG TTS CSG TAC CAG TA – 3`) (Ayuso-Sacido and Genilloud, 2005) , set 2: (Ketela-KS α-F: 5` – TSG CST GCT TCG AYG CSA TC – 3`) and (Ketela- KS β-R: 5` –TGG AAN CCG CCG AAB CCT CT – 3`) (Ketela et al., 2002; Reddy et al., 2012) and set 3: (A3F: 5`- GCS TAC SYS ATS TAC ACS TCS GG - 3`) and (A7F: 5`– SAS GTC VCC SGT SCG GTA S - 3`) (Ayuso-Sacido and Genilloud, 2005).

Amplification

A PCR Reagent kit (HotStartTaq DNA polymerase) was used to amplify the region of interest in all DNA samples. The amplification was performed in a Microseal 96-well PCR plate with a total volume of 25µl for each well. Each well comprised 23µl of the master mix including 2µl of the DNA sample. For control 23µl of the master mix and 2µl of distilled water was added to a well instead of DNA; the Microseal 96-well PCR plate was then placed in the thermal cycler and, the program being set for each primer separately. Gradient PCR was carried out in order to determine the correct annealing temperatures for each pair of primers, The thermal cycler program settings used for DNA amplification of the three genes are shown in (Table 2), (Table 3) and (Table 4).

Amplifications were performed in a Thermal Cycler, according to the following profile: 5 min at 95oC, 35 cycles of 30 s (seconds) at 95oC, 35 cycles for 2 min at 55oC for K1F/M6R, 58oC for Ketela-KSα-F / Ketela-KSβ-R and 59oC for A3F / A7R, 35 cycles for 4 min at 72oC, followed by 10 min at 72oC. Amplification products were then analyzed by electrophoresis in 1% (w/v) agarose gels stained with ethidium bromide.

Table 1. The master mix contents, concentration and amounts

Components	**Concentrations**	**Amounts 1X**	**25X**
Distilled water	-	16.3 µl	407.5 µl
10X PCR buffer	-	2.5 µl	62.5 µl
dNTP	2.5 µM	2 µl	50 µl
Forward & Revers primers	5 µM	2 µl	50 µl
DNA	10	…2 µl	-
Hot start taq	-	0.2 µl	5 µl
Total volume		25 µl	-

The master mix was distributed into PCR tubes and DNA was added after calculating the correct individual dilution required.

Table 2.Thermal cycler program settings for PKSI

Step	Temperatures	Time mm:ss	Cycles
Denaturation	95°C	5:00	1
	95°C	00:30	35
Annealing	55°C	2:00	35
Extension	72°C	4:00	35
	72°C	10:00	1
	4°C	∞	1

Table 3. Thermal cycler program settings for PKSII

Step	Temperatures	Time mm:ss	Cycles
Denaturation	94°C	5:00	1
	94°C	00:30	35
Annealing	58°C	2:00	35
Extension	72°C	4:00	35
	72°C	10:00	1
	4°C	∞	1

Table 4. Thermal cycler program settings for NRPS

Step	Temperatures	Time mm:ss	Cycles
Denaturation	95oC	5:00	1
	95oC	00:30	35
Annealing	59oC	2:00	35
Extension	72oC	4:00	35
	72oC	10:00	35
	4oC	∞	1

Gel Electrophoresis

After completing the PCR amplification 2% agarose solution was made in 100 ml 1X TBE buffer in a flask. The flask was then heated in the microwave until all the agarose was completely dissolved. The gel was removed from the microwave and allowed to cool until 60^{o}C then 5µl of the dye ethidium bromide was added to the gel this was handled with care because it is a carcinogenic substance.

After preparing the gel, the DNA ladder (2µl of 1000 bp) was injected in to the first well in the gel, an orange dye was applied to the samples and pippeted to mix then 2µl of each sample containing PCR product was injected into the remaining wells after labeling them. A current of 120V was applied for the DNA to move toward the positive anode charge which was running for one hour. After running the gel, it was placed under UV light to visualize the DNA bands. Visualized bands of the DNA and desired amplified DNA segment were then photographed.

3. Results

Cell wall structure analysis

Isomers of diaminopimelic acid (DAP) in the cell wall hydrolysates of the studied strains were determined by thin layer chromatography following the standard methods of (Waksman and Henrici, 1943) and (Boone and Pine, 1968)

DNA extraction

Bacterial DNA was successfully extracted from all twenty isolates; (Figure 1) shows the visualized gel bands photographs for the extracted DNA.

Figure 1. Photograph of the gel showing the bands of the extracted DNA

PCR and Gel electrophoresis analysis

Using the genomic DNA as a template, PCR products with predicted sizes of about 680 bp, 613 bp and 700 bp were amplified from most of the twenty strains under the study using degenerate primers designed based on the conserved region of the KS domain of PKSI, PKSII and NRPS. Only five strains did not show any expression of the three studied antibiotic producing genes. Expression of the genes expression is shown in Table 5 and the gel bands results are shown in Figure 2, Figure 3 and Figure 4.

Table 5. Expression of antibiotic producing genes

Strain	PKSI	PKSII	NRPS
M3	-	+	+
M7	-	-	+
M10	-	+	-
M11	-	+	+
M12	+	+	+
M13	+	-	+
M15	-	+	-
M16	-	+	+
M19	-	-	+
M20	-	-	-
M24	-	-	-
M25	-	-	-
M27	+	-	+
M28	-	+	+
M29	-	+	-
M33	-	-	-
M36	-	+	-
M38	-	-	+
M42	-	+	+
M47	-	+	+

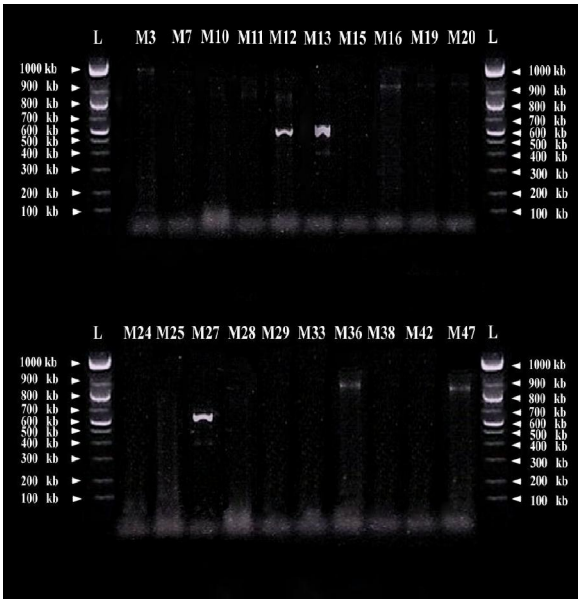

Figure 2. Gel results showing the expression of PKSI gene

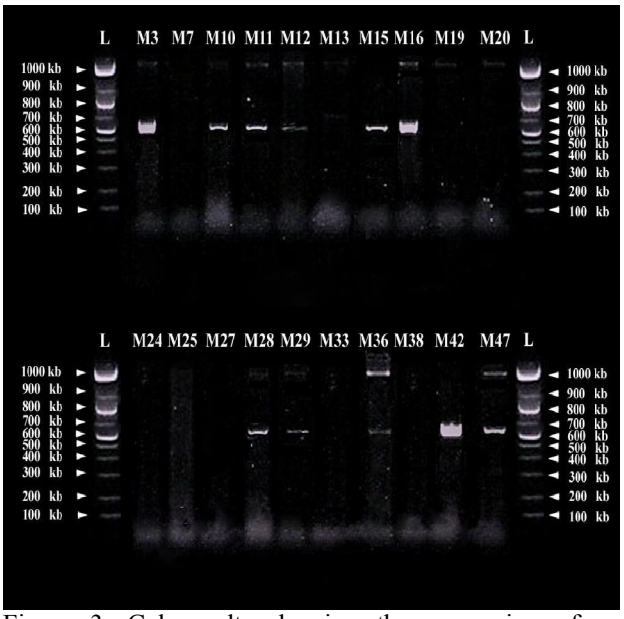

Figure 3. Gel results showing the expression of PKSII gene

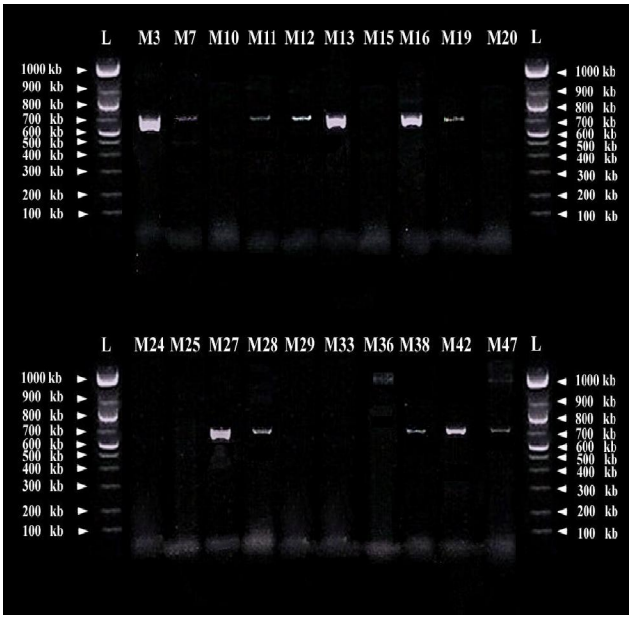

Figure 4. Gel results showing the expression of NRPS gene

4. Discussions

Diaminopimelic acid (DAP) is an amino acid that is a derivative of lysine. DAP, a characteristic of certain cell walls of some bacteria. Bacteria exhibit normal growth when provided with this compound, while in its absence they continue to grow, but are unable to make new cell-wall peptidoglycan.

Diaminopimelic acid (DAP) exists in three stereoisomeric forms: the LL-, DD- and meso-isomers (Work, 1963). The genus *Streptomyces* is characterized by the LL- DAP isomer of diaminopimelic acid in its cell wall which showed that all twenty strains under this study contained the LL-DAP as diagnostic acid of the cell-wall. Our results show that peptidoglycan, was found in all of the isolates and as result, we can confident that they are species of *Streptomyces*. This finding is similar to that of (Xianwen et al., 2010) who studied the presence of LL-DAP in the whole –cell hydrolysates of the strain ACMA006.

The *Streptomyses* isolates studied here were screened for the presence of three biosynthetic genes, namely PKS-I, PKS-II and NRPS via specific primers. High frequencies of positive PCR amplification were obtained for PKS-I (15%), PKS-II (50%) and NRPS (50%). The highest detection levels of biosynthetic systems were for PKSII and NRPS and were observed in most isolates. However, some strains did not express any of the three genes although they did express antimicrobial activity. These percentages differed slightly from those reported by Peng *et al*., (2009), who found PKS I, PKS II, and NRPS amplicons in 79.6, 70.4, and 57.4%, respectively. In the study reported by Houbo et al., (2012) the highest percentage for antibiotic producing genes was for NRPS and lowest was for PKSI ; i.e..findings which are similar to those found to be present in this study.

Acknowledgements:
This research was supported by a grant from King Abdulaziz City for Science and Technology (KACST), General Administration of Research Grants, (Saudi Arabia); Also this research project was supported by a grant from the "Research Center of the Center for Female Scientific and Medical Colleges", Deanship of Scientific Research, King Saud University (Saudi Arabia).

Corresponding Author:
Dr. Sulaiman Ali Alharbi
Department of Botany and Microbiology
College of Science, Building Number:05
King Saud University
P.O.Box: 2455; Riyadh-11451
Kingdom of Saudi Arabia
E-mail: sharbi@ksu.edu.sa

References

1. Dowding JE, Vivian A, Chater KF, Hopwood DA. Advances in *Streptomyces coelicolor* genetics. Bacteriology Rev 1973; 37, 371-405.
2. Waksman SA. *The Actinomyctes. Their Nature, Occurrence, Activities and Importance*. Chronica Botanica Company, Waltham, Mass. 1095; USA.
3. Austin MB, Noel JP. The chalcone synthase superfamily of type III polyketide synthases, Nat. Prod. Rep 2003; 20, 79–110.
4. Staunton J, Weissman KJ. Polyketide biosynthesis: a millennium review, *Nat. Prod. Rep* 2001; 18, 380–416.
5. Mootz HD, Schwarzer D, Marahiel MA. Ways of assembling complex natural products on modular nonribosomal peptide synthetases. Chem. BioChem 2001; 3, 490–504.
6. Schwarzer D, Finking R, Marahiel MA. Nonribosomal peptides: From genes to products. *Nat. Prod. Rep* 2003; 20, 275–287.
7. Cane DE, Walsh CT. The parallel and convergent universes of polyketide synthases and nonribosomal peptide synthetases. Chem. Biol 1999; 6, 319–325.
8. Mansson M, Gram L, Larsen TO. Production of bioactive secondary metabolites by marine vibrionaceae. Mar. Drugs 2011; 9, 1440–1468.
9. Crosa JH., Walsh CT. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. Microbiol. Mol. Biol. Rev. 2002. 66, 223–249.
10. Janso JE, Carter GT. Biosynthetic potential of phylogenetically unique endophytic actinomycetes from tropical plants. *Appl. Environ. Microbiol* 2010; 76, 4377–4386.
11. Johnson R., Voisey C, Johnson L, Pratt J, Fleetwood D, Khan A., Bryan G. Distribution of NRPS gene families within the *Neotyphodium/Epichloe* complex. Fungal Genet. Biol 2007; 44,1180–1190.
12. Schneemann I, Nagel K, Kajahn I, Labes A, Wiese J, Imhoff JF. Comprehensive investigation of marine Actinobacteria associated with the sponge *Halichondria panicea*. Appl. Environ. Microbiol 2010; 76, 3702–3714.
13. Jiang S, Sun W, Chen M, Dai S, Zhang L, Liu Y, Lee KJ, Li X . Diversity of culturable actinobacteria isolated from marine sponge *Haliclona sp*. Antonie Van Leeuwenhoek 2007; 92, 405–416.
14. Zhang W, Li Z, Miao X, Zhang F. The screening of antimicrobial bacteria with diverse novel nonribosomal peptide synthetase (NRPS) genes from South China Sea sponges. Mar. Biotechnol 2009; 11, 346–355.
15. Zhou K, Zhang X, Zhang F, Li Z. Phylogenetically diverse cultivable fungal community and polyketide synthase (PKS), non-ribosomal peptide synthase (NRPS) genes associated with the South China Sea sponges. Microb. Ecol 2011; 62, 644–654.
16. Ehrenreich IM, Waterbury JB, Webb EA. Distribution and diversity of natural product genes in marine and freshwater cyanobacterial cultures and genomes. Appl. Environ. Microbiol 2005; 71, 7401–7413.
17. Gontang EA, Gaudencio SP, Fenical W, Jensen PR. Sequence-based analysis of secondary-metabolite biosynthesis in marine actinobacteria. Appl. Environ. Microbiol 2010; 76, 2487–2499.
18. Hodges TW, Slattery M, Olson JB. Unique actinomycetes from marine caves and coral reef sediments provide novel PKS and NRPS biosynthetic gene clusters. Mar. Biotechnol 2012; 14, 270–280.
19. Schirmer A, Gadkari R, Reeves CD, Ibrahim F, DeLong EF, Hutchinson CR. Metagenomic analysis reveals diverse polyketide synthase gene clusters in microorganisms associated with the marine sponge *Discodermia dissoluta*. Appl. Environ. Microbiol 2005; 71, 4840–4849.
20. Siegl AM, Hentschel U. PKS and NRPS gene clusters from microbial symbiont cells of marine sponges by whole genome amplification. *Environ. Microbiol. Rep* 2010; 2, 507–513.
21. Hasegawa T, Takizawa M, Tanida S. A rapid analysis for Chemical grouping of aerobic actinomycetes. J. Gen.App. Micorbiol 1983; 29, 319-322.
22. Staneck JL, Roberts GD. Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. App.Microbiol 1974;28, 226-231.
23. Ara I, Kudo TL. Three novel species of the genus *Catellatospora* , *Catellatospora chokoriensis sp*. Nov., *Catellatospora coxensis* sp. nov. and *Catellatospora bangladeshensis* sp , nov and transfer of *Catellatospora citrea* subsp. Methionotrophica Asano and Kawamoto 1988 to *Catllatospora methionotrophica* sp. Nov. comb. Nov. int. J. Syst. Evol. Microbiol 2006; 56, 393-400.
24. Ketela MM, Halo L, Munukka E, Hakala J, Mantsala, P, Ylihonko K. Molecular evolution of aromatic polyketides and comparative sequence analysis of polyketide ketosynthase and 16S ribosomal DNA genes from various streptomyces species. Appl. Environ.Microbiol 2002; 68, 4472–4479.
25. Reddy BVB, Kallifidas D, Kim JH, Powers ZC, Feng Z, Brady SF. Natural product biosynthetic

gene diversity in geographically distinct soil microbiomes. Appl.Environ.Microbiol 2012; 3744–3752.
26. Ayuso-Sacido A, Genilloud O. New PCR primers for the screening of NRPS and PKS-I systems in actinomycetes: detection and distribution of these biosynthetic gene sequences in major taxonomic groups. Microb Ecol 2005; 49, 10–24.
27. Waksman SA, Henrici AT. The nomenclature and classification of the actinomycetes. J Bacteriol 1943; 46: 337-4.
28. Boone KE, Pine L. Rapid method for characterization of actinomycetes by cell wall composition. Appl Microbiol 1968; 16: 279-84.
29. Work E. (1963). A, e-Diaminopimelic acid. In *Methods in Enzymology*, 634. Edited by S. P. Colowick and N. 0. Kaplan. New York: Academic Press. 1963; 6: 624-630.
30. Xianwen S, Ailing L, Yanping L, Xiaoqi H, Lin D, Jun M, Guifa X, Ying L, Hongwei G, Guansheng M. (2010). The association of weight status with physical fitness among Chinese children. Int. J. Pediatr 2010; 1-6.
31. Peng QZ, Chen J, Zhang YQ, Chen Q, Peng DJ. *Virgibacillus zhanjiangensis* sp. nov., a marine bacterium isolated from sea water. Antonie van Leeuwenhoek 2009; 96: 645-652.
32. Houbo W, Wen C, Guanghua W, Shikun D, Danyan Z, Hengzhi Z, Yatao G, Yongchang O, Xiang L. Culture-dependent diversity of Actinobacteria associated with seagrass (*Thalassia hemprichii*). Afr. J. Microbiol. Res. 2012; 6,. 87-94.

10/21/2013