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Genetic analysis of antibiotic production and other phenotypic traits from *Streptomyces* associated with seaweeds

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The Gram-positive bacterium such as streptomycetes known for its production of a diverse array of biotechnologically important secondary metabolites, have major application in health, nutrition and economics of our society. There are limited studies on the genetics of streptomycetes, especially seaweed associated *Streptomyces* sp. So, the present study made an attempt to study the genetics of production of antibiotic and other phenotypic properties was demonstrated by plasmid DNA curing analysis. The DNA-intercalating agent ethidium bromide was used to eliminate plasmid DNA from streptomycetes and effects of curing agent (EB) on the antibiotic production and loss of other phenotypic traits such as aerial and substrate mycelial production, biomass production, protein synthesis were studied. The study demonstrates that the ethidium bromide is potent and probably region-specific mutagens that are capable of inducing high rates of plasmid loss (curing), production of antibiotics was not eliminated, but was reduced by 20.2-79.8% and extracellular protein of 26 KDa mol.wt. was unaffected by curing agents. Data suggests that production of antibiotics and other phenotypic traits likely chromosomally encoded in marine *Streptomyces* species. The study concludes that the new methodologies such as mutasynthesis have contributed substantially to the discovery of additional antibiotics as an added feather to the scope of antibiotic industry.

Key words: Plasmids, genetics of *Streptomyces*, curing, phenotypic traits, antibiotic production.

INTRODUCTION

Microbial molecular genetics is gaining its momentum and popularity over the conventional methods in the classification of microbes. Genetic manipulation and conventional genetic analysis of actinomycetes (mainly streptomycetes) producing antibiotics and other secondary metabolites have high-lightened their possible

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involvement in the biosynthesis of plasmids, clusters of antibiotic biosynthesis gene and inter-specific gene-exchange. There are few studies regarding the genetic basis for the biosynthesis of these antibiotic compounds and other biologically active secondary metabolites. The genetic determinants for antibiotic compounds as well as other secondary metabolites in *Streptomyces* are carried on plasmids (Ishibashi, 1992; Kawachi et al., 2000).

Plasmids have been identified in many *Streptomyces* spp. and have been implicated in the control of a wide range of phenotypic properties, including the transfer of chromosomal markers ('fertility'), antibiotic biosynthesis and resistance, differentiation and melanin formation (Hopwood et al., 1986a; Chater and Kinashi, 2007) and production of secondary metabolites with variety of applications (Dharma raj and Dhevendaran, 2012). It has also been shown that various antibiotic-producing streptomycetes harbor plasmids, which vary in size and/or form depending on the antibiotic production (Hayakawa et al., 1979). The loss of antibiotic and other secondary metabolite production can occur upon treatment with "curing agents" (Coyne et al., 1984). However, with certain exceptions, genetic evidence for the existence of *Streptomyces* plasmids has been limited to studies on the effect of plasmid-curing agents on the stability of particular phenotypes, notably antibiotic production (Ismail et al., 1998; Hopwood, 2006). The biosynthetic genes for antibiotics in *Streptomyces,* which form a gene cluster, are usually located on chromosome (Keasling, 2008). In *Streptomyces coelicolor* A3 (2), the antibiotic biosynthetic genes for methylneomycin is located in extrachromosome (Marnix et al., 2010; Bentley et al., 2004). In some cases, the plasmid genes may encode 'regulatory function' while the structural genes are located on chromosomes (Ravel et al., 2000). The morphological differentiations that exist within their colonies, phenotypic characteristics and their genetic control mechanisms that regulate these events have been of great biological interest. The genetic determinants for the production of antibiotics and other phenotypic traits have not been extensively studied. Considering the above facts, the present study aimed at analyzing the effects of curing agent (EB) on the production of antibiotics, loss of other phenotypic traits and plasmid DNA from *Streptomyces* species. Therefore, the purpose of this study was to ascertain whether the genes for antibiotic and other phenotypic traits are either plasmid-borne or encoded on the chromosome in *Streptomyces* isolated from seaweeds.

MATERIALS AND METHODS

In the present study, seaweeds were collected from Muttom coast which is situated in the south coast of India located at a distance of 34 km from Cape Comorin at (8° 7' 15" N: 77° 1'E) in India. Seaweeds were collected from the substratum along with the holdfast using a blunt knife and chiser. Seaweeds were transported to the lab in sterile polyethene bags immediately for further study.

Identification of seaweeds

The seaweed specimen was preserved in 4% formalin-seawater solution for further investigations. Some material was preserved in the form of herbarium sheets and kept in the Herbarium, Department of Botany, Holy Cross College. Cross sections (C. S.) of the material were obtained by free hands with the help of shaving blades, which were then stained with iodine, mounted in glycerine and sealed with the help of nail polish. Prepared slides were examined under microscope, and photographs were taken.

Isolation of *Streptomyces* from seaweeds

The seaweed extract were obtained by using the following method: 1 g mantle of live specimen was weighed, washed with distilled water and treated with 0.5% phenol for 5 min to inhibit the bacterial and fungal colonies present as contaminants. After washing with distilled water, the mantle was macerated. Aliquots of 1 ml of each seaweed extract was serially diluted with seawater to a dilution of 10^{-2}. A quantity of 1 ml of the dilutions were mixed with 20 ml of glycerol asparagine agar medium and incubated at room temperature (28 ± 2°C) for seven days. The *Streptomyces* colonies were counted and expressed as CFU/gm dry weight of visceral mass. The isolated strains were stored in ISP5 (glycerol asparagine agar Base) medium as agar slant cultures at 28 ± 2°C.

Characterization and identification of *Streptomyces* strain

Microscopic, cultural and physiological characteristics of *Streptomyces* strains were carried out by adapting the methods described by Shirling and Gottlieb (1966). Based on the characteristics, six strains were identified with the help of Nonomura keys (1974) and Actinobase database (Ugawa et al., 1989).

Isolation of plasmids from *Streptomyces*

Isolation of plasmid DNA from *Streptomyces* strains was carried out by alkaline lysis and potassium acetate precipitation. 2 ml of a 2- 4 day-old culture in FM medium were harvested by centrifugation (17,000× *g*, 4°C, 1 min). After washing with 1 ml of solution MP1 (Tris HCl-50 mM, EDTA-10 mM, RNase A 100 µg/ml), the cells were resuspended in 500 µl of solution MP1GL (Glucose-50 mM, Tris-HCl-25 mM, EDTA-10 mM, RNase A-100 µg/ml, Lysozyme-2-4 mg/ml) by vortexing. The suspension was incubated at 37°C for 30-60 min, then mixed with 500 µl of solution MP2 (NaOH-0.2 M, SDS-1%(w/v) by inversion and incubated at RT for 10 min. 400 µl solution MP3 (KAc.3H_2O-5 M) and 40 µl Rotiphenol® were added and mixed by inversion. The mixture was incubated on ice for 5 min. After 20 min centrifugation (20,000× **g**, 4°C), the supernatant was poured into a fresh microfuge tube and extracted twice with 300 µl Rotiphenol®. DNA was precipitated by addition of 0.8-fold volume of isopropanol and centrifugation (20,000× *g*, 4°C, 20 min). DNA pellet was washed with 500 µl 70% cooled ethanol, air dried and resuspended in 20-50 µl distilled water or TE buffer.

Agarose gel electrophoresis of DNA

Gel electrophoresis with 0.8-1% (w/v) agarose was used to separate DNA fragments. The buffer system employed was 1×TBE buffer. After running the gels, bands were detected under the UV light and photographed.

Plasmid DNA curing

Curing of plasmid was attempted by inoculating the suspension of

cultures into the Fermentation medium containing 10 mM EB. Broth cultures were incubated at 28°C in a shaker with 200 rpm up to sufficient growth. After sufficient growth, serial dilutions of cultures were made, each dilutions were plated onto GA agar medium and incubated at 28°C for 2-4 days. Presumptive aerial mycelium-negative colonies were picked up aseptically and re-placed onto same medium under the same conditions (modified protocol of Ismail et al., 1998)

Mycelial color determination

The cured and non-cured *Streptomyces* isolates were streaked on Petri dish containing glycerol asparagine agar, incubated at room temperature (28 ± 2°C) for seven days and mature sporulating aerial mycelium production was noticed. The cultures were classified into white, grey, red, green, blue, yellow and violet series depending on the aerial mycelial color. The color of the reverse side of *Streptomyces* colonies was observed; the strains were classified into pale yellow, olive or yellowish brown, cream depending on the substrate mycelial color.

Biomass production

The cured and non-cured isolates were cultured in broth medium under above said conditions. After sufficient growth, the cells were harvested by filtration. The wet minus dry weight of the filtrate was taken as growth.

Antimicrobial bioassay

Antibiotic activity of cured and non-cured *Streptomyces* strains was done against four different *Vibrio* spp. by disc diffusion method. Ten microlitre of *Streptomyces* culture was drawn in sterile discs and placed over the vibrio agar plates pre-seeded with V*ibrio* spp. The zone of inhibition was measured after 48 h in mm.

Drug resistance bioassay

The antibiotic sensitivity was tested against each cured and non-cured *Streptomyces* strain. Twelve antibiotic discs viz., gentamicin (10 µg), lincomycin (10 µg), penicillin-G (10 units), rifampicin (5 µg), streptomycin (10 µg), vancomycin (30 µg), amphotericin-B (100 units, 20 µg), chloramphenicol (30 µg), Erythromycin (15 µg), kanamycin (30 µg), nystatin (100 units), and tobramycin (10 µg) used in this piece of study were obtained from Hi-Media Pvt. Ltd., India. As per the specification, the concentration of each antibiotic was maintained. A suspension of the *Streptomyces* isolate was prepared to a particular McFarland standard (0.5 mcf), and then spread evenly onto a Muller-Hinton agar (M173) in a Petri dish. The commericial antibiotic disc was impregnated onto the medium and each plate was incubated at 28±2°C for 48 h during the study. After incubation, the occurrences and sizes of inhibition zones around the discs of the different antibiotics were tabulated (modified protocol of Rajput et al., 2012).

Extracellular protein profile

The strains of cured and non-cured isolates were inoculated onto GA broth medium, and incubated under submerged condition for four days. After four days, the twelve ml of culture was centrifuged at 1000 rpm for 10 min. The supernatant was collected and equal volume of 10% TCA (Tri Chloro acetic acid) solution was added. They were kept under refrigeration for over-night incubation (for precipitation). The precipitate was centrifuged at 1000 rpm for 15 min. Then, 50 or 100 µl of 1x PBS solution was added to the pellet and mixed well. This extracellular protein was stored at -20°C for further use (modified protocol of Subashkumar et al., 2007). Extracellular protein separation was made by 12.5% of SDS-gel SDS-PAGE electrophoresis (Lamelli, 1970).

RESULTS

Isolation and characterization of *Streptomyces* isolates

In the present study, 16 seaweeds were collected at monthly interval from Muttom coast, South India. The seaweeds were identified as *Gracillaria corticata, Chnoospora minima, Sargassum weightii, Spyridia hypnoides, Enteromorpha intestinalis and Hypnea valentiae, Gelidium microptera, Chatetomorpha media, Sargassum longifolium* etc., belonging to the order of Rhodophyta, Phaeophyta, Chlorophyta. For identification of Seaweeds, fixed in formalin, herbarium sheets were prepared and photographed and classified using guide (Dhargalkar, 2004). Seaweeds are the rich source of protein than cereals, egg and fish. Marine algae are not only the primary and major producers of organic matter in the sea, but they also exert profound effects on the density and distribution of other inhabitants of the marine environment. An understanding of the wide range of behavioral relationships that exist among organisms would provide us with clues to substances of biomedical interest. Secondary metabolites produced by the seaweeds and host organism to protect themselves and to maintain homeostasis in their environment (Sheeja, 1994; Dhevendaran et al., 2004; Kolanjinathan and Stella, 2011)

The maximum streptomycetes population (from 73-68 × 10^2) was observed in *Sargassum weightii*, *Enteromorpha intestinalis* and *Ulva lactuca*. The minimum *Streptomyces* population was (14- 5 × 10^2) observed in seaweeds such as *Chaetomorpha media* and *Hypnea valentiae* (Figure 1). Moreover, the streptomycetes strains were isolated using four different media such as Kusters agar, actinomyces agar, glycerol asparaginase agar and potato dextrose agar, and among the four media, GA medium obtained maximum number of streptomycetes isolates compared to other media (Figure 1). The present study is correlated with the findings of Anithakumary and Dhevendaran (2004) and Prasheetha (2008). Forty-five strains of *Streptomyces* different in aerial and substrate mycelial coloration were selected for the study. Among them, 15 isolates, which showed antimicrobial activity (Figure 2) against any one of four different *Vibrio* species (*V. harveyji*, *V.parahemolyticus*, *V. alginolyticus*, *V. vulnificus*) were tested for the presence of plasmids; out of them, six isolates having plasmids were further characterized by adopting the methods of International *Streptomyces* Project (ISP). The colonies of *Streptomyces* were slow-growing and had powdery

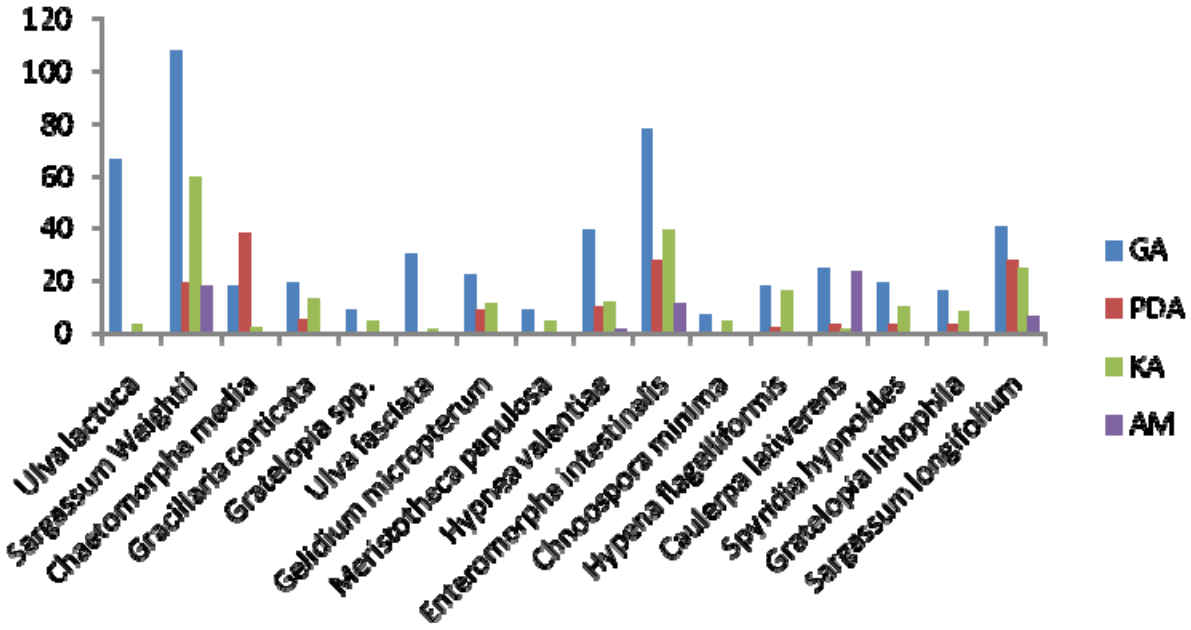


Figure 1. *Streptomyces* population recorded in different seaweeds in different months.

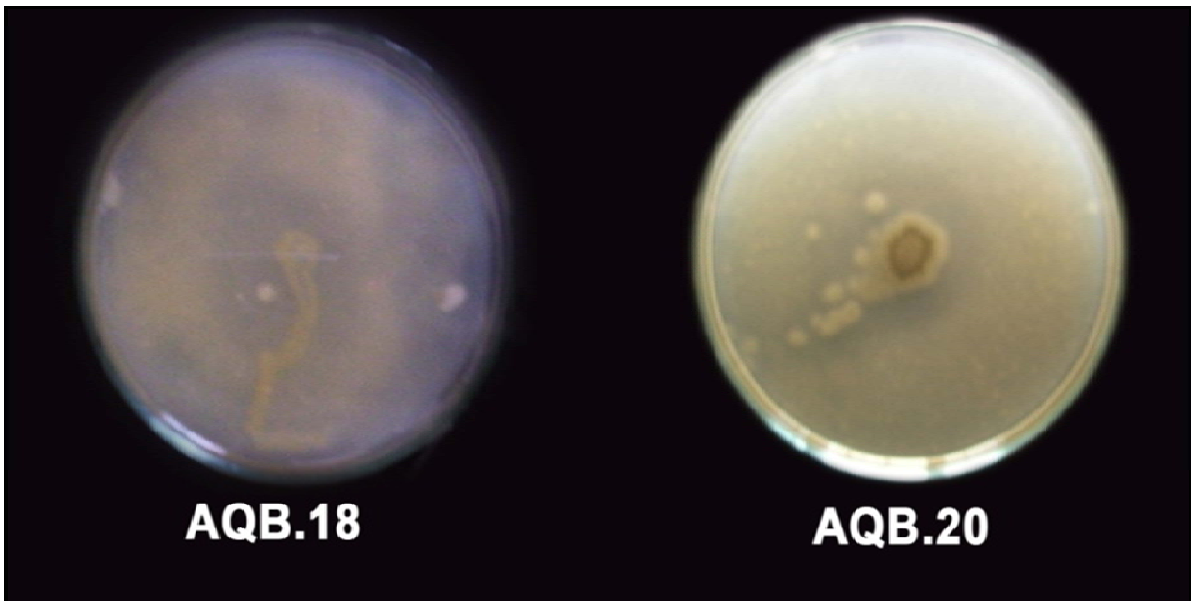


Figure 2. Antimicrobial activity of *Streptomyces* isolates against *Vibrio* sp. by double layer method.

appearance. Aerial mycelial colors of isolated strains were yellow, green, grey, white and bluish green and substrate mycelial color of each strain was very different like brown, reddish brown, red and yellow (Table 1). The different aerial and substrate mycelial color were due to the utilization of different carbon source for the growth (Vanajakumar, 1981). Most of the strains showed Rectiflexibles spore morphology; the strain AQB.SKKU8 showed hock smooth spore morphology (Figure 3) and among the six strains, only one strain produced melanoid and soluble pigments (Table 1).

Less than 10% of various sources of *Streptomyces* are known for melanoid production and grey colour series usually produce smooth spore surface compared to other (Mathew, 1995; Lakshmanaperumalsamy, 1978). The glucose, arabinose and xylose were well utilized and

Table 1. Phenotypic characteristics of *Streptomyces* spp. isolated from seaweeds.

Name of closely related species	Strain number	Seaweed source	Spore chain morphology	Spore surface	Aerial mass color	Reverse side color	Melanoid Pigment	Soluble Pigment	Carbon utilization								
									Glu	Ara	Xyl	Ino	Man	Fru	Rha	Suc	Raf
S. coelicolor	AQB.SKKU8	*Gracillaria corticata*	hock	Smooth	Yellow/ Green	Brown /lavender	-	-	+	+	+	-	+	+	±	±	-
S. autotrophicus	AQB.SKKU10	*Chnoospora minima*	RF	Smooth	Pale yellow	ND/ cream	-	-	+	+	+	+	+	+	+	+	-
S. pedanensis	AQB.SKKU18	*Sargassum weightii*	RF	Smooth	White	Brown	-	-	+	+	+	±	+	-	±	+	-
S. deccanensis	AQB.SKKU20	*Spyridia hypnoides*	SC	Hairy	Grey	Yellow	+	+	+	+	+	+	+	+	+	+	+
S. vinaceus	AQB.SKKU25	*Enteromorpha intestinalis*	RF	Smooth	Yellowish brown	Reddish brown	-	-	+	-	+	-	+	+	-	+	-
Streptomyces Nov sp.	AQB.SKKU37	*Hypnea valentiae*	RF	Smooth	Bluish green	Reddish Brown/red	-	-	+	±	+/	+	+/	+/	+	+	±

Abbreviations: RF,Rectiflexibles; SC, Straight chains; ND, not distinctive; Glc, D-glucose; Ara,L-arabinose, Fru, D-fructose; Ino, iinositol; Man, D-Mannitol; Raf, raffinose, Rha, L-rhamnose. Suc, sucrose; Xyl, D-xylose, "+": well utilized;" +/_": poorly utilized; "_": not utilized; S, *Streptomyces; Nov* sp.

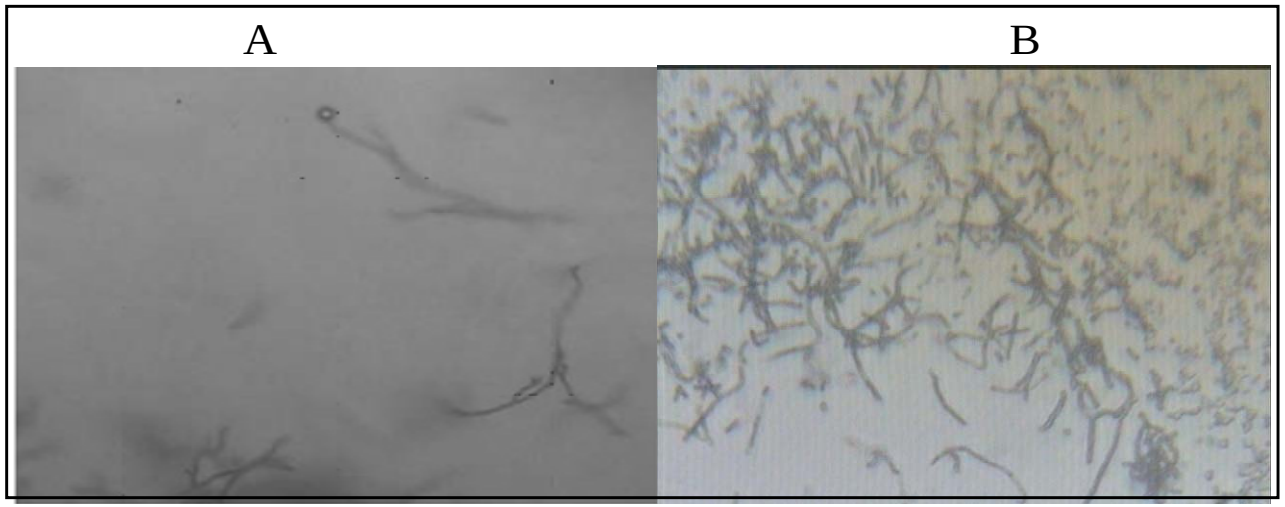


Figure 3. Gram-staining photograph smooth morphology of **A.** hock (strain AQB.SKKU 8) **B.** Rectiflexibile (strainAQB.SKKU18).

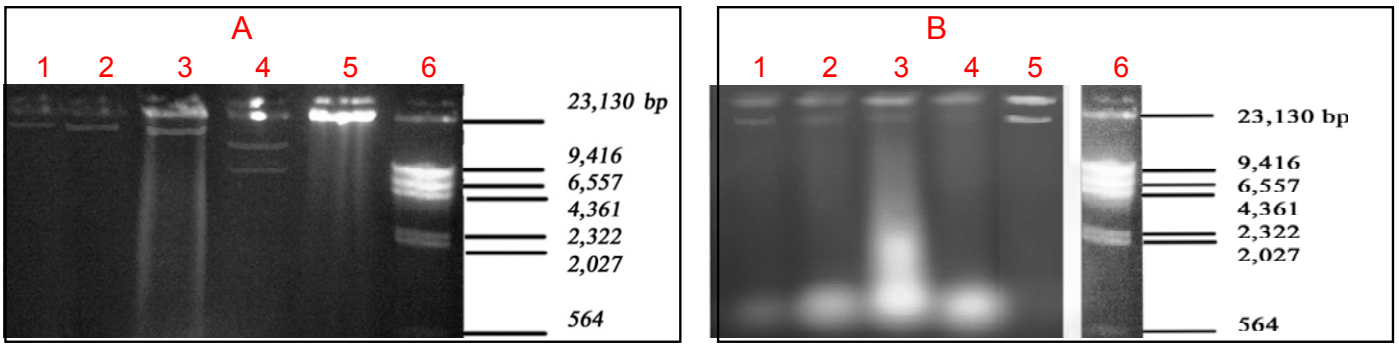


Figure 4. Plasmid DNA separated in Agarose gel electrophoresis**. (A)** Before curing; **(B)** After Curing; Lane 1, Strain AQB.SKKU8*;* Lane 2, MTCC 1540; Lane 3, Strain AQB.SKKU18; Lane 4, Strain AQB.SKKU25*;* Lane 5, Strain AQB.SKKU37; Lane 6, Marker (Hind III digest).

raffinose was not utilized by some strain (AQB.SKKU 8, 10, 18, 20); the rhamnose and sucrose was moderately utilized by the strain (AQB.SKKU 8, 18, 25). The strain AQB.SKKU 37 poorly utilized the entire carbon source. The *Streptomyces* isolates of marine seaweeds did not utilized the carbon source such as raffinose. Strepto-mycetes isolated from marine sponge *Mycale mytilorum* (Annandale), and *Tendania anhelans* (Lieberkuhn) showed abundant mycelium with glucose and xylose, moderate growth in medium containing arabinose, rhamnose, galactose, raffinose, mannitol and inositol, whereas growth was doubtful on media with fructose and sucrose (Selvakumar et al., 2010).

Plasmid profile before and after curing

Fifteen *Streptomyces* colonies associated with seaweeds were screened for plasmids. Of these, seven strains showed plasmids of varying sizes as their extra-chromosomal genetic material. Omura and coworkers (1981) from macrolide antibiotic producers conducted similar survey, and they found that only five of the 21 strains examined contained plasmids. The plasmid profile of *Streptomyces* species were resolved by agarose gel electrophoresis and it expressed multiple plasmids viz: major plasmids of 26and 23 kb and two minor plasmids of 17 and 8.8 kb (Figure 4A). Comparison of plasmid DNA profile of the six field strains of *Streptomyces* among themselves and with the reference strain (MTCC 1540) to catalogue the extent of homology indicated that, while strainAQB.SKKU.8 and strain AQB.SKKU18 carried a plasmids of 23 and 17 kb (Figure 4A) like that of reference strain. *Streptomyces* AQB.SKKU25 hosted multiple plasmids namely 26, 23.7, 14 and 8.8 kb. The strain AQB.SKKU10 showed the plasmids range from 0.8, 0.9 to 26 kb. The strain AQB.SKKU8, strain AQB.SKKU20 and strain *A*QB.SKKU18 showed unique plasmid patterns, which had homology with the reference strain *Streptomyces* MTCC 1540 before curing treatment (Figure 4A). Hayakawa et al. (1979) was found 17 kb linear plasmid in antibiotic resistance isolates of *Streptomyces*. The above results correlated with the previous findings in which the molecular weight 23.7 Kb plasmids were detected in antibiotic resistance *Streptomyces* species (Kinashi et al., 1987). The presence of multiple plasmids indicates the multiple antibiotic resistances and loss of plasmid after curing indicates the instability of plasmids treated with curing agents (Imran, 2009).

After curing treatment with EB, *Streptomyces* isolates and reference strain (MTCC 1540) lost the previously harbored plasmid. The strain AQB.SKKU8 and strain AQB.SKKU37 did not lose the high molecular weight plasmid (Figure 4B). The antibiotic production was also lost with high frequency from cultures treated with curing agents known to cause elimination of plasmids (Okanishi and Umezawa, 1978). In another report, plasmid-borne genes cured by dye treatment played a regulatory role (Akagawa et al., 1979).

Aerial and substrate mycelial coloration in wild and cured strains

The aerial and substrate mycelia production and coloration of *Streptomyces* strains before and after curing was attempted. The selected *Streptomyces* strains lost aerial mycelium after curing. The white color aerial mycelium was changed as pinkish white in AQB.SKKU18 and no color change was observed in strain AQB.SKKU25. The aerial mycelial color was different in each strain as shown in Figure 5A. The substrate mycelia color of *Streptomyces* strains were changed as pink, red and orange color after curing treatment (Figure 5B). The loss of aerial mycelium and different substrate mycelium production in *Streptomyces* may be due to the secondary metabolite production during germination of spores while treated with curing agents (Schaeffer, 1969). It has also been reported that the formation of genetically mapped

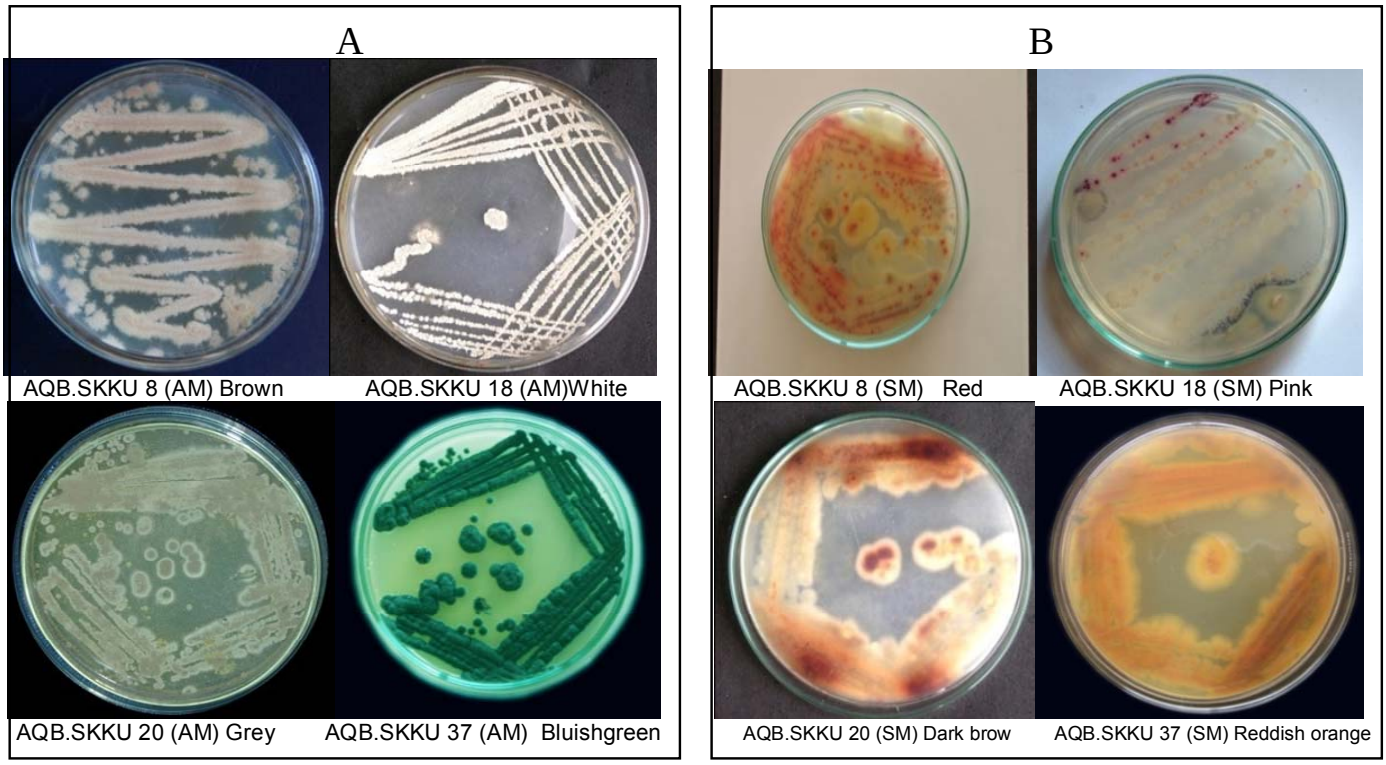


Figure 5. Different Mycelial coloration of *Streptomyces* isolates. **(A)** Before Curing; **(B)** After Curing; AM, Aerial mycelium; SM,Substrate mycelium.

mutants lacking in aerial mycelium is controlled by chromosomal genes rather than plasmids (Merrick, 1979; Imran, 2009).

Biomass production in cured and non-cured strains

The variation in biomass production was observed in all strains before and after curing treatment. The biomass production was decreased after curing and it was increased in strain AQB.SKKU25 (Figure 6). It indicated a re-directing of cell-energy toward primary metabolism (Ikeda et al., 1981; Ismail et al., 1998).

Antimicrobial bioassay before curing and after curing

Among the plasmid-isolated strains, experiments were performed in cured and non-cured *Streptomyces* isolates to find the presence or absence of antibiotic activity against three different *Vibrio* spp. Among the seven strains, all the *Streptomyces* isolates and reference strain MTCC 1540 showed similar antagonism against pathogenic *Vibrio* spp. In the case of strain AQB.SKKU20, the antagonistic activity was expressed towards *V. alginolyticus* only after curing treatment (Figure 7). The present also supported by Hopwood (1999), he described in a separate experiment, he picked seven *Streptomyces* strains that had failed to reveal any antimicrobial activity. After irradiating them with X-ray, five gave rise to antibiotic-producing variants. Thus, the study demonstrates that mutation stimulates antibiotic production in some cases (Flickinger et al., 1990; Imran, 2009; Kim et al., 2011).

Drug resistance bioassay in non-cured and cured isolates

The antibiotic resistance of six isolates and one reference strain MTCC 1540 (*S. griseus*) was tested before and after curing treatments. The resistance pattern of *Streptomyces coelicolor* AQB.SKKU8 and reference strain was similar. The isolated strains showed resistance to most of the tested antibiotics before curing. The strain AQB.SKKU18 and strain AQB.SKKU20 was resistant to all antibiotics used. Strain AQB.SKKU10 and strain AQB.SKKU37 were sensitive to the antibiotics such as Amphotericin-B and Nystatin before curing treatment. The strain AQB.SKKU25 showed sensitivity to Gentamycin, Streptomycin and Chloramphenicol before curing treatment (Table 2). The reference strains were homology with *Streptomyces* isolates in the resistance pattern of antibiotics such as Lincomycin, Penicillin-G

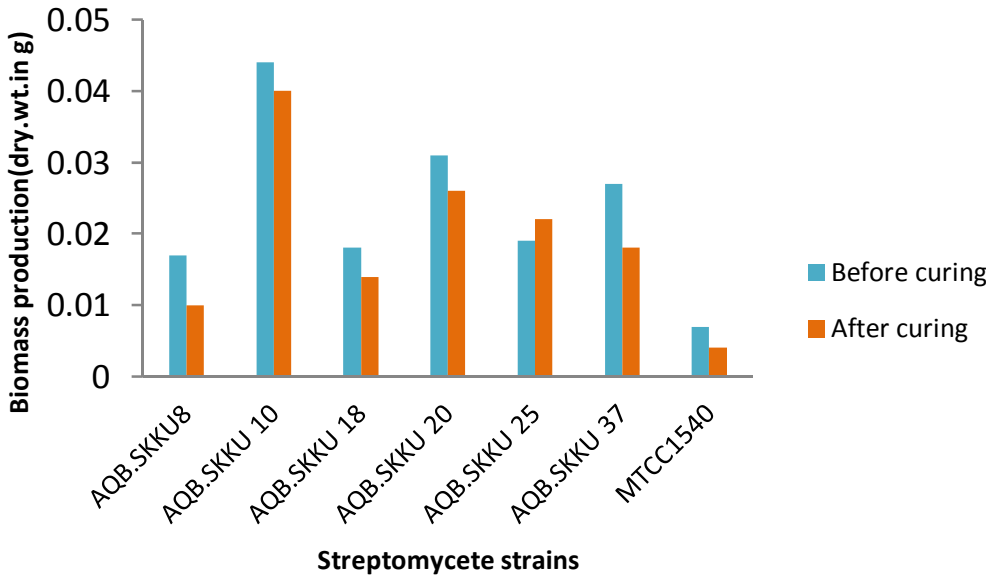


Figure 6. Biomass production of *Streptomyces* spp. before and after curing.

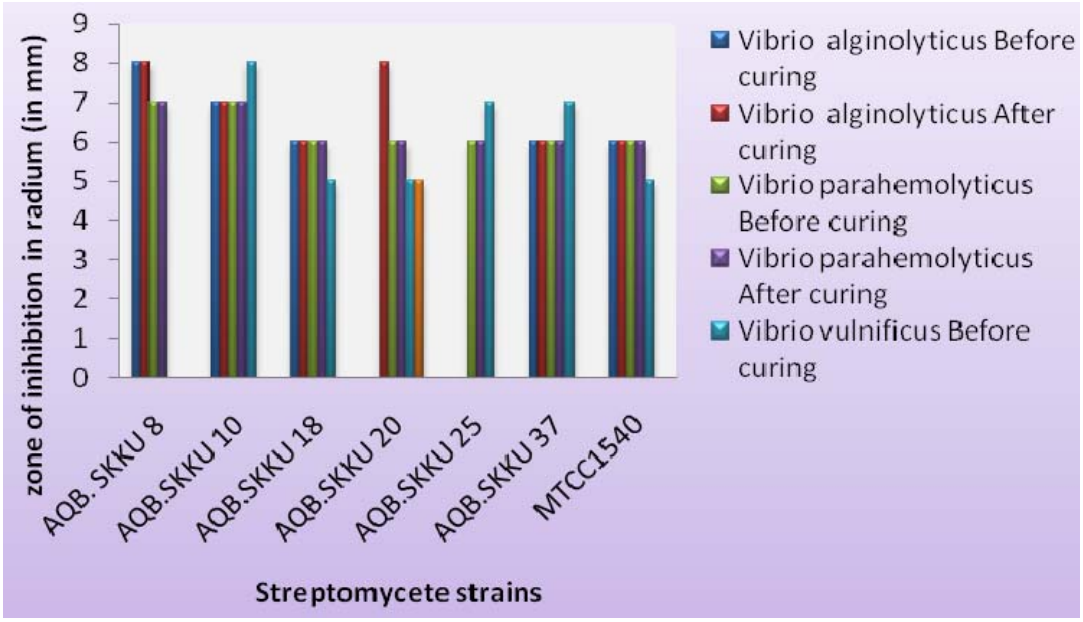


Figure 7. Antibacterial effect of *Streptomyces* sp. against pathogenic *Vibrio* spp. before and after curing.

and vancomycin. These are the resistance pattern of *Streptomyces* isolates and reference strain before curing. After curing, the resistance of *Streptomyces* species decreased compared to selected antibiotics before curing; it was reduced gradually after curing. The reference strain (MTCC1540) and the isolates of streptomycetes diameter of inhibition zone was reduced or increased and in some cases, sensitive strain become resistant and vice versa after curing which depends on the strain. The isolated *Streptomyces* also showed similar pattern (Table 2 and Figure 8B), which had homology with the plasmid profile also (Table 2). Characterization of *Streptomyces* strains in terms of their resistance to certain ranges of antibiotics makes it possible to avoid the repeated screening of isolates with the production of known antibiotics. It has been observed that *Streptomyces* species with mutagenic agents (also curing agents such as EB, AF and AO) cause strains to

Table 2. Antibiotic resistance of *Streptomyces* isolated from seaweeds.

Name of the antibiotics	Zone of Inhibition (mm)													
	Strain*SKKU8*		Strain*SKKU10*		Strain*SKKU18*		Strain*SKKU20*		Strain*SKKU25*		Strain*SKKU37*		*MTCC1540*	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II
Gentamicin	23	13	R	16	R	26	R	23	22	28	R	26	26	20
Lincomycin	R	R	R	R	R	R	R	R	R	R	R	21	R	R
Penicillin-G	R	R	R	R	R	R	R	R	R	R	R	25	R	R
Rifampicin	25	15	R	15	R	21	R	13	R	16	R	26	17	23
Streptomycin	16	R	R	15	R	27	R	22	11	20	R	R	28	34
Vancomycin	R	R	R	R	R	R	R	R	R	R	R	26	R	R
Amphotericin-B	R	R	11	22	R	R	R	R	R	R	22	20	R	R
Chloramphenicol	19	23	R	27	R	30	R	25	12	24	R	31	33	15
Erythromycin	R	15	R	23	R	R	R	14	R	16	R	30	28	R
Kanamycin	R	22	R	14	R	26	R	23	R	25	R	25	25	27
Nystatin	R	R	12	R	R	R	R	R	R	R	21	R	R	R
Tobramycin	R	14	R	21	R	16	R	20	R	12	R	12	R	15

I- Zone of inhibition before curing; II-Zone of inhibition after curing; R-resistant.

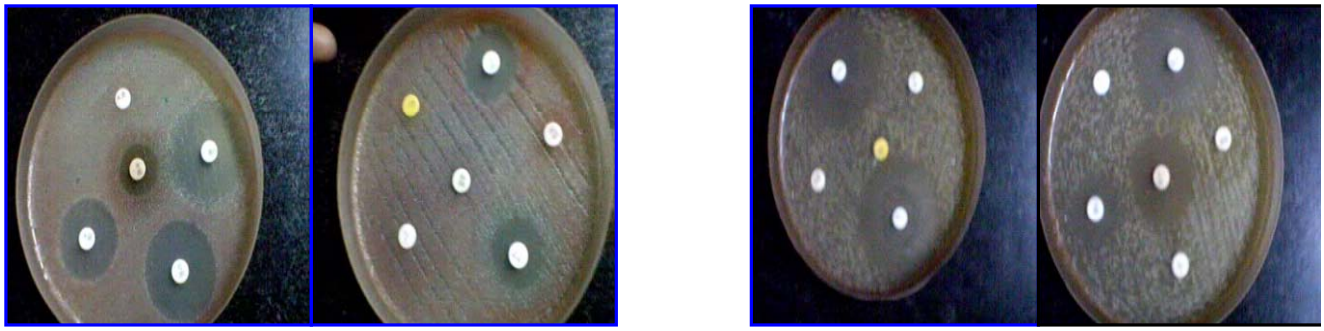
lose the ability to synthesize the secondary metabolites (Imran, 2009). Saadoun et al. (1998) suggested that antibiotic production is likely to be chromosomally encoded.

Extracellular protein expression in pre and post cured strains

The extracellular protein profiles of six strains of seaweed origins and one reference strain of MTCC 1540 (*S. griseus*) were studied. *Streptomyces* isolates exhibited below 26,000 and 43,000 Da as their extracellular protein. The similar ranges of proteins were also expressed in the reference strain (MTCC 1540) with SDS-PAGE separation. However, the expressed high molecular weight protein ranged between 43,000 to 66,000Da and above 66,000 to 97,4000Da and it differed in all pre-cured *Streptomyces* strains (Figure 9A). *Streptomyces* isolates and reference strain did not lose the molecular weight of 26,000Da protein during post-curing treatment (Figure 9B). The proteins present of *Streptomyces* strains in pre-curing was not expressed after curing, instead of that, new protein of high molecular weight (nearly 180,000 Da) was expressed in all *Streptomyces* isolate after curing (Figure 5B). Yang et al. (2012) reported the butanolide binding protein BarA of *S. virginiae* and has a molecular weight of 26,000 Da. In *S. griseus*, an A-factor binding protein had an apparent molecular weight of 26,000 Da (Miyake et al., 1989; Kawachi et al., 2000).

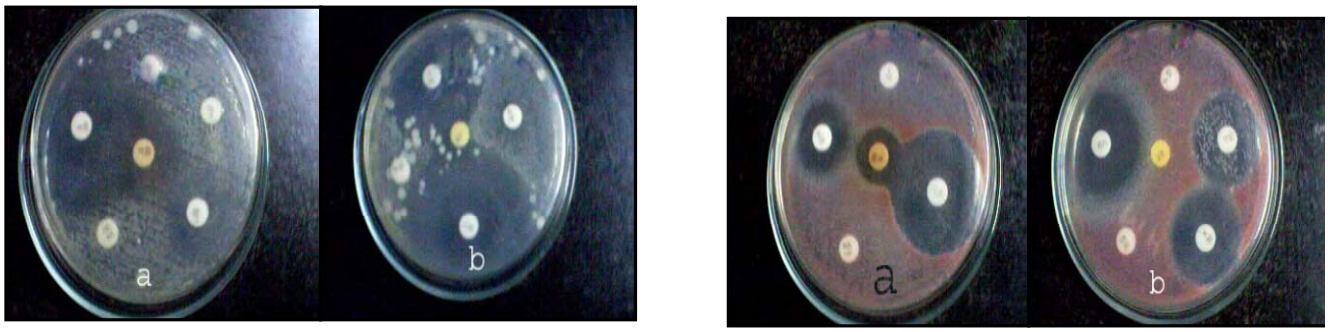
DISCUSSION

As secondary metabolites are frequently the result of complex, highly regulated biosynthetic process, a variety of changes in the genome may be necessary for the selection of high yielding derivatives of a wild strain. Baltz (2001) suggested that in many cases strain improvement have been achieved using natural methods of genetic recombination, which bring together genetic elements from two different genomes into one unit to form new genotypes; however, the most effective strategy is mutagenesis. A variety of chemical mutagens like ethidium bromide, N-methyl-N'-nitro-Nnitrosoguanidine (MNNG or NTG) are used for antibiotic and other secondary metabolite yield improvement in *Streptomyces* (Mamatha, 2009: Imran, 2009). These mutagens induce modifications of the base sequences of DNA that result in base pair substi-tutions, frame shift mutations, or large deletions that go unrepaired (Kieser et al., 2000). Little attention has made on the aspect of genetics study for the strain improvement and increase the yield of

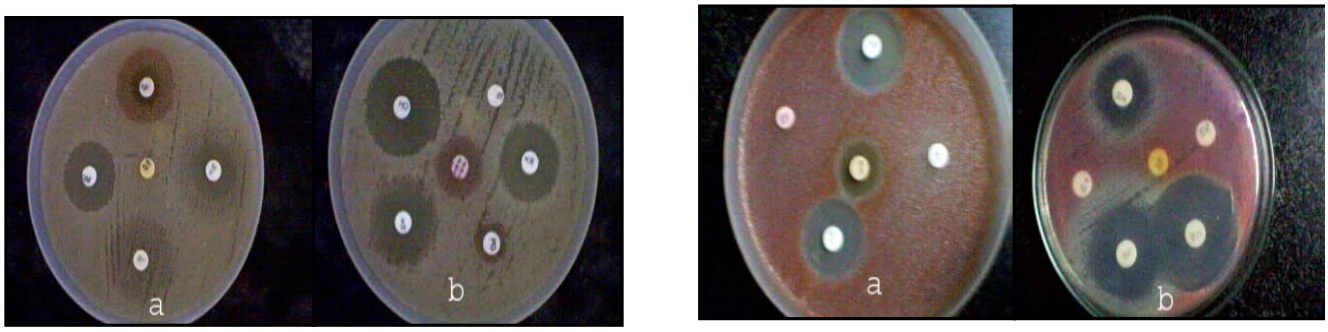


Streptomyces MTCC 1540 *Streptomyces* AQB.SKKU 8

(A)



Streptomyces MTCC 1540 *Streptomyces* AQB.SKKU 8



Streptomyces AQB.SKKU10 *Streptomyces* AQB.SKKU20

(B)

Figure 8. (A) Antibiotic sensitivity of the selected *Streptomyces* isolates before curing. **(B)** Antibiotic sensitivity of the selected *Streptomyces* isolates after curing

biotechnology industry, especially *Streptomyces* from marine source other than soil such as seaweeds. Marine seaweeds have been in recent focus because they form close association with a wide variety of microbes and are rich source of biologically active secondary metabolites (Prasheetha, 2008). In this respect, *Streptomyces* strains were isolated from marine seaweeds and the genetic determinant for antibiotic production and phenotypic characteristics were analyzed by curing test. In the present study, *Streptomyces* species associated with marine seaweeds were identified with the help of Nonomura keys (1974) and Actinobase database

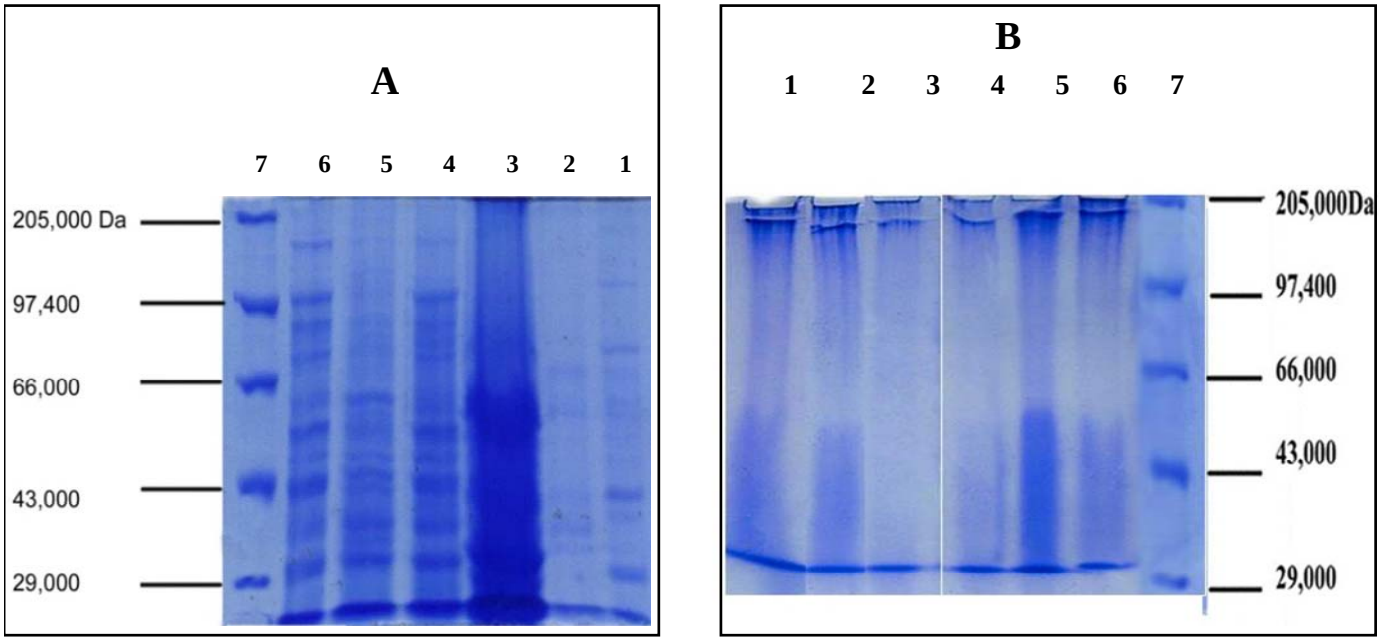


Figure 9. SDS-PAGE Separation of Extracellular protein from *Streptomyces* isolates. **(A)** Before curing; **(B)** After curing: Lane 1: Strain*SKKU8*; Lane 2, MTCC 1540; Lane 3, Strain*SKKU18*; Lane 4, Strain SKKU 20; Lane 5, Strain*SKKU37*; Lane 6, Strain*SKKU25;* Lane 7, Marker.

(Ugawa et al., 1989). Further molecular characterization is needed for taxonomic position. According to Shirling and Gottlieb (1966), unknown streptomycetes isolates can be characterized by the morphology, microscopic appearance, biochemistry, physiology, pattern of proteins and molecular genetics. It has also been reported that the exudates of seaweeds and slimes of the fish attracted microbes as the nutrient source and hambered them (Sheeja et al., 2011).

Detection of plasmid from various antibiotic producing strains strengthened the case for plasmid involvement in antibiotic production (Saadoun et al., 1998; Jorgensen et al., 2009). The study isolation and detection of plasmids were carried out in *Streptomyces* associated with seaweeds. After curing, *Streptomyces* isolates and reference strain (MTCC 1540) lost the previously harbored plasmid. Plasmidless variants had an altered secondary metabolism and a changed antibiotic resistance pattern (Akagawa et al., 1979). The *Streptomyces* colonies derived by treatment with EB appeared soft and did not form any characteristic aerial mycelium. The loss of plasmid after curing did not affect aerial mycelial production in all strains and it lost in some of the isolates indicates the genetic instability plasmid. It also indicates that aerial mycelium production is not plasmid mediated, but chromosomally mediated (Imran Sajid, 2009). The biomass production was decreased after curing. The studies on anticancer compound producing *Streptomyces* from marine algae reported decreasing biomass production after treatment with different concentration of pesticides (Prasheetha, 2008). The antagonism against *V. alginolyticus* was expressed only after curing in strain AQB.SKKU20; indicates the mutation stimulates the antibiotic production (Butler et al., 2002). Characterization of *Streptomyces* strains in terms of their resistance to certain ranges of antibiotics are used to avoid the repeated screening of isolates with the production of known antibiotics. In the present study, the streptomycetes isolates were resistant to antibiotics such as rifampicin and streptomycin. Earlier, antibiotic resistance of pesticide treated actinomycetes were studied, found rifampicin resistance actinomycetes from marine algae (Prasheetha, 2008). The diameter of inhibition reduced or increased after curing in some cases. The deduction is supported by Chater and Hopwood (1973). Volf and Altenbuchner (1998), supports the present study, who found genetic instability affect all the phenotypic properties and production of secondary metabolites and genes for primary metabolism.

An attempt on extracellular protein isolation of seaweed associated *Streptomyces* before and after curing was made in the present study. Previously harbored extracellular protein was lost after curing in *Streptomyces* sp. and expressed new protein. However, extracellular protein of 26000da (26KDa) was unaffected by curing treatment. It indicates that the 26KDa protein is genetically stable (Okamato et al., 1995). Butler (2003) also proved that extracellular molecules influenced antibiotic production in many streptomycetes. Furthermore, the experiments of plasmid curing with SDS

revealed that some catabolic genes were apparently plasmid-associated (AI Haixin, 2008). The present study on the effects of curing agents (Ethidium bromide) on the loss of linear plasmid DNA and further the generation of antibiotics in *Streptomyces* were revealed that the production of antibiotics was not eliminated, and isolates reduced by 20.2-79.8% in the plasmid cured strain. Similar survey conducted by Michaelson and Vinning (1978), who reported that exposure to Acriflavine and Ethidium bromide gave relatively large numbers of progeny that failed to produce the antibiotic or produced it in a much smaller quantities. Relationships between the genetic and phenotypic characteristics examined may provide preliminary insight into the distinct strategies that microbes use in optimizing their fitness in natural environments (Anita et al., 2006).

This is the first report on the genetic characterization of *Streptomyces* isolated from marine seaweeds based on the phenotypic characteristics. Multiple antibiotic resistance patterns can be regarded as useful marker phenotype for predicting the types of antibiotics and other metabolites which streptomycete produced. The data of the present study suggest that antibiotic and other phenotypic characteristics are chromosomally encoded in seaweed associated *Streptomyces* species. The study offers the new promises to modern molecular genetics world for the productivity and yield of secondary metabolites, which could be increased to many folds by selectively mutating the target genes for possible exploration of industrial strain for biotechnological application.

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