Isolation, Screening, Statistical media optimization, Purification and Structural characterization of bioactive compound from marine derived Streptomyces sp JS-S6.

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Abstract- Exploitation and overuse of antibiotics in the health sectors leads to the development of resistance mechanism in microbes against antibiotics. As a consequence, there is a vital need to develop effective lead compound from natural sources**.** Compounds derived from marine habitat have a remarkable nature of unique chemical structures and exhibit bioactivity, so it can be used in the treatment against antibiotic resistant clinical pathogens. The present study was mainly focused on isolation and screening of novel *Streptomyces* species against clinical pathogens from unexplored marine environment. In this study, thirty five actinomycetes isolates were identified. Among these, the isolate JS-S6 exhibits a strong antagonistic effect against the pathogens used. The 16S rRNA gene analysis of the isolate JS-S6 showed that it was similar to *Streptomyces viridobrunneus*^{T} LMG20317 and named as *Streptomyces* sp JS-S6. The Scanning Electron Microscopy (SEM) studies revealed that the spores were highly abundant, irregular in shape and distributed unequally. One Factor at a time (OFAT) medium optimization revealed that starch, ammonium sulphate and seven days of incubation period as efficient factor to emphasis the maximum biomass production and highest antibacterial activity. Placket Burman method and central composite design of response surface methodology in statistical media optimization disclosed the significant factors were starch, ammonium sulphate and Sodium chloride and their optimum concentration were 1%, 0.2% and 0.1 % respectively for maximum biomass production and antibacterial activity. Mass fermentation was carried out with optimized medium and purification of crude extract was done by silica column chromatography. The UV-Visible spectroscopy analysis showed that the compound has maximum absorption at 260.0 nm and FTIR analysis revealed the presence of methoxy functional group. The GC-MS analysis also strongly suggest that the bioactive metabolite was found to be Methyl N-hydroxybenzenecarboximidate (Oxime-Methoxy phenyl compound) when compared with mass spectra of Wiley and NIST95 database. The compound exhibited strong antibacterial activity against clinical pathogens without exhibiting cytotoxicity potential against H9C2 cell line. From this study it was clear that the bioactive compound from *Streptomyces* sp. JS-S6 have antibacterial activity against clinical pathogens. In future, it can be developed as potential lead molecule against pathogens that develop resistance against antibiotics.

Index Terms- Marine *Streptomyces*, media optimization, Spectroscopy studies.

I. Introduction

Combating drug resistant bacteria was found to be nightmare scenario for most of the clinicians. In order to overcome this issue, the lead bioactive molecule derived from natural source can be used as an effective drug candidate against the emerging drug resistance pathogens. Currently, most of the available drugs were originated from the microbial source (Singh *et al*., 2014). Bioactive molecules from marine microbes with unique chemical entity have the ability to tackle the drug resistant bacteria and can be developed as broad spectrum antibiotics in future (Mangamuri *et al.,* 2016). In the marine microbial world, *Streptomyces* genus of actinomycetales order made headway as the lead bioactive molecule producer in the synthesis of commercially important pharmaceutical products (Manivasagan *et al*., 2014). *Streptomyces* are filamentous gram positive bacteria with inimitable and complicated secondary metabolite pathways that synthesize antibiotics, antitumor agents, immunosuppressants and enzyme inhibitors (Khattab *et al.*, 2016). Most of the antibiotics that are currently in usage were obtained from the genus *Streptomyces.* In Microbial natural product, surplus amount of starting material for the drug candidate can be obtained from the fermentation process (Carter, 2011). In the present study, among 35 isolates, the isolate JS-S6 showed antagonistic effect against the clinical pathogens was selected. It was then subjected to media optimization, mass fermentation, purification and spectral studies of the purified compound. Cytotoxicity of the bioactive compound was verified with the H9C2 normal cell line .

II. Materials Methods

Isolation and screening for antagonistic isolate:

The sediment sample used in the study was collected at a depth of 2.0 m from the mangrove estuary of Arabian Sea, Kanyakumari district, located in southern part of India (8.0883° N, 77.5385° E). The sediment samples were transported to the laboratory in aseptic condition. Isolation was carried out using 1.0 g of marine sediment sample by standard serial dilution and plating method in Actinomycetes Isolation medium that constitutes of Soluble Starch 20 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO4 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 20 µM, Agar 15 g in 1000 ml of brackish water from mangrove habitat (Santhi and Jebakumar , 2011). Nalidixic acid (50.0 µg mL^{-1}) and Ketoconozole (50.0 µg mL^{-1}) were added to inhibit the growth of

gram-negative bacteria and fungi respectively. The plates were incubated at 28ºC for about 10-14 days. Pure cultures were obtained by repeatedly re-streaking the colonies with different morphology from the Actinomycetes Isolation Medium. Antibacterial potential of compounds from the marine isolates were tested against a panel of clinical pathogens.

Determination of Antibacterial activity for the compound produced by marine isolates

Clinical pathogens were obtained from Kovai Medical Center Hospital, Coimbatore and they were used to determine the antibacterial activity of compound produced by marine isolates. Clinical pathogens were swapped on molten MHA plates in the Biosafety Level II laminar air flow chamber and then the bioactive compounds impregnated discs were placed on plates inoculated with a test pathogens. The disc loaded with the chloroform was maintained as a negative control. After overnight incubation at 37ºC, the plates were observed for zone of inhibition and measured. The isolate with the antibacterial activity was selected for the further study.

Morphological characteristics of isolate with antibacterial activity

Morphological characteristics of the isolate with antibacterial activity were studied in different International *Streptomyces* Project (ISP) medium. The media used were (ISP-1- Tryptone Yeast Extract agar media), (ISP-2- Yeast Extract Malt Extract agar medium), (ISP-3- Oat-Meal agar medium), (ISP-4- Inorganic Salt Starch agar medium), (ISP-5- Glycerol-Asparagine agar media), (ISP-6- Peptone Yeast extract iron agar media), (ISP-7- Tyrosine agar medium) and Starch casein agar medium (Shirling and Gottleib, 1966). The culture plates were incubated at room temperature for 5-7 days. The colour of both substrate and aerial mycelia, together with the production of soluble pigments, were determined by comparison with chips from the ISCC-NBS colour charts (Kelly, 1964).

Genomic DNA isolation, 16S rRNA gene amplification and sequencing

Genomic DNA was isolated as described by Hopwood *et al.*, 1985. 16S **r**RNA gene was amplified using specific universal primers 27F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R 5'-GGT TAC CTT GTT ACG ACT T-3' (Sigma Genosys, India). The PCR reaction mixture consist of 1X reaction Buffer (10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), 200 μM dNTPs, 0.05 U of Taq DNA polymerase enzyme (Sigma, USA), 10 pM of each primer and 30 ng template DNA. The conditions for initial denaturation were 94°C , 5 min ; 31 cycles of 30 s at 95°C, 1 min 30 s at 54°C, 2 min at 72°C, and a final extension for 5 min at 72°C. The amplification reaction was performed with a thermal cycler (MyCycler, Bio-Rad, USA). The phylogenetic analysis of 16S rRNA gene was performed by PHYLIP 3.68 (Felsenstein, 2008) using bootstrapping over 1000 replicates.

Media optimization for the antagonistic isolate
Optimization of Carbon and Nitrogen sources

To determine the effective carbon source, eight different carbon sources such as sucrose, glucose, fructose, mannitol, xylose, lactose, starch and maltose were supplemented at 1% w/v to the minimal medium. The influence of various nitrogen sources such as sodium nitrate, potassium nitrate, ammonium sulphate, ammonium chloride, ammonium dihydrogen orthophosphate and urea were studied by independently adding nitrogen source at 0.2% w/v to the minimal medium containing optimized carbon source at 1% w/v. Effective carbon and nitrogen source was selected based on biomass production and antibacterial activity of the bioactive molecule synthesized by the antagonistic isolate.

Optimization of incubation period

The fermentation medium containing optimized carbon and nitrogen source in the minimal media were incubated for fifteen days. Biomass production and antibacterial activity were recorded to determine the suitable incubation period.

Statistical Media Optimization

Statistical approach such as placket Burmann design was acclimatized to identify the factors which persuade the significant effect on the biomass and bioactive compound production in the antagonistic isolate. A total of 11 factors were screened in 12 combinations which consists equal number of high level concentration (coded as +) and low level concentration (coded as -) of media components as indicated in table 1. Response surface methodology mainly central composite design was carried out to optimize the concentration of the significant factors identified by placket burman method. CCD method consists of five concentration (coded as +α, +1, 0,-1 and –α) executed in 20 different experimental runs for three significant factors as shown in table 2. Design-Expert ® Version 8 software (Stat-Ease, Inc., Minneopolis, USA) was used to evaluate response of both placket burmann and CCD method.

Table 1: Eleven factors with its high and low level concentration used in Plackett-Burman design

S.No	Code	Name of the Factor	High Level concentration (+)	Low level Concentration(-)
1	A	Starch	12 g/l	8 g/l
2	B	K_2HPO_4	1.5 g/l	0.5 g/l
3	C	$MgSO_4$	1.5 g/l	0.5 g/l
4	D	NaCl	1.5 g/l	0.5 g/l
5	E	$CaCO_3$	1.5 g/l	0.5 g/l
6	F	$(NH_4)_2SO_4$	3 g/l	1 g/l
7	G	Ferrous Sulphate	22 µg/l	18 µg/l
8	H	Asparigine	30 µg/l	15 µg/l
9	J	pH	8	6
10	K	Temperature	37ºC	28º C
11	L	RPM	160	120

Table 2: Three significant factors with its high and low level concentration in RSM (Central Composite design).

S.No	Code	Name of the factor	Five concentration coded for CCD				
			+α,	+1	0	-1	–α
1	A	Starch (g/l)	13.36	12	10	8	6.64
2	B	NaCl (g/l)	3.68	3	2	1	0.32
3	C	$(NH_4)_2SO_4$	4.68	4	3	2	1.32

Mass fermentation and purification of the antagonistic isolate

The isolate with antagonistic activity was cultured in the optimized fermentation medium containing 10.0 g/l Starch, 1.0 g/l NaCl, 2.0 g/l $(NH_4)_2SO_4$ in the minimal media and incubated at 28ºC, 120 rpm for 7 days. The fermented broth was centrifuged at 10,000 rpm for 10 min. The cell- free culture filtrate was then extracted with equal volume of chloroform. The organic phase was collected and concentrated by vacuum drier. Concentrated compound was again dissolved in chloroform and the crude extract was subjected to purification by silica gel column chromatography. The crude compound was purified by silica gel column (mesh size- 60-120) chromatography with gradient elution. Initially, non-polar solvent, Hexane (100%) was used as the mobile phase. Hexane followed by chloroform, acetone and finally polar organic solvent methanol was used for gradient elution of compounds. The Purity of the eluted compound was determined through TLC. The antibacterial activity was tested against clinical pathogens for each fractions eluted through column chromatography. The fractions that exhibit the antibacterial activity were subjected to various spectroscopic studies.

Spectroscopic Studies of the bioactive compound

UV-Visible spectral analysis

The absorption spectrum of the compound from the bioactive fraction was determined in the UV-VIS region (235-650 nm) by using a UV-VIS Spectrophotometer (UV-1800 Shimadzu). Wavelength was plotted in X-axis and absorbance intensity of the compound was plotted in the Y-axis.

FT-IR spectroscopic analysis

The FT- IR spectrum analysis was carried out in between the region (500-5000 $cm^{-1,}$) to determine the functional groups present in the compound obtained from the antagonistic isolate. The FT-IR spectra intensity was measured in X-axis and wavelength was plotted in Y-axis.

GC-MS analysis

The bioactive fraction was subjected to GC-MS analysis in GC-Trace Ultra Ver 5.0, MS DSQ II (Thermo Scientific, MA, USA). The DB35-MS capillary standard non-polar column with a dimension of 30 m, inner diameter of 0.25 nm and film thickness of 0.25 um was used. Electron Bombardment (EI) ion source was performed at 70 ev. Helium was used a carrier gas with a flow rate of 1.0 ml min^{-1}. Initial oven temperature was 50ºC and further it was increased to 260ºC at 10ºC min^{-1}. The spectrum was scanned in the range of 50-650 amu.

Bioassay of the Purified compound

Minimum inhibitory concentration (MIC):

The MIC of the bioactive fraction was determined against clinical pathogens by broth microdilution method as per the

guidelines of Clinical and Laboratory Standards Institute (CLSI) formerly, the National Committee for Clinical Laboratory Standards.

Cytotoxicity assay:

The cytotoxic effect of the purified compound was determined by MTT assay in mice cardiomycetes cell line H9C2. Various concentrations from 10 µg to 80 µg/ml were used along with DMSO as a control. The cell survival was determined by plotting the concentration of compound versus percentage of cell viability (Aliabadi *et al.*, 2010)

III. Results and Discussion:

Morphological characteristics of the antagonistic isolate JS-S6

Thirty five isolates with different morphology were identified from the marine mangrove sediment sample. Among the thirty five isolates, the crude compound obtained from isolate JS-S6 exhibited strong antibacterial activity against the clinical pathogens shown in table 3. The antagonistic isolate exhibited different morphological pattern on various ISP medium (International *Streptomyces* Project) were shown in table 4.

Table 3: Antibacterial activity of compound produced by the antagonistic isolate JS-S6

S.NO	Clinical Pathogen	Zone of inhibition (mm)
1	MRSA	26.0 ± 0.1
2	*Salmonella typhi*	24.0 ± 0.3
3	*Candida albicans*	11.0 ± 0.2
4	*Proteus vulgaris*	18.0 ± 0.5
5	*Pseudomonas sp*	9.0 ± 0.1
6	*E.coli*	24.0 ± 0.4
7	*Klebsiella pneumoniae*	25.0 ± 0.2

Table 4: ISP medium exhibiting different morphological character of isolate JS-S6.

Medium	Aerial mycelium	Substrate mycelium	Diffusible pigment	Elevation
Starch-casein	White Powdery	Greenish yellow	Yellow	Elevated
ISP-1	Halo white	Pale Yellow	Greenish yellow	Elevated
ISP-2	Grey	Olive	Olive Brown	Elevated
ISP-3	White	Brown	Olive Brown	Not elevated
ISP-4	Whitish grey	Grey	No	Centroid
ISP-5	White	Tangerine	Olive brown	Elevated
ISP-6	Brown, no spore	Brown	Olive Brown	Not elevated
ISP-7	Pearl anthracite	Pine green	No	Elevated

The spores were abundant and highly condensed, flexous in a particular region. The spores were irregular, lobulate in shape and the substrate mycelium gives rise to aerial hyphae which is highly branched and consists of spores with smooth surface indicated in figure1.

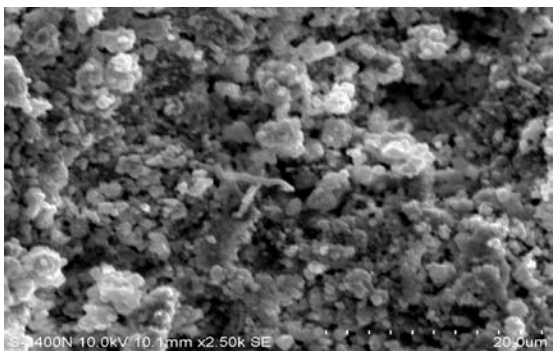

Figure 1: Spore of isolate JS-S6 visualized by SEM

Molecular 16S rRNA gene analysis of the isolate

Genomic DNA of the isolate JS-S6 was used as a template for the amplification of 16S rRNA gene with eubacterial universal primers (27F and 1492R). The expected size of 16S rDNA gene with 1482 bp was obtained. The phylogenetic tree analysis from the fig 2 showed that the isolate was similar to *Streptomyces viridobrunneus*[T] LMG20317 (AJ781372), *Streptomyces showdoensis*[T] NBRC13417 (AB184389) and *Streptomyces roseoviridis*[T] NBRC12911 (AB184389). Therefore, the isolate was further named as *Streptomyces* sp. JS-S6. The *Streptomyces* sp. JS-S6 exhibited similarities like grey color and floxous smooth spore surface in the ISP medium (Zhu *et al.*, 2007) as that of the *Streptomyces viridobrunneus* and *Streptomyces showdoensis*, which were closely related and falls in same cluster .

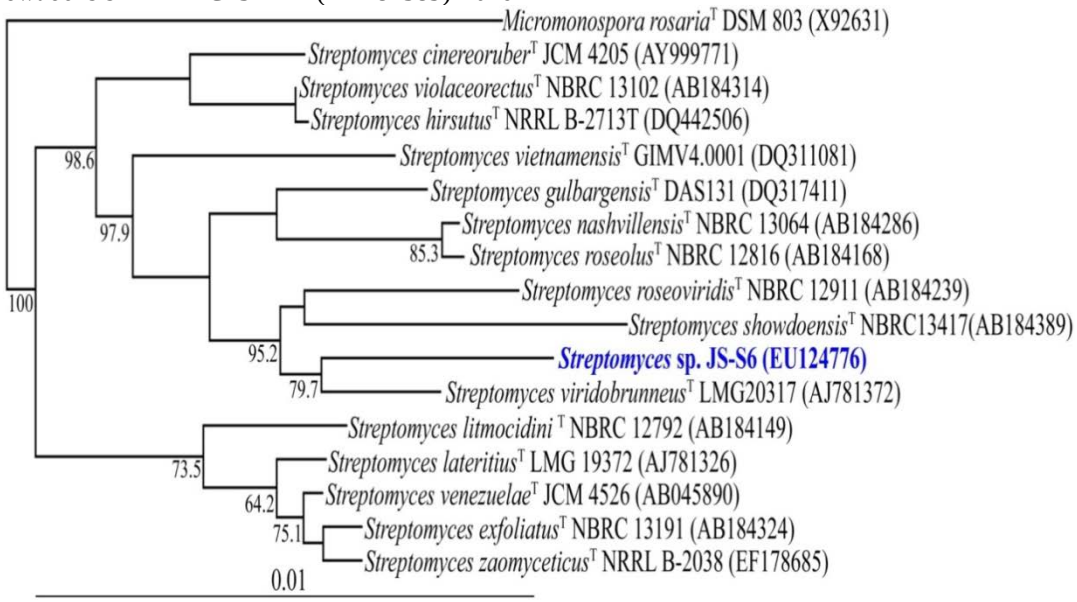

Figure 2: 16S rRNA phylogenetic analysis of *Streptomyces* spp JS-S6 revealed that the isolate JS-S6 was similar to *Streptomyces viridobrunneus* [T] LMG20317(AJ781372), *Streptomyces showdoensis* [T] NBRC13417(AB184389) and *Streptomyces roseoviridis*[T] NBRC12911(AB184389).

Media optimization for the antagonistic isolate:
Optimization of carbon and nitrogen source

The bioactive metabolite synthesis in *Streptomyces* species, depend on the nutrients used in the fermentation medium (Yu *et al.*, 2008). Starch is the preferred carbon source for most of the *Streptomyces* species in the production of secondary metabolites (Gupte and Naik, 1998; Jia *et al.*, 2008; Syed *et al.*, 2009). The growth and antibacterial compound production of *Streptomyces* sp JS-S6 were studied using eight different carbon sources. The production medium containing starch exhibited highest antimicrobial activity against different pathogens used,when compared to other carbon sources. Maltose had similar effects as starch but inhibition for MRSA, *Staphylococcus aureus* and *Enterococcus* sp was slightly lower than that of starch. Fructose, Dextrose and sucrose subsequently had comparable inhibition effects. In xylose, MRSA was highly inhibited compared to other pathogens. Antimicrobial activity was not observed in the medium containing mannitol, lactose and medium devoid of carbon source. In the case of Biomass production, medium containing starch had highest biomass production followed by Maltose and Lactose. In the case of lactose and mannitol, the isolate showed good growth but failed to produce antibacterial compound. Lowest growth was obtained in medium containing fructose and without carbon source. Dextrose, Xylose, Sucrose and Mannitol displayed the analogous growth pattern. Based on the surveilance from fig 3a & 3b, starch with utmost inhibitory property and effective biomass production was selected as the appropriate carbon source. Nitrogen source also play an important role in the production of antibacterial compound. The complex nitrogen sources could increase the production of antibiotic compounds by the *Streptomyces* species . Several studies have shown that nitrogen incorporation is crucial for the regulation of antibiotic production but the mechanisms involved is not clearly understood (Voelker and Altaba, 2001). In case of biomass production, ammonium sulphate had highest antimicrobial compound production. Potassium nitrate, ammonium dihydrogen orthophosphate, urea and ammonium chloride also have a good deed for the biomass production. Moderate growth was obtained in sodium nitrate and ammonium molybdate supplemented medium. In case of antibacterial activity, ammonium siulphate and ammonium chloride only displayed inhibitory effect against tested pathogens . Ammnonium molybdate exhibited antibacterial activity against six pathogens excluding *Corynebacterium diptheriea*. Nitrogen sources such as potassium nitrate, ammonuium dihydrogen orthophosphate , urea and medium lacking nitrogen does not diplay inhibition against pathogens. Maximum inhibitory effect against clinical pathogens and maximum highest biomass production were recorded in the presence of ammonium sulphate shown in fig 4a & 4b and thus it was selected as effective nitrogen source.

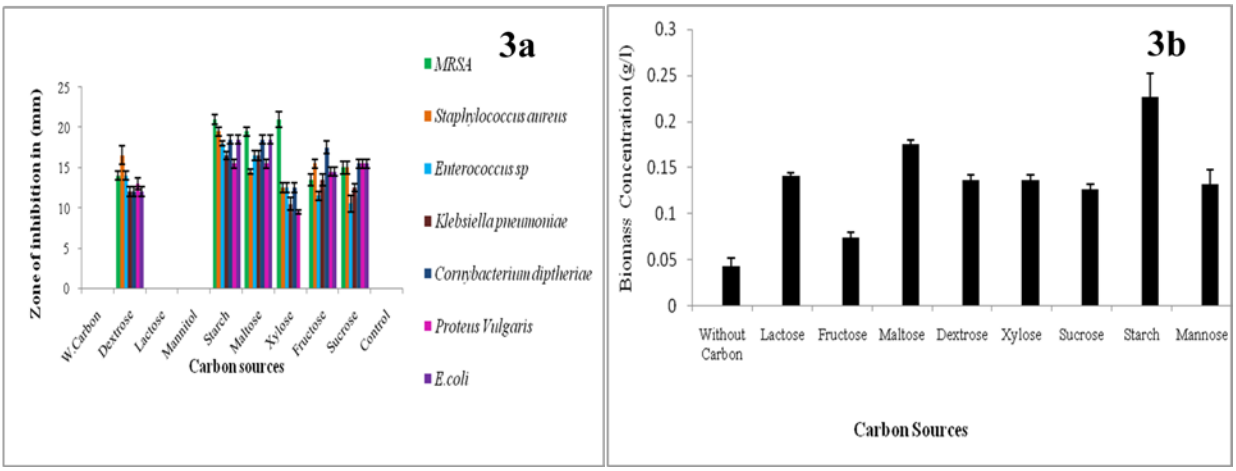

Figure 3a & 3b : Antibacterial activity and Biomass production in different carbon sources

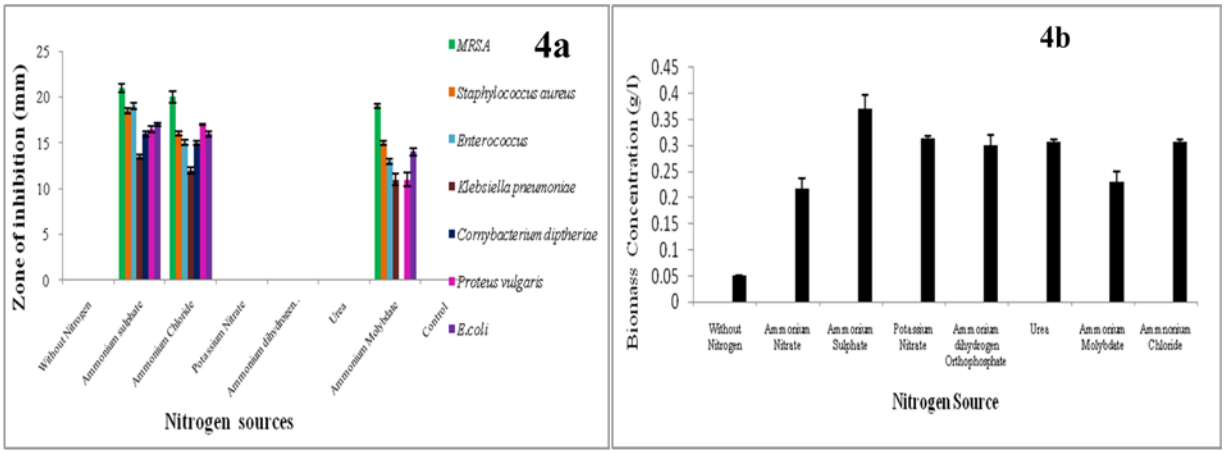

Figure 4a & 4b: Antibacterial activity and Biomass production in different nitrogen sources

IV. Optimization of Incubation Period

Incubation period also had a major effect on biomass and bioactive compound production. *Streptomyces* spp. VITSVK9 showed the maximum growth with highest biomass at the incubation period of nine days (Sauvrav et al.,2010). From the time course study of *Streptomyces* sp JS-S6, over 15 days period of incubation, 8th day of incubation was found to be ideal for better biomass yield. Biomass production was maximum at the stationary phase. From the 8th day of incubation onwards, there is no increase or decrease in the biomass production was observed. Antibacterial activity was not witnessed until the fourth day of incubation. At the fifth day, inhibition was observed in MRSA, *Staphylococcus aureus, Corynebacterium diptheriae and E.coli.* Antibacterial acivity against seven pathogens was progressively increased from the sixth day itself. Beyond 10 days of incubation, there was a slight loss of antibacterial activity. Hence from the experimental investigation, incubation till eight days was found to be suitable incubation period as shown in fig 5a & 5b.

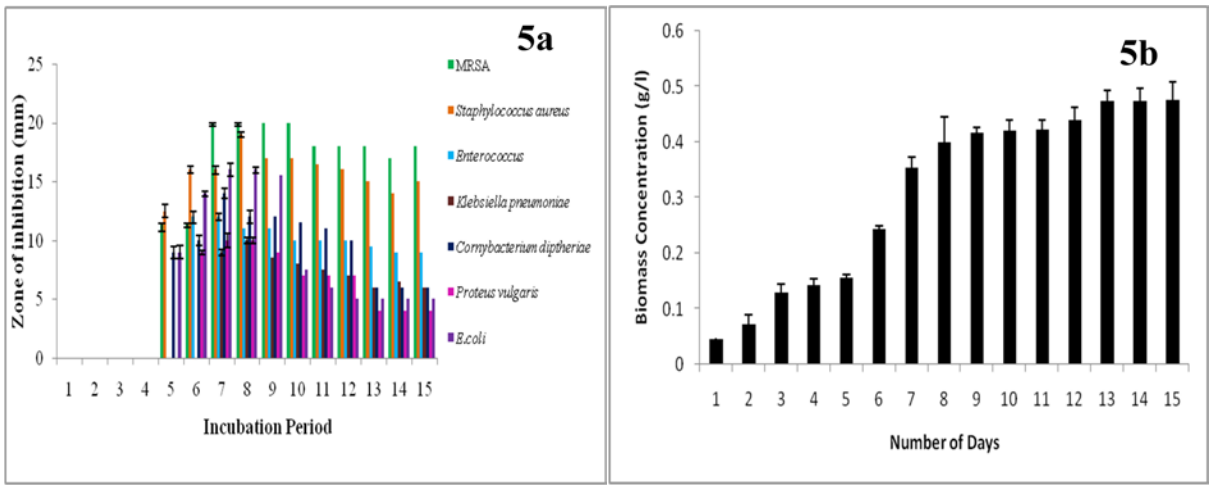

Figure 5a & 5b: Antibacterial activity and Biomass production in different incubation period.

V. Statistical Media Optimization

One factor at a time approach leads to the assortment of appropriate carbon, nitrogen source and suitable incubation period respectively. Apart from carbon and nitrogen source various media components and physiological factors also have influence in the antibacterial activity and production of biomass. In placket Burmann method, eleven factors were selected and it was evaluated in twelve experiments with equal number of high and low concentration for each factor. Biomass production and antibacterial activity was recorded as response for each of the experimental runs in placket burman design and indicated in table 5

The Model F-value of 332.64 from the anova analysis of biomass as response in Placket bumann method implies the model is significant. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case Model with the p-value of 0.0426, factor A (starch) with p-vaue of 0.0127, D (Sodium Chlroide) with p-value of 0.0474 and J (ammonium sulphate) with p-value of 0.0356 were significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The Pred R-Squared of 0.9567 was in reasonable agreement with the Adj R-Squared of 0.9967. Adj R-Squared of 0.9967 was in reasonable agreement with the R-Squared of 0.9997. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 54.799 indicates that it was an adequate signal. In the anova analysis for Zone of inhibition, the Model F-value of 566.60 implies the model was significant. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case, the p-value of model was 0.0327, the p-value of the factor A (Starch) was 0.0101, factor D was 0.0374, factor F was 0.0205, factor G was 0.0424 were significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The Pred R-Squared of 0.9746 was in reasonable agreement with the Adj R-Squared of 0.9981. Adj R-Squared of 0.9981 was in reasonable agreement with the R-Squared of 0.9998.Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 79.599 indicates that it was an adequate signal.

Table 5: Plackett burman design in experimental concentration and its responses as Biomass and Zone of inhibition.

Run	A	B	C	D	E	F	G	H	J	K	L	Biomass(g/l)	Zone of Inhibition (mm)
1	12	1.5	0.5	1.5	1.5	3	18	15	6	37	120	3.662	16
2	8	1.5	1.5	0.5	1.5	3	22	15	6	28	160	1.782	09
3	12	0.5	1.5	1.5	0.5	3	22	30	6	28	120	3.616	14
4	8	1.5	0.5	1.5	1.5	1	22	30	8	28	120	1.702	12
5	8	0.5	1.5	0.5	1.5	3	18	30	8	37	120	1.269	09
6	8	0.5	0.5	1.5	0.5	3	22	15	8	37	160	1.815	10
7	12	0.5	0.5	0.5	1.5	1	22	30	6	37	160	2.894	16
8	12	1.5	0.5	0.5	0.5	3	18	30	8	28	160	2.822	14.5
9	12	1.5	1.5	0.5	0.5	1	22	15	8	37	120	2.565	15.5
10	8	1.5	1.5	1.5	0.5	1	18	30	6	37	160	1.849	12.5
11	12	0.5	1.5	1.5	1.5	1	18	15	8	28	160	2.391	20
12	8	0.5	0.5	0.5	0.5	1	18	15	6	28	120	1.583	12

Apart from anova analysis, pareto chart also played a major role in selecting significant factors for maximum biomass production and antibacterial activity. The factors starch (A), ammonium sulphate (F), sodium chloride (D), pH (J), ferrous sulphate (G) was found to be significant factor because of it t-value limit as indicated in the fig 6a & 6b. Among the five significant factors, the factors starch (A), ammonium sulphate (F) and sodium chloride (D) was found to be common in both biomass production and antibacterial activity. The optimum concentration of the significant factors, namely Starch, Sodium chloride and ammonium sulphate was determined by the central composite design of the response surface methodology.

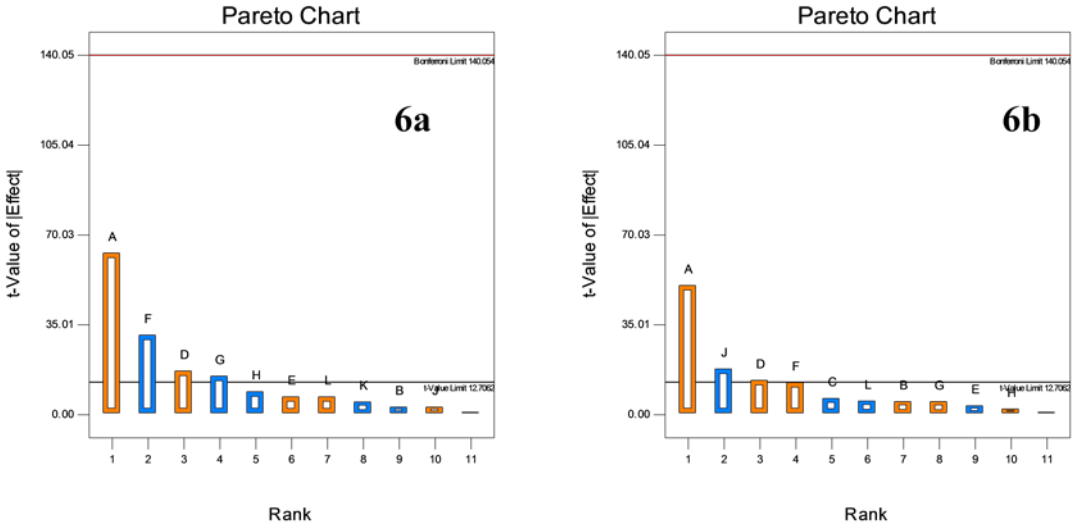

Figure 6a & 6b : Pareto chart for Antibacterial activity and Biomass production in placket burmann method.

In response surface methodology, the three significant factors were executed in twenty different experimental runs. In Central composite design, biomass and antibacterial activity was recorded as responses as indicated in table 6. Anova analysis were carried out for biomass production and antibacterial activity and listed in the table 7 & 8. In case of Biomass production, the Model F-value 21.09 implies the model is significant. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The Pred R-Squared of 0.8463 is in reasonable agreement with the Adj R-Squared of 0.9095. Adj R-Squared of 0.9095 is in reasonable agreement with the R-Squared of 0.9547. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 13.108 indicates that it is an adequate signal. In case of antibacterial activity, the Model F-value 36.49 implies the model is significant. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, C, AB, BC, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The Pred R-Squared of 0.7759 is in reasonable agreement with the Adj R-Squared of 0.9467. The Adj R-Squared of 0.7759 is in reasonable agreement with the R-Squared of 0.9733. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 15.738 indicates that it is an adequate signal.

Table 6: Central composite design in experimental concentration and its response as Biomass and Zone of inhibition

S.No	Factor 1 A: Starch (g/l)	Factor 2 B: Sodium Chloride (g/l)	Factor 3 C: Ammonium Sulphate (g/l)	Response 1 Biomass (g/l)	Response 2 Zone of Inhibition (mm)
1	8	1	2	1.238	8.5
2	12	1	2	2.458	14
3	8	3	2	1.127	16
4	12	3	2	2.321	16
5	8	1	4	1.283	09
6	12	1	4	2.992	15
7	8	3	4	1.674	11.5
8	12	3	4	2.756	8.5
9	10	2	3	3.016	21
10	10	2	3	3.987	22
11	10	2	3	3.992	22
12	10	2	3	3.932	21.5
13	6.64	2	3	1.013	8

14	13.36	2	3	2.991	15.5
15	10	0.32	3	2.661	15
16	10	3.68	3	2.145	17
17	10	2	1.32	2.432	17.5
18	10	2	4.68	2.686	10
19	10	2	3	3.924	20.5
20	10	2	3	3.924	22

Table 7 : Anova analysis for biomass production in central composite design of Response Surface methodology

S.No	Source	Sum of squres	Degree of freedom	Mean Square	F-Value	p-value prob>F
1	Block	0.12	1	0.12		
2	Model	18.13	9	2.01	21.09	<0.0001-Significant
3	A-Starch	5.33	1	5.33	55.80	<0.0001
4	B-Sodium Chloride	0.068	1	0.068	0.71	0.4220
5	C- Ammonium Sulphate	0.29	1	0.29	3.03	0.1157
6	AB	0.053	1	0.053	0.56	0.4741
7	AC	0.018	1	0.018	0.19	0.6764
8	BC	0.020	1	0.020	0.21	0.6557
9	A^2	6.87	1	6.87	71.94	<0.0001
10	B^2	4.34	1	4.34	45.44	<0.0001
11	C^2	3.51	1	3.51	36.77	0.0002
12	Residual	0.86	9	0.096		
13	Lack of fit	0.17	5	0.035	0.20	0.9445-Not signficnt
14	Pure Error	0.69	4	0.17		
15	Cor Total	19.11	19			

Table 8 : Anova analysis for antibacterial activity in central composite design of Response Surface methodology.

S.No	Source	Sum of squres	Degree of freedom	Mean Square	F-Value	p-value prob>F
1	Block	0.35	1	0.35		
2	Model	475.35	9	50.82	36.49	<0.0001-Significant
3	A-Starch	32.64	1	32.64	23.44	0.0009
4	B-Sodium Chloride	5.75	1	5.75	4.13	0.0726
5	C- Ammonium Sulphate	39.12	1	39.12	28.09	0.0005
6	AB	26.28	1	26.28	18.87	0.0019
7	AC	0.78	1	0.78	0.56	0.4730
8	BC	22.78	1	22.78	16.36	0.0029
9	A^2	195.02	1	195.02	140.04	<0.0001
10	B^2	68.28	1	68.28	49.03	<0.0001
11	C^2	127.28	1	127.28	91.39	<0.0001
12	Residual	12.53	9	12.53		
13	Lack of fit	10.72	5	1.39	4.73	0.0786-Not signficnt
14	Pure Error	1.81	4	2.14		
15	Cor Total	470.24	19	0.45		

From the 3D response surface plot, the interaction between the three significant factors were clearly studied and indicated in fig 7a-7c & 8a-8c. The optimum concentration of the significant factors starch, ammonium sulphate and sodium chloride was found to be 1%, 0. 2% and 0.1% respectively.

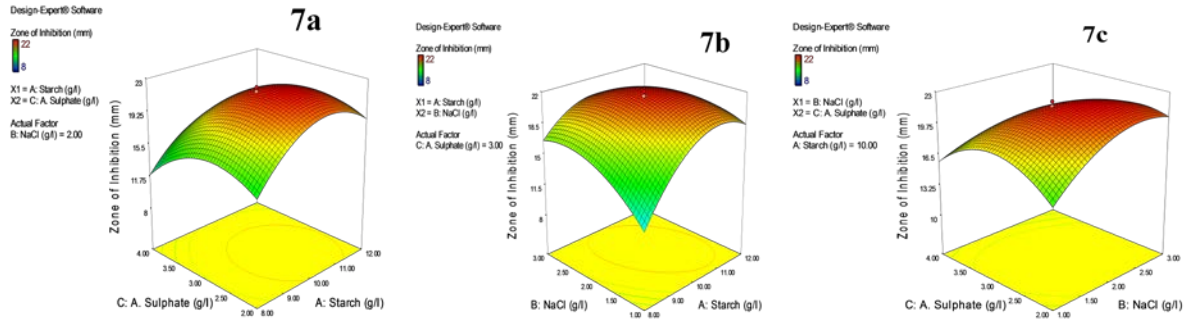

Figure 7a-7c: 3-D surface view of Interaction between the three significant factors based on antibacterial activity

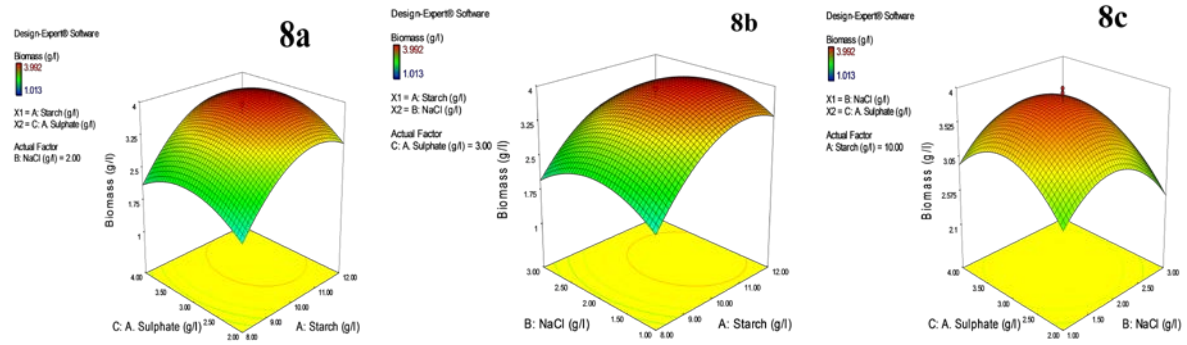

Figure 8a-8c: 3-D surface view of Interaction between the three significant factors based on Biomass Production.

Spectral analysis of the active compound

The maximum absorption range of bioactive compound in UV-Vis spectrum was found to be 260 nm, as indicated in fig 9. The characteristic absorption peak at 260 nm range indicates that the compounds are highly polyene in nature (*Slavica et al.*, 2005).

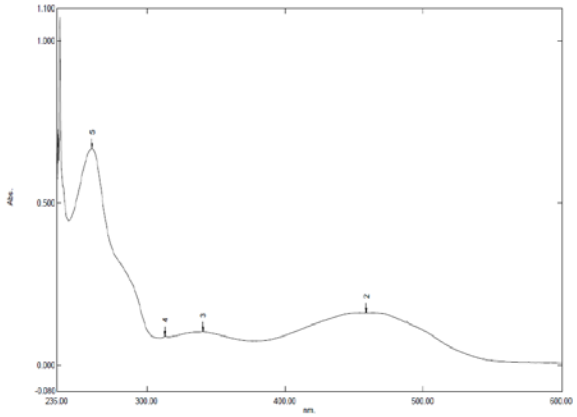

Figure 9: UV- Visible Spectrum of bioactive compound from *Streptomyces* sp JS-S6

In the FTIR spectrum shown in fig 10, absorption bands at 3,437.26 cm^{-1}, 3452.70 cm^{-1} indicates the presence of Hydroxy group, H-bonded OH stretch, carboxylic group and Heterocyclic amine, with the NH stretching. The absorption peak at 1,633.76 cm^{-1}, indicates the presence of conjugated ketone functional group with the presence of secondary amine and NH bend. The FTIR spectrum of purified compound showed one absorption peak (2922.25 cm^{-1}) in the region between 3500 cm^{-1} and 1730 cm^{-1}. The absorption peak between the range 1300 to 700 cm^{-1} in the FT-IR spectrum indicates the presence of skeletal C-C vibrations. The presence of the absorption peak at 1633.7 cm^{-1} indicates the presence Alkenyl C=C stretch. The absorption peak that ranges from 1225 to 950 cm^{-1} in both the FTIR spectrum indicates the presence of Aromatic C-H in-plane bend (John Coates, 2000). The presence of ketone and carboxylic acid group denotes that the compound belongs to polyketide nature.

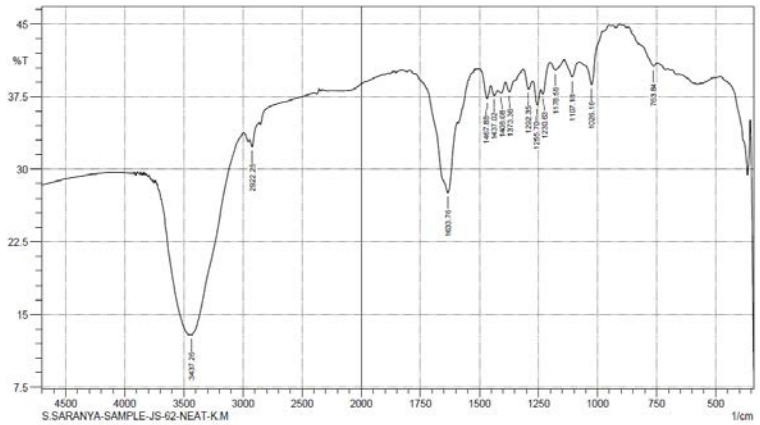

Figure 10: FTIR Spectrum of bioactive compound from *Streptomyces* sp JS-S6

The bioactive compound at the retention time of 3.99 min in GC-MS spectrum was identified by comparison with the mass spectra of Wiley NIST95 database and Pub chem database. From the Mass spectra of Wiley and NIST95 database, the structure of the bioactive compound from the *Streptomyces* sp JS-S6 was identified as Methyl N-hydroxybenzenecarboximidate (Oxime – methoxy phenyl). The fragmentation pattern due to hard ionization in the spectrum at 68 (15 %), 133 (100 %) and 151 (65 %) with the molecular weight of 151 and molecular formula $C_8H_9NO_2$ also suggest that it is Oxime-methoxyl-phenyl. Previously, the Oxime-methoxyl-phenyl compound has been reported as a volatile compound from terrestrial *Streptomyces globisporus* JK-1 produced on 7 day and 14 day old wheat seed culture (Eddy et al, 2012). Oxime-methoxyl-phenyl was also identified in plant source such as *Azadirachta indica* A.Juss in n-hexane leaves extract with the antifungal activity (Akpuaka *et al*.,2012) . Oxime-methoxyl-phenyl has also been reported in the crude extract of *Sedum palladium* leaves which exhibited antibacterial activity against pathogens (Rahdary and Sobati, 2012). Hence, in this study, Oxime-methoxy-phenyl compound was first reported in *Streptomyces* sp JS-S6, derived from the marine environment. The compound also has strong inhibitory effect against clinical pathogens.

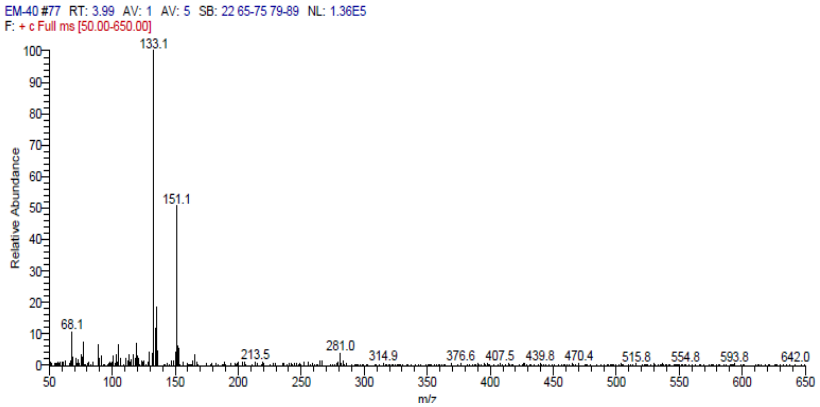

Figure 11. GC-MS spectrum of the bioactive compound with molecular weight 151 and ionization peaks at 68, 133 and 151.

Bioassays of the purified compound

The Minimum inhibitory concentration (MIC) of the purified compound was listed in the table 9. The Purified compound was active against Gram-positive and Gram-negative bacteria. The purified compound exhibited low MICs against the tested microorganisms including MRSA.

Table 9: Antibacterial activity and MIC of the bioactive compound from *Streptomyces* sp. JS-S6.

S.NO	**Microorganisms**	**Zone of Inhibition (mm)**	**Compound MIC (µg mL^{-1})**
1	*E.coli*	24±0.10	15.625
2	*Klebsiella pneumoniae*	25±0.15	15.625
3	MRSA	26±0.10	31.25

- The data shown are mean value of three replicates ± standard deviation

The compound in bioactive fraction does not exhibit cytotoxicity effect against the mice cardimomycetes H9C2 cell line. The cell viability of 99.5 % was observed at 80 µg mL^{-1} of the purified compound shown in fig 12, indicating the bioactive compound inhibits clinical pathogens but does not have any effect cardiomycetes cell line.

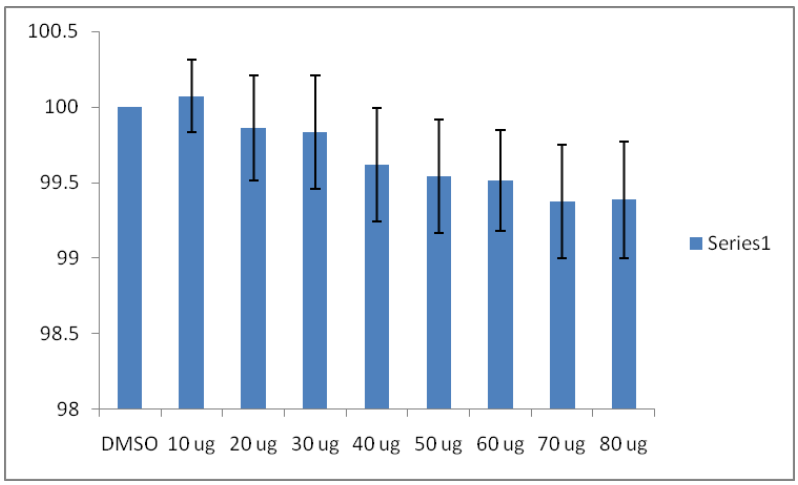

Figure 12: Cytotoxicity assay in mice cardiomycetes cell line H9C2

VI. Conclusion

The marine environment based *Streptomyces* sp JS-S6 exhibited antagonistic activity against clinical microorganisms including the MDR pathogens. The bioactive compound has high antibacterial activity against a set of clinical pathogens and low cytotoxicity against the normal cell line. From these prospects it can be a lead molecule for pharmaceutical industries for controlling the uncontrolled emergence of pathogens which is becoming resistant to antibiotic which is available currently in the pharmaceutical market.

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