Studies on Optimization of Growth Parameters for Enhanced Production of Antibiotic Alkaloids by Isolated Marine actinomycetes

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ABSTRACT
The aim of the present study is to optimize the growth conditions for improved production of alkaloids by promising marine actinomycetes isolated from marine sediments collected on Kakinada coast. The bioactive compounds were extracted from the isolated actinomycete using organic solvents and screened for alkaloids using qualitative tests. The presence of alkaloids in the crude methanol extract was confirmed by UV spectroscopic analysis and quantified by BCG method. The effect of pH, temperature, carbon and nitrogen sources on the growth and fermentative production of alkaloids was optimized. The strain was improved for enhanced production of alkaloids by physical and chemical mutagenesis. The antimicrobial activity of the crude alkaloid extract was determined by the well diffusion method. The isolated strain exhibited the highest growth and alkaloid production at pH 6 and temperature 30°C in 7 days. The alkaloid production was significantly increased 4.5-folds with UV treatment for 30 min and further, 5.5-folds with ethidium bromide treatment (30µg/mL) for 1 hr. The resultant double mutant strain exhibited significantly high antimicrobial activity against S. aureus compared to other bacterial strains with MIC index less than 4. The isolated double mutant strain of actinomycetes can be a potential source of antibiotic alkaloids.

INTRODUCTION
Alkaloids are naturally occurring nitrogenous basic compounds that occur in plants, microorganisms, marine organisms, and animals. The majority of these compounds display biological activity. Recent past, alkaloids become the focus of research in academia, industry and joint ventures for their development as antibacterial agents (Oluosla et al., 2015). Marine bioactive alkaloids are emerging as novel leads (Urban et al., 2000). The first alkaloid isolated from marine sources is hordenine (Kasım et al., 2010). Subsequently, several potent alkaloids were isolated from marine sources. Lodopyridone is a unique alkaloid isolated from marine actinomycetes collected at the mouth of the La Jolla coast (Maloney et al., 2009). A novel alkaloid with broad-spectrum of antibacterial and cytotoxic activities was isolated from marine derived actinomycete (Wence et al., 2013). Two indolocarbazole alkaloids with apoptotic activity were isolated from marine derived actinomycete Z2039-2 (Rui et al., 2007). Later, different types of alkaloids were isolated from the marine source like Tyramine, Dopamine, caulerpin, denticine, martefragine, fragilamide and almazolone and martensine (Kasım et al., 2010).

Actinomycetes are the diverse group of filamentous prokaryotes, which are usually Gram positive. Marine actinomycetes produce secondary metabolites including terpenes, sterols, peptide, alkaloids, fatty acids and amino acid derivatives (Jakeline et al., 2009). These secondary metabolites reported to possess novel biological activities and have the potential to develop antibiotics (Nalinee et al., 2015). Marine actinomycetes are capable of producing clinically important alkaloids, which have significant antimicrobial activity (Romila et al., 2004). Marine alkaloids have distant properties compared to terrestrial alkaloids. Actinomycetes are of special commercial importance because they produce alkaloids with antibiotic activity.
Therefore, isolation of alkaloids from marine actinomycetes and their potential antimicrobial activity is paramount importance of this study. Ramesh et al., (2012) demonstrated the distribution of several unique forms of actinomycetes in marine sediments. Chemical diversity of marine actinomycetes is remarkable due to unique adaptation characteristics of actinomycetes (Maldonado et al. 2005a). Therefore, the marine environment is a promising source of interesting research for new species and pharmaceutically important antibiotics (Fenical et al., 2006). Recently, our group isolated a novel isolate with antioxidant and antiproliferative activity from marine sediments collected near Kakinada coast, Bay of Bengal using starch casein agar. This isolate was characterized and identified as novel actinomycetes strain (Nagaseshu et al., 2016).

The concentration of alkaloids produced by wild strains is very low due to the complicated economical procedures (Samia et al., 2104). Optimization of physico-chemical conditions of actinomycetes, which influence the growth and production of alkaloids with antibiotic property is necessary for commercialization. The yield of bioactive alkaloids can be enhanced through strain improvement techniques (Barrios et al. 2003).

Among the strain improvement procedures, induction of mutagenesis using UV radiation and chemical treatment is the most reliable and widely used. This study involves the extraction of bioactive compounds, screening of secondary metabolites, quantification of alkaloids, optimization of conditions for maximum production of alkaloids and determination of antimicrobial activity.

MATERIALS AND METHODS

Chemicals and bacterial strains used

Eschericia coli, Bacillus cereus, Pseudomonas aeruginosa and Staphylococcus aureus were obtained from MTCC, Chandigarh and maintained on nutrient agar at 27 °C in an incubator. Starch casein agar, rifampicin, ketocozanole, and nutrient agar from HiMedia, Mumbai. Petroleum ether, methanol, chloroform from Merck, India. Ethidium bromide from Sigma, USA.

Actinomycetes strain and media

Actinomycetes strain (KU375127) isolated from the marine sediment samples collected at Kakinada coast, Bay of Bengal was used for this study. The starch casein agar medium (soluble starch, 10.0 g; vitamin free casein, 0.3 g; KNO₃, 2.0 g; NaCl, 2.0 g; KH₂PO₄, 2.0 g; MgSO₄.7H₂O, 0.05 g; CaCO₃, 0.02 g; FeSO₄.7H₂O, 0.01 g; agar, 20.0 g; sterilized 50% sea water in distilled, 1.0 L; pH, 7.0) supplemented with antibiotics, rifampicin(10µg/mL) and ketoconazole (75 µg/mL) to prevent bacteria and fungi growth, respectively (Nagaseshu et al., 2016).

Fermentative production and Extraction of bioactive compounds

The isolated actinomycetes strain was inoculated into starch casein broth and incubated at 37°C for 2 weeks. The culture was centrifuged at 5000 rpm for 10 min. The supernatant was mixed with acidified water (3:7) to adjust the pH to 4.7 and heated in a water bath at 50°C for 4hrs. After filtration, the pH of filtrate was adjusted to 10 using 2N NaOH. The bioactive compounds were extracted by double the volume of petroleum ether and warmed in a water bath for 20 min at 50°C. After evaporation of petroleum ether, it was re-extracted with an equal volume of aqueous methanol (9:1) and concentrated under vacuum at room temperature. Then concentrated aqueous methanol was extracted with an equal volume of chloroform. These solvent extracts were used for screening of secondary metabolites.

Screening of solvent extracts from actinomycetes for secondary metabolites

The solvent extracts were subjected to phytochemical analysis for alkaloids, flavonoids and terpenoids using standard protocols (Abdul et al., 2013; Prashant et al., 2011). The UV spectrum of the crude methanol extract was recorded from 200-500nm with a spectrophotometer (Biodrop, Cambridge, UK).

Quantification of alkaloids

The total alkaloid content of crude methanol extract was determined by the Fazet al. (2008). The aqueous methanol was dissolved in 2N HCl, filtered and condensed. Methanol fraction (100µg) was transferred to a separating funnel and washed three times with 10mLchloroform and the pH was adjusted to neutral with 0.1N NaOH.

Then 5mL of each of bromocresol green solution and phosphate buffer was added. The contents were mixed well and the complex formed was extracted with chloroform by vigorous shaking. The extract was collected into a 10mLvolumetric flask and diluted to mark with chloroform and the optical density was measured at 470nm. Calibration curve was prepared by using atropine standard (40-200 µg/mL).

Optimization of growth for the production of alkaloids

Determination of growth

The growth of isolated actinomycetes was determined by measuring absorbance of inoculated sample at 600nm on a spectrophotometer (Biodrop) using un-inoculated medium as blank.

Effect of pH

The impact of pH on the growth and alkaloid production was evaluated by culturing the isolated actinomycetes in starch casein broth, adjusted to various pH levels ranging from 2.0 to 8. The broth was inoculated with isolated actinomycetes strain and kept in a orbital shaker at 200rpm for 7 days. After incubation, the absorbance of broth was determined at 600nm and % growth was
determined using un-inoculated medium as blank. The alkaloids were extracted and its concentration was determined using the BCG method as described earlier.

**Effect of Temperature**

The optimum temperature was determined by varying the incubation temperatures to 10, 20, 30, 40 and 50°C. The isolated *Actinomycetes* was inoculated in the optimized media, pH 6.0 and kept in a rotary shaker at 200rpm for 7 days. After incubation, the absorbance of broth was determined at 600nm using spectrophotometer and % growth was determined using un-inoculated medium as blank. After extraction, the concentration of alkaloids was determined using the BCG method.

**Strain Improvement by UV exposure**

The strain improvement of isolated *Actinomycetes* was done by sequential treatment of UV radiation and ethidium bromide. The isolated *Actinomycetes* strain was inoculated into culture tubes containing 5mL of starch casein broth, pH 6.0 and incubated for overnight at optimum temperature, 30°C on a rotary shaker set at 200 rpm for 48 hrs. After incubation, the tubes were exposed to UV-radiation at a distance of 30 cm for 5, 10, 15, 20, 25 and 30 min. Non-radiated tubes were served as controls (Munazzah et al., 2012). After UV exposure, the culture broth (100µL) from each tube was spread onto starch casein agar and incubated at 30°C for 48 hrs. The number of colonies in each plate was counted using colony counter (Maloy et al., 2007). The survival rate was calculated as follows: surviving colonies in UV treated plate/Total colonies in control plate x100. The plate with the lowest survival rate was selected and colonies from this plate was inoculated onto starch casein agar plate and incubated for 48 hrs at 30°C. The selected mutant colonies were further used for quantification of alkaloid production using the BCG method.

**Strain Improvement by chemical mutagen (Ethidium bromide)**

The *Actinomycetes* samples with the lowest survival rate and highest alkaloid produced from UV treatment was inoculated into tubes containing 30 mL starch casein broth, pH 6.0 and incubated at 30°C for 48 hrs in a rotary shaker at 200 rpm. After incubation, 5mL culture was distributed into each tube and either untreated (control) or treated with ethidium bromide (5, 10, 15, 20, 25 and 30 µg/mL) for 1 h. After treatment, the sample was shaken well and 100µL of the sample was spread on the starch casein agar plate and incubated at 30°C for 48 hrs. The number of colonies on each plate was counted using the colony counter. The survival rate was calculated as follows: survival colonies in ethidium bromide treated plate/total colonies in the control plate x100.

The selected colonies from the plate with lower survival rate were inoculated onto starch casein agar and incubated for 48 hrs at 30°C. The selected mutant colonies were further used for quantification of alkaloid production using the BCG method.

**Effect of carbon sources on growth and alkaloid production**

To study the effect of different carbon sources on growth and alkaloid production by *Actinomycetes*, dextrose, galactose, sucrose and maltose were separately added to starch casein broth, pH 6.0 at a final concentration of 1g/100mL, inoculated with a double mutant strain of *Actinomycetes* sample and incubated at 30°C for 48 hrs. After incubation, the growth of *Actinomycetes* and alkaloid content in broth was determined.

**Effect of Nitrogen sources on growth and alkaloid production**

To study the effect of different nitrogen sources on growth and alkaloid production, alanine, leucine, glycine and urea were separately added to starch casein broth, pH 6.0 containing dextrose (1%) at a final concentration of 1g/100mL, inoculated with a double mutant strain of *Actinomycetes* sample and incubated at 30°C for 48 hrs. After incubation, growth of *Actinomycetes* and alkaloid content was determined.

**Antimicrobial activity**

**Well diffusion method**

The growth curve of test organisms *E. coli*, *B. cereus*, *P. aeruginosa* and *S. aureus* was observed for 24 hrs at an interval of 30 min by measuring the absorbance at 600nm. The antimicrobial activity of the crude alkaloid extract obtained from a double mutant strain of isolated *Actinomycetes* against log phase *B. subtilis* and *S. aureus* and *P. aeruginosa* and *E. coli*. The test bacterial strain (500 µL) was inoculated on nutrient agar plates by spreading using the L-shaped rod. The wells are made using the well cutter and 5, 10 and 15µL of crude alkaloid extract (1mg/mL) was added to each wells and incubated for 24hrs at 30°C. 30µL of streptomycin or rifampicin were added to each well used as positive controls (1mg/mL). After incubation, the zone of inhibition was measured and expressed as millimeter (Mohanraj et al., 2011).

**Minimum inhibitory concentration (MIC)**

To determine the minimum inhibitory activity of crude methanolic extracts against *S. aureus*, 50 µL of Mueller–Hinton broth was taken in 96-well plate, and 10 µL of crude methanol extract (1mg/mL) was added to the first row of the plate. Then, serially diluted to reduce the concentration from 10, 5, 2.5, 1.25, 0.6, 0.3 µg/mL & then 20µL of inoculum was added to each well. Standard antibiotic, streptomycin under similar concentrations was used as positive control. Wells without extract was served as negative control. The plates were incubated for 24hrs at 37°C. After incubation, 50 µL of 2, 3, 5-triphenyl tetrazoliumchloride (TTC) solution (0.01%) was added to each well and again incubated for 1h. Least concentration of extract, which showing no color change was considered as MIC (Bevara et al., 2016).

**Minimum bactericidal concentration (MBC)**

MBC of the crude alkaloid extract was determined using standard protocols (Bevara et al., 2016). Agar blocks from the
plates that showed no growth in the MIC test was transferred to nutrient broth and incubated at 32°C for 24hrs. Finally, the growth of bacteria was determined by turbidimetry. Bactericidal or bacteriostatic nature of the crude alkaloid extract and the standard was determined using MIC index.

Statistical analysis

All the experiments were carried out three times. Values are shown as means ± SD of at least three independent experiments. Significance set as p<0.001.

RESULTS AND DISCUSSION

Fermentative production of bioactive compounds

Historically, natural products from bacteria, plants and animals have been used as a source of antibacterial drugs. Alkaloids are a structurally diverse group of natural products, used as scaffold substructures for the development of several antibacterial drugs (Oluosola et al., 2015). Therefore, alkaloids are recognized as a potential precursor of antibiotics from unexplored environmental niches (Imhoff et al., 2011). Marine actinomycetes are well known to synthesize antibacterial, antifungal, antiviral, antiinflammatory, anti-parasitic, anti-infective, anticancer (Panchanathan et al., 2014) and antioxidant compounds (Nagaseshu et al., 2016). Hence, marine actinomycetes are widely recognized as industrially important microorganisms. In the present study, bioactive compounds were extracted into different solvents which differ in polarity from 21 days old starch casein broth culture of isolated marine actinomycetes. The extracts were screened for alkaloids, flavonoids and terpenoids using widely accepted protocols. The petroleum ether and chloroform extracts were negative to Mayer’s, Wagner’s, Hagner’s and Dragendroof’s reagents, but aqueous methanol extract was positive, indicating the presence of alkaloids like compounds (Table.1). However, petroleum ether, chloroform and aqueous methanol extracts were negative for both flavonoids and terpenoids.

![Fig. 1: UV absorption spectrum of aqueous methanolic extract of isolated actinomycetes.](image)

Table 1: Analysis of different solvent extracts of isolated actinomycetes.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of the test</th>
<th>Petroleum ether</th>
<th>Aqueous methanol</th>
<th>chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mayer’s</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Wagner’s</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Hagner’s</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Dragendroof’s</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ferric Chloride</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Alkaline Reagent</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>Terpenoids</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

All the experiments were repeated three times.

The UV absorption spectrum of the aqueous methanol extract was observed using UV-visible spectrophotometer in the range of 200-500nm with an interval of 5nm (Figure 1). The spectrum showed the peak value at 240nm confirming the presence of alkaloids. Similarly, Koyama et al., (1981) and Stadler et al., (1998) observed the alkaloids in the solvent extracts by measuring the UV spectrum in the range of 200-500nm. Previously, Naik et al., (2001) also tested the presence of alkaloids from Streptomyces sp, in the UV spectral range between 200-280nm.

Optimization growth parameters for the production of alkaloid

The environmental and cultural conditions are critically important for the cell growth and production of bioactive compounds (Mustafa et al., 2011). Parameters like initial pH, temperature, etc., have a profound effect on the production of bioactive metabolites. An appropriate initial pH is critical for successful fermentation process, however, it varies from strain to strain. The effect of pH and temperature on growth and production of bioactive compounds by the isolated actinomycetes was studied for 24, 48 and 72hrs in starch casein broth. The maximum growth (82%) and alkaloid production (240µg/mL) was observed at pH 6 (Figure 2A). It was noted that an initial increase in pH (2-6), the growth and alkaloid production was also increased, and it decreased significantly above pH 6. Studies have shown that pH effect the growth by influencing the uptake of nitrogen and other ions (Lang et al., 1994). Subathra Devi et al., (2015) reported that the pH plays an important role in growth of S.erythraea and erythromycin production.

Normally actinomycetes are sensitive to temperature (Subathra Devi et al., 2015). The temperature is one of the critical factors that influence the growth of actinomycetes and production of bioactive compounds. Maximum growth (56%) and alkaloids production (190µg/mL) was obtained at temperature 30°C (Figure 2B). It was noticed that an increase in temperature from10 to 30°C increased the growth and alkaloid production. However, beyond 30°C, the growth and alkaloid production was decreased. The increase in alkaloid production with an increase in temperature up to 30°C might be due to the formation of chaperones in response to temperature that supports the high alkaloid formation at higher growth and permissible temperature. In terms of its optimum temperature for growth, the actinomycetes appears to be
mesophilic (Mangamuri et al., 2011). It is noteworthy that a parallelism may exist between growth and alkaloid production (Hanane and Mostefa, 2012).

Screening of industrially important actinomyces is very important for the development of antibiotics. Therefore, to improve the productivity, the parental isolated actinomyces strain was treated with mutagens like UV radiation and Ethidium bromide. The survival rate of actinomyces was decreased with increased the time of UV exposure. UV exposure for 5, 10, 15, 20, 25 and 30 min resulted in 86, 68, 54, 40.9, 27.2 and 22.7%, survival, respectively, compared to UV untreated control. The maximum increase in alkaloid production was observed at exposure time of 30 min. It was found that the alkaloid production was increased 4.5 folds at 30 min in UV exposed strain compared non UV exposed controls (Phillips, 1960) also reported that UV-mutated actinomyces increased antibiotic production compared to non-mutated actinomyces. The UV treated strain of Streptomyces albus exhibited increased antimicrobial activity against all the tested human bacterial and fungal pathogens (Ashok et al., 2014).

The mutant strain of actinomyces obtained at 30 min exposure of UV light was subcultured for five generations after exposure to light and tested in alkaloid production. The results show that the mutant strain was stable for two culture cycles (Data not shown). The effect of alternate treatment of different mutagens are stronger than single mutagen. Hence, chemical mutagenesis was induced. Moreover, chemical mutagen like ethidium bromide gives stable and viable mutants (Venkatanagaraju et al., 2013). In double mutagen studies, the mutant strain obtained from UV treatment was further treated with ethidium bromide from 5 to 30µg/mL for 60 min. The survival rate of the double mutant strain of actinomyces was 90, 65, 55, 45, 35 and 25% at 5, 10, 15, 20, 25 and 30µg/mL concentration of ethidium bromide. The results indicate the survival rate of mutant actinomyces was gradually decreasing with the increased concentration of ethidium bromide, however, alkaloid production was increased. The results show that the amount of alkaloid produced in 90, 65, 55, 45, 35 and 25% was 130, 140, 170, 200, 210 and 220 µg/mL, respectively (Figure 3B). The increased alkaloid production with ethidium bromide may be due to intercalation and the elimination of plasmid DNA, which interfere with secondary metabolite production, from isolated actinomyces (Sridevi and Devendran, 2014).

Supplementation of carbon and nitrogen sources affects growth and alkaloid production

The nutritional sources of carbon and nitrogen are known to have a profound effect on the antibiotic production by actinomyces (Himabindu et al., 2006). In the present study, different carbon sources such as dextrose, galatose, maltose and sucrose were supplemented into starch casein broth to determine their impact on growth and alkaloid production. Compared to other carbon sources, alkaloid production as well as growth was significantly higher in the presence of dextrose (Figure 4A). These
results indicate that starch casein media supplemented with dextrose as a sole source of carbon exhibited highest production of alkaloids (270 µg/mL). However, the media supplemented with other carbon sources such as galactose, maltose and sucrose showed a decreased order of production of alkaloids. Previously, Sunita et al., (2015) reported that monosaccharides are suitable sources for the growth of actinomycetes. Several studies have demonstrated that the excess glucose may repress the production of secondary metabolites (Demain, 1989). Ripa et al., (2009) found that supplementation of medium with glucose (2%) as sole carbon source produced high levels of antimicrobial metabolites by new Streptomyces species (RUPA-08PR) isolated from Bangladeshi soil. Jonsbu et al., (2002) reported that due to species specific variation, different Streptomyces species require different types of carbon sources for cell growth and secondary metabolite production.

Antimicrobial activity

Antimicrobial activity of crude alkaloid extracts obtained from a double mutant strain of isolated actinomycetes was evaluated against P.aeruginosa, S.aureus, B.cereus and E.coli using well diffusion method (Figure 5).

A vast number of studies have demonstrated that the addition of excess nitrogen sources can lead to the better microbial growth and antibiotic production (Lee et al., 1997; De Queiroz. Sousa et al., 2001; Tripathi et al., 2004). The medium supplemented with the different nitrogen sources like alanine, leucine, urea and glycine enhanced the growth and produced different levels of alkaloids (Figure 4B). The results indicate that the maximum growth and alkaloid production was observed by leucine at 72h incubation. Methionine prompted the growth of Streptomyces spp. VITSVK9 (Kumar and Kannabiran, 2010). Antibiotic production was maximum with amino acid alanine in the fermentation medium of actinomycetes (Jagan Mohan et al., 2013). Zhang et al., (2012) demonstrated that supplementation of amino acids to the fermentation broth efficiently produced biomolecules. Aleksandra et al.,(2015) studied the stimulatory effect of amino acids on secondary metabolite production in Streptomyces species 8812 and found a concentration dependent stimulatory effect of L- tyrosine on secondary metabolite activity.

Table 2: Antimicrobial activity of crude methanolic extract of isolated actinomycetes and standard antibiotics.

<table>
<thead>
<tr>
<th>S no</th>
<th>Strain</th>
<th>Zone of inhibition (mm in diameter) with 15µg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CME</td>
</tr>
<tr>
<td>1</td>
<td>P. aeruginosa</td>
<td>16±0.02</td>
</tr>
<tr>
<td>2</td>
<td>S.aureus</td>
<td>26±0.01</td>
</tr>
<tr>
<td>3</td>
<td>B.cereus</td>
<td>17±0.02</td>
</tr>
<tr>
<td>4</td>
<td>E.coli</td>
<td>18±0.02</td>
</tr>
</tbody>
</table>

All the experiments were repeated three times (n=3). Mean ± SE.

At similar concentration, standard antibiotic, rifampicin showed 23mm of zone of inhibition against S.aureus, P.aeruginosa and B.cereus, but 22mm against E.coli. However, streptomycin showed highest zone of inhibition against S.aureus, (32mm) but lowest against B.cereus (27mm) at similar concentrations. Hotam et al., (2013) reported that the actinomycetes strains AS-14,-27 and -28 isolated from soil exhibited 8, 5 and 12 mm zone of inhibition, respectively, against
**S. aureus. Parthasarathi et al., (2012)** reported that the methanol extract from marine Streptomyces hygroscopicus BDUS 49 exhibited 24mm zone of inhibition against S. aureus and 14mm against E. coli.

*S. aureus* is a persistent nosocomial and community acquired pathogen. It became a global health concern, due to remarkable capability of developing drug resistant mechanisms against most antimicrobial agents (Montefiore et al., 1989). Hence, the antibacterial efficacy of the crude alkaloid extract was generally evaluated in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The results showed that MIC of the crude alkaloid extract and streptomycin was 3.5±0.1 and 3.1±0.2 μg/mL against S. aureus (Table 3). The MBC of the crude alkaloid extract and streptomycin was 13.5±0.09, 11.5±0.04 μg/mL against S. aureus. In the present study, the crude alkaloid extract showed significantly lower MIC and MBC values against *S. aureus* comparable to standard antibiotic, streptomycin. As per NCCLS protocol, MIC value is an index of bactericidal or bacteriostatic of action of compounds. The bactericidal compounds kill the microorganisms, however, bacteriostatic compounds inhibit cell division. If the MIC index is ≤ 4, the compound will be considered as bactericidal, whereas if>4 and<32, the compound will be considered as bacteriostatic. The results of the present study indicate that the crude alkaloid extract have strong bactericidal activity against *S. aureus*. Generally, bactericidal compounds are more potent than bacteriostatic compounds. It is well established that bactericidal activity is an important determinant of clinical outcome (Jianbo, 2015). Therefore, the present results suggest that the crude alkaloid extract can be a potential source of antibiotic compounds.

**Table 3:** MIC, MBC and MIC index of crude alkaloid extract.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MIC (μg/ml)</th>
<th>MBC (μl/ml)</th>
<th>MIC index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude alkaloid extract</td>
<td>3.5±0.1</td>
<td>13.5±0.09</td>
<td>3.9±0.02</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>3.1±0.2</td>
<td>11.5±0.04</td>
<td>3.7±0.02</td>
</tr>
</tbody>
</table>

All the experiments were repeated three (n=3). Mean ± SE

**CONCLUSION**

Actinomycetes are filamentous prokaryotes produce a variety of secondary metabolites with novel biological activities. In the present study, alkaloids were extracted from the isolated actinomycete using aqueous methanol. The growth conditions for enhanced production of alkaloids was evaluated. The isolated strain exhibited the highest growth and alkaloid production at pH 6 and temperature 30°C in 7 days. The alkaloid production was increased 4.5-fold with UV treatment for 30min. Further treatment with ethidium bromide at 30μg/ml for 1h increased the alkaloid production by 5.5 folds. The double mutated strain produced high concentrations of alkaloids also exhibited significantly high antimicrobial activity against *S. aureus*. The crude alkaloid extract exhibited MIC index less than 4 indicating a bactericidal action against *S. aureus*. Therefore, the double mutant strain of isolated actinomycetes can be a potential source of antibiotic alkaloids.

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**DECLARATION**

Isolation of alkaloids from marine actinomycetes, optimization of growth parameters and strain improvement was carried out in GITAM University, Visakhapatnam, Andhra Pradesh, India.

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**REFERENCES**


