

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 (30 µg/mL) for 1hr. 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 (Olusola *et al.*, 2015). Marine bioactive alkaloids are emerging as novel leads (Urban *et al.*, 2000). The first alkaloid isolated from marine sources is hordenine (Kasim *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 (Kasim *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.

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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

Escherichia 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, ketoconazole, 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; K₂HPO₄, 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 (10 µg/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 Fazel *et al.* (2008). The aqueous methanol was dissolved in 2N HCl, filtered and condensed. Methanol fraction (100 µg) was transferred to a separating funnel and washed three times with 10mL chloroform 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 10mL volumetric 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 orbital 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 48hrs. 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 48hrs 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* sample 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 rotatory 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 48hrs 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 24hrs at an interval of 30min 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 \pm 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 (Olusola *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, anti-inflammatory, 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.

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

S. No.	Name of the test	Different solvent extracts		
		Petroleum ether	Aqueous methanol	chloroform
1	Alkaloids			
	Mayer's	Negative	Positive	Negative
	Wagner's	Negative	Positive	Negative
	Hagner's	Negative	Positive	Negative
	Dragendroof's	Negative	Positive	Negative
2	Flavonoids			
	Ferric Chloride	Negative	Negative	Negative
	Alkaline Reagent	Negative	Negative	Negative
	Lead Acetate	Negative	Negative	Negative
3	Terpenoids	Negative	Negative	Negative

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.

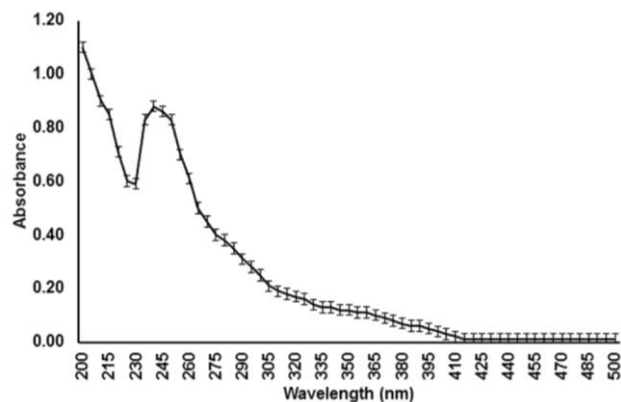


Fig. 1: UV absorption spectrum of aqueous methanolic extract of isolated *actinomycetes*.

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 (240 μ g/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 (190 μ g/mL) was obtained at temperature 30°C (Figure 2B). It was noticed that an increase in temperature from 10 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).

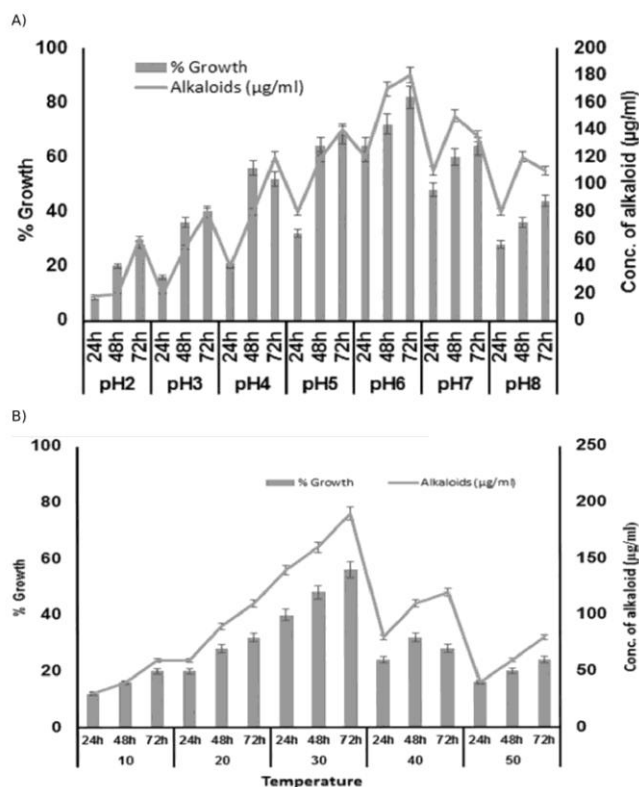


Fig. 2: Effect of initial Ph (A) and different levels of incubation temperature (B) of the medium on production of alkaloids from isolated *actinomycetes*.

Screening of industrially important *actinomycetes* is very important for the development of antibiotics. Therefore, to improve the productivity, the parental isolated *actinomycetes* strain was treated with mutagens like UV radiation and Ethidium bromide. The survival rate of *actinomycetes* 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 (Figure 3A). Phillips (1960) also reported that UV-mutated *actinomycetes* increased antibiotic production compared to non-mutated *actinomycetes*. 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 *actinomycetes* 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 30 µg/mL for 60 min. The survival rate of the double mutant strain of *actinomycetes* was 90, 65, 55, 45, 35 and 25% at 5, 10, 15, 20, 25 and 30 µg/mL concentration of ethidium bromide. The results indicate the survival rate of mutant *actinomycetes* 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 *actinomycetes* (Sridevi and Devendaran, 2014).

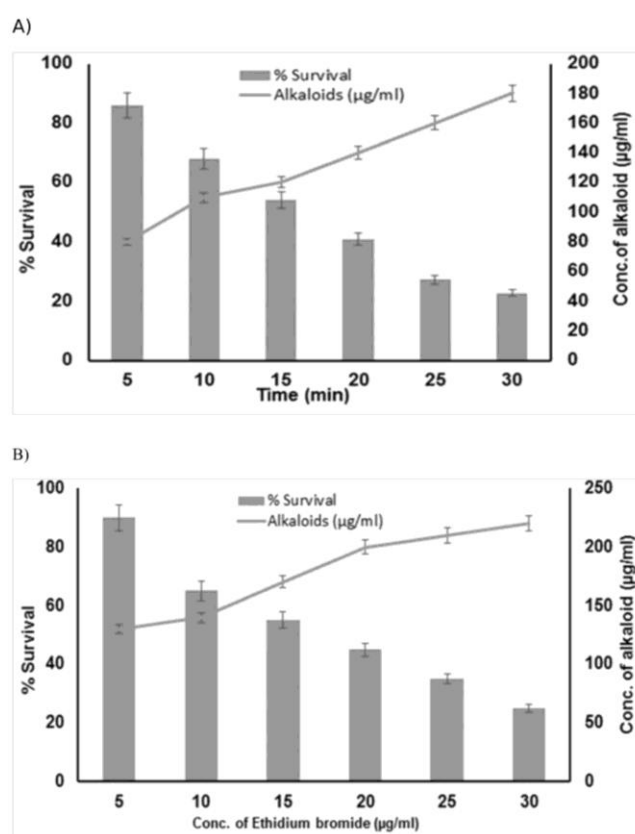


Fig. 3: Effect of different UV radiation times (A) and Ethidium bromide concentrations (B) on survival (Bar) and total alkaloid content (line) of isolated *actinomycetes*.

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 *actinomycetes* (Himabindu *et al.*, 2006). In the present study, different carbon sources such as dextrose, galactose, 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 $\mu\text{g}/\text{mL}$). 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.

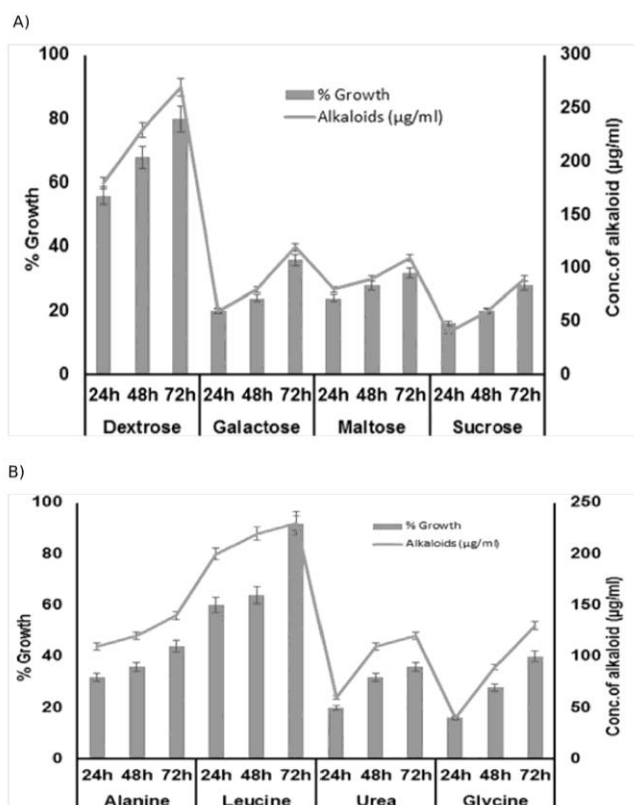


Fig. 4: Effect of various carbon (A) and nitrogen sources (B) on growth (Bar) and production alkaloids (line) by isolated *actinomycetes*.

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.

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).

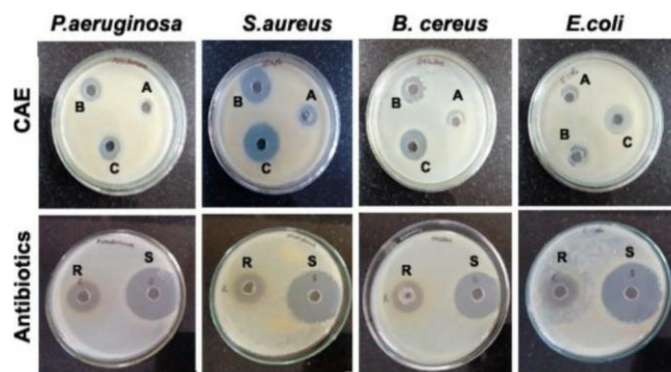


Fig. 5: antimicrobial activity of crude alkaloid extract from double mutant strain of isolated *actinomycetes* and antibiotics. Panel-1: Antimicrobial activity with 5 (A), 10 (B) and 15 μl (C) of crude alkaloid extract (CAE) at 1mg/ml; Panel -2: Antimicrobial activity with 15 μl of rifampicin (R) and streptomycin (S) at concentration of 1mg/ml.

The results show that a crude alkaloid extract exhibited high zone of inhibition against gram positive bacteria, *S.aureus* (26mm) compared to other bacteria tested at 3 μg (Table 2).

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

S no	Strain	Zone of inhibition (mm in diameter) with 15 μg		
		CME	Rifampicin	streptomycin
1	<i>P. aeruginosa</i>	16 \pm 0.02	23 \pm 0.02	29 \pm 0.02
2	<i>S.aureus</i>	26 \pm 0.01	23 \pm 0.03	32 \pm 0.03
3	<i>B.cereus</i>	17 \pm 0.02	23 \pm 0.02	27 \pm 0.02
4	<i>E.coli</i>	18 \pm 0.02	22 \pm 0.01	31 \pm 0.02

All the experiments were repeated three times (n=3). Mean \pm 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 $\mu\text{g/mL}$ against *S. aureus* (Table 3). The MBC of the crude alkaloid extract and streptomycin was 13.5 ± 0.09 , 11.5 ± 0.04 $\mu\text{g/mL}$ 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.

Treatment	MIC ($\mu\text{g/ml}$)	MBC ($\mu\text{l/ml}$)	MIC index
Crude alkaloid extract	3.5 ± 0.1	13.5 ± 0.09	3.9 ± 0.02
Streptomycin	3.1 ± 0.2	11.5 ± 0.04	3.7 ± 0.02

All the experiments were repeated three (n=3). Mean \pm 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-folds with UV treatment for 30min. Further treatment with ethidium bromide at $30\mu\text{g/ml}$ for 1h increased the alkaloid production by 5.5folds. 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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Conflict of Interests: There are no conflicts of interest.

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