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A STUDY ON POTENTIAL ANTIBACTERIAL PRODUCING STREPTOMYCES SP. ISOLATED FROM AN ESTUARINE LAKE SEDIMENT

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ABSTRACT

The antimicrobial agent production potential of a Streptomyces strain MM1AG7 was evaluated. The selective isolation of the strain was carriedout on starch casein agar. The primary screening of the Streptomyces isolate was done by cross strak method againt pathogenic test strains *Escherichia.coli* MTCC 82, *Staphylococcus aureus* MTCC 96, *Bacillus cereus* IP406 and *Salmonella typhi* MTCC 734 and *Micrococcus leuteus*. The secondary screening was carried out by using the culture supernatant against the test strain by agar well diffusion method. The growth and antimicrobial production ability against *Micrococcus leuteus* was studied. The antimicrobial agent production was also observed till pH11 and NaCl concentration 3% (w/v).

KEYWORDS: *Escherichia.coli* MTCC 82, *Staphylococcus aureus* MTCC 96, *Bacillus cereus* IP406 and *Salmonella typhi* MTCC 734 and *Micrococcus leuteus*.

The genus *Streptomyces* consists of Gram-positive filamentous bacteria that grows in aquatic and terrestrial environments. Growth of *Streptomyces* involves the process of development of a layer of hyphae that can further differentiate into a chain of spores for which a specialized and synchronised metabolism is required.[1] Streptomyces are well known for their property of production of bioactive secondary metabolites such as, antitumoral, antivirals, antifungals, anti-hypertensives, and mainly antibiotics and immuno suppressives.

Streptomyces are the largest known antibiotic producing genus in the microbial world.[1,2] Most *Streptomyces* and other Actinomycetes produce a diverse array of anti-biotics including aminoglycosides, anthracyclins, glycol-peptides, β-lactams, macrolides, nucleosides, peptides. Estuarine habitats are good sources for bio-prospecting of micro-organisms with halophilic and alkaline microbes with industrial applications. Microbes which belong to genus *Streptomyces*, isolated from marine and estuarine habitat have been widely recognized as a potential source of antifungal, anti-tumour, anti-bacterial compounds.[3,4,5] Natural products can be defined as "the chemical (carbon) compounds isolated from diverse living organisms. They may be derived by primary or secondary metabolism of living organisms. The primary metabolites (polysaccharides, proteins, nucleic acids, fatty acids) are common in all. The secondary metabolites are rather of low molecular weight (MW<3000); chemically and structurally extremely diverse with obscure functions. They seem to be completely unnecessary for the producer and do not have any role in their life cycle. The biochemistry of *Streptomyces* is truly remarkable, considering their production of secondary metabolites, many of which account for almost half of all known antibiotics. 55% of them were produced by the genus *Streptomyces*, 11% from other *Actinomycetes*, 12% from non-filamentous bacteria and 22% from filamentous fungi. The fame of *Streptomycetes* as versatile producers of secondary metabolites started with the discovery of Actinomycin in 1940, followed by streptomycin in 1943. Two-thirds of the marketed microbial drugs are produced by *Streptomycetes*. Recent estimates indicate that nearly 50% of the 20,000 bioactive secondary metabolites described from 1900 onwards are produced by filamentous Actinomycetes that originated in the soil. Among them, the easiest to isolate from soils are *Streptomyces* species. It follows that they have been extensively isolated since 1940, and today the chance of rediscovering known antibiotics from them. Many of these compounds have important applications in human medicine as antibacterial, antitumor and antifungal agents. Also, in agriculture these compounds act as growth promoters, agents for plant protection, anti-parasitic agents and herbicides. The onset of antibiotic production of *Streptomyces* cultures grown on agar usually coincides with the early stages of morphological

differentiation.

MATERIAL AND METHOD

Sampling

Sediment were collected from the shore lines of 21 different sampling stations of Chilika lake. Sediment samples were collected as Streptomyces and Actinobacteria are generally soil and sediment dwellers.(**Figure 1)**. Samples were collected aseptically in zip lock packets and stored at 4^{o}C until further analyzed. Temperature and pH of the water and soil samples were measured at the time of collection.

Enrichment and isolation of Streptomyces

The enrichment was done on starch casein media (Table 4, Appendix I) broth supplemented with nystatin (stock: 50mg/L; working: 50ug/ml)) at 30^{o}C at 120 rpm. Five grams of sediment were added to 250 ml conical flasks containing 50 ml of sterile starch casein medium. The flasks were incubated on a rotary shaker (120 rpm) at 30^{0}C for 7 days. 1ml of this culture was transferred to fresh broth and this was repeated 4 times for 1.5 months. Isolates were obtained by plating 100ul of serially diluted enrichment culture, after 7 days on starch casein agar medium supplemented with nystatin.

Primary Screening of isolates for antimicrobial activities

The typical Streptomyces colony obtained on Starch casein media at the end of incubation period were then re-streaked to obtain pure culture. The pure colonies were maintained on Starch casein agar plate and slants. For long term storage the strains were grown in starch casein broth. The spore suspension of the grown culture along with a cryopreserver glycerol (50% spore suspension+50% sterile glycerol*) were then stored at -80°C. Antimicrobial activity of isolates were analysed on seawater (50 % v/v) LB for 7 days against indicator strains *Escherichia.coli* MTCC 82, *Staphylococcus aureus* MTCC 96, *Bacillus cereus* IP406 and *Salmonella typhi* MTCC 734 and *Micrococcus luteus* procured from Microbial Type Culture Collection (MTCC, Chandigarh) by cross streak method.

Secondary screening of isolates for antimicrobial activities

The secondary screening of the isolate was carried out by determining the antimicrobial activity of the culture supernatant of Streptomyces strains against test bacterial strains *Escherichia.coli* MTCC 82, *Staphylococcus aureus* MTCC 96, *Bacillus cereus* IP406 and *Salmonella typhi* MTCC 734 and *Micrococcus luteus* by agar well diffusion assay.[6] In brief, the indicator strains were streaked on LB agar plate. 50 microlitres of the Streptomyces culture supernatant was then incorporated into the wells prepared in the same agar plate. The plates were incubated at 37°C for 24 hours. The antibacterial activity was determined by measuring the diameter of the zone of inhibition formed around the well.

Morphological characterization

Morphological and culture characteristics of spore chain morphology, spore surface, and spore chain ornamentation were observed by light and phase contrast (Leica DM300, Leica microsystem, Germany) and scanning electron microscopy (Zeiss Scanning Electron Microscope, Carl Zeiss AG, EVO®40, GmbH, Germany) after incubation on ISP 3 agar medium for 3-4 weeks at 30^{o}C. Culture traits of strain RC1832^{T} were recorded after 21 days incubation at 30 ^{0}C on several ISP (International Streptomyces Project;[7]) media : Glucose yeast extract malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts starch agar (ISP 4), glycerol asparagine agar (ISP 5), peptone yeast extract iron agar (ISP 6) and tyrosine agar (ISP 7). The growth was also recorded on modified ISP fortified with sea water (50%, v/v). Colour evaluations for diffusible pigments and substrate mycelia were also observed on ISP media by comparing the cultures with colour chips from ISCC-NBS COLOR CHARTS standards no.2 2106.[8]

A range of biochemical and physiological tests were conducted at 30^{o}C as described by,.[7,9,10,11,12] Production of hydrolysing enzymes for hydrolysis of starch, cellulose, gelatin liquefaction, casein, Tween 80, lecithin, tributyrin, chitin and utilization of urea and citrate were performed as described by **[13,1,14]**. Utilization of D-glucose, D-fructose, maltose, D-xylose, D-cellobiose, L-arabinose, L-rhamnose, D-raffinose, sucrose, D-galactose, D-mannose and inositol as sole carbon source (1% w/v) were tested on basal salt media fortified with sea water (50% v/v). Methods for estimation of melanin production and nitrate reduction were performed on modified ISP media as described by Shirling and Gottleib.[15,9] Catalase activity was determined by oxygen bubble formation on addition of 4% H_2O_2 to well grown colonies and oxidase activity was determined by change in colour of the oxidase disk (Hi Media, Pvt. Ltd. Mumbai, India). The growth and pH tolerance of RC1832^{T} was studied over a temperature range of 20-50^{0}C and pH 6-10 on SWLB and salt tolerance of the strain was observed on various NaCl concentrations (0-18 %, w/v) in LB media. Antibiotic susceptibility was investigated using antibiotic discs (Hi Media Pvt. Ltd. Mumbai) as described by **[10]** on SWLB. Antimicrobial activity of strain RC 1832^{T} was also examined on SWLB for 7 days against indicator strains *Staphylococcus aureus* MTCC 96, *Salmonella typhi* MTCC 734, procured from Microbial Type Culture Collection (MTCC, Chandigarh), *Micrococcus luteus* LBG B4291 and *Bacillus cereus* IP406 (a kind gift from Dr. Peter Luethy, Institute of Microbiology, ETH, Zurich), by observing formation of zone of inhibition.

Optimization of culture conditions for antimicrobial compound production by the isolate MM1AG7

For large scale production of antimicrobial compound by MM1AG7, the experiments were conducted to determine the optimum culture condition for the same including the effect of incubation period, culture media, temperature,

pH, carbon source, nitrogen source, NaCl concentration. Cell growth was observed by calculation of cfu/ml and antimicrobial activity was tested by the well diffusion assay.[6]

Effect of various carbon source on growth and antibacterial agent production

Preculture of isolates MM1AG7 was grown in Starch casein media. 1% (v/v) of precultures (containing approx.1.2-1.8 x10^6 cells/ml) were inoculated to 50 ml of MSM supplemented with appropriate carbon source (glycerol, starch, sucrose, glucose, and fructose) separately in flasks. Aliquots of 1ml were taken at every 24h interval for 5 days for measurement of antimicrobial activity and methods described earlier. All the experiments were carried out in triplicates**.** Cell growth was evaluated by plating 100 microlitres of the aliquot on starch casein media and incubated. The developed colonies were counted to determine cfu/ml and antimicrobial activity was tested by the classical well diffusion assay.[6]

Effect of different Nitrogen source on growth and antibacterial agent production

Preculture of isolates MM1AG7 was grown in Starch casein media. 1% (v/v) of precultures (containing approx.1.2-1.8 x10^6 cells/ml) were inoculated to 50 ml of MSM supplemented with appropriate carbon source having different nitrogen sources (ammonium sulphate, potassium nitrate, peptone, yeast extract) separately in flasks. Aliquots of 1ml were taken at every 24h interval for 5 days for measurement of antimicrobial activity and methods described earlier. All the experiments were carried out in triplicates and comparative study was done. Cell growth was observed by calculation of cfu/ml and antimicrobial activity was tested by the classical well diffusion assay.[6]

Effect of different pH on growth and antibacterial agent production

The experiment was carried out by analysing activity and growth of the strain MM1AG7 in media with a pH range of 4-11 The preculture of isolates MM1AG7 was grown in Starch casein media. 1% (v/v) of this (containing approx.1.2-1.8 x10^6 cells/ml) was inoculated to 50 ml of MSM supplemented with appropriate carbon source having different pH (5, 7, 8, 9, and 11) separately in flasks. Aliquots of 1ml were taken at every 24h interval for 5 days for measurement of antimicrobial activity and methods described earlier. All the experiments were carried out in triplicates**.** Cell growth was observed by calculation of cfu/ml and antimicrobial activity was tested by the classical well diffusion assay.[6]

Effect of different NaCl concentration on growth and antibacterial agent production

Preculture of isolates MM1AG7 was grown in Starch casein media. 1% (v/v) of precultures (containing approx.1.2-1.8 x10^6 cells/ml) were inoculated to 50 ml of MSM supplemented with appropriate carbon source with different NaCl conc. (0.5%, 1%, 2%, 4% 65 and 8% w/v) separately in flasks. Aliquots of 1ml were taken at every 24h interval for 5 days for measurement of antimicrobial activity and methods described earlier. All the experiments were carried out in triplicates and comparative study was done. Cell growth was observed by calculation of cfu/ml and antimicrobial activity was tested by the classical well diffusion assay.[16]

Purification of the antimicrobial compound by column chromatography

MM1AG7 strain was inoculated in 500ml SCA media and incubated at 37^{O}C for 5 days. Fully grown culture was centrifuged at 6500rpm for 10 minutes. The culture supernatant obtained was extracted twice with ethyl acetate, concentrated in rota-vapour and again dissolved in 5ml of ethyl acetate. 5ml of this sample is loaded onto a silica gel column and eluted successively with 50ml of 100% hexane, 50ml of linear gradient hexane: ethyl acetate (v/v, 75:25, 50:50, 25:75), 50ml of 100% ethyl acetate, 50ml of 100% methanol; resulting in 31 fractions of 10ml each. All the fractions were tested for antimicrobial activity by well diffusion assay against *M.leuteus*.

Confirmation of the purified antimicrobial compound by HPLC

A modified method was developed from the studies so far was used to determine the presence of secondary metabolite in terms of antibiotic. In brief, sample MM1AG7 was extracted twice using ethyl acetate in an equal volume ratio (1:1, v/v). after concentrating the sample by using rotary evaporator (Eyela CCA-1141, Tokyo) residues dissolved in methanol (HPLC grade) were determined by HPLC (Agilent Technologies 1260 Infinity) equipped with a Ascentis@ C18 reversed phase column (4.6 nm × 5 nm) with detection at 230nm with a manual injector. A mixture of methanol and water (85:15) was used as the mobile phase at a flow rate of 1.2ml/min. the injection volume was 20µl.

Determination of the Minimum Inhibitory Concentration (MIC) of the extract of the Isolates

Minimum inhibitory Concentration is the lowest concentration of antibiotic that completely inhibits bacterial growth[17]. Quantitative assay was carried out to determine the MIC of the antibacterial agent present in the culture supernatant of isolated *Streptomyces* strain. The experiment was carried out by adding and serially diluting the antibacterial extract of the *Streptomyces* strains to tubes containing 5ml of Luria Bertanii medium followed by addition of 5 µl of overgrown test strains such as *Micrococcus luteus*. The tubes were then incubated at 37^{0}C for 24h. The absorbance was then measured at 600nm. The obtained and pooled 500 microlitres of purified extract of MM1AG7 culture was considered as 100 %. Different concentration of the extract was prepared by adding different volumes i.e. 500µl, 400µl, 300µl, 200µl, 100µl, 50µl, 40µl, 30µl, 20µl and 10µlof the same to tubes containing 5ml of LB,

followed by addition to 5µl *M.leuteus* culture in each. Culture tubes were incubated at 37^{O}C for 24hrs and then OD was measured for each.

Characterisation of the purified antimicrobial compound

Effect of temperature on activity of the antimicrobial agent

For determining the effect of temperature on stability of the antibacterial agent, 1ml of supernatant was harvested from broth culture and treated at -20^{O}C, 4^{O}C, 25^{O}C, 37^{O}C, 56^{O}C, 70^{O}C, 90^{O}C for 1hr. The residual antibacterial activity of heat-treated samples was determined by measurement of zone of inhibition against *M. luteus.*

Effect of pH on activity of the antimicrobial agent

For determining the effect of pH on stability of the antibiotic, 1ml of supernatant was harvested from broth culture and treated with pH 5, 6, 7, 8, 10 for 1 hour. The residual antibacterial activity of pH-treated samples was determined by measurement of zone of inhibition against *M. luteus.*

Effect of mutation on activity of the antimicrobial agent

MM1AG7 strain was inoculated in 4, 100ml conical flasks containing 50 ml SCA broth in each. Ethidium-bromide was added in 3 of them in different concentrations (5µg/ml, 10µg/ml, and 15µg/ml) and one was left without ethidium-bromide as control. All were incubated for 120 hours. Cfu/ml count and antimicrobial activity was observed in every 24 hours to see the post-mutation effect.

Qualitative Determination of the antimicrobial agent by Thin Layer Chromatography (TLC)

For TLC analysis was carried according to the method of Darabarpur *et al* (2012) 300µl of reaction mixture was spotted on TLC plate (Silica gel 60, F_{254} (20 x20cm); E. Merck, Darmstadt, Germany) using micro-capillary (Drummond, Scientific company, USA), along with respective chito-oligosaccharide standards. The plates were air dried. Mobile phase (butanol:acetic acid: water (v/v 4:4:1)) was allowed to run along the TLC plate till solvent front reached more than 3/4th of its length after which the plate was marked and dried. The plates were then developed by spraying with developing reagent: aniline diphenylamine reagent (Table-6, Appendix I) and heated at 120°C for 10-15 minutes. Dark brown-greenish spots were identified and Rf value was compared to that of standards.

Rf value =

Distance travelled by the sample from the origin

Distance travelled by the solvent front from the origin

RESULTS

A total of 161 Actinomycetes strains were obtained from 11 sampling stations of Chilika lake. The strains were checked for their antimicrobial production ability by cross streak method against Antimicrobial activity of isolates were analysed on seawater (50 % v/v) LB for 7 days against indicator strains *Escherichia.coli* MTCC 82, *Staphylococcus aureus* MTCC 96, *Bacillus cereus* IP406 and *Salmonella typhi* MTCC 734 and *Micrococcus luteus* procured from Microbial Type Culture Collection (MTCC, Chandigarh) . 67 *Streptomyces* strains were then selected for secondary screening. The Actinobacterial strains were grown in Starch casein broth for 5-7 days. The culture supernatant were then checked for their potential by agar well diffusion method against the test strains (Fig 1). Eventually the strain MM1AG7 was selected for further studies.

The 16srRNA sequencing and phylogenetic analysis showed that the strainMM1AG7 may belong to a novel species of Streptomyces i. e *Streptomyces cinereoruber subsp. cinereoruber* NBRC 12756^{T} (Fig 2). The novelty of the strain can be confirmed by polyphasic taxonomy (Result not shown for all).

Effects of mutation by different concentration of ethidium bromide on cell growth and antimicrobial activity was studied. Among them only the MM1AG7 culture mutated with 5µg/ml conc. of EtBr showed cell growth and productivity of antimicrobial agent against *M.luteus*; whereas with the increasing conc. of the mutagenic agent cell growth and antimicrobial activity both were prevented (Fig 7).

Effects of different nitrogen sources on cell growth and antimicrobial activity were studied. Among the nitrogen sources tested, the potassium nitrate was the best for the productivity of antimicrobial agent by MM1AG7 against *M.luteus* followed by peptone, whereas the other nitrogen source yeast extract used in this study reduced both cell growth and antimicrobial activity. Ammonium sulphate completely prevented the cell growth and antimicrobial activity (Fig 6).

Effects of different NaCl conc. to the media, on cell growth and antimicrobial activity were studied. Among the different NaCl conc.used, 0.5% (w/v) NaCl conc. was the best for the productivity of antimicrobial agent by MM1AG7against *M.luteus* followed by 1% and 2% NaCl conc., whereas the other concentrations used in this study reduced cell growth and completely prevented its antimicrobial activity (Fig 5).

Effects of supplying different pH of the media, on cell growth and antimicrobial activity were studied. Among the different NaCl conc.used, 0.5% NaCl conc. was the best for the productivity of antimicrobial agent by MM1AG7 against *M.luteus* followed by 1% and 2% NaCl conc., whereas the other concentrations used in this study reduced cell growth and completely prevented its antimicrobial activity.

Effects of supplying different carbon sources, on cell growth and antimicrobial activity were studied. Among the different carbon sources used, glycerol was the best for the productivity of antimicrobial agent by MM1AG7against*M.luteus* followed starch; whereas the carbon sources used in this study reduced cell growth and completely prevented its antimicrobial activity.

Streptomyces have been recognized as the potential producers of metabolite such as antibiotics, antitumor, anticancer compounds and many more. Isolation of new microbial species from unexplored areas is one of the more efficient approaches for the development of novel bioactive metabolite. () Optimization of culture conditions for MM1AG7 strain was observed. Glycerol was found to be the best carbon source in comparison to starch, glucose, sucrose, and fructose. It was able to grow in a broad pH range starting from pH 5 to10. But it showed the highest activity at pH 11; thus concluding it to be an alkalophilic in nature and also tolerance at higher pH. Potassium nitrate was found to be the best nitrogen source; while best NaCl concentration is 0.5% making it a mild-halophile.

The antimicrobial compound was found to be a highly polar compound as it was eluted by methanol in column purification process.

Purified antimicrobial compound was found to be heat, enzyme (lysozyme, proteinase k) and pH tolerant. It showed its activity efficiently in all the temperature ranges; but maximum activity was found in 90ºC (Fig 10). It also efficiently showed activity in pH ranging from 5-9; but there was no activity starting from pH 11 (Fig 9) and highest activity at NaCl concentration 0.5%(w/v) (Fig 11). The compound when analysed in TLC under UV showed an evident spot for the antimicrobial agent (Fig 12). HPLC analysis showed an significant peak for the antimicrobial compound (Fig 13).

The minimum inhibitory concentration (MIC) for the strain MM1AG7 antimicrobial extract againt *Micrococcus luteus* was 40%. (Fig 14)

Table 1: secondary screening of Streptomyces strains for determination of efficiency of antimicrobial agent production against the test strains.

Strains\Test organisms	*S.typhi*	*S.aureus*	*B.subtilis*	*M.luteus*	*B.cereus*
MK1	-	-	+	-	+
MK2	-	-	+	+	+
MK4	-	-	+	-	+
MK5	-	-	- (z)	-(z)	+
MK6	-	-	- (z)	- (z)	+
MK8	-	-	+	- (z)	+
MK9	-	-	- (z)	- (z)	- (z)
MM1AG7	-	-	- (z)	-	+
MM1AG9	-	-	- (z)	-	- (z)
PAT1AG	-	+	- (z)	+	+
PAT2AG	-	-	+	-	+
BM1AG	-	-	+	-	+
BM2AG	-	-	+	+	+
KJ3AG	-	-	- (z)	-	+
GH1AG	-	-	- (z)	+	+

MK1,MK2, MK4, MK5, MK6, MK8, MK9, MM1AH7, MM1AG9, PAT1AG, PAT2AG, BM1AG, BM2AG, KJ3AG and GH1AG are Streptomyces strains.

Table 2: Growth and culture characteristics of strain MM1AG7 on different ISP media. No diffusible pigment was observed for either of the strains on any of the media. All the data presented are from this study.

Colony character on ISP media	MM1AG7
Yeast extract malt extract agar (ISP 2)A	G++
S	W++
Oat meal agar (ISP 3) A	-
S	BR+
Inorganic Salt Starch agar (ISP 4) A	G++
S	B++
Glycerol asparagine agar (ISP 5) A	W+
S	W++
Peptone yeast extract iron agar (ISP 6) A	-
S	-
Tyrosine agar (ISP 7) A	G++
S	B++
Tryptone -Yeast extract (ISP 1) A	W+
S	BR+++

Key: +++ abundant growth, ++ moderate growth, + poor growth, -no growth G, Grey; W, White; BR, Brown;

Table 3: Physiological and Biochemical test of strain MM1AG7. All the data are from this study.

Biochemical property	**MM1AG7**
Degradation of [#]	
Starch	+
Casein	-
Tributyrin	-
Gelatin	+
Chitin	+
Pectin	+
Carboxymethyl cellulose	+
Tween80	+
Utilization of [#]	
Citrate	+
Growth on sole carbon source (1% w/v)[∞ #]	
Sucrose	+
Glucose	+
Mannose	+
Xylose	+
Fructose	+
Fermentation/utilization of sugars[∞]	
Arabinose	+
Cellobiose	+
Mellibiose	+
Mannitol	+
Rhamnose	+
Salicin	-
Trehalose	+
Tolerance to antibiotics	
Amikacin (Ak)	+
Amoxycilin (Am)	-
Bacitracin(B)	+
Carbenicilin(Cb)	+
Co-trimazole(Co)	-
Cephalexin(Cp)	-
Cephadroxil(Cq)	-
Erythromycin€	+
Furazalidone(Fr)	-
Nalidixic acid(Na)	-
Nitrofuratoin(Nf)	+
Norfloxacin(Nx)	-
Oxytetracyclin(O)	+
Growth in Presence of NaCl (%, w/v)[§]	
1,2	+
Growth in pH[#]	
5, 7, 9, 11, 13	+

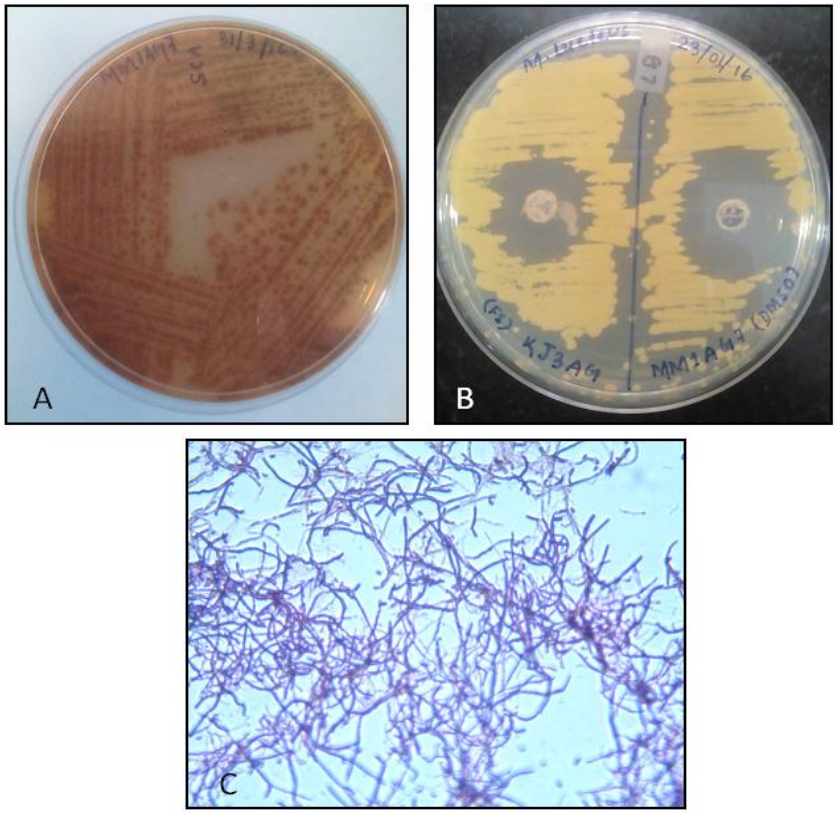
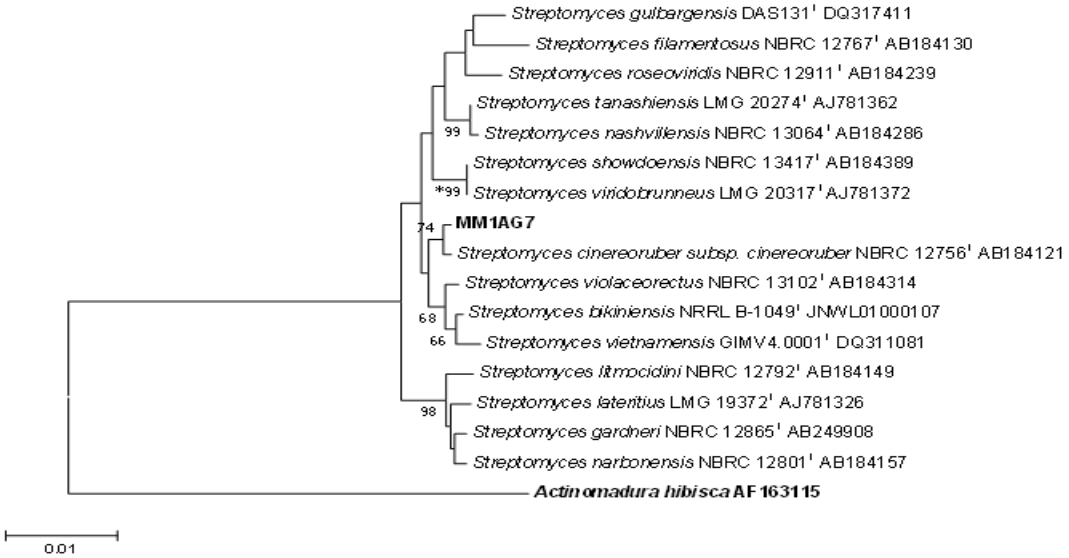


Fig. 1 (A): Colony morphology of the isolate MM1AG7 on starch casein plates.
Fig. 1 (B): Agar well diffusion assay for the antimicrobial agent produced by MM1AG7.
Fig. 1 C: 100X oil immersion microscopic view of MM1AG7.



*****All the other phylogenetic tree type are given as supplementary data.**

Figure 2: Evolutionary relationships of taxa for MM1AG7 inferred by Neighbor Joining method

The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 0.14154585 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [3] and are in the units of the number of base substitutions per site. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1321 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [4].

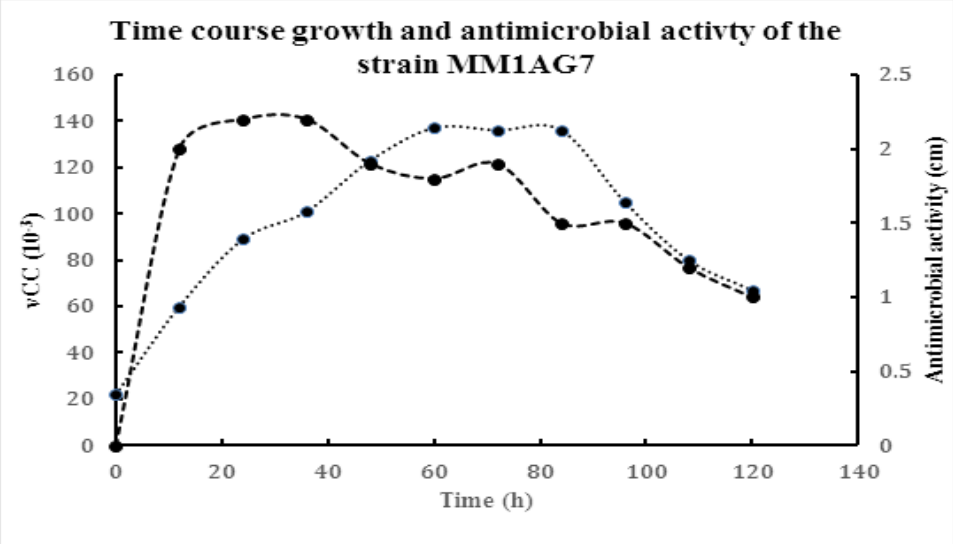


Fig. 3: Time course growth curve and antimicrobial activity of the strain MM1AG7.

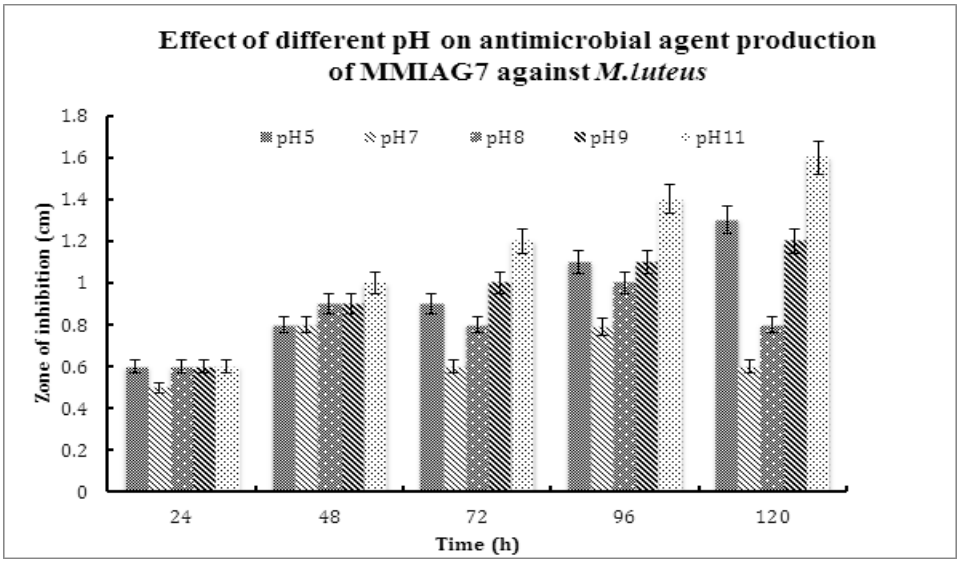


Fig. 4: Effect of different pH on antimicrobial agent production of MMIAG7 against *M.luteus.*

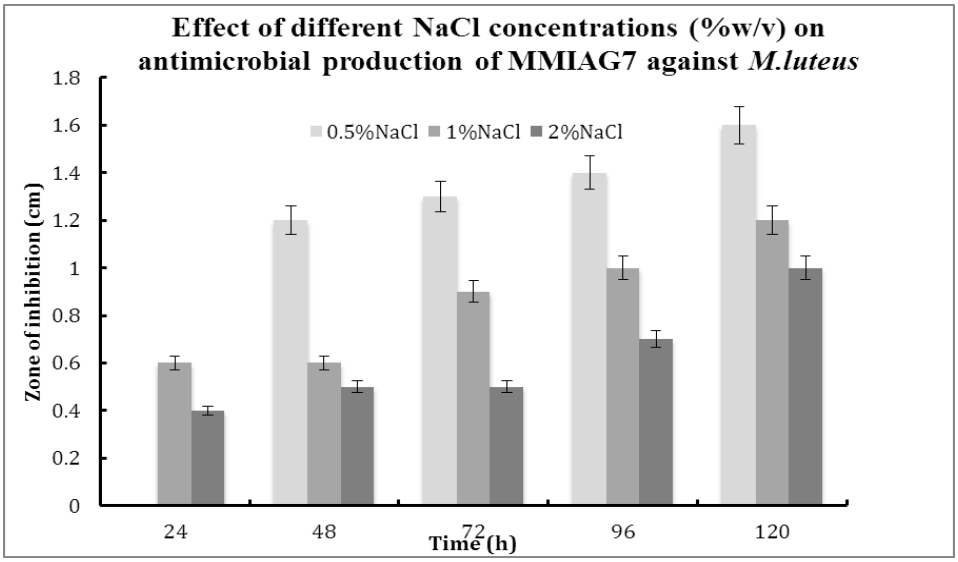


Fig. 5: Effect of different NaCl concentrations (%w/v) on antimicrobial production of MMIAG7 against *M.luteus*.

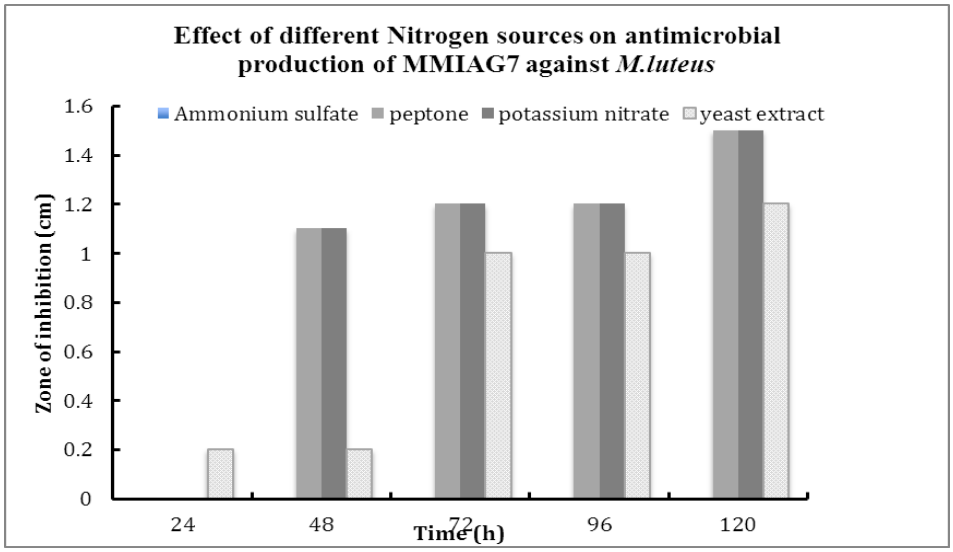


Fig. 6: Effect of different Nitrogen sources on antimicrobial production of MMIAG7 against *M.luteus*.

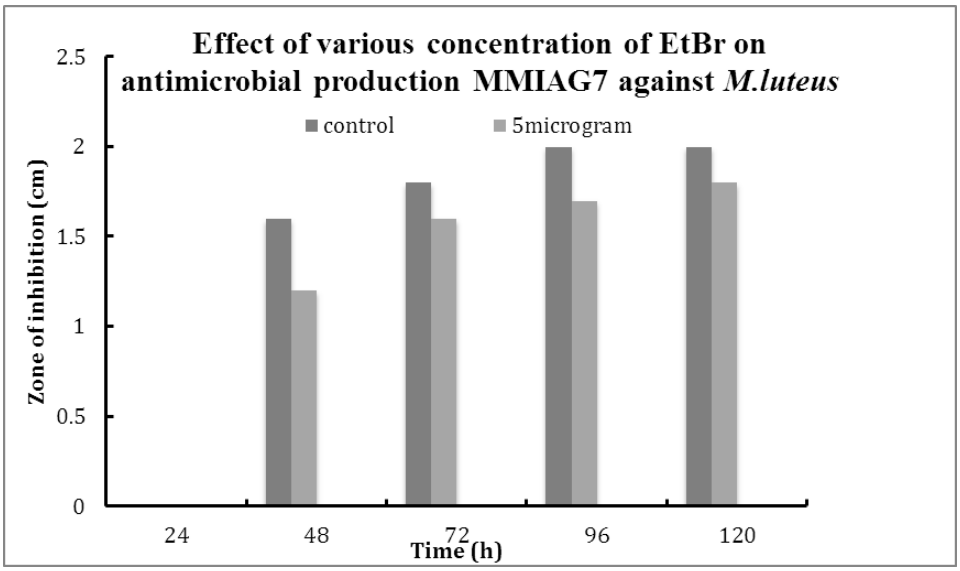


Fig. 7: Effect of various concentration of EtBr on antimicrobial production MMIAG7 against *M.luteus*.

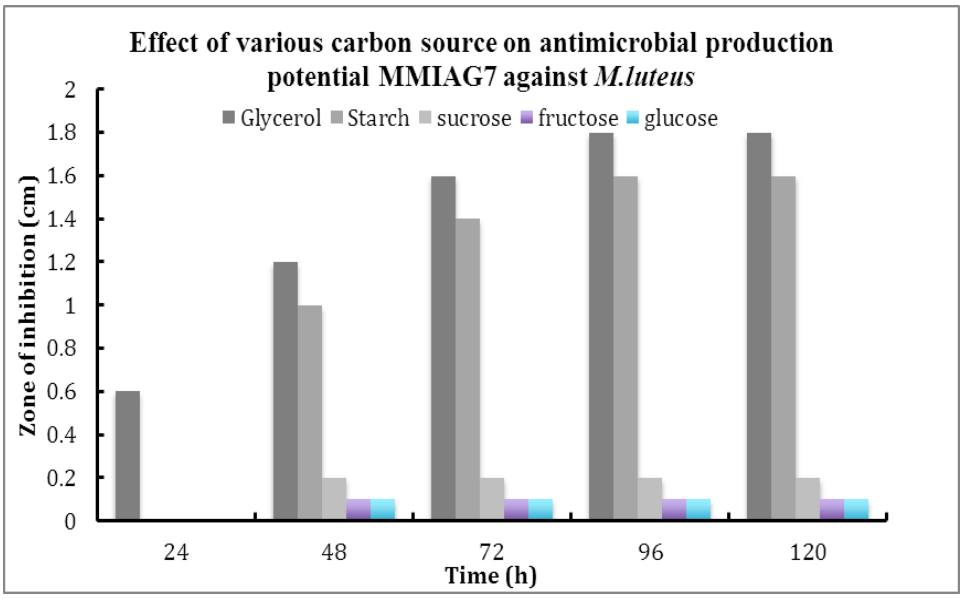


Fig. 8: Effect of various carbon source on antimicrobial production potential MMIAG7 against *M.luteus*.

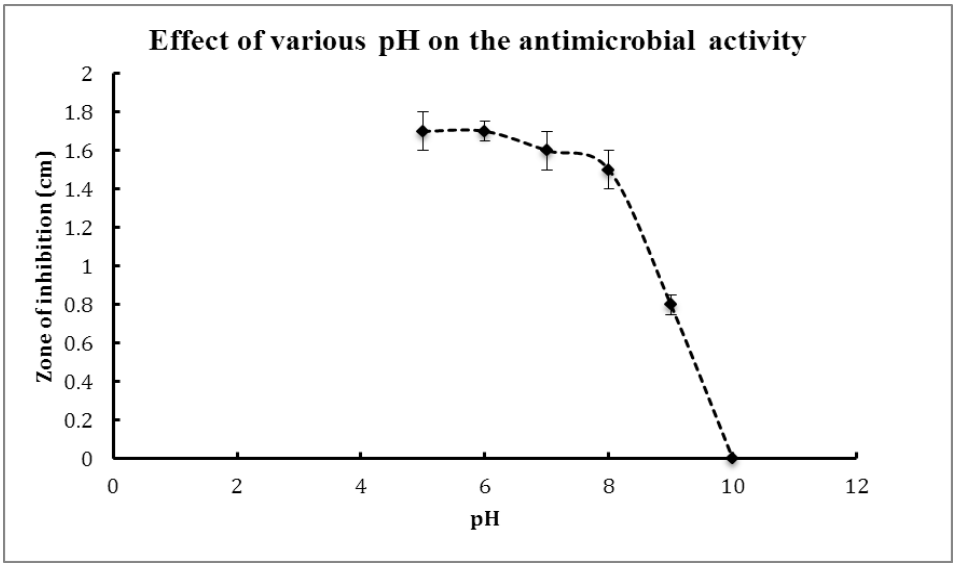


Fig. 9: Effect of different pH on the activity of the antimicrobial agent partially purified from MM1AG7.

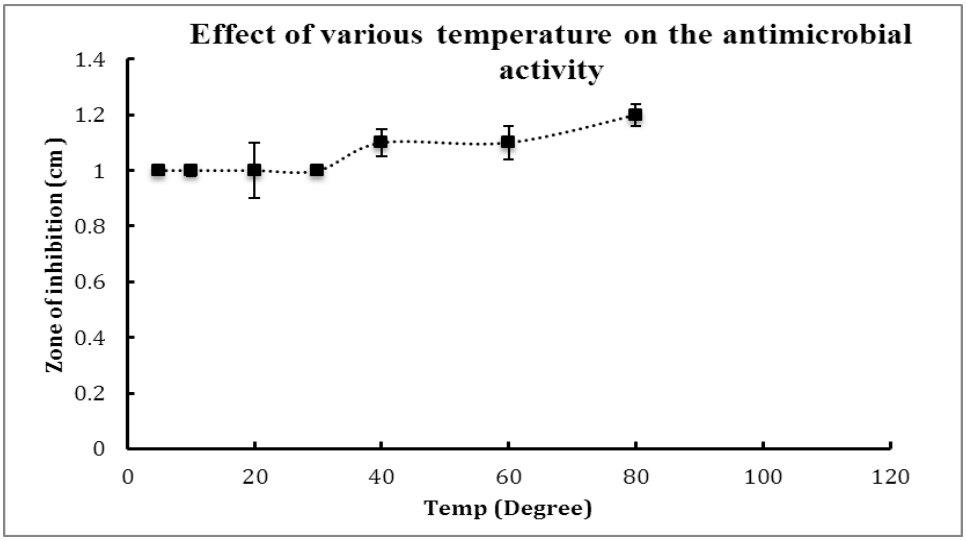


Fig. 10: Effect of various temperature on the activity of the partially purified antimicrobial agent from MM1AG7.

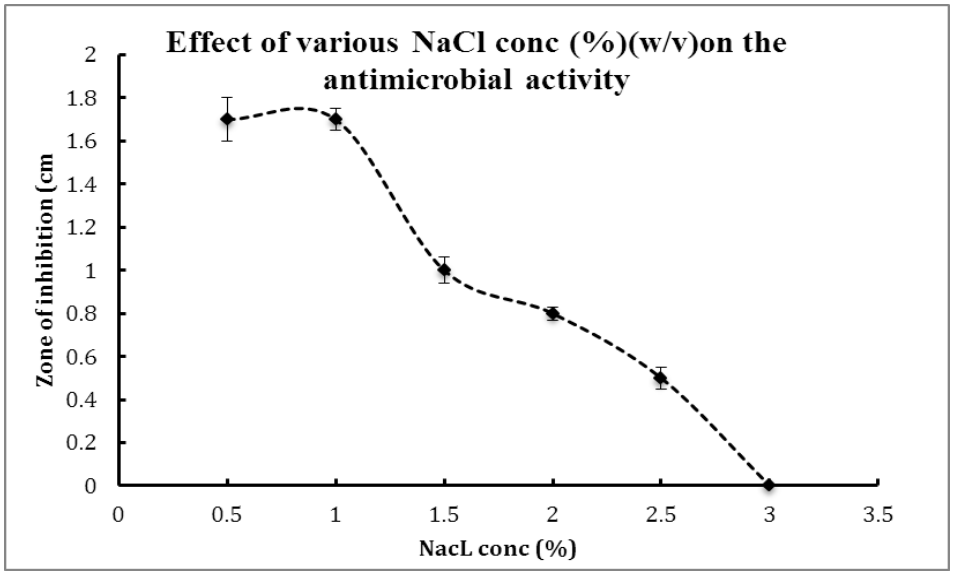


Fig. 11: Effect of varying NaCl concentrations on the activity of the antimicrobial agent.

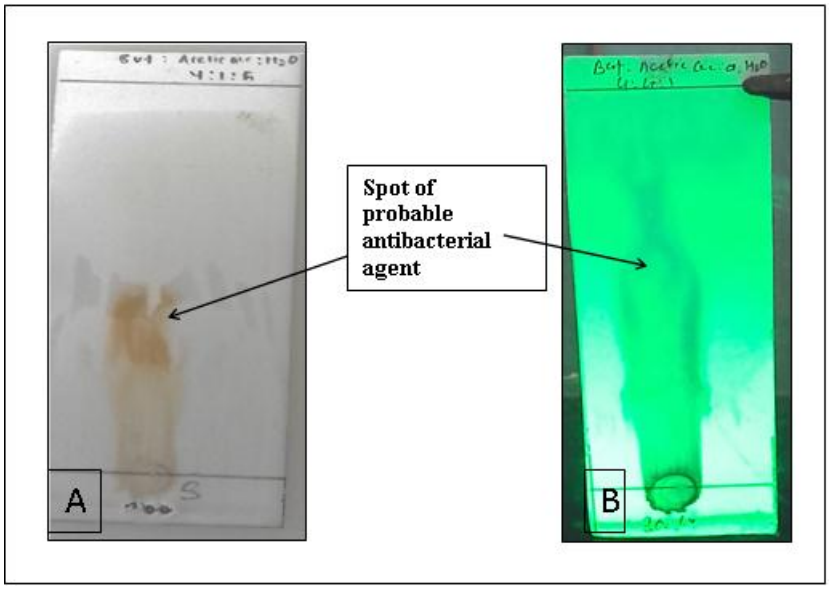


Fig. 12: Thin layer chromatography of the antibacterial agent.

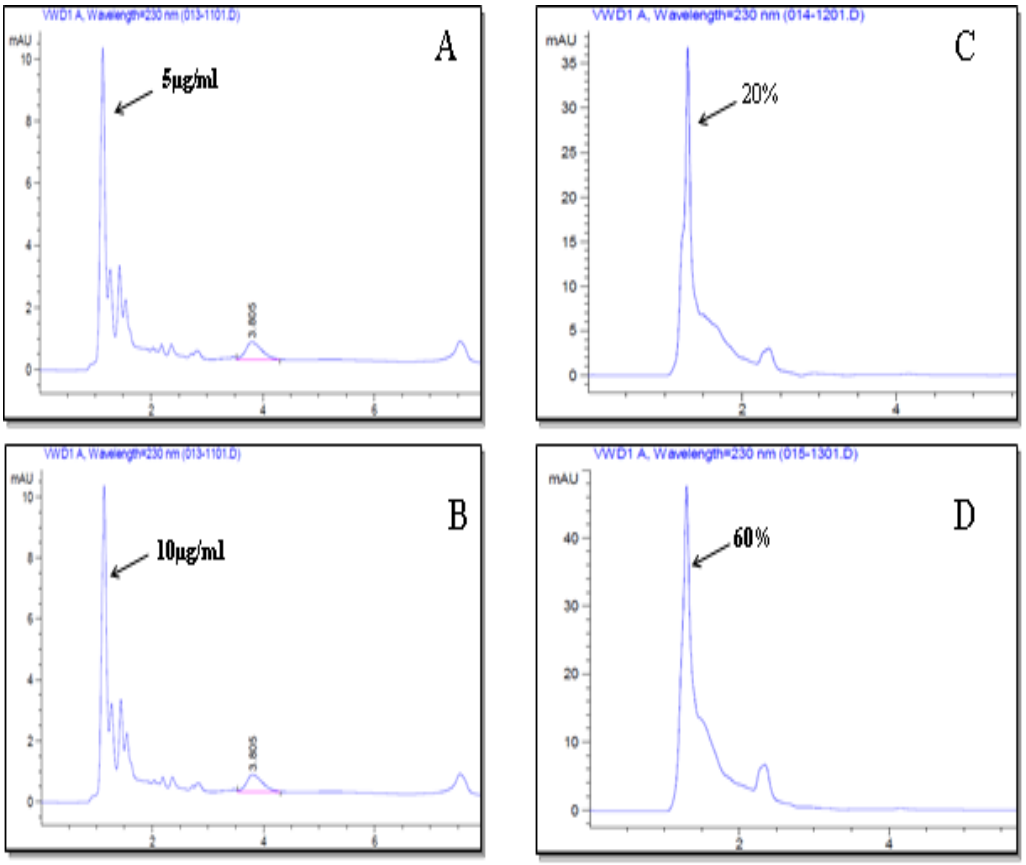


Fig. 13: HPLC analysis shows the peak of the antimicrobial agent similar to Streptomycin.

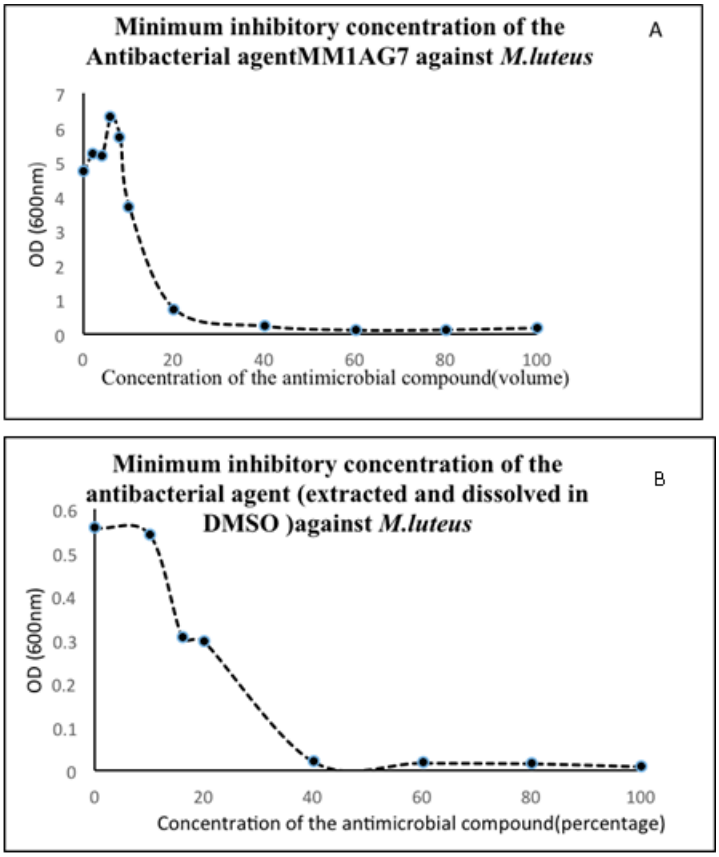


Fig 14: Determination of Minimum Inhibitory Concentration of the antibacterial agent.

DISCUSSION

Actinomycetes particularly genus Streptomyces are well known for their ability to produce various antimicrobial compound and other secondary metabolites. Chilika with its unique habitat harbours many such Streptomyces. The isolate MM1AG7 which showed similarity with *Streptomyces cinereoruber subsp. cinereoruber* NBRC 12756^{T} was found to produce a potent antimirobial compound against *Micrococcus luteus.* The antimicrobial agent produced to the supernatant was extracted with ethyl acetate and the minimum inhibitory concentration was determined to be 40%. Since the extract was partially pure and cannot be represented as gram or milligram as far as efficiency is concerned thus the extract was considered as 100% efficient and further dilutions were made to determine the MIC. The preliminary HPLC analysis showed that the antimicrobial agent may have similarity with Streptomycin. The antimicrobial agent was effective at pH 5 to pH 11 which shows that its acid tolerant and alkali tolerent as well. The NaCl tolerance was found to be 0.5% (w/v).The temperature tolerance was from 10° C-80° C which indicates the efficieny of the antimicrobial agent at a higher temperature. Glycerol was the most effective carbon source for the production of the antimicrobial agent, which shows the possibility of using glycerol waste for the production of the antimicrobial agent, that is an cost effective process.

CONCLUSION

The study thus shows that strain MM1AG7 is a novel Streptomyces strain with potential antimicrobial agent production which is active at high pH 11. Ability of the microorganisms to utilise glycerol for best amount production of antimicrobial agent shows that it can be utilised for degradation of glycerol containing waste. HPLC analysis showed that this antimicrobial agent may be similar to Streptomycin. This study further can be conducted for detailed characterization of the antibiotic and upscaling of the production of the same by using oil waste.

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