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Optimization for anti-MRSA activity by *Streptomyces geysiriensis* lk8 isolated from Saline Dessert soil of Kutchha

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Abstract

The emergence of Methicillin-resistant Staphylococcus aureus (MRSA) as a formidable pathogen necessitates the discovery of novel antibiotics. Streptomyces geysiriensis lk8, isolated from the saline desert soil of Kutchha, exhibits promising anti-MRSA activity. This study aims to optimize the cultural and environmental conditions for maximizing the anti-MRSA potential of this strain. Initial screening identified significant antibacterial activity against MRSA, prompting further optimization efforts .A systematic approach was employed to enhance antibiotic production, starting with the optimization of media components. Various carbon (glucose, sucrose, starch) and nitrogen sources (peptone, yeast extract, ammonium sulfate) were tested. The influence of pH (6.0-9.0), and salinity (0%-10% NaCl) on antibiotic production was assessed. This comprehensive optimization strategy highlights the significant promise of S. geysiriensis lk8 in addressing the urgent need for effective MRSA treatments.

Keyword: *Streptomyces, Antiiotic, Optimization, Marine Environment, MRSA, Saline desert anti-MRSA, Caron,Nitrogen and Ph*

1. INTRODUCTION

Grampositive aerobic mycelial bacteria are grouped into a large and varied category called actinomycetes. They are a widely

dispersed group that live in the earth in particular. About two thirds of antibiotics and several significant bioactive compounds with high medicinal properties have been created by them. It is estimated that actinomycetes, mostly those belonging to the genera Streptomyces and Micromonospora, generate over 80% of the antibiotics used worldwide. The World Health Organization claims that several bacterial infections have developed antibiotic resistance as a result of overprescribing and ongoing antibiotic usage. Drug-resistant pathogenic microorganisms have been emerging more swiftly recently than new antibiotics and medications are being discovered. As a result, the pharmaceutical industry and other academics have been aggressively searching for alternative sources of antibiotic manufacturing. Actinomycetes are continuously isolated from various harsh environments and screened for the generation of new antibiotics. Severe infections brought on by bacteria that are resistant to standard antibiotics have grown to be a significant worldwide health concern in the twenty-first century. The broad-spectrum disease-causing Staphylococcus aureus and other nosocomial infection bacteria are now resistant to the majority of antibiotic classes. Clinicians and public health officials have faced difficulties due to the prevalence of multidrug-resistant bacteria, such as Pseudomonas aeruginosa and hospital-acquired drug-resistant S. aureus. Therefore, it is critical to find new drugs that can combat these illnesses that are resistant to existing ones. Goodfellow and Haynes reviewed the literature and found that just 10% of actinomycetes were found in soil. Despite soil being an important source of drugs, most of these actinomycetes do not live in soil, making it impossible to find new antibiotics. The majority of antibiotics now in use are secondary metabolites that are created by fungi and actinomycetes. It is possible to isolate actinomycetes from both marine and soil sediments. After screening soils for around fifty years, the pharmaceutical sector has only tested a small portion of the world's surface and found a small portion of actinomycetes taxa. In order to find actinomycetes strains that are active against several drug-resistant infections and produce novel antibiotics that have not yet been found, we must screen an increasing number of actinomycetes from various environments for antimicrobial sources. The genus Streptomyces comprises filamentous soil bacteria that are widely acknowledged as industrially significant microorganisms due to their capacity to generate a diverse range of unique secondary metabolites, such as enzymes and antibiotics. In fact, almost 75% of antibiotics that are used in medicine and commerce are produced by various Streptomyces species. Culturing conditions may be fine-tuned by altering nutritional, chemical, and physical factors to enhance Streptomyces growth and secondary metabolite synthesis. Streptomyces' antibiotic production might go up or down depending on the environment. The optimization of the cultural factor has a substantial influence on the productivity and economics of the critical process. Therefore, the goal of our research was to isolate Streptomyces sp. capable

of producing antibacterial metabolites and to optimize a few environmental factors for superior synthesis of antimicrobial metabolites.

2. LITERATURE REVIEW

Osman, M. E., Elnasr, et.al., (2024) filled in these gaps by using statistical optimization to increase the synthesis of metabolites from Streptomyces thinghirensis WAE1. One potential source of new bioactive metabolites for drug development is the isolation of strains of Streptomyces. However, because of duplicate findings, traditional screening methods are not as effective in finding fresh leads. Culture conditions should be optimized, but this has always been done one element at a time without taking interactions into account. Numerous physical and chemical elements influencing the synthesis of metabolites were found. In order to optimize important parameters including carbon supply, nitrogen source, inoculum size, pH, temperature, and incubation length, response surface approach with a central composite design was used. By optimizing manufacturing, antibacterial activity against Streptococcus pneumoniae was increased by 74.92%. Nineteen bioactive substances, including 1,25-dihydroxyvitamin D3, which inhibits the growth of cell walls, were discovered using gas chromatography-mass spectrometry. the potential of S. thinghirensis WAE1 as a bioresource and highlights the need to research the metabolite synthesis from newer Streptomyces strains in order to find innovative antibacterial medications.

Atallah, B. M., et.al., (2023) As the number of germs that are resistant to several drugs continues to rise, the development of new antibiotics has become one of the top priorities. This study is the first to report the isolation of the marine actinomycete Streptomyces sp. Sp1 from Egypt's Nile Delta lake Burullus. Using both morphological and biochemical techniques, Streptomyces sp. Sp1 was identified. The 16S rRNA gene sequence, which was analyzed with the help of the BLAST-N tool (Basic Local Alignment Search program-Nucleotides) developed by the National Center for Biotechnology, also played a role in the determination. Streptomyces sp. Sp1 produced potent antibacterial effects against Listeria monocytogenes, Staphylococcus aureus, and Pseudomonas aeruginosa, three harmful multidrug resistant organisms. Streptomyces sp. Sp1 was tested for its potential antibacterial compounds using gas chromatography-mass spectroscopy (GC-MS). It was discovered that five bioactive substances—ricinoleic acid, broxyquinoline, 9,10-anthracenedione, harmine—were present, each with its own unique antibacterial mechanism. The several antimicrobial mechanisms mediated by these chemicals may explain the strong antibacterial action of Streptomyces sp. Sp1.

Kumar, P. S., et. al., (2020) has demonstrated that Streptomyces sp. strain SCSIO-L054, an untapped reservoir of PTMs, might pave the way for the discovery of novel PTM families

exhibiting a wide range of functions. The endless store of bioactive compounds with potential medicinal and industrial uses is considered to be held by marine actinobacteria in particular. A total of 126 marine actinomycetes strains, originating from various invertebrates, sponges, corals, and gastropod mollusks, were found in Daya Bay and the Nansha Islands. Using HPLC-UV fingerprinting, we assessed the chemical diversity and antibacterial activity of the metabolites that were extracted from the strains. Antibacterial activity and early chemical screening have demonstrated that actinomycetes, which are prevalent in marine invertebrates, are a rich source of new physiologically active chemicals. The distinctive metabolic profile and antibacterial characteristics of the SCSIO-PTE-L054 strain were the subject of substantial investigation. An essential chemical component with strong antibacterial activity was identified by bioassay-guided isolation as a polycyclic tetramate macrolactam (PTM). In light of this assessment, the current study provides support for using marine actinomycetes from Daya Bay and the Nansha Islands as a source for the production of powerful chemicals that might be used in future pharmaceutical research.

Vellingiri, M. M., et.al., (2021) Using statistical methods, the antibacterial activity of Streptomyces hygroscopicus AVS7 was measured, and its maximal production of crude pigmented secondary metabolites (CPSM) was enhanced. The current focus of research is on finding new ways to use statistics to increase the yield of bioactive microbial metabolites with possible medical applications. With time, this may provide a practical answer to several health problems. While grown in starch casein nitrate (SCN) medium, the isolate peaked at 2.92 g/L on day nine. The study used a factor-by-factor (OFT) approach to determine which physical, biochemical, and physiological aspects were most crucial. The Plackett-Burman (PB) design was employed to further investigate the significance of the variables that were filtered by OFT. Results from the PB analysis revealed the presence of potassium nitrate, glycerol, glucose, casein, and urea, among other significant components. In order to determine how the parameters impacting the maximum yield of CPSM interact with one another, experiments were conducted using the Box-Behnken (BB) design-RSM, and the model was verified. The optimized medium was superior to seed media in two ways: it allowed S. hygroscopicus AVS7 to grow to its full potential and produce the highest concentration of CPSM extract. It is straightforward and inexpensive to use this statistical strategy to give the most CPSM for industrial and medical applications. The CPSM that was made possesses antibacterial characteristics. The pure form of the bioactive secondary metabolite was also studied in both laboratory and live creature settings as part of the reviewed study.

Betancur, L. A., et, al., (2020) Linking Streptomyces sp. metabolite production by the use of MVDA and RNN-metabolic profiling. As a rich source of bioactive secondary metabolites, actinobacteria are a prime target for antibacterial drug discovery efforts. The biological effect of Dictyota sp. algae PNM-9 against Burkholderia spp., a rice phytopathogen... A 15-day LB medium culture of Streptomyces sp. PNM-9 was found to include 2-methyl-N-(2′-phenylethyl)-butanamide and 3-methyl-N-(2′-phenylethyl)-butanamide, according to MVDA and 2D NMR examinations. Following their identification, mass spectrometry (MS) and one- and two-dimensional nuclear magnetic resonance (NMR) data provided further confirmation of the structures of compounds 1 and 2. Compound 2 had a minimum inhibitory concentration (MIC) of 1.21 mM while compound 1 had a MIC of 2.43 mM against the burkholderia glumae (ATCC 33,617) rice pathogenic bacterium. Marine microbial bioprospecting using a metabolomics-guided method with NMR-metabolic profiling revealed Streptomyces sp. The PNM-9 strain and its components may provide protection against bacteria that cause harm to plants.

3. RESEARCH METHODOLOGY

3.1 Biochemical characterization of the actinomyceteisolates

Actinomycete colonies were studied for their morphological features using a cover-slip culture technique to verify architectural features of mycelia and spores. The arrangement of spore and spore structure usually helps in preliminary identification of actinomycetes (Binayke *et al.,* 2018). The isolates examined for cell shape & arrangement.

3.1.1 Microscopic examination

Gram reaction was performed by standard methods given by Cappuccino and Sherman in the Microbiology laboratory manual (Cappuccino *et al.,* 1996).Gram staining was performed for all the isolates and observed under a light microscope (1000x magnification).

3.1.2 Biochemical characterization

The biochemical characterization gives brief confirmation about the possibilities for the genus of the organisms. The following biochemical tests viz., carbohydrates fermentation testfor (sucrose, maltose, lactose, glucose, dextrose, and fructose) sugar

utilization, M-R and V-P test, indole production,citrate utilization,nitrate utilization, ammonia production, triple sugar iron test, H_2S production, litmus milk test, catalase activity, urea utilization were carried out for biochemical characterization of all selected isolates according to methods given in cappuccino .

3.2 Extracellular enzyme profiling

Saline actinomycetes made a range of biologically active enzymes than the terrestrial actinomycetes (Janaki *et al*., 2017). Microbial enzymes generally regarded as safe, and they are functional at a wide range of temperature, pH, salinity, or other extreme conditions (Mukhtar *et al*., 2017).

3.2.1 Gelatinase

The gelatin hydrolysis test was detected the proteolytic activity of the enzyme gelatinase. The 24-hour old actinomycete culture was inoculated on nutrient gelatin agar tubes then incubated at 28ºC temperature for 48 to 72 hours and observed for the liquid stateeven after refrigeration(Singh *et al*., 2014).

3.2.2 Caseinase

Casein hydrolysis test was done to examine the activity of caseinase. The 24-hour old actinomycete isolates were streaked on casein agar plates and incubated at 28ºC temperature for 48 to 72 hours Development of zone of clearance surrounding the growth indicates the production of caseinase(Tang *et al*., 2017).

3.2.3 Amylase

Starch hydrolysis test was done to examine the activity of amylase. The 24-hour old actinomycete isolates were streaked on starch agar plates and incubated at 28ºC temperature for 48 to 72 hours. Flood the plate with Lugol's Iodine to observed hydrolysis of starch with the presence of zone of clearance surrounding the colonies (Attimarad *et al*., 2012).

3.2.4 Lipase

The production of the enzyme lipase by actinomycete isolates was tested using tributyrin agar plate. The selected isolates (24 hours old) aseptically inoculated on plates by streaking, and the plates incubated at 28ºC for 48to 72 hours. The formation of zone of clearance was due to solubilization of calcium carbonate indicating the production of lipase enzyme (Dhanasekaran *et al*., 2009).

3.3 Optimization of culturing condition of selected isolates for the production of antibiotic

Actinomycetes have antibacterial action that is typically influenced by the habitat's characteristics and variations in substrate composition. Additionally, there may be differences between test organisms and strains.

3.3.1 Effect of carbon source on antibiotic production

The many carbon sources that were utilized in this investigation were sucrose, xylose, lactose, fructose, galactose, and maltose. Instead of starch, each carbon source was added to the production medium (SCB) at a rate of 1.0% w/v. The base production medium was supplemented with 10% v/v of inoculums, and the fermentations were conducted for seven days at 28°C with a rotary shaker operating at 100 rpm. After fermentation was complete, MRSA test organisms were used in an agar well diffusion technique to assess the antibiotic activity of the centrifuged broth samples.

3.3.2 Effect of nitrogen source on antibiotic production

The study employed casein, urea, NH4No3, NaNo3, NH4Cl, peptone, yeast extract, and meat extract as sources of nitrogen. In lieu of casein, each nitrogen source was added to the production medium (SCB) at a rate of 0.3% w/v. The procedure outlined was followed for the fermentation and assessment of their antibacterial activity.

3.3.3 Effect of pH on antibiotic production

One of the factors influencing microorganisms' growth, ability to generate products, and metabolism is the ideal pH of the medium. The concentrations of H+ may act directly on the cell or indirectly. The effects of the medium's pH at7.0,8.0,9.0, and 10.0 were investigated. After the

fermentation process was completed, the antibiotic that was generated was tested for the formation of antimicrobial metabolites.

3.3.4 Effect of NaCl concentration

1% to 10% (w/v) concentration range of sodium chloride was used to examine the effects of various concentrations on the synthesis of the antimicrobial metabolite. The MRSA strain was tested using the agar well diffusion technique for bioassay.

Results and Discussion

1 Macroscopic examination

Using a coverslip culture technique, morphological characteristics of actinomycete colonies were examined to confirm the architectural elements of mycelia and spores. Actinomycetes may often be identified in part by their spore arrangement and spore structure (Binayke et al., 2018). The isolates' cell structure and organization were analyzed.

2 Microscopic examination

The Microbiology laboratory handbook (Cappuccino et al., 1996) provided standard procedures for performing the Gram reaction, which were developed by Cappuccino and Sherman.All of the isolates underwent Gram staining, which was seen under a light microscope with a 1000x magnification.

3.**Biochemical characterization**

The organisms' genus may be tentatively confirmed based on the biochemical characterisation. The biochemical characterization of all the isolates that were chosen was done using the following techniques: the triple sugar iron test, the litmus milk test, the fermentation test for carbohydrates (sucrose, maltose, lactose, glucose, dextrose, and fructose), sugar utilization, M-R and V-P test, indole production, citrate utilization, nitrate utilization, ammonia production, catalase activity, and urea utilization.

Table 1 : Biochemical and Enzyme Profile Results

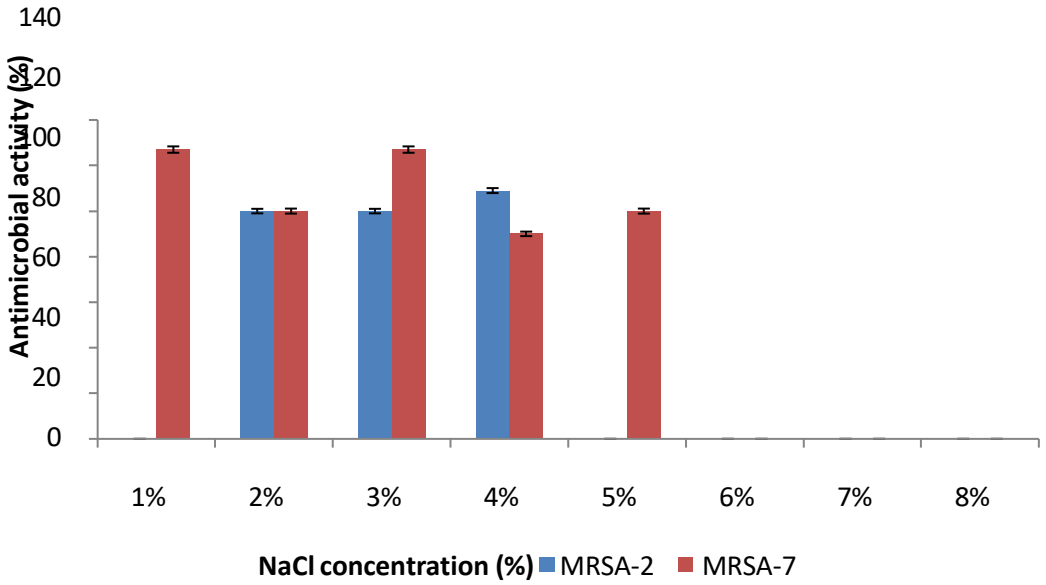
Sr.no	Biochemical test	Ik2	Ik5	Ik7	Ik8	Ik9	Ik10	st1	st5	vb1	vb2	mb1
1	Catalase	+	+	+	-	+	+	+	+	+	+	+
2	H2S production	-	-	-	-	+	-	-	-	-	-	-
3	Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-
4	Ammonia	+	+	+	+	+	+	+	-	+	-	+
5	MR test	-	-	+	+	-	-	+	-	+	-	-
6	VP color	-	-	+	+	+	-	-	-	-	-	-
7	Indole production	+	+	+	+	+	+	+	+	+	+	+
8	Gelatin hydrolysis	-	-	-	-	-	-	-	-	-	-	-
9	Casein hydrolysis	-	+	+	+	-	+	-	+	+	-	-
10	Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+
11	Litmus milk test	+	+	+	-	+	+	+	-	+	+	-
12	Lipid hydrolysis	+	+	-	+	+	+	-	-	+		+
13	Citrate utilization	+	+	+	+	+	+	+	+	+	+	+
14	Triple sugar iron	-	-	-	-	-	-	-	-	-	-	-
15	Glucose	+	+	+	+	+	+	+	+	+	+	+
16	Fructose	+	+	+	+	+	+	+	+	+	-	+
17	Maltose	+	+	+	+	+	-	+	+	+	+	+
18	Lactose	+	+	+	+	+	+	+	+	+	+	-
19	Sucrose	+	+	+	+	+	-	+	+	-	+	+
20	Dextrose	+	+	+	+	+	+	+	-	+	+	+

4 Optimization for anti-MRSA activity of lk8 using carbon source, nitrogen source, NaCl and pH.

4.1 Effect of different concentration of NaCl on the antimicrobial activity of *Streptomyces geysiriensis (lk8)*

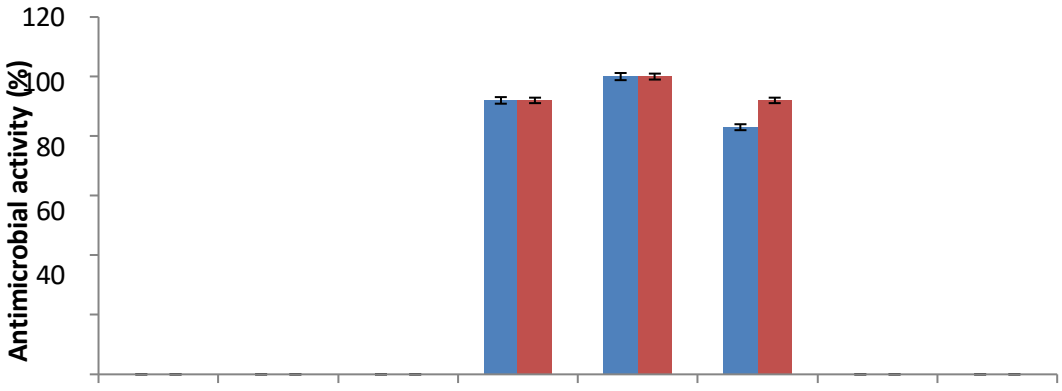
For the observation of profound effect of Nacl, different concentration of NaCl added up to 8% in the production media. Optimum NaCl concentration required for the production

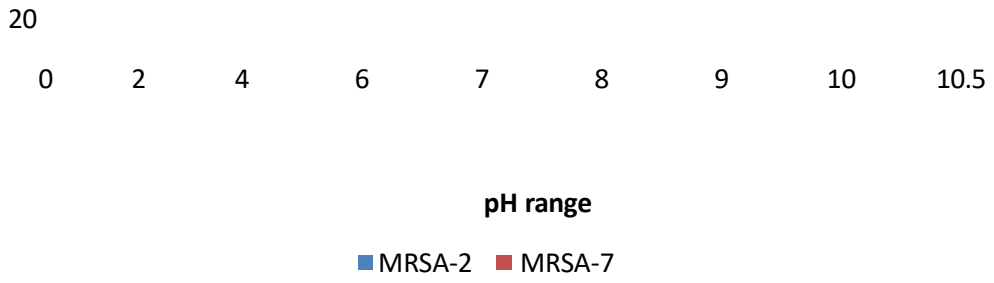
of antimicrobial compound was 5%. Further increase in NaCl concentration showed a drastic decrease in the production of antimicrobial compound.9% increase in antimicrobial activity was observed against MRSA-2 and 27% against MRSA-7 (Figure- 4.24). Salt concentration has a profound effect on the production of antibiotic from microorganism due to its effect on the osmotic pressure to the medium salt concentration (Ripa *et al*., 2009). Similar result was reported by Ng *et al*., that twofold increase in antibiotic yield obtained from 3% NaCl (Ng *et al*., 2014).



4.2 Effect of pH range on the antimicrobial activity of *Streptomyces geysiriensis (lk8)*

pH 8.0 reveal 100% activity against MRSA-2 and MRSA-7. 8% less activity was recorded from 7.0 pH against MRSA-2 and MRSA-7 (Figure-4.25). Most of the commercially used *Streptomyces* species showed optimum pH range from 7.0 to 8.0 (Augustine et al., 2005). The hydrogen or hydroxyl ion concentration may have a direct effect on cell or it may act indirectly during fermentation (Venkata et al.,2011).





3.3 Effect of different carbon sources on the antimicrobial activity of Streptomyces geysiriensis (lk8)

to investigate how various carbon sources affect *Streptomyces geysiriensis (lk8)*'s ability to fight two isolates of MRSA-2 and MRSA-7. In place of starch, eight carbon sources—maltose, glucose, xylose, fructose, lactose, galactose, and sucrose—were introduced to the SCB medium at a rate of 1.0% w/v. Figure 1.

Table 2. Impact of carbon source on lk8's antibacterial activity

Carbon Source	MRSA-2 (Zone of Inhibition %)	MRSA-7 (Zone of Inhibition %)
Starch	100	100
Maltose	150	100
Glucose	100	100
Xylose	100	50
Fructose	100	100
Lactose	150	100
Galactose	180	120
Sucrose	100	0

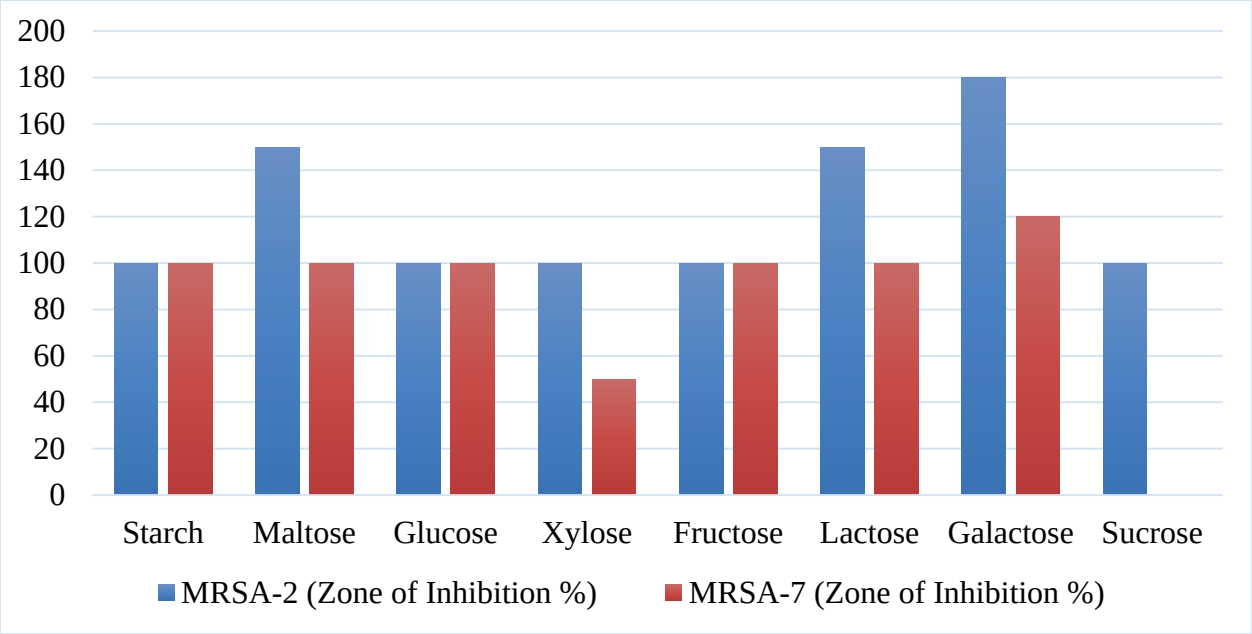


Figure 1. Impact of carbon source on lk8's antibacterial activity

The results indicate that lactose increases the synthesis of antibiotics against MRSA-2 by 40% and against MRSA-7 by 20% when compared to starch. The anti-MRSA activity was also enhanced by adding additional carbon sources to the SCB medium, but it was not as effective as it was with lactose. It is possible for maltose, sucrose, and xylose to obstruct the production of antibiotics. However, the sensitivity of each gene related to secondary metabolite to carbon source regulatory control varies. According to Sanchez et al., one of the key elements needed to regulate secondary metabolism is carbon source modulation. Singh et al. also reported on optimization, finding that the optimal concentration of 1.0% w/v sucrose was determined to be the largest amount of antibiotic production from *Streptomyces griseus* in starch casein broth. The generation of antibiotics and the development of microorganisms may be impacted by the external introduction of various carbon sources into production medium.

3.4 Effect of different nitrogen sources on the antimicrobial activity of Streptomyces geysiriensis (lk8)

Nitrogen sources included ammonium nitrate, sodium nitrate, peptone, meat extract, urea, casein, and ammonium chloride. In lieu of casein, each nitrogen source was added to the production medium at a concentration of 0.3% w/v.

An important component of the production of antibiotics was the availability of nitrogen. The findings show that peptone exhibited the highest level of antibacterial activity, whereas all other examined sources of nitrogen shown only moderate levels of anti-MRSA activity (figure 2).

Table 3. Impact of nitrogen supply on lk8's antibacterial activity

Nitrogen Source	MRSA-2 (Zone of Inhibition %)	MRSA-7 (Zone of Inhibition %)
Casein	100	100
Urea	0	0
NH4NO3	0	0
NaNO3	120	100
Meat extract	0	0
Yeast extract	0	0
Peptone	150	120
NH4Cl	0	0

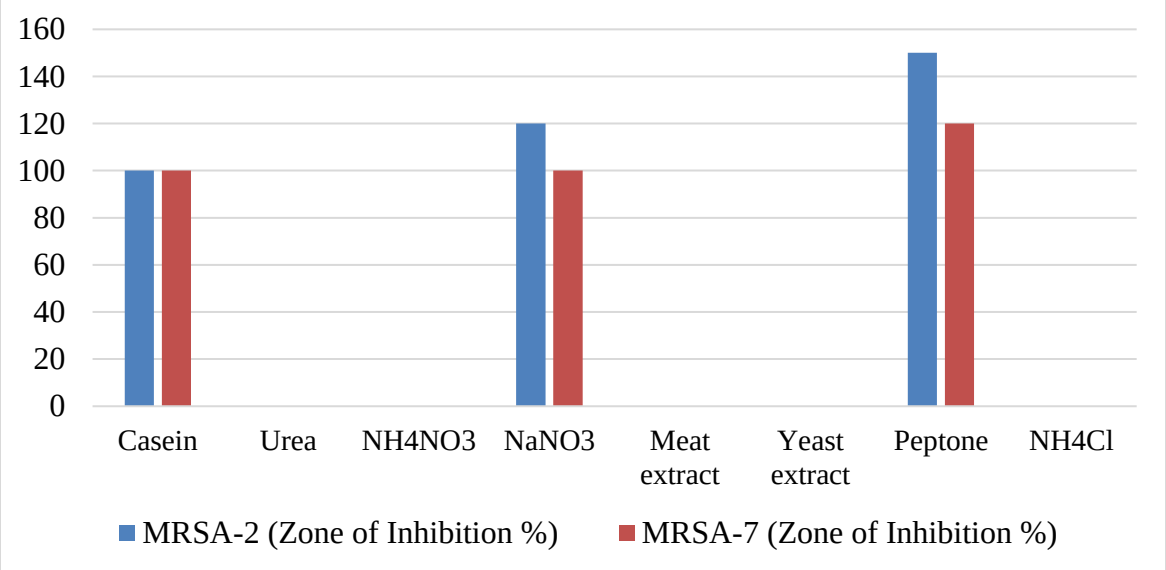


Figure 2. Impact of nitrogen supply on lk8's antibacterial activity

Surprisingly, compared to casein as a control, SCB medium supplemented with 0.3% peptone as a nitrogen source showed 50% higher antibacterial activity against MRSA-2. Although the

antibacterial activity was nevertheless enhanced by adding additional sources to the medium, it was less effective than when peptone was used.

Furthermore, when SCB medium was supplemented with 0.3% NaNO3 as a nitrogen source, 17% more antibacterial activity against MRSA-7 was demonstrated. Other carbon sources added to the SCB medium also enhanced the anti-MRSA activity, albeit not as much as when NaNO3 was used as a comparison.

Whichever biosynthetic route is involved, nitrogen supplies may have an impact on the synthesis of antibiotics. Malt extract was shown to be the most effective source of nitrogen to enhance the development of antimicrobials.

The study investigated how to maximize *Streptomyces geysiriensis (lk8)*'s anti-MRSA action by adjusting parameters including pH, carbon and nitrogen sources, sodium chloride, and others. Lactose outperformed starch in terms of carbon source-induced antibacterial activity against MRSA-2 and MRSA-7, with 40% and 20% increases, respectively. Antibiotic production seemed to be inhibited by maltose, sucrose, and xylose, indicating that carbon sources impact secondary metabolite synthesis through a complicated regulatory regulation. Similarly, nitrogen sources were crucial; peptone, in comparison to casein, considerably increased antibacterial activity against MRSA-2 by 50%. Additionally, NaNO3 showed a significant improvement in the fight against MRSA-7. These results show that the availability of nitrogen is critical for antibiotic production, and that the best source of nitrogen is malt extract.
Looking at the bigger picture, the study highlights how environmental conditions and microbial metabolite synthesis are always interacting. By fine-tuning these aspects, *Streptomyces geysiriensis (lk8)* may be made much more effective against microbes that resist antibiotics, opening up new possibilities in the fight against MRSA and other antibiotic-resistant infections. We need further studies to figure out how different environmental signals control the production of secondary metabolites. The results of this study add to what is already known about the need of microbial biotechnology in the treatment of disease and the prevention of antibiotic resistance.

3 CONCULSION

To sum up, improving antimicrobial activity requires optimizing culture conditions for the synthesis of antibiotics from specific isolates of Streptomyces species. Many variables, including

pH, NaCl content, and sources of carbon and nitrogen, have been studied in order to provide important insights into the synthesis of antibiotics. Different sources of carbon and nitrogen were shown to have a substantial impact on antibacterial activity; lactose and peptone, in particular, demonstrated encouraging findings. Furthermore, the investigation of the impact of pH and NaCl concentration on antibiotic synthesis underscores the significance of environmental conditions in maximizing the production of antimicrobial metabolites. These results address a critical need in healthcare by highlighting the potential of Streptomyces species as useful sources of new antibiotics against drug-resistant bacteria. More investigation in this field may yield more effective antibacterial substances with wider uses in business and medicine.

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