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SCREENING OF BIOACTIVE COMPOUNDS FROM ACTINOMYCETES FOR ANTIMICROBIAL & ANTIOXIDANT PROPERTIES

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L asparaginase, antibacterial resistance, antioxidant

Abstract

Globally, antibiotic resistance is on the rise. In a similar vein, improper antioxidant synthesis has led to the production of oxygen radicals, which has caused numerous ailments. As a result, the search for antioxidants and antibacterial agents from natural sources like plants and microorganisms has become increasingly important. Actinomycetes, manufacture over 10,000 bioactive chemicals and make up around 45% of all known microbial metabolites. Therefore, the present study focused on isolation of actinomycetes for production of bioactive compounds. Total 75 isolates were successfully isolated from different areas in Maharashtra. Appropriate isolation and standard treatment were given to the collected samples. The characterization of the purified isolates was carried out by slide culture method and were screened for their Antioxidant and Antibacterial properties. The IS73 showed the highest antioxidant (97.2%) and antibacterial property against UTI pathogens. It also showed antibacterial activity against S. aureus & S. pyogens with MBC of 128 µg/ml & 32 µg/ml respectively. The growth parameters of IS73 have been optimized, revealing that the most suitable carbon source is starch, nitrogen source is casein hydrolysate, and t temperature and pH are 30˚C and 8, respectively. Hence this IS73 can be used as a potential Antioxidant and Antimicrobial agent.

Introduction

Antibacterial resistance is a global threat to health and development. Misuse and overuse of antibacterial are the main causes of the development of drug-resistant pathogens. As a result of drug resistance, antibiotics, and other antibacterial drugs become ineffective, and treating infections becomes increasingly difficult or impossible. Without effective antibacterial, the success of modern medicine in treating infections, including major surgery and cancer chemotherapy, would be severely compromised. Oxidative stress arises from the overproduction of reactive oxygen species (ROS), leading to cellular damage and contributing to the progression of numerous chronic conditions. The human body's antioxidant defence mechanisms are effective, but are often insufficient under pathophysiological conditions, necessitating the need for additional antioxidants. Huy et al., 2008). Therefore, it is now more crucial than ever to look for antioxidants and antibacterial agents from natural sources like plants and microbes. These natural compounds are currently being investigated as safe and effective therapeutic alternatives (M. Suriyavatana et al., 2010). Among the prolific producers of these secondary metabolites are the actinomycetes, filamentous bacteria responsible for over 10,000 bioactive compounds, accounting for approximately 45% of all known microbial

metabolites (Subathra et al., 2013). These bacteria are ubiquitously present in various environments, including soil, water, plant debris, compost, and food, existing in either spore or vegetative forms. They interact with humans, animals, and plants, contributing to the biocontrol of other microorganisms by producing antibacterial compounds, degrading complex polymers, and causing the deterioration of stored food products (Sindhi et al., 2013). Furthermore, Actinomycetes are known for their production of antiviral, antifungal, antitumor, insecticidal, antioxidant, anti-inflammatory, anti-biofouling, immunosuppressive, antiparasitic, plant growth-promoting, herbicidal compounds, enzyme inhibitors, and industrially significant enzymes (Janardhan et al., 2014). Many of these bioactive compounds are being developed into drugs to treat a wide range of human, veterinary, and agricultural diseases. The present study aims to explore the potential of Actinomycetes isolated from soil and water samples in Maharashtra as sources of antioxidants and antibacterial agents. By isolating and characterizing these bioactive compounds, this research seeks to contribute to the development of novel therapeutic agents and enhance our understanding of the role of Actinomycetes in health and disease prevention.

Materials And Methods

Enrichment, Isolation of Actinomycetes.

Samples such as soil, water, & mangrove area were collected from different areas of Raigad and plated onto Starch Casen agar plates as per protocol. The colonies which showed dry powdery nature were further confirmed to be actinomycetes by morphological & biochemical analysis.

Production & Extraction of bioactive compounds from the isolates

The isolates confirmed to be actinomycetes were cultivated in 200ml of starch casein broth at room temperature at shaker condition for 8-12 days. The broth was centrifuged at 5000rpm for 15 min, supernatant was collected, filtered with Whatman filter paper and subsequently bioactive compounds were extracted by using different solvents (Chloroform, Methanol, Ethyl acetate) in 1:1 proportion). It was left undisturbed for 5-7 days. Residual solvent was evaporated at 40^0C to obtain purified bioactive compound.

Determine antioxidant activity of isolates using DPPH scavenging assay.

Solvent extract and DPPH were prepared in Ethyl acetate. Approximately, 2ml of DPPH solution (0.002% in ethyl acetate) was mixed with 2ml of solvent extracts and reference standard (ascorbic acid) subsequently in separate tubes. The tubes were then incubated in dark at room temperature for 30 minutes. Optical density was measured at 517 nm. Absorbance of DPPH control was recorded. Scavenging activity was calculated as per formula.

Scavenging activity (%) = [(A – B) / A] x 100, where A= Absorbance of DPPH control, B= absorbance of DPPH in the presence of extract/standard.

Determination of the Antibacterial activity of the isolates

4.1 Primary Screening (Cross streak method):

Purified isolates were streaked onto Muller Hinton plates as straight line in centre. The test organisms (UTI pathogens S. *aureus, S. pyogens, P. aeruginosa, E. Coli, P. mirabilis, K. pneumoniae*) were streaked at 90^0C from the center and plates were incubated at 37^0C for 24hrs.

4.2 Secondary screening (Agar cup method):

Quantitative assay was done by agar well diffusion assay by spread plating the test organisms on Muller Hinton plates and 50 µl of crude extract of bioactive compound extracted from actinomycetes were added in the agar wells. The plates were incubated at 37^0C for 24 hrs.

Optimization of studies of carbon and nitrogen sources:

The most potent isolates were further optimized for pH, carbon source, nitrogen source, and temperature.

5.1 Carbon source Optimization:
Purified isolates of the most efficient actinomycetes were inoculated in Starch casein broth with varied carbon sources. (Starch, Maltose, Dextrose, Sucrose & Cellulose). The broth was incubated at 28^0C for 5 days. The growth was monitored by measuring the optical density.

5.2 Nitrogen source optimization:
Purified isolates of the most efficient actinomycetes were inoculated in starch casein medium containing different nitrogen sources (Casein Hydrolysate, Peptone, Beef extract, Yeast extract, Meat extract) and incubated at 28°C for 5 days. The broth was incubated at 28^0C for 5 days. The growth was monitored by measuring the optical density.

5.3 pH optimization:
Purified isolates of the most efficient Actinomycetes were inoculated in starch casein medium having different pH ranging from (6.5- 8.5) and was incubated at 28°C for 4-5 days. The growth was monitored by measuring the optical density.

5.4 Temperature optimization
Purified isolates of the most efficient Actinomycetes were inoculated in starch casein medium and were incubated at different temperatures ranging from (15 °C-40°C) for 4-5 days. The growth was monitored by measuring the optical density.

Determination of MIC and MBC;
Determination of MIC & MBC (minimum inhibitory concentration & minimum bactericidal concentration) was performed by preparing 24hrs suspension of the test organism. Dilution of bioactive compound was prepared by using Muller Hinton broth ranging from(1-256µg/ml) in which of test organisms was the inoculated. The tubes were incubated at 37°C for 24hrs, and further bactericidal activity was performed by subculturing from the MIC no growth tubes onto Muller Hinton agar plates.

Comparative account of the antioxidant and antibacterial activity of the isolates.
All the isolates were compared for the highest Antioxidant and Antibacterial activity.

Results and Discussions: -

1. Enrichment, Isolation of Actinomycetes.

Samples were collected from ten different locations across Raigad, yielding a total of 75 isolates. These isolates were selected on the basis of their initial characteristics, such as powdery colony appearance and Gram-positive filamentous morphology. The selected isolates were further examined using the slide culture method. Microscopic observation focused on identifying features like aerial hyphae, mycelium, and spores. Of the 75 isolates, 50 tested positive for actinomycetes. The most potent isolate, exhibiting strong antibacterial and antioxidant activities, was subjected to molecular characterization and was found to share similarity with *Streptomyces.*

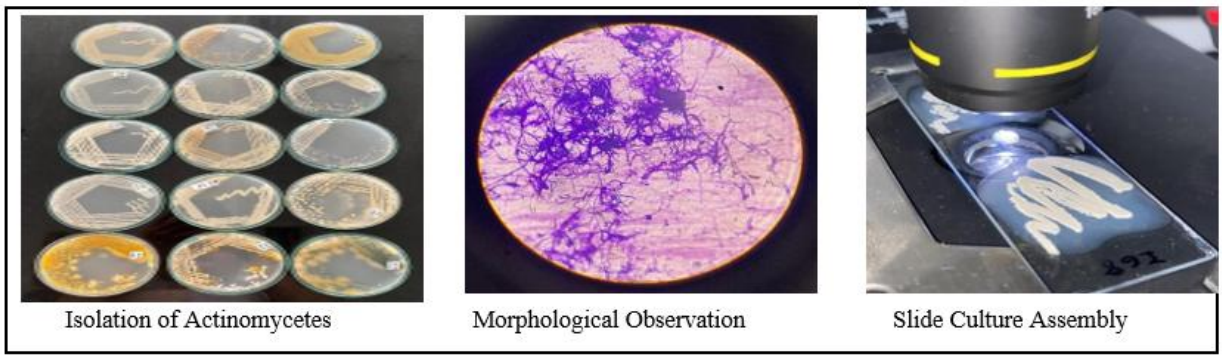


Fig 1: Isolation, Morphological Characterization

2. Production & Extraction of bioactive compounds from the isolates.

The isolates were separately inoculated in sterile starch casein broth incubated at 28ºC with shaking conditions for 8-12 days. After incubation the medium was centrifuged, filtered and bioactive compound was extracted by using solvent in 1:1 proportion. Out of different solvents tested ethyl acetate was found to be most suitable and hence the broth was left undisturbed with ethyl acetate for 5-7 days. Residual ethyl acetate was evaporated at 40°C temperature to get purified extract of the bioactive compound which was further analyzed by using GCMS.

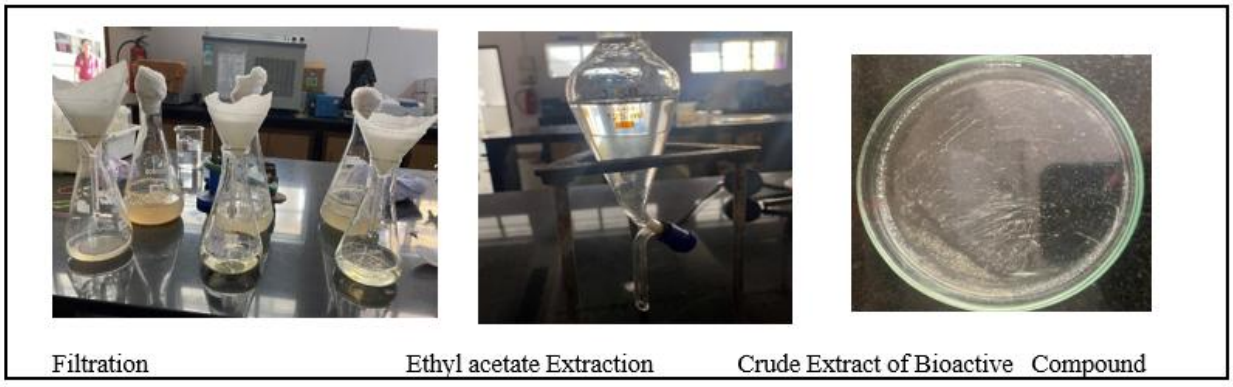


Fig 2: Production & Extraction of Bioactive Compound

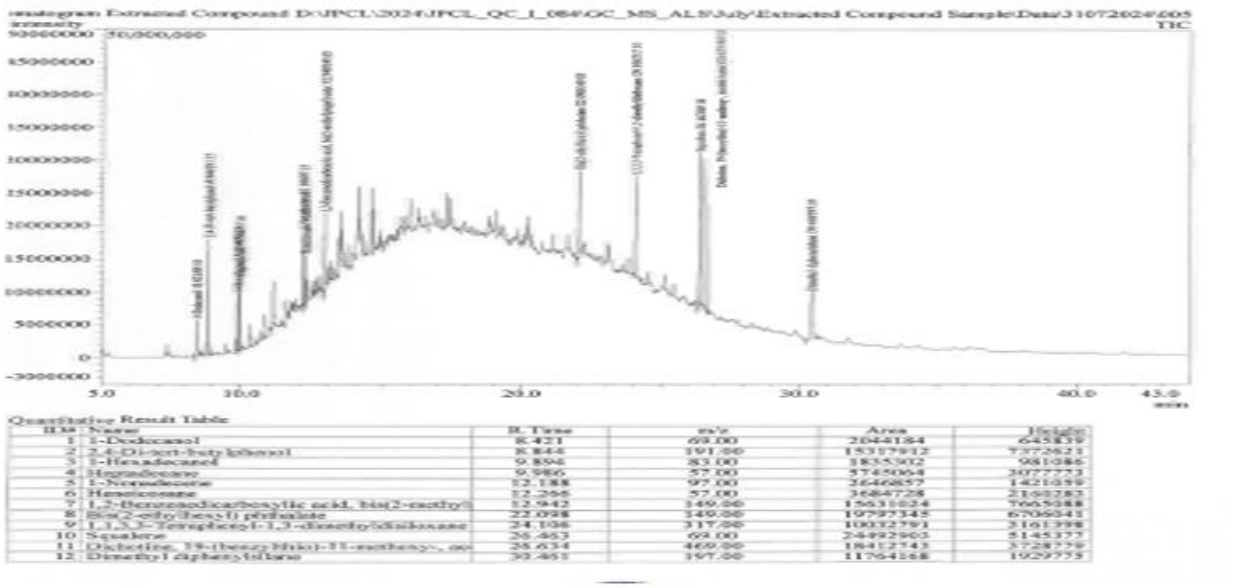


Fig :3 GCMS analysis of extracted bioactive compound

Determine antioxidant activity of isolates using DPPH scavenging assay:

Standard graph was performed by using ascorbic acid as standard. All the isolates were tested for antioxidant properties isolate I- 73 showed the highest activity. Antioxidant property was calculated as per the formula.

Table :1 Comparative Table of Antioxidant properties of Isolates

Sr No.	Scavenging Activity %	IS no.	Scavenging Activity %
IS 1	90.6	**IS 39**	5.70
IS 2	85.6	**IS 40**	70.1
IS 3	94.3	**IS 45**	40.7
IS 5	88.3	**IS 49**	93.6
IS 6	78.7	**IS 50**	93.0
IS 8	94.7	**IS 51**	95.3
IS 9	94.5	**IS 52**	66.4
IS 11	92.3	**IS 56**	94.6
IS 13	89.1	**IS 57**	67.8
IS 14	90.8	**IS 58**	95.1
IS 15	94.6	**IS 60**	19.8
IS 17	93.4	**IS 61**	96.5
IS 20	83.9	**IS 62**	40.7
IS 23	86.0	**IS 64**	93.3
IS 24	94.8	**IS 65**	89.7
IS 26	92.7	**IS 66**	75.9
IS 28	75.2	**IS 68**	96.3
IS 29	45.6	**IS 69**	95.7
IS 30	92.7	**IS 70**	92.8
IS 32	89.8	**IS 71**	96.1
IS 36	79.8	**IS 73**	**97.2**

IS 37	88.9	**IS 74**	87.3
IS 38	74.9	**IS 75**	93.2

Determination of the Antibacterial activity of the isolates

4.1 Primary screening of the isolates by using agar streak method

After incubation at 37°C for 24 hours the level of growth inhibition was assessed by the no growth around the streaked lines. Out of total 50 isolates two of them showed inhibition pattern and hence proceeded for quantitative assay

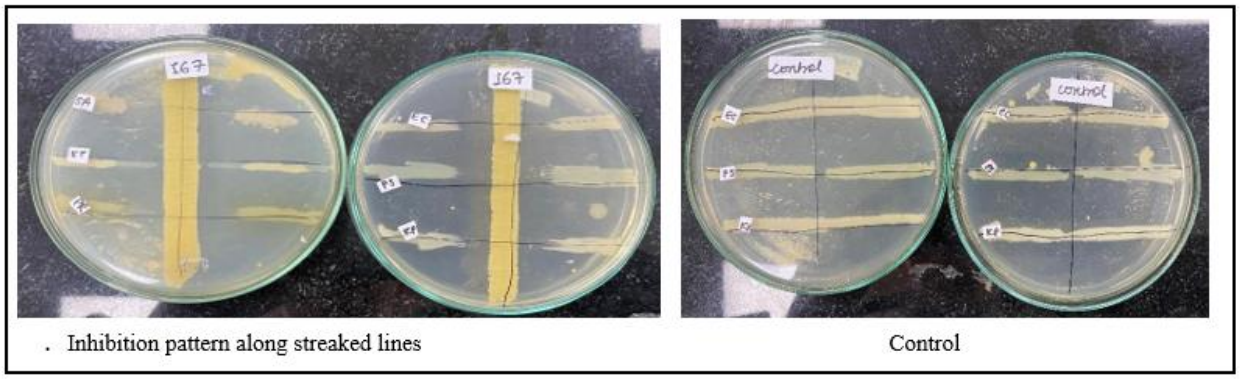


Fig 4: Agar Streak plate results of bioactive compound against different test organisms

4.2 Secondary screening of the by using Agar Cup Method.

The bioactive extract was further analyzed for a quantitative assay using the agar well diffusion technique. The level of bacterial growth inhibition was evaluated by measuring the average diameter of the zones of inhibition. Two isolates (I- 3) exhibited inhibitory activity against *Staphylococcus aureus* and *Streptococcus* species, while isolate I-73 showed an inhibition pattern against *Klebsiella* species.

Table 2: Zone of Inhibition in mm against Urinary pathogens.

Organism	I-3(mm)	I-73(mm)	Streptomycin	Organism	I-3(mm)	I-73(mm)	Streptomycin
S aureus	--	21	24	***E coli***	---	--	15
S pyogenes	---	30	31	***Pmirabilis***	-----	---	24
P aeruginosa	----	---	26	***K pneumonae***	25	----	10

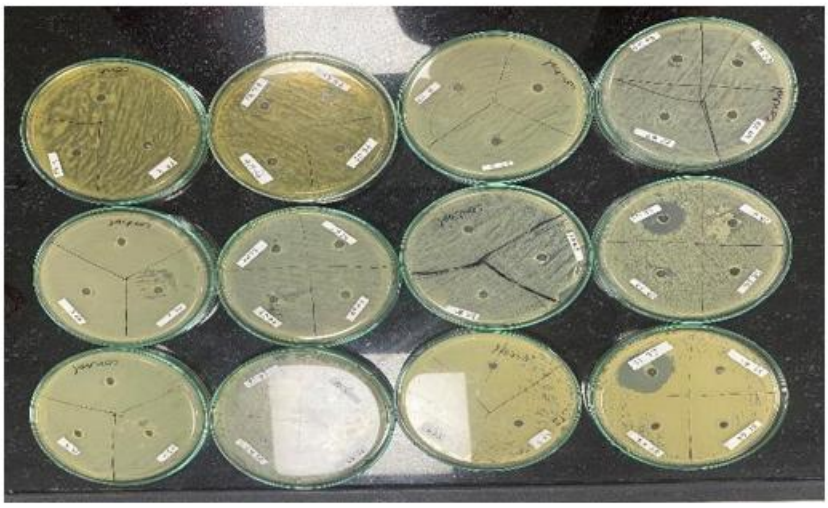
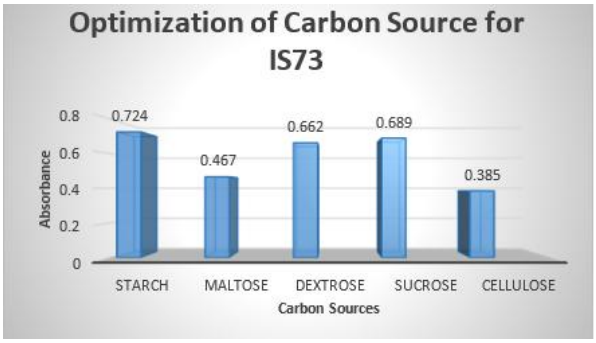


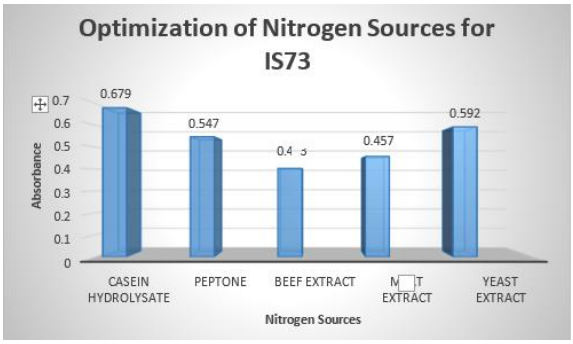
***Fig: 5* Evaluation of Antibacterial Activity of Microbial Extracts Against Test Pathogens by Agar Well Diffusion Assay"**

Optimization of the Isolate

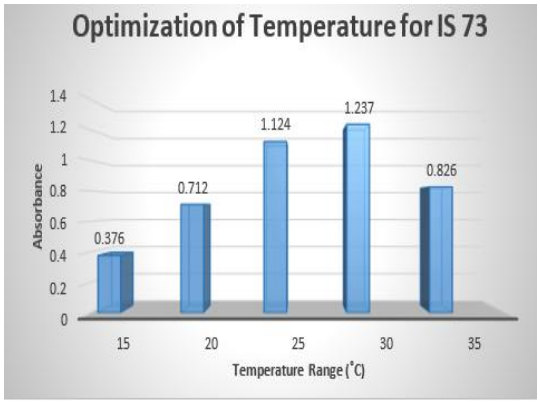
Optimization studies were conducted for the isolate which showed good results for antimicrobial & antioxidant properties. Isolate 73 was subjected to further optimization of carbon sources (Starch, Maltose, Dextrose, Sucrose, Cellulose0, further nitrogen sources (Casein hydrolysate, Peptone, Beef extract, meat extract, yeast extract). pH (6.5,7.0,8.08.5), & temperature (15°C, 20°C, 25°C,30°C & 35°C. The results conclude that the optimum carbon, nitrogen, pH & temperature parameters for isolate I 73 are starch, Casein hydrolysate, pH 8 & temperature 30^0Crespectively



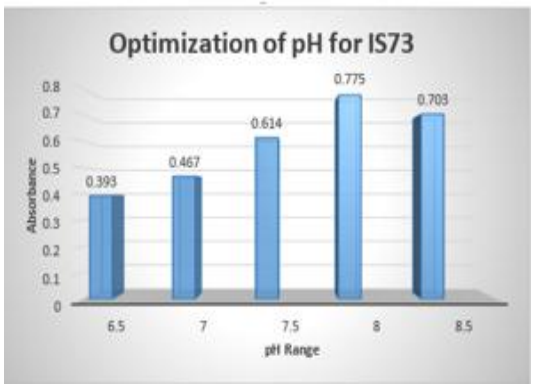
Graph: 1 Optimization of Carbon Source for IS73



Graph:2 Optimization of Nitrogen Source



Graph:3 Optimization of Temperature



Graph:4 Optimization of pH

Determination of MIC & MBC of Bioactive compound:

The MIC of bioactive compound was performed by dilution Method thereafter MBC was tested for bacterial viability by sub-culturing on agar media prepared without Crude extract (Bioactive Compounds). Minimum inhibitory concentration of bioactive compound for *S aureus was* found to be 32 µg/ml & *S pyogenes* 64 µg/ml & MBC (minimum bactericidal concentration as 128 µg/ml.

Table 3: Minimum Inhibitory Concentration of Bioactive compound against test organism

Concentration (µg/ml)	Turbidity	
	S. aureus	*S. pyogens*
1	+	+
2	+	+
4	+	+

8	+	+
16	+	+
32	-	+
64	-	+
128	-	-
256	-	-
+ve control	+	+
-ve control	-	-
Medium control	-	-

Table 4: Minimum Bactericidal concentration of Bioactive compound. Against test organism

Concentration (µg/ml)	**Turbidity**	
	S. aureus	*S. pyogens*
32	+	+
64	+	+
128	-	-
256	-	-

Comparative account of the Antioxidant and Antibacterial activity of the isolates.

Table 5: Observation table of Comparative account of the Antioxidant and Antibacterial

Isolate No.	Antioxidant Activity (%)	Antibacterial Activity (mm)		
		Sa	**Sp**	**Kp**
IS 3	**94.3**	**-**	**-**	**25**
IS 61	**96.5**	**-**	**-**	
IS 68	**96.3**	**-**	**-**	**-**
IS 73	**97.2**	**21**	**30**	**-**

Conclusion

Oxidative stress is an imbalance between free radicals and antioxidants in your body which can lead to lifelong diseases such as neurodegenerative diseases, diabetes, hypercholesterolemia, atherosclerosis, and other conditions linked with obesity. Antioxidants are substances that can prevent or slow cell damage caused by free radicals. Synthetic antioxidants such as BHT, BHA, and PG are thought to be toxic or carcinogenic. Concerns about the harmful health effects of utilizing synthetic antioxidants have brought a lot of focus to natural antioxidants. Along with oxidative stress, Antibacterial resistance is a global threat to health and development. Misuse and overuse of antibacterial are the main causes of the development of drug-resistant pathogens. As a result, the search for Antioxidants and antibacterial in natural sources such as plants and microbes is very intriguing. Natural antioxidants and Antibacterial derived from plants and microorganisms have received a lot of attention due to their beneficial biological activities, especially for their positive effects on human health. Total 75 isolates were successfully isolated from different areas in Maharashtra. Appropriate isolation and standard treatment were given to the collected samples. The characterization of the purified isolates was carried out by slide culture method. The isolates were screened for their Antioxidant and Antibacterial properties. The IS73 showed the highest antioxidant (97.2%) and antibacterial property against UTI pathogens. The MIC and MBC of the isolate was determined. It also showed antibacterial activity against *S. aureus & S. pyogens* with MIC of 128 µg/ml & 32 µg/ml respectively. The growth parameters of IS73 were optimized. The optimum Carbon source was found out to be Starch, while the substrate concentration was found out to be 3g/l. The optimum Nitrogen source was found to be Casein Hydrolysate. The optimum temperature and pH were found out to be 30˚C & 8 pH

respectively. The investigation leads to the conclusion that Maharashtra is the potential ecosystem for Antioxidant and Antibacterial properties possessing Actinomycetes. Hence this IS73 can be used as a potential Antioxidant and Antimicrobial agent.

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