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Isolation, Identification, Purification, and Characterization of Antibiotic-Producing Bacteria from Different Soil Samples

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Abstract: Streptococcus and Micrococcus luteus are the best source of antibiotic. In the present study 4 culture (Bacillus cereus (S6), Pseudomonas fluorescence (S1), Streptococcus (S2), Micrococcus luteus(S3) were isolated from 6 soil sample collected from Lucknow, Kanpur, and Unnao. Isolation of microbes was done by serial dilution method. Primary screening of the culture was done by streaking. Gram's staining was done to check the culture was pure or not. Identification was done by Bergey's manual, further media optimization was done. Production media was prepared for this 4 culture in which the best carbon sources was Sucrose, Glucose, Dextrose, Sucrose best nitrogen sources were Yeast extract, the best temperature was 37^{0}C and best pH was 11,7,7,7(Micrococcus luteus, Streptococcus, pseudomonas fluorescens, Bacillus cereus) respectively. Further extraction of seconda ry metabolite was done by using one solvent: (Chloroform) for extracellular component and (Methanol) for the intracellular component. Further purification of the antimicrobial compound using TLC (thin layer chromatography) and a spectrophotometer was performed resulting Quercetin compound.

Keywords: Soil samples, Secondary Metabolites, Extracellular, Intracellular, MDR Pathogens, drug Resistance, Pathogens, Solvent, Quercetin.

INTRODUCTION

The role of Antibiotic drug is used to cure infections caused by bacteria and another infectious microorganism. Mostly treatments regarding cancer destroy white blood cells, in this manner reduces the body's immunity because white blood cells kill the disease-causing organism. For example, chemotherapy is used for the treatment of bladder, pulmonary, and urinary tract infections. However, they can effect serious infections in persons with low white blood cell counts. Since the dangerous infections present in cancer patients, antibiotic treatment is started before the accurate nature of the infection has been determined. Therefore the type of antibiotic for a particular disease may depend on the site of the infection and the organism that is probable to be the cause. An antibiotic that kills a broad spectrum of bacteria is chosen and several antibiotics may be used together [1].

An **antibacterial** is an agent that inhibits bacterial growth or kills bacteria. Many antibacterial compounds are relatively small molecules with low molecular weight than 2000 atomic mass units like beta-lactam antibacterial, which include the penicillin's , the cephalosporins, and the carbapenems. Aminoglycosides compounds that are still isolated from living organisms whereas the sulfonamides, the quinolones, and the oxazolidinones are produced solely by chemical synthesis [2][3][4]. More recent antibiosis between microorganisms was observed in the laboratory for the discovery of natural antibacterial compounds produced by microorganisms. Antagonistic activities by fungi against bacteria are also observed in the laboratory [5][6]. Antibiotics, also known as antimicrobial drugs, are drugs that fight against infection caused by bacteria. Antibiotics are low-molecular-weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil [7][8].

The present study is carried out by isolation, Identification, production, and characterization of the anti-bacterial compound from soil microbes of the soil sample.

1. MATERIAL & METHOD

Sterilization of glassware and media: glassware is sterilized in hot air oven at160^{0}C for 50-60 minutes. And all the media are firstly prepared in Erlenmayer flask and then kept in an autoclave for sterilization at 15 psi pressure, the 121^{0}C temperature for 15-20 minutes.

Collection of Sample
The soil sample was collected from the garden soil of different places in Lucknow such as domestic Wastage area Gomtinagar, Ram Manohar Lohiya Hospital & OPD Gomtinagar, Vibhuti Khand, Gomtinagar. Viraj Khand Gomtinagar, Industrial area Unnao and Industrial area Kanpur.

Isolation of antibiotic-producing microbes from soil (Serial dilution)
Serial dilution method to get 10^{-5} dilution soil sample and their growth was checked by agar plating method. Plates were incubated for 24 hrs at 37^0c. This is commonly used the procedure for isolation and enrichment of most prevalent microorganisms. This method is used to get reduce no. of bacterial colonies in order get a pure form of colonies.

Primary screening for antibacterial nature: Screening technology involves detection and isolation of high yielding species from natural sources such as soil containing heterogeneous microbial population through screening. After incubated overnight four types of colonies present in plates and zone of inhibition were observed around the colony. The culture showing zone of inhibition was said to be positive in primary screening and was used for further study [9].

Subculturing: Subculturing was done by quadrant streaking method on Nutrient agar plates.

Pure culturing: Pure culturing is done to preserve the single type of culture in the form of broth from the streak plate.

Secondary screening for antibacterial nature: Secondary screening includes the growth kinetics study of the culture by inoculating the culture into autoclaved NB medium to know about the growth rate of the culture. This is useful for the extraction of a secondary metabolite of the microbes. Microbes produce the secondary metabolite at the end of the log phase. The extraction of extracellular and intracellular secondary metabolite by the treatment of the organic solvent is also the part of the secondary screening. Finally, it is followed by the antibiogram test to check the activity of extracted intracellular and extracellular secondary metabolites after treatment with organic solvent [10].

Staining and biochemical characterization sample: Isolates were identified based on culture morphology, Gram's staining and then biochemical test according to the Bergey's manual [11].
Optimization: Optimization refers to the suitable condition for the growth of culture. Various sources like carbon source, nitrogen sources, pH, and temperature are calculated at which O.D showing best growth for each bacterial culture.

Extraction of secondary metabolite from production media
Secondary metabolite has been extracted using solvent chloroform and ethanol. Intracellular secondary metabolite extraction is carried out by dissolving it into ethanol and extracellular secondary metabolites have been extracted by dissolving it into the chloroform, on the basis of their polarity and their dissolving power. The treatment of the culture with an organic solvent enhances the activity of the secondary metabolite.

Procedure: After incubation production media was transferred to 6 eppendorf tube (1 ml each). The tubes were centrifuged at 10,000 rpm for 10 minutes. After centrifugation, stored supernatant, and pellets. The supernatant is extracellular component and pellet is an intracellular component.

Extraction of extracellular compound
After collecting supernatant, add an equal volume of chloroform and mixed gently by inversion method for one hour. Then centrifuged at 10,000 rpm for 10 minutes. The top layer was discarded and transfer the second layer in pre-weighted empty bowl. Now bowl was allowed to dry and again took the weight and the amount of metabolite was calculated by subtracting empty weight from the weight of bowl after dried. And then added 50µl 100mM tris HCL in all the tubes.

Extraction of intracellular compound
Add 0.5ml ethanol to the pellet and mixed gently by inversion method for one hour. Then centrifuged at 10,000 rpm for 10 minutes. Transfer supernatant (top layer) in a pre-weighted empty bowl. Now bowl was allowed to dry and again took the weight and the amount of metabolite was calculated by subtracting empty weight from the weight of bowl after dried. And then added 50µl 100mM tris HCL in all the tubes.

Antibacterial sensitivity test (AST) of purified antibacterial metabolites
For AST, agar well diffusion method was used wherein NA plates were prepared. 20 ul of the selected pathogen was spread (*Staphylococcus aureus, Pseudomonas auriginosa, E.coli, B.subtilis*) onto the solidified agar plate. 4 wells were made at an appropriate distance onto the plate with the help of gel puncture. Wells were loaded with 50 ml extract, standard antibiotic and distilled water as a blank. Plates were incubated at 37^0c for the overnight and observed result.

RESULTS

Total 6 cultures (S1, S2, S3, S, S5, S6) were isolated from the soil sample and all 6 have been characterized.

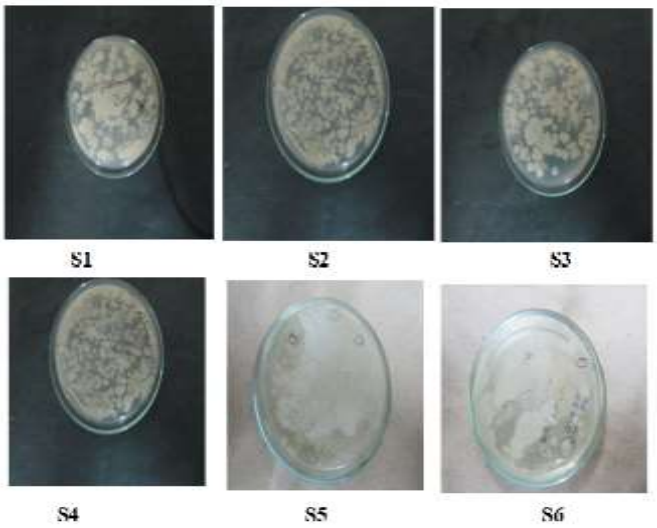


Fig 1: Isolates of microbes from different soil sample

Subculturing:

Fig 2: a Streaked plate of 6 different colonies obtained.

Gram's staining for characterization of pure culture:

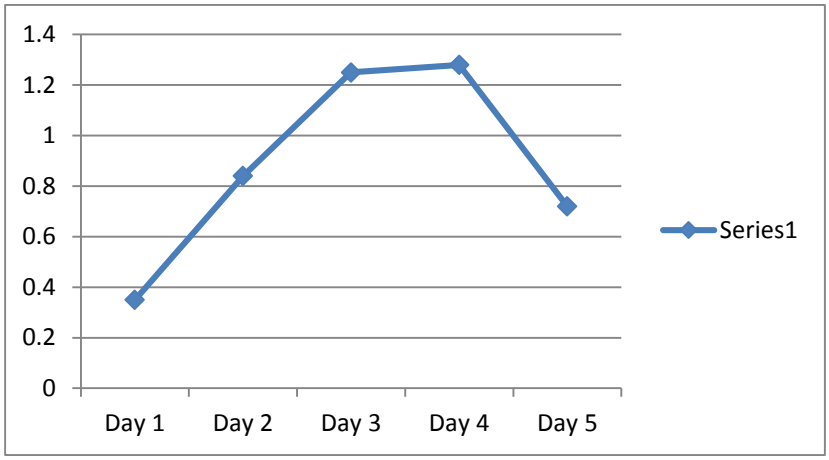
Name of culture	Gram –ve/+ve
C1	Gram +ve cocci
C2	Gram +ve rods
C3	Gram –ve rods
C4	Gram –ve cocci
C5	Actinomycetes
C6	Actinomycetes

Biochemical Test:

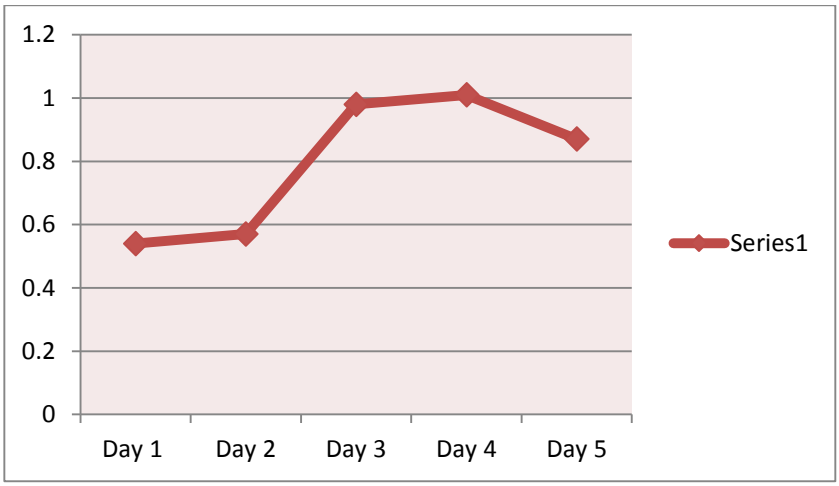
Biochemical tests	**S6**	**S1**	**S3**	**S2**
Glucose fermentation tests	-	+ve	+ve	+ve
Mannitol test	-	-ve	-ve	-ve
Catalase test	+ve	-	+ve	+ve
Oxidase test	-	+ve	-	-

Growth kinetics: O.D showing growth kinetics.

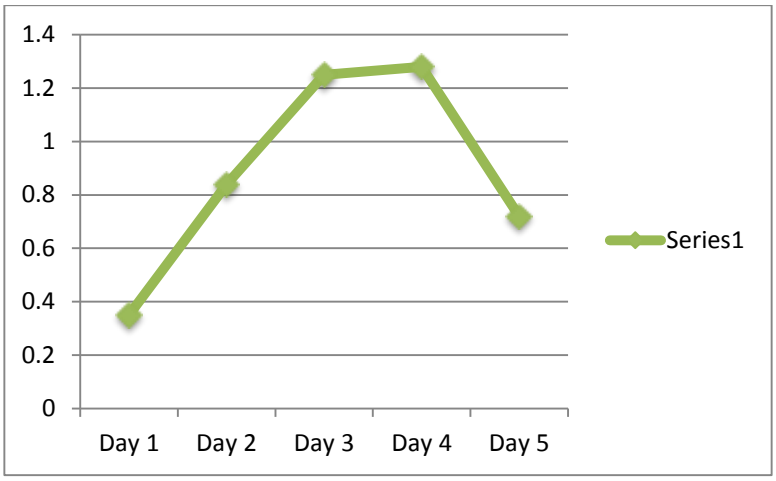
No of days	O.D at 620nm			
	S1	**S3**	**S1**	**S2**
Day 1	0.32	0.54	0.64	0.35
Day 2	0.56	0.57	1.00	0.84
Day 3	0.88	0.98	1.13	1.25
Day 4	1.16	1.01	1.23	1.28
Day 5	0.94	0.87	0.84	0.72



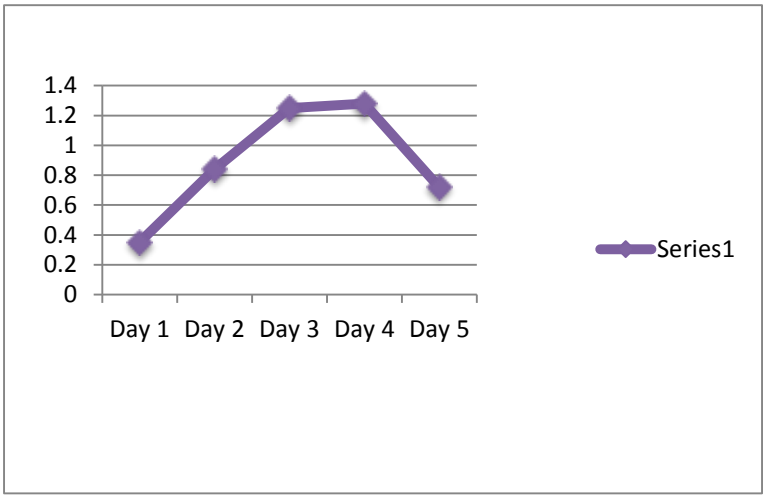
Graph1: ***Bacillus cereus***



Graph 2: ***Micrococcus luteus***



Graph3: Pseudomonas fluoresces



Graph 4: *Streptococcus*

Optimization:

pH	***S6***	**S2**	**S1**	**S3**
5	0.01	0.03	0.01	0.02
7	0.47	0.43	0.49	0.40
9	0.11	0.02	0.12	0.09
11	0.45	0.35	0.34	0.45

Antibacterial sensitive test (AST)

Result showing Isolate S1 (S. fluorescent) against B.subtitis, S2 (Streptococcus) against P.auriginosa, S3 (Micrococcus) against S.aureus, S6 (B.cereus) against E.coli respectively.

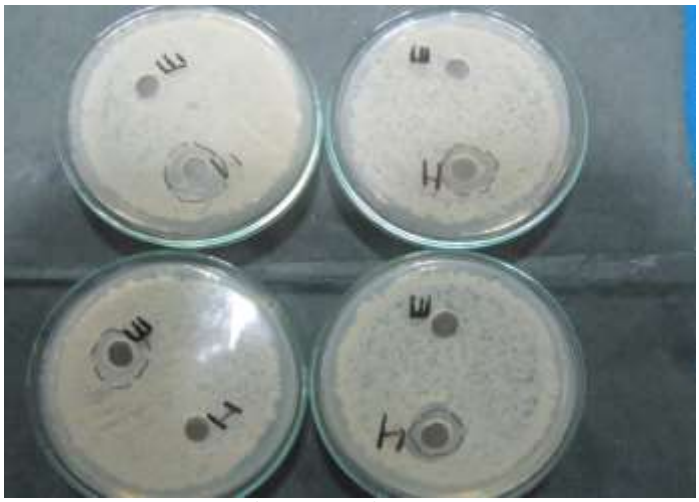


Fig 7: Zone of inhibition after solvent extraction

Result showing Isolate S1 (S. fluorescent) against S.aureus, S2 (Streptococcus) against S.aureus, S1 (S. fluorescent) against E.coli, S3 (Micrococcus) against E.coli respectively.

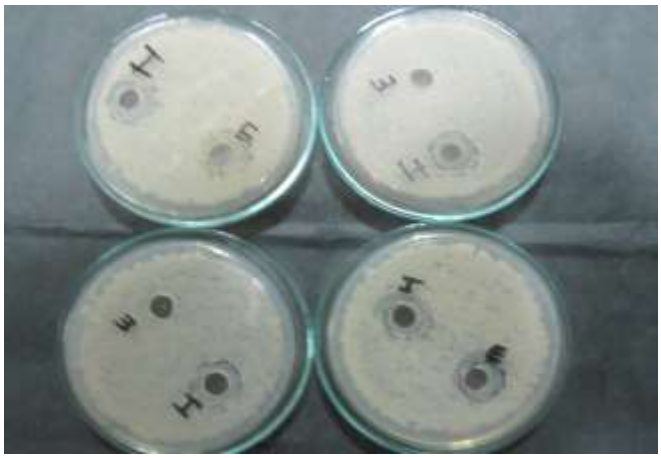


Fig8: Zone of inhibition after solvent extraction

Result showing Isolate S6 (B.cereus) against P.auriginosa.

Fig 9: Zone of inhibition after solvent extraction

TABLE NO 4: ZONE OF INHIBITION

Culture	Pathogen	Intracellular(Methanol)	Extracellular(Chloroform)
S1	*B.subtilis*	Positive	Negative
S1	*S.aureus*	Positive	Negative
S6	*E.coli*	Positive	Negative
S1	*E.coli*	Negative	Positive
S2	*P.auriginosa*	Positive	Positive
S6	*P.auriginosa*	Positive	Negative
S2	*S.aureus*	Positive	Negative
S6	*S.aureus*	Negative	Positive
S3	*S.aureus*	Positive	Positive
S2	*E.coli*	Negative	Negative
S6	*E.coli*	Negative	Negative
S3	*P.auriginosa*	Negative	Negative

Table no.5: Zone of inhibition using chloroform and methanol

Pathogen	Culture	Intracellular (dia)mm	Extracellular (dia)mm
Bacillus subtilis	S1	22	0
Staphylococcus aureus	S1	23	0
	S6	0	22
	S2	23	0
	S6	21	0
	S3	24	22
E.coli	S1	0	21
	S2	20	0
	S3	24	0
Pseudomonas auriginosa	S2	20	23
	S3	21	0

Table 6: Retention Factor (Rf) values of various intracellular bacterial samples in TLC.

Intracellular bacterial sample	Retention factor values of various colour spots	
	yellow(cm)	purple(cm)
B.cereus	10	9
P. aeruginosa	10.5	9.5
S. faecalis	11	0
M.luteus	11.5	0

Table 7: Retention Factor values of various extracellular bacterial samples in TLC.

Extracellular bacterial sample	Retention factor values of various colour spots	
	yellow(cm)	purple(cm)
B.cereus	12.5	0
P. aeruginosa	12	0
S. faecalis	13	12
M.luteus	12	0

Distance travelled by the solvent was15 cm

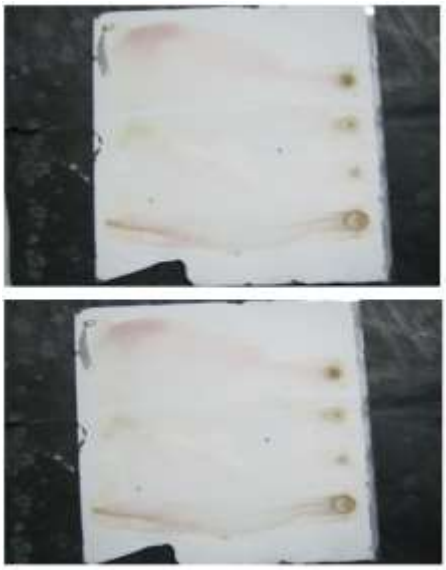


Figure 10: TLC result of intracellular and extracellular bacterial sample respectively.

U.V Spectroscopy result of various bacterial samples:

Table 8: Value of O.D of various intracellular and extracellular bacterial samples for the estimation of protein by Bradford assay**.**

Intracellular samples	O.D at 595 nm	Extracellular samples	O.D at 595 nm
B.cereus	1.807	P. aeruginosa	1.381
P.aeruginosa	1.666	M. luteus	1.360
S.faecalis	1.830	S. faecalis	1.163
M.luteus	1.828	B. cereus	1.581

DISCUSSION

In the present study, the bacterial cultures were isolated from the different region of Lucknow, Kanpur, Unnao. In total 6 soil sample were used and-and 6 culture were isolated and only 4 culture were characterized on the basis of Bergey's manual. The culture was: ***Bacillus cereus, Pseudomonas fluorescens, Streptococcus, Micrococcus luteus*** **and** ***Actinomycetes***. Optimization was done for

four culture in which the best carbon source are **Sucrose, Dextrose,** and **Glucose** and best nitrogen sources are **Yeast extract** and the best temperature was **37^{0}C** and best pH was **7** for ***B.cereus, Streptococcus, Pseudomonas fluoresces*** and **11** for ***micrococcus luteus.*** Further growth kinetics was carried out for all the four culture which gave the log phase for each culture which was 5 days for all culture.

The extraction of intracellular and extracellular secondary metabolites was done by solvent extraction method in which two solvent was used for each **chloroform** and **ethanol.** Further Antibiogram analysis was performed by agar well diffusion method using four different pathogens ***E.coli, B.subtilis, S.aureus, Pseudomonas auriginosa.***

CONCLUSION

Screening of antibiotic has been widely performed for about last 50 years and new antibiotic are still being formed. In the screening of new antibiotics, new approaches are required and following three-factor must be considered i.e. detection of antibiotic-producing microbes, selection of producing microbes and cultivation method.

Finally, it can be concluded that ***micrococcus luteus*** and ***Streptococcus*** are the best sources of antibiotic isolation.As the antibiotic is secondary metabolite, they are synthesized in trace amount. The synthesis of antibiotic is regulated by tight metabolic and genetic regulation. Therefore it is the task of a microbiologist to modify the wild-type strain and to provide culture condition to improve the productivity of antibiotic. Improvement of the microbial strain offers the greatest opportunity for cost reduction without significant capital investment

FUTURE PROSPECTS

Traditional antibiotics are now the mainstay of drug discovery for the treatment of emerging & old diseases. The present research work includes isolation & purification of therapeutic antimicrobials from the bacteria and carried out a further pharmacological evaluation by several methods like NMR, GC-MS, HPLC to screen and isolate bioactive agents which can be used as a potential source of drugs against diseases.

However, there is a need to ensure that, what is known is not made use for financial gain. For improvement of the health of people, there is a need to establish the necessary expertise for the development of traditional antibiotics & deliberate efforts should be made to encourage local industrial production of antibiotics so that cultivation may become possible and hence contribute to poverty reduction.

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