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PRODUCTION AND PURIFICATION OF ANTIBACTERIAL METABOLITES FROM SOIL ISOLATE

[1]Kaushik C, [2]Srivastava P

School of Life Sciences, Invertis University, Bareilly, UP, India.

***Corresponding Author:** Chandani Kaushik

Email ID: achandami7@gmail.com

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ABSTRACT

Secondary metabolites acquired from various microorganisms provide a potential source of many unique compounds with antimicrobial and other medicinal properties. The majority of these compounds are widely used as medicines for combating antibacterial metabolites against various strains. Supporters of *Bacillus* species are profile procedures of previously-known secondary metabolites producers. The active culture has been isolated from terrestrial soils as well as from the marine sediments. The production of the active antibacterial metabolites was carried out by submerged fermentation. After purification the best activity has been shown by the acetone extracted metabolite.

Key words: Secondary Metabolites, Medicinal, *Bacillus*, Submerged Fermentation.

INTRODUCTION

Antibiotics is a Greek term which means, against life and used to denote any compound which can be used against microorganisms, which cause harm to living organisms **[1]**. Antibiotics can be synonymous with antimicrobial or antibacterial but few references differentiate between the antibiotics and antibacterial as antibacterial is used in soaps and disinfectants whereas the antibiotics are used as medicines **[2]**.

This can be specified as a preventive measure (prophytactic) measure, or can also be used to cure diseases caused by the infection or other means. Antibiotics can also be used to combat protozoan diseases, such as metronidazole, which is an important antibiotic drug against a variety of parasites infections **[3,4]**. In 1941, Selman Waksman identified these antibiotics and identified them as any small molecule produced by a microbe that antagonizes the growth of other microbes. Thus the term antibiotic is applied to any drug that destroys the bacteria or prevents the growth of the bacteria irrespective of whether or not the medicine is created by microorganisms **[5]**.

Antibiotics can be derived from the soil microorganisms, such as bacteria from Actinomycetes. Up to 70% of the antibiotics are derived or removed from the bacteria of Actinomycetes, the remainder is derived or removed from non-filamentous fungi or non-actinomycete bacteria [**5**]. Microorganism produces a large variety of bioactive compounds and estimates of plate counts give a value range of 10.4 to 10.8 per gram of soil that is susceptible to low pH 1.e., pH 6.5 to pH 8.0 **[6,7]**. Compared to chemical sources, the manufacture of antibiotics from a natural source is relatively straightforward since the natural sources are easy to access **[8]**. Natural source isolated microorganism (e.g. soil) develops secondary metabolites that act as antibiotic compounds. Secondary metabolites are the compounds that the body produces but not utilized by itself **[9]**.

Actinomycetes are probably the most suitable microorganism during the development of antibiotics because they are simple to distinguish and their characteristics that are essential for a more successful antibiotic [**10]**.

As antimicrobials a wide variety of chemical and natural compounds are used. Chemical acid is commonly used in food products as antimicrobials. Such as lactic acid, citric acid, acetic acid and its salts, eithers as additives or as disinfectants **[11]**.

METHODOLOGY

Collection of soil sample:

Samples are collected from the respective places **[12]**.

Isolation of antibiotic producing bacteria from soil sample:

The samples were serially diluted, after spreading the diluted samples in sterilized nutrient agar plates, the bacterial colonies were observed. The mixed cultures were purified by streak plate method, the cultures were selected on the basis of colony morphology.

The cultures were screened by agar well diffusion method for the analysis of the production of antibacterial metabolites by bacterial cultures. The cultures were loaded to wells in nutrient agar plates along with pathogenic organisms. Further the zone of inhibition was measured **[13]**.

Identification of bacteria:

For identification, Gram staining and few other biochemical tests were performed based on Bergy's manual **[14]**.

Selection and optimization of the production media:

The media selection and optimization were done on the basis of one factor at a time and the best components were selected by observing the bacterial and the zone of inhibition **[15]**.

Fermentation and purification of active metabolites:

The sterilized selected media was prepared for the batch fermentation. Further the culture was inoculated and incubated at 37^0C for a week at shaker incubator. Once the fermentation completed the purification of active metabolite was carried out by solvent extraction method. The activity of purified component was carried out by antibacterial sensitivity test **[16,17]**.

RESULTS

Collection of soil sample:

The samples were collected from various places of Gomtinagar, Lucknow, as described in below table 1.

Table 1: - Sites of sample collections

Soil Samples	Location of Soil Collection
Sample no. 1	 Rhizospheric Soil of tree
Sample no. 2	 Field
Sample no. 3	 Park
Sample no. 4	 Pond side

Isolation of bacteria from soil sample:

Microbes from soil were isolated by serial dilution method and mixture culture was obtained by spreading, as shown below in the **figure 1.** The colonies showing inhibitory zone around themselves were selected and named tentatively as C01, C02, C03, C04, C05, C06, C07, C08, C09, C10, C11 and C12 as shown in figure 1 and 2.

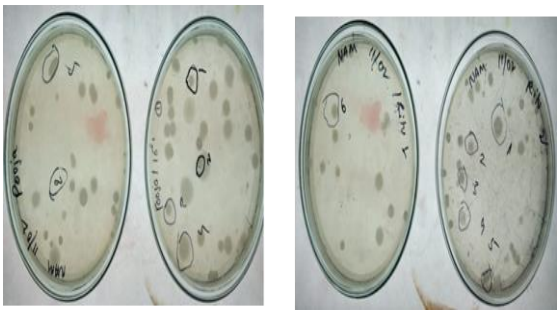

Figure 1:-Above figure represent the Serial dilution plates of soil samples

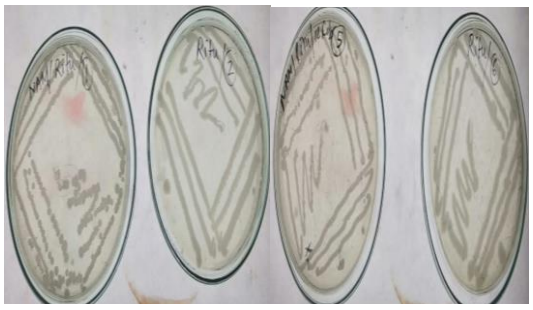

Figure 2:- Above figure represent the few pure cultures of all isolated antibacterial bacterial colonies

Secondary screening of isolates for the production of antibacterial components

Antibacterial susceptibility assay of crude antibacterial *extracts* from C01 to C09.was performed by Agar well diffusion method against several pathogens such as *Salmonella typhi, Pseudomonas aeruginosa* and *Escherichia coli.* The results were obtained after 24hrs. The inhibitory zone of clearance was seen around the well which was measured in mm. The results of the same can be seen below in figure 3 and 4.

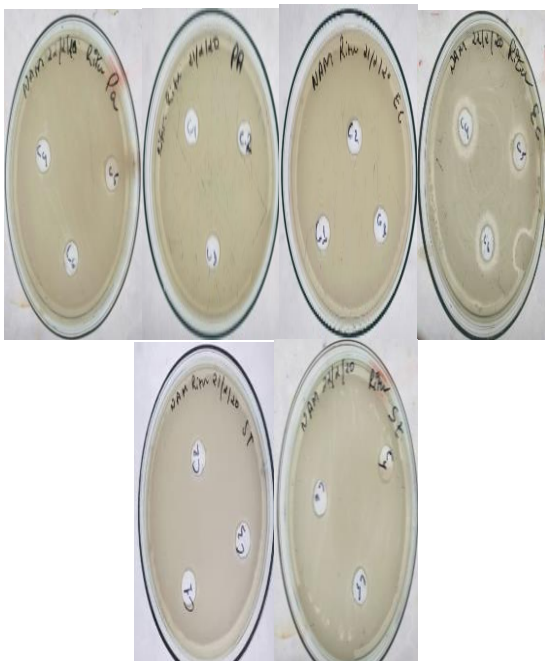

Figure 3: - Above figures represent the results of secondary screening of few isolated bacterial colonies for the production of Antibiotics.

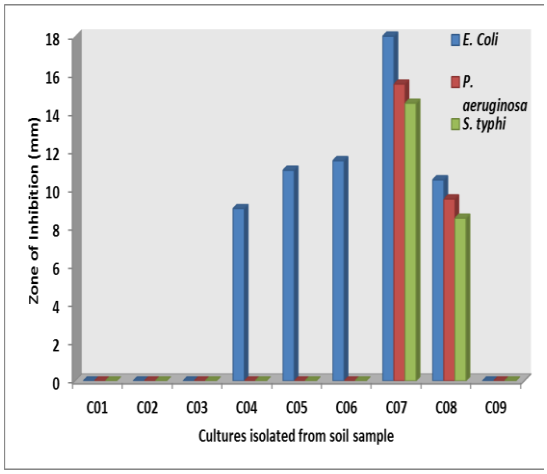

Figure 4: Antibacterial Susceptibility assay of C01 to C09

Optimization of Physio-Chemical factors for maximum yield of antimicrobial component:

Table 2: Effect of temperature on growth of C07

Incubation temperature	Remark
4^0C	-
22^0C	-
37^0C	+++
50^0C	++

Table 3: Effect of pH on growth of C07

pH	O.D at 620 nm
4.5	0.02
7.5	**1.07**
9.5	0.03
11.5	0.03

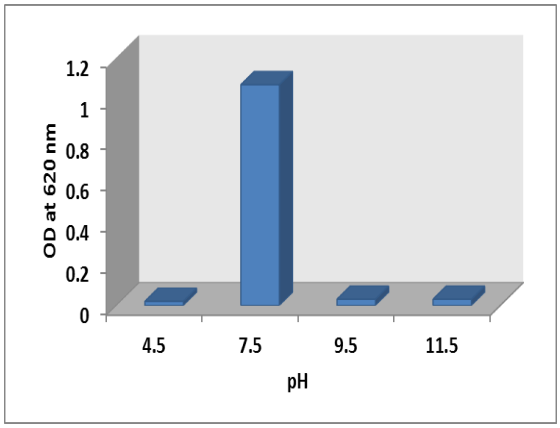

Figure 5. Above graph represent the results of the effect of pH such as 4.5, 7.5, 9.5 and 11.5 on the growth of the C07

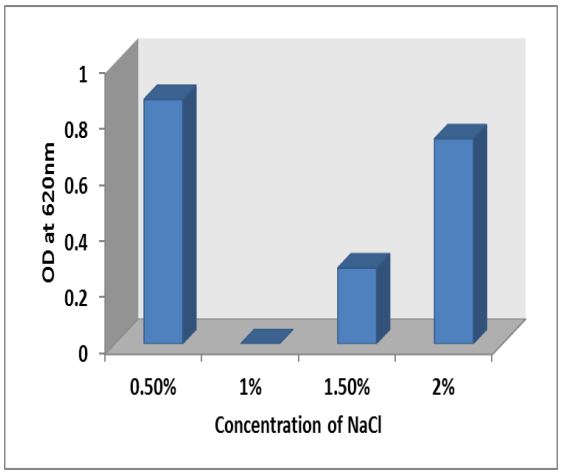

Figure 6. Above graph represent the results of the effect of concentration of NaCl such as 0.5%, 1%, 1.5% and 2% on the growth of the C07

Optimization of Nitrogen sources in the selected production media II:

After giving the incubation of 24 hours at 37^0C, it was done to observe the maximum growth of the bacterial culture at different nitrogen sources. The best Nitrogen source for C07 was Yeast as shown in figure 7.

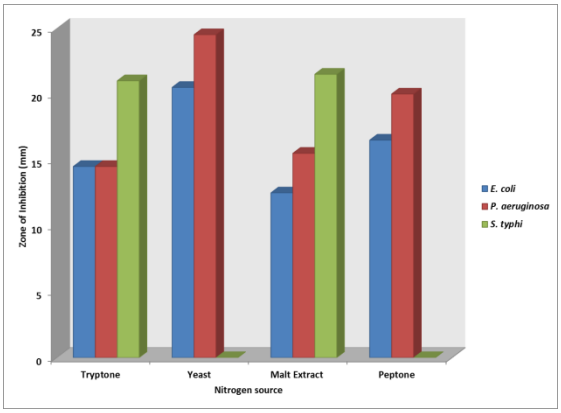

Figure 7. Above graph represent the Antibacterial susceptibility assay of antibiotic produce by C07 in selected production media that contain different nitrogen source, such as tryptone, yeast, malt extract and peptone that shown maximum zone of inhibition against three different pathogen.

Supplementation of carbon sources for bacteriaC07:

After giving the incubation of 24 hours at 37^0C, it was done to observe the maximum growth of the bacterial culture at different carbon sources. The best carbon source for the bacteria is Mannitol as expressed in figure 8.

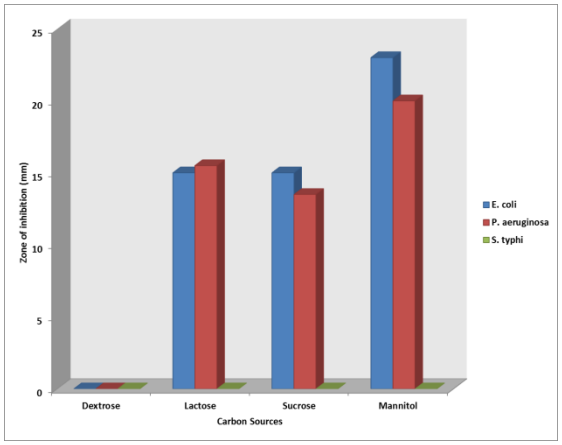

Figure 8. Above graph represent the Antibacterial susceptibility assay of antibiotic produce by C07in selected production media that contain different carbon sources such as dextrose, lactose, sucrose and mannitol that shown maximum zone of inhibition against three different pathogen.

Supplementation of Metal ions for bacteriaC07:

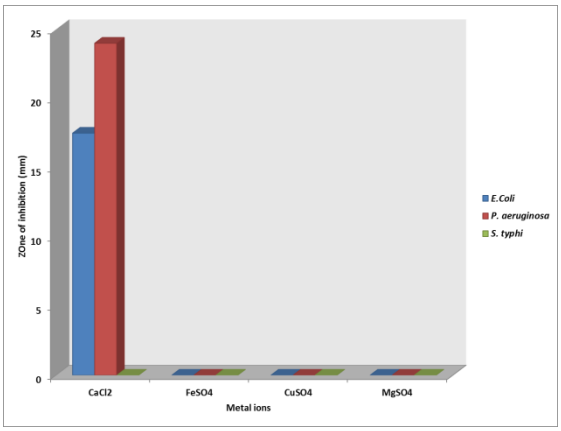

Figure 9. Above graph represents the Antibacterial susceptibility assay of antibiotic produce by C07 in selected production media that contain different metal ion sources that shown maximum zone of inhibition against three different pathogens.

Optimization of $CaCl_2$ in different concentration:

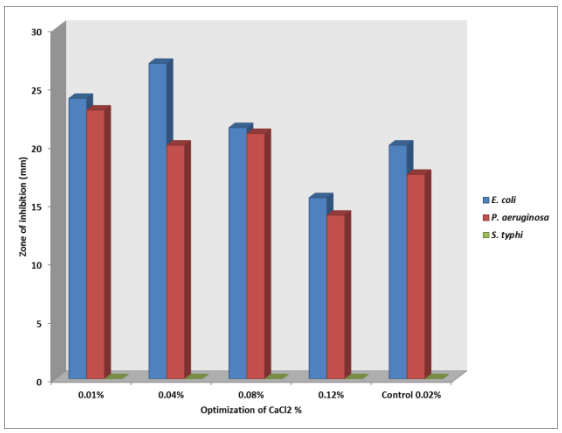

Figure 10. Above graph represent the Antibacterial susceptibility assay of antibiotic produce by C07 in selected production media that contain different concentration of $CaCl_2$metal ion source, in which due to 0.02% $CaCl_2$ content in production media that shown maximum zone of inhibition against three different pathogen.

Fermentation and purification of metabolite:

The purification was done by solvent extraction (Acetone, Chloroform, Petroleum ether and Ethyl acetate). The amount of antibacterial component left after air drying was measured as below in figure.

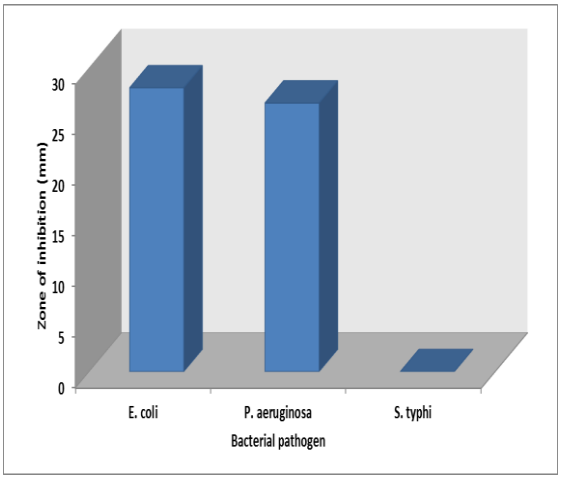

Figure 11. Above graph represent result of Antibiotic susceptibility assay of crude antibiotic produced after fermentation in optimized media

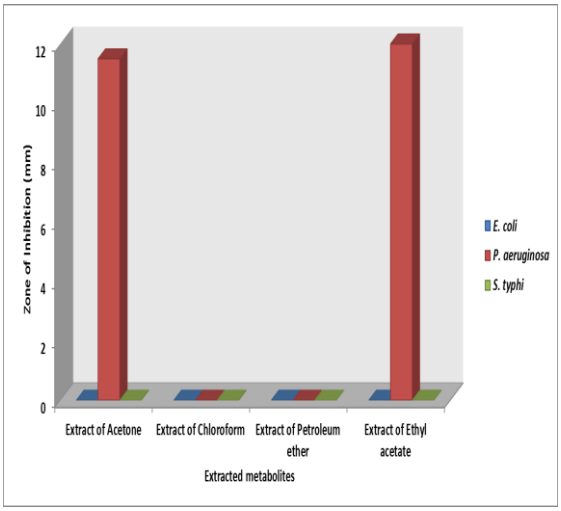

Figure 12. Above graph represent result of Antibiotic susceptibility assay of pure antibiotic produced after fermentation in optimized media

Identification of isolated C07:

The isolate C07 was identified as +ve Rods in chain based on Bergey's manual by performing various staining and biochemical activities, **Table** below show results of various staining and biochemical activities. Below show the result of biochemical test.

Table4:- Results of Identification test

S. No.	Identification test	Remark
1.	Gram's staining	Positive
2.	Mannitol test	Positive
3.	VP test	Positive
5.	Endospore test	Negative
6.	Catalase test	Negative

DISCUSSION

The need of antibiotics has always been a highly important for human welfare. Some recent studies on antibiotics has made with the increase in the piece of emergence. It was found that the soil of field area of the Lucknow, has the maximum number of antibiotic producing bacteria. When isolates found after performing the primary screening were subjected to secondary screening, 9 isolates were found that are potentially antibiotic producing bacteria. Analyzing the result of secondary screening it was found that C07has the maximum zone of inhibition 11.5mm against *S. typhi* while it produces zone of inhibition of 8.5mm against *P. aeruginosa* and 10mm against *E. coli.* On the basis of zone of inhibition formed, the C07 was selected for strain improvement and identification. After the identification of C07, it was found *Bacillus spp*.

On strain improvement of C07, its antimicrobial activity was increases in case of *E .coli.* In order to characterize the optimum fermentation media for C07the late stationary phase is obtained on 48 hours with the temperature of 37º C at pH7.5. During optimizing the production media the mannitol as carbon source, yeast as the nitrogen source and 0.2% $CaCl_2$ as the metal ion had been found best in inducing the production of antimicrobial components. It has enhanced the production by increasing the zone of inhibition. After the identification and optimization of the production media, the production of the antimicrobial metabolite was done by the wild strain by using submerged fermentation.

Further the purification of extract done by the solvent extraction method using chloroform, petroleum ether, ethyl acetate and acetone as polar and nonpolar solvent. In which the best result of wild strain was obtained by the extract of the acetone.

CONCLUSION

From the above work it can be evaluated that bacterial strain C07 i.e. belongs to *Bacillus Spp.* is able to produce secondary metabolites in a good amount which can be able to inhibit the growth of various pathogenic microorganisms. Four soil samples were studied in which nine bacterial cultures were isolated on the basis of primary screening in which maximum Zone of Inhibition was found in culture C07. Further optimization and supplementation of media was done with various Carbon sources such as Dextrose, Lactose, Sucrose and Mannitol in which Mannitol was proved to be the best Carbon source in the media. Similarly various Nitrogen sources such as Tryptone, Peptone, Malt extract and yeast was used in which Yeast was to be the best source for the Nitrogen source. Further various metal ion sources are used such as $FeSO_4$, $MgSO_4$, $CaCl_2$ and $CuSO_4$ was used in which the $CaCL_2$ was proved to be the most active form of metal for the metal ions in the optimized and supplemented media. The enhancement for the production of antimicrobial metabolite was done by submerged fermentation. Then purification was carried out by using various solvents such as Acetone, Chloroform, Petroleum ether and Ethyl acetate, in which acetone was found to be best solvent for the extraction of the compound that shows antimicrobial property. The metabolites extracted here can be used as an antibiotic drug after proper and complete pharmacological evaluation.

FUTURE ASPECTS

Future work of this present study would be its characterization and identification of the chemical structure of the extracted secondary metabolites (antibiotic components), evaluation of physical and chemical properties of the extracted metabolites. Also in the current problem with multiple antibiotics resistant in clinical strains and the potential for increasing resistance to antibiotics whose use is increasing in the community, it is clear that the use of available and as yet effective antimicrobial is called for, hence for resolving all such problems. There is a huge requirement of the antibiotics now a days which cannot be resistance to the strains.

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