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MICROBIOLOGY

Optimization of Microbial culture for Improved Production of Secondary Metabolites

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ABSTRACT

The present study is carried out by optimization of microbial culture for improved production of secondary metabolites. The secondary metabolites work as an antibiotics that are responsible for inhibition of growth of bacterial pathogens. In this method total 14 soil samples were collected at different – different places from Lucknow according to the geographical location. Total 13 bacterial strains were isolated after pure culturing (C1- C13) and further they were tested against *E. coli*, *S. aureus* and *P. aeruginosa* by disc diffusion method. Out of 13 cultures best results were obtained for 4 cultures (C1, C11, C12 and C13). Further work involved biochemical tests by Bergey's manual for identification of bacteria and optimization of culture conditions in which the best carbon source, best nitrogen source, best pH and best temperature were observed for improved production of secondary metabolites. The best carbon source was obtained as glucose, best nitrogen source was obtained as an ammonium chloride, best pH obtained was 7 and the best temperature was 37 °C for C1, C11, C12 and C13. Identification of bacterial isolates was performed by Bergey's manual which indicates the presence of *Bacillus* culture.

Highlights

- The optimized conditions for the growth of antimicrobial metabolites producing bacteria have been presented in this article.
- It was found that these antimicrobial metabolite producing bacteria grow at an optimum:
  - pH – 7
  - Temperature - 37 °C
- Best carbon source was obtained as glucose,
- Best nitrogen source was obtained as ammonium chloride

Keywords: Optimization, antibacterial activity, multidrug resistant pathogens, geographical location, Disc diffusion method

Some microorganisms are responsible for the production of secondary metabolites and their production can be increased by optimization of culture conditions. Optimization refers to the suitable condition that favour the growth of microbes and improve the secondary metabolite production. Optimization involves suitable carbon, nitrogen sources, suitable conditions of and pH, temperature as well as salt tolerance. Secondary metabolites can act as an antibiotics, which can inhibit the growth of bacterial pathogens (Sanchez and Demain 2008).

Antibiotics are substances used against infection caused by bacteria. Antibiotics act by inhibiting or destroying bacterial cells that cause certain diseases (Fair and Tor 2014). In nature, antibiotics are secondary metabolites produced by bacteria in order to maintain their niche and territory. Secondary metabolite that includes antibiotics is produced in nature and also serves as survival functions for

the organisms producing them (Demain and Fang 2000). Only limited groups of microorganisms are responsible for the sources of clinically useable antibiotics. As stated by (Russell and Furr 1977) only those antibiotics that have an effect on bacterial cells but not on the host cells like human are categorized as useful antibiotics. Besides, there are only limited number of antifungal antibiotics that can be used to treat fungal infections (Leekha *et al.* 2011).

An antibiotic can be found easily but only few are useful. Hence, concerted efforts have been carried out by many scientists for screening novel antibiotic producing microbes (Stadler and Teuber 2002). Through their efforts, many antibiotics have been successfully discovered in order to combat pathogenic bacteria that cause diseases. However, the emergence of new diseases and reemergence of multiple-antibiotic resistant pathogens have rendered the existing used antibiotics ineffective. This problem has spurred the needs for the discovery of new antibiotics (Wang *et al.* 2013).

MATERIALS AND METHODS

Collection of soil sample

A total of 14 soil samples of 20 grams each were obtained from different places in Lucknow located at latitude 26˚55′ N and longitude 80˚59′ E. The list of soil samples are shown in Table 1. Each soil sample was put into respective plastic bag under aseptic condition. The plastic bag containing the soil samples were labelled specifically and kept at 4 ˚C for further use.

Table 1: pH variation in soil of isolated bacterial cultures

Location	pH of soil	Bacterial isolates
Malihabad	6.5	C1, C2
Bakshi ka Talab	7.8	C3, C4
Kakori	6.0	C5
Chinhat	7.0	C6, C7
Sarojini nagar	5.8	C8, C9
Gosainganj	6.7	C10, C11
Mohanlalaganj	7.5	C12, C13

Isolation and screening of cultures

Soil samples collected from different geographical areas were pre-treated with physical and chemical methods before plating to eliminate various common microbes. One gram of each soil sample was suspended in 10 ml of normal saline and distributed in aliquots, one aliquot was treated with heat (1 hr at 120 ˚C) and the other was treated with 1.5% phenol (30 ˚C, 30 min) (Rachid *et al.* 2012). The pre-treated soil samples were plated by serial dilution method on nutrient agar (NA). Plates were incubated at 37 ˚C for 24-48 hours. The colonies selected on the basis of morphological features were purified and subjected to screening for antimicrobial activity.

Antimicrobial activities of the axenic cultures were determined by perpendicular streak method against different strain of one gram positive culture *Staphylococcus aureus* (MTCC 2940) and two gram negative cultures- *Pseudomonas aeruginosa* (MTCC 2453) and *E. coli* (MTCC 739). Strains showing moderate to good activity were selected for secondary screening, which was performed by agar well and disc diffusion methods using 100 µl of their fermented broth against different test organisms. Activities of the strains were compared with that of Ampicillin (Amp), Tetracycline (Tet) and Ofloxacin (Ofl) (Munoz-Atienza *et al.* 2013).

Characterization and identification of selected strains

The cultural characteristics of the producer strains were studied based upon their intensity of growth, growth pattern and colony colour. The strains were characterized by streaking the culture(s) on the nutrient agar plates and were observed after 24 - 48 hours of incubation at 37˚C for the given characteristics. Physiological and biochemical tests were performed as described by Williams and Bergey's manual (Singh *et al.* 2016b, Berdy 2005). The results were observed after 24 - 48 hours of incubation of plates at 37 ˚C. In addition, the strains were tested for assimilation, utilization, tolerance to NaCl, pH tolerance and temperature tolerance.

Antibiotic sensitivity test

Antibiotic sensitivity test is done to check the activity of antibiotics against pathogens. Antibiotic sensitivity is a term used to describe the susceptibility of bacteria to antibiotics. Antibiotic sensitivity testing (AST) is usually carried out to determine which antibiotic will be the most successful in

treating a bacterial infection *in vivo* (Jenkins and Schuetz 2012). This process can be performed *in vitro* by 2 methods:

Disc diffusion method

The Nutrient agar medium was prepared and poured in to sterile petriplates. After solidification spreading was done for 3 bacterial pathogens that include *E. coli, P. aeruginosa* and *S. aureus*. Later at the surface of nutrient agar medium the antibiotic discs were placed. All these plates were incubated at 37°C overnight (Fujikawa and Morozumi 2005).

Optimization of Culture conditions

Medium optimization commonly involves the addition of various supplements to an existing basal medium formulation. It defines the parameters of the culture media that should be manipulated for optimization of production. The culture process development and optimization is essential for the viable production of microbes and microbial products (Singh *et al*. 2016a).

Assimilation: Five types of carbon sources were selected-glucose, sucrose, lactose, mannitol, galactose; 1% of each was added to separate test tubes along with NB. 1 test tube was kept as the control test tube.

Utilization: Five types of nitrogen sources were selected-urea, glycine, ammonium sulphate, potassium nitrate, ammonium chloride; 1% of each was added to separate test tubes along with N.B. 1 test tube was kept as the control test tube.

NaCl tolerance: 1%, 3%, 5% and 7%NaCl were used to test for salt tolerance based on previous reports.

pH tolerance: Four types of pH values were selected-5, 7, 9 and 11; each of them were maintained separately in test tubes containing N.B media. 1 test tube was kept as the control test tube.

Temperature: Based on the previous reports three temperature ranges were selected -4 °C, 37 °C and 55 °C; the test tubes containing N.B were maintained at these temperatures after the inoculation of cultures. (1) The test tubes containing suitable media were autoclaved and were inoculated with bacterial isolates. (2) The carbon, nitrogen and pH-range sources were incubated overnight at 37 °C. (3) The results were obtained by taking OD at 620nm.

RESULTS AND DISCUSSION

Screening of the Active Strains: During the screening, thirteen bacterial cultures were isolated from seven different niches of northern India. Microbial colonies showing distinct morphological characters were selected for the primary screening. The isolation of bacterial cultures was done using serial dilution method. The antibiotic sensitivity test was performed through agar well diffusion method and disc diffusion method against bacterial pathogens like *E. coli, S. aureus* and *P. aeruginosa,* which also showed MDR activity which indicates that such type of cultures can survive in the presence of the existing antibiotics. Out of 13 bacterial isolates, four showed moderate to strong antibacterial activity against gram positive (*S. aureus* MTCC 2940), gram negative (*E. coli* MTCC 739 and *P. aeruginosa* MTCC 2453). Out of 13 active isolates, four showed strong antimicrobial activity and were selected for detailed taxonomic, physiological, and biochemical studies (Table 2 and 3).

Table 2: Characterization of bacterial isolates having antibacterial activity against MDR pathogens

Tests	C1	C11	C12	C13
Grams staining	+	–	–	+
Endospore staining	+	–	+	+
Catalase test	+	–	+	+
Glucose fermentation	+	–	–	+
Mannitol fermentation	+	+	–	+
Methyl red-Voges Proskauer test	+	–	+	+

+ sign indicates positive result for test and – sign indicates negative result

Table 3: Antibiotic Sensitivity Test by Disc diffusion method

Cultures	(ZOI in mm) *E. coli*	(ZOI in mm) *S. aureus*	(ZOI in mm) *P. aeruginosa*
C1	10	15	24
C2	0	0	0
C3	0	0	0
C4	0	0	0
C5	0	0	0
C6	0	0	0
C7	0	0	15
C8	0	20	0
C9	0	15	0

C10	0	0	0
C11	18	23	14
C12	20	14	13
C13	20	15	23

Result indicates that out of 13 bacterial isolates only 4 cultures were showing positive result.

Result of optimization of culture conditions for bacterial strains C1, C11, C12, C13

Optimization of culture conditions involved best carbon source, nitrogen source, pH and temperature for isolates C1, C11, C12 and C13, and it was observed that glucose and ammonium chloride were found to be the best sources of carbon and nitrogen respectively. In addition to this, pH 7 and 37 ˚C temperature were found to be the best for optimum growth of these four bacterial cultures, which is mentioned in Table 4.

CONCLUSION

In summary, optimization refers to provide suitable condition for the growth of bacterial isolates in the form of best carbon, nitrogen, pH and temperature. According to the result, the best carbon source obtained was glucose, best nitrogen source was ammonium chloride, best pH was 7 and best temp was 37 ˚C, which was different from the previous study related to antibiotics isolation from soil bacteria. The future work involved the identification of metabolites, which inhibit the growth of bacterial pathogens. The major sources that are involved for isolation of secondary metabolites can be

Table 4: Biochemical and physiological characteristics of the producer strains

Characteristics	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13
Assimilation													
Glucose	+3	--	--	--	--	--	--	--	--	--	+3	+3	+3
Sucrose	+3	--	--	--	--	--	--	--	--	--	+2	+2	+3
Lactose	+2	--	--	--	--	--	--	--	--	--	+2	+2	+2
Mannitol	+2	--	--	--	--	--	--	--	--	--	+2	+2	+2
Galactose	+2	--	--	--	--	--	--	--	--	--	+2	+2	+2
Utilization													
Urea	+2	--	--	--	--	--	--	--	--	--	+2	+2	+2
Glycine	+2	--	--	--	--	--	--	--	--	--	+2	+2	+2
Potassium Nitrate	+2	--	--	--	--	--	--	--	--	--	+2	+2	+2
Ammonium Chloride	+3	--	--	--	--	--	--	--	--	--	+3	+3	+3
pH tolerance													
5	+2	--	--	--	--	--	--	--	--	--	+2	+2	+2
7	+3	--	--	--	--	--	--	--	--	--	+3	+3	+3
9	+2	--	--	--	--	--	--	--	--	--	+2	+2	+2
11	+	--	--	--	--	--	--	--	--	--	+	+	+
NaCl tolerance													
1%	+3	--	--	--	--	--	--	--	--	--	+3	+3	+3
3%	+2	--	--	--	--	--	--	--	--	--	+2	+2	+2
5%	+	--	--	--	--	--	--	--	--	--	+	+	+
7%	_	--	--	--	--	--	--	--	--	--	_	_	_
Growth at													
4^0 C	_	--	--	--	--	--	--	--	--	--	_	_	_
37^0 C	+3	--	--	--	--	--	--	--	--	--	+3	+3	+3
55^0 C	_	--	--	--	--	--	--	--	--	--	_	_	_

(-), No growth, (+), Poor growth, (+2), Moderate growth, (+3), Heavy growth (--), Optimization of these cultures was not done as there was no antibacterial activity as evidence by zone of inhibition in table no. 3.

microorganisms in the form of bacteria, fungi or *Actinomycetes.*

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Conflict of interest: None

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