

## OPTIMIZATION OF CULTURING CONDITIONS FOR THE PRODUCTION OF ANTIMICROBIAL METABOLITE BY *BACILLUS CAROTARUM*

SAIFUN NAHAR, FERDOUSI ALI AND MOHAMMAD ABUL MANCHUR\*

Department of Microbiology, University of Chittagong, Chittagong-4331, Bangladesh.

### ABSTRACT

Forest soil antagonistic bacteria *Bacillus carotarum* was isolated and tested for optimum antimicrobial metabolite production. Maximum antimicrobial metabolite production was found at temperature 30°C and pH 7.0 and on 1st day of incubation at stationary condition. The medium consisting of beef extract - 0.3%, peptone - 0.5% and NaCl - 0.05%, was screened out as a suitable medium for maximum antimicrobial production supplemented individually with four carbon sources of which starch was found as the best carbon source. The active agent was best extracted with petroleum ether. Homogeneity of the crude bioactive metabolites were also tested by paper chromatographic analysis where a single type of bioactive metabolite was found with an R<sub>f</sub> value of 0.94 in the solvent system n-butanol : acetic acid : water = 4 : 1 : 5. The antimicrobial spectrum of the metabolite was wide and showed activity against *Shigella dysenteriae* (AE14612), *Salmonella typhi* (AE14296), *Vibrio cholerae* (AE14748), *Pseudomonas aeruginosa* (CRL, ICDDR' B), *Bacillus cereus* (BTCC19), and *Bacillus subtilis* (BTTC17).

**Key words:** Antimicrobial metabolite, optimum production, *Bacillus carotarum*.

### INTRODUCTION

There is a growing recognition of the pressing need for new antimicrobial agents for the treatment of infectious diseases (Bush 2004 and Shlaes *et al.* 2004). In addition, providing effective and affordable antibiotics to people in epidemic-prone developing countries remains a major challenge (Sarkar *et al.* 2006).

Antagonism is an inter-population relationship in which one population has a deleterious (negative) effect on another. These inhibitory organisms are called 'antagonistic' microorganisms (Atlas 1997). Such organisms may be of great practical importance, since they often produce antibiotics or other inhibitory substances, which affect the normal growth processes or survival of other organisms (Pelczar *et al.* 1974). Soil is a diverse medium composed of many minerals and substrates essential for metabolic pathways of prokaryotic and eukaryotic inhabitants (Dakora and Phillips 2002). There are great opportunities for discovering new groups of microorganisms with industrial and clinical importance in soil (Rondon *et al.* 1999).

Microorganisms like Gram-positive, Gram-negative bacteria and fungi have the ability of synthesizing antimicrobial agents. A compilation of the microbial sources of antibiotics in the soil discovered in the United States and Japan between 1953 and 1970

---

\* Corresponding author, E-mail – manchur.mbio@cu.ac.bd



revealed that approximately 85% are produced by actinomycetes, 11% by fungi and 4% by bacteria (Tyler *et al.* 1988). *Bacillus* species occur mainly in the soil because of their spore forming capability. These microbes exhibit a vast metabolic versatility. They can complete many physiological cycles that produce intermediate molecules such as enzymes or secondary metabolites with antibacterial, antifungal and antiviral capabilities. Considering above-mentioned facts, the present study was undertaken to find out and optimize the growth of anti-microbial metabolite producing bacterial isolates.

## MATERIALS AND METHODS

### *Isolation and purification of the antimicrobial metabolite producing microorganisms*

#### *Media used for isolation*

The media used for the isolation of antibiotic producing microorganisms were nutrient agar medium and starch casein medium (Kuster and Williams 1964).

#### *Isolation and purification of microorganism*

Samples (soil and compost) were collected from different forests and farm yards in Chittagong, Bangladesh. Ten mg of collected sample was added to 90 ml sterile enrichment media in a sterile 250 ml conical flask, shaken well by stirrer and then allowed to stand for 30 minutes for sedimentation. Necessary dilution (up to  $10^{-5}$ ) was made with this mother solution. The samples (0.1 ml) were used for inoculating 15 ml molten medium at 45°C - 50°C in Petri plates. Then the Petri plates were incubated at 37°C for about 4 to 5 days. Observation was made at 24 hours intervals to detect any colony surrounded by a clear zone of inhibition. Both the organisms (inhibiting and inhibited) involved in antagonistic reaction were isolated aseptically and transferred to slant of the same medium. The isolated colonies were purified through repeated streak plate method on nutrient agar plate.

The colonies appeared were checked for the presence of spore, performed gram staining and finally streaked onto agar slants for further characterization. The cultures were kept at 4°C until used.

#### *Test organisms*

In the present study, to screen the anti-microbial activity of microbial secondary metabolites, 9 human pathogenic bacteria were used as test organisms. Among the 9 human pathogens, 5 were Gram negative - *Shigella dysenteriae* (AE 14612), *Salmonella typhi* (AE14296), *Vibrio cholerae* (AE14748), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (CRL, ICDDR' B) and 4 were Gram positive - *Bacillus cereus* (BTCC19), *Staphylococcus aureus* (ATCC6538), *Bacillus subtilis* (BTTC17), *Bacillus megaterium* (BTTC18).

#### *Primary screening*

Primary screening was made by sensitivity spectrum analyses test against 9 test organisms. Perpendicular streak plate method (Florey *et al.* 1949) was used for this purpose.



## OPTIMIZATION OF CULTURING CONDITIONS FOR THE PRODUCTION OF ANTIMICROBIAL METABOLITE BY *BACILLUS CAROTARUM*

The isolate was streaked across the surface of the nutrient agar medium at the middle position of the plate and incubated at 37°C for growth. After growth, the test organisms were streaked perpendicularly. A space of 2-3 mm was kept between two streaks and the plates were incubated at 37°C for the growth of the test organisms. The plates were then observed for organisms sensitive to the metabolites produced by the isolates.

### *Identification of the Isolated Bacteria*

#### *Cultural characterization*

The isolate was observed under the microscope, the colony morphology was noted with respect to color, shape, size, nature of colony and pigmentation.

#### *Microscopic observation*

The bacterial isolate was Gram stained and observed under a light microscope. Endospore staining and motility tests were performed to observe the morphology and motility of the cells.

#### *Biochemical characterization*

The bacterial isolate was characterized biochemically by indole test, methyl red test, Vogues Proskauer test, catalase test, oxidase test, urease test, nitrate reduction test, gelatin hydrolysis test, Starch hydrolysis test, H<sub>2</sub>S production and carbohydrate fermentation test (glucose, sucrose and lactose).

The organism, which showed a considerable inhibition to the test organisms in primary screening, was selected for mass culture in liquid media. In a solid medium, an organism may produce an antimicrobial metabolite but it may not do so in liquid medium. The production of an antibiotic may be influenced by different types of factors such as composition and pH of media, incubation period, temperature and incubation condition (Shaking or stationary). Therefore, the maximum yield of an antibiotic depends on the optimization of these factors.

#### *Antibiotic assay by agar cup plate method*

In performing the sensitivity spectrum analyses by agar cup plate method, nutrient agar plates were heavily seeded ( $2.7 \times 10^3$  cells/ml) uniformly with the test organisms. Then a hole was made in media by gel cutter in sterile condition. Then one drop of melted agar was poured into hole and allowed to solidify to make a base layer. After that specific amount of culture filtrate (0.1 ml) was poured into the hole. Then plates were kept at low temperature (4°C) for 2-4 hours to allow maximum diffusion. The plates were then incubated at 37°C for 24 hours at inverted position to allow maximum growth of the organisms. The antibacterial activity of the test agent was determined by measuring the zone of inhibition expressed in millimeter in diameter (Barry 1976). The experiment was carried out more than once and mean of reading was recorded.



### *Optimization of Incubation Period*

The effect of incubation periods on the antimicrobial metabolite production by the selected isolate was studied. For this, 50 ml of nutrient broth medium was taken in each 100 ml conical flask and autoclaved. After cooling, the broth medium was inoculated with equal quantity of inocula, incubated at 37°C for 1, 2 and 3 days. Then the culture filtrate was assayed for antimicrobial metabolite production.

### *Optimization of incubation temperature*

After inoculation, the culture medium was incubated at different temperatures such as 27°C, 30°C and 37°C for optimum incubation period. The culture filtrate was assayed for maximum antimicrobial metabolite production by agar cup plate method.

### *Optimization of Media pH*

To observe the effect of pH on antimicrobial metabolite production, the selected medium was prepared at different pH (6.0, 7.0 and 8.0), dispensed 50 ml per 100 ml conical flask and autoclaved. Then the medium was inoculated and incubated at 37°C for optimum period. After incubation, the culture filtrate was assayed for antimicrobial metabolite production.

### *Optimization of carbon sources*

The production of active agent under different carbon sources was studied in the nutrient broth medium. Four carbon sources (glucose, fructose, sucrose, starch) were added to the selected medium. Keeping other cultural condition optimum and after specific period of incubation the culture media were filtered and assayed for antimicrobial metabolite production.

### *Optimization of stationary and shaking incubation condition*

To determine the effect of stationary and shaking conditions broth medium was inoculated and incubated in both stationary and shaking conditions keeping all other experimental conditions optimum. After specific period, the culture media were filtered and assayed for antimicrobial metabolite production.

### *Production and recovery of antimicrobial metabolite*

#### *Fermentation*

For fermentation, selected medium was prepared and sterilized. Then it was inoculated with 5-10% (v/v) of inocula of the selected isolates. All other optimum conditions were maintained for maximum production.

#### *Isolation of antimicrobial agent by solvent extraction*

The isolation of the antibiotic from the culture broth was achieved by extraction with an organic solvent not completely miscible with water. In this case, the solubility of the antibiotic in different organic solvents was first investigated in order to find out a suitable solvent. The solvents used were petroleum ether, n-butanol, chloroform and benzene. For solvent-solvent partitioning of product, the fermented product was gently



## OPTIMIZATION OF CULTURING CONDITIONS FOR THE PRODUCTION OF ANTIMICROBIAL METABOLITE BY *BACILLUS CAROTARUM*

shaken in a separating funnel with almost equal volume of pure petroleum ether, which is immiscible with aqueous alcohol. The mixture was then kept for several minutes for separating the organic layer from the aqueous phase. The materials of the crude extract were partitioned between the two phases depending on their affinity towards their respective solvents. The organic layer was separated and collected in a conical flask and the process was repeated thrice. After collection of organic phase, the aqueous phase thus obtained was further extracted with other organic solvents such as n-butanol, chloroform and benzene in the same way usually of increasing polarity. Finally, all the fractions were collected separately and dried and tested their anti-microbial efficacy.

### *Paper chromatographic analysis*

Homogeneity of the crude bioactive metabolites was tested by paper chromatographic analysis using four types of solvent system. These are-n-butanol : acetic acid : water = 4 : 1 : 5, acetone : acetic acid : water = 50 : 3 : 47, chloroform : methanol = 2 : 1 and benzene : methanol = 9 : 1. Then this paper was assayed for bioactive agent production. After that Rf value of active spot was determined.

## RESULTS AND DISCUSSION

Primarily, soil samples were collected from different forests and farm yards in Chittagong, Bangladesh. Then the soil samples were examined by pour plating technique on two different media namely- nutrient agar medium and starch casein medium to find out the presence of any antagonistic microorganisms. Following incubation, 15 antagonistic bacteria surrounded by a zone of inhibition and the inhibited ones were isolated. These were purified and preserved and the inhibiting isolates were tested for their antimicrobial activity.

### *Primary screening of the isolates*

Based on their better antimicrobial activity against 9 pathogenic test organisms in nutrient agar medium, the bacterial isolate R12 was finally selected for detailed study. The sensitivity spectrum analyses test on nutrient agar medium revealed that the antimicrobial agent produced by the isolate *Bacillus carotarum* was active strongly against *Salmonella typhi*, *Shigella dysenteriae*, *Bacillus subtilis*, *Bacillus cereus*, and weakly against *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Bacillus megaterium* and inactive against *Escherichia coli* and *Staphylococcus aureus*. The results of screening revealed that Gram-positive bacteria are more susceptible to bioactive agents than Gram-negative ones. The outer membrane of Gram-negative bacteria serves as effective barrier (Nikaido 1999), while Gram-positive bacteria lack outer membrane. Hence, the outer membrane of Gram-negative bacteria might be the determinant of their less susceptibility to the bioactive agents.

### *Identification of selected isolate*

On the basis of their morphological, cultural and biochemical characteristics, the bacterial isolate R12 was found to belong to the genus *Bacillus*. It was provisionally identified as *Bacillus carotarum*. The identification was made according to Bergey's manual of determinative bacteriology (Buchanan and Gibbons 1974).



*Optimization of culture conditions for maximum production of antimicrobial metabolite*

*Optimum incubation period*

During the optimization of various parameters, it was observed that maximum antimicrobial metabolite production was found at 24 hours of incubation suggesting that bacteria secreted bioactive agent in their idiophase (Table 1). It is very closely related to the observation of Awais *et al.* (2007) in *Bacillus* sp.

*Effect of incubation temperature and pH on antimicrobial metabolite production*

The effects of temperature and pH on antimicrobial metabolite production by *Bacillus carotarum* are presented in tables 2 and 3, respectively. The bacterial strain *Bacillus carotarum* exhibited maximum antimicrobial metabolite production at 30°C (Table 2), which is the usual temperature of soil from where the organism was isolated. It also showed maximum antimicrobial activity against *Salmonella typhi* (zone of inhibition 24 mm) at pH 7.0 (Table 3) which coincides with the findings of Yousaf (1997) in *Bacillus* sp.

*Effect of carbon sources on the production of antimicrobial metabolite*

In order to design the effective medium, the roles of different carbon sources were evaluated for their impact on growth and bioactive metabolite production. Among the various carbon sources tested, starch was the best carbon source for bioactive metabolite production which results in maximum activity against *Bacillus subtilis* (zone of inhibition 22.5 mm) (Table 4), whereas in the study of Awais *et al.* (2007), glucose was the best carbon source for the production of bioactive metabolite by *Bacillus* sp.

*Effect of incubation condition (stationary/shaking) on antimicrobial metabolite production*

Stationary incubation condition was found optimum for the production of antimicrobial metabolite as compared to shaking culture (Table 5). It corresponds to the report of Lim *et al.* (2010) in *Phomopsis longicolla*.

*Solvent extraction of the antibiotic from culture filtrate*

The active metabolites from fermented broth of the isolate R12 were extracted in different organic solvents: n-butanol, chloroform, petroleum ether and benzene. Petroleum ether was found to be the best solvent for the extraction of the antibiotic (Table 6). Other solvents were unable to extract the metabolites to detectable level. The possible reason could be the presence of polar functional groups in the metabolites that made them instantly soluble in water than other less polar organic solvent, inadequate shaking of the mixture and lack of appropriate solvent.

*Paper chromatographic analysis*

The solvent system (n-butanol : acetic acid : water = 4 : 1 : 5) was found to be efficient for the separation of bioactive metabolites. One type of active substance was detected which was brown in color and R<sub>f</sub> value of the active substance was found to be 0.94.

OPTIMIZATION OF CULTURING CONDITIONS FOR THE PRODUCTION OF  
ANTIMICROBIAL METABOLITE BY *BACILLUS CAROTARUM*

In this study, an attempt was made to purify partially the antimicrobial metabolite produced by *Bacillus carotarum*. Further purification and identification of that antimicrobial metabolite may make it possible to meet up the growing need of newer antibiotic.

TABLE 1: PRODUCTION OF BIOACTIVE AGENTS BY *BACILLUS CAROTARUM* WITH DIFFERENT INCUBATION PERIODS

Test organism	Inhibition zone (diameter in mm)		
	* 1 day	2 days	3 days
<i>Salmonella typhi</i>	24	23	20
<i>Bacillus subtilis</i>	23	20	20
<i>Bacillus cereus</i>	22	20	20
<i>Shigella dysenteriae</i>	22	19	18
<i>Pseudomonas</i>	20	18	16

Note: Assay method = Agar cup method; Medium used = Nutrient agar;  
\* = Selected for further studies

TABLE 2: PRODUCTION OF BIOACTIVE AGENTS BY *BACILLUS CAROTARUM* WITH INCUBATION TEMPERATURE

Test organism	Inhibition zone (diameter in mm)		
	27 <sup>0</sup> C	*30 <sup>0</sup> C	37 <sup>0</sup> C
<i>Salmonella typhi</i>	20	23	19
<i>Bacillus subtilis</i>	20	22	15
<i>Bacillus cereus</i>	19	23	20
<i>Shigella dysenteriae</i>	20	24	20
<i>Pseudomonas</i>	16	19	18



TABLE 3: PRODUCTION OF BIOACTIVE AGENTS BY *BACILLUS CAROTARUM* WITH DIFFERENT pH

Test organism	Inhibition zone (diameter in mm)		
	pH 6	* pH 7	pH 8
<i>Salmonella typhi</i>	20	24	22
<i>Bacillus subtilis</i>	20	23	21
<i>Bacillus cereus</i>	19	22	21
<i>Shigella dysenteriae</i>	19	22	20
<i>Pseudomonas</i>	16	20	18

TABLE 4: PRODUCTION OF BIOACTIVE AGENTS BY *BACILLUS CAROTARUM* IN PRESENCE OF DIFFERENT CARBON SOURCES

Pathogen	Zone of inhibition (diameter in mm)			
	Glucose	Fructose	Sucrose	* Starch
<i>Bacillus subtilis</i>	22	12	20	22.5
<i>Bacillus cereus</i>	14	15	15	16
<i>Pseudomonas</i>	14	12	15	16
<i>Shigella dysenteriae</i>	19	16	15	18
<i>Salmonella typhi</i>	16	18	15	21

TABLE 5: PRODUCTION OF BIOACTIVE AGENTS BY *BACILLUS CAROTARUM* WITH STATIONARY AND SHAKING CONDITION

Test organism	Inhibition zone (diameter in mm)	
	*Stationary	Shaking
<i>Salmonella typhi</i>	23	20
<i>Bacillus subtilis</i>	21	18
<i>Bacillus cereus</i>	21.5	20
<i>Shigella dysenteriae</i>	22	20
<i>Pseudomonas</i>	20	18



OPTIMIZATION OF CULTURING CONDITIONS FOR THE PRODUCTION OF  
ANTIMICROBIAL METABOLITE BY *BACILLUS CAROTARUM*

TABLE 6: SOLVENT EXTRACTION OF THE ANTIBIOTIC FROM CULTURE FILTRATE OF *BACILLUS CAROTARUM* (THE ACTIVITY WAS EXPRESSED IN TERMS OF DIAMETER OF INHIBITION ZONE IN MM)

Initial color of the medium	Final color of the medium	Test organism	Inhibition zone in diameter in mm							
			n-butanol		*Petroleum ether		Chloroform		Benzene	
			C	T	C	T	C	T	C	T
Light yellow	Yellowish Brown	<i>Salmonella typhi</i>	19	12	—	20	15	—	12	—
		<i>Bacillus subtilis</i>	16	14	—	22	12	—	—	—
		<i>Bacillus cereus</i>	12	11	—	22	13	—	15	—
		<i>Shigella dysenteriae</i>	15	—	—	22	12	—	12	—
		<i>Pseudomonas</i>	15	—	—	20	14	—	12	—

Note: C= Control; and T= Treatment

#### REFERENCES

- ATLAS R.M. 1997. *Principles of Microbiology*. 2<sup>nd</sup> ed., WCB/McGraw-Hill, New York. 751pp.
- AWAIS, M., SHAH, A.A., HAMEED, A. AND HASAN, F. 2007. Isolation, identification and optimization of bacitracin produced by *Bacillus* sp. *Pak. J. Bot.* **39**(4): 1303-1312
- BARRY, A.L. 1976. *The Antimicrobial Susceptibility Test, Principle and Practices*, 4th edition, ELBS, London. 180 pp.
- BUCHANAN, R.E. AND GIBBONS, N.E. 1974. *Bergey's Manual of Determinative Bacteriology*, 8<sup>th</sup> ed. Williams and Wilkins Co. Baltimore. 759 pp.
- BUSH, K. 2004. Why it is important to continue antibacterial drug discovery? *ASM News* **70**: 282-287.
- DAKORA F., AND PHILLIPS, D. 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil* **245**: 35-47.
- FLOREY, H.W., CHAIN, E.B., HEATLY, N.G., JENNININGS, M.A., SAUNDERS, A. G., ABRAHAM, E.P. AND FLOREY, M.E. 1949. *Antibiotics* Oxford University Press. **1**: 273-287.
- KUSTER, E. AND WILLIAMS, S.T. 1964. Selection of media for isolation of *Streptomyces*. *Nature*, London. **202**: 928-929.



- LIM, CHASEUNG, KIM, J., CHOI, J.N., PONNUSAMI, K., JEON, Y., KIM, S., KIM, J. G. AND LEE, S.H. 2010. Identification, fermentation and bioactivity against *Xanthomonas oryzae* of antimicrobial metabolite isolated from *Phomopsis longicolla*. *J. Microbiol. Biotechnol.* **20**(3): 494-500.
- NIKAIDO, H. 1999. *Microdermatology: Cell surface in the interaction of microbes with the external world.* *J. Bacteriol.* **181**: 4-8.
- PELCZAR, M.J. AND REID, R.D. 1974. *Microbiology.* Tata McGraw-Hill Pub. Co. Ltd. pp. 71-72.
- RONDON, M., RAFFEL, S., GOODMAN, R. AND HANDELSMAN. J. 1999. Toward functional genomics in bacteria: analysis of gene expression in *Escherichia coli* from a bacterial artificial chromosome library of *Bacillus cereus*. *Microbiology* **96**: 6451-6455.
- SARKAR, P. AND GOULD, I.M. 2006. Antimicrobial agents are societal drugs: How should this influence prescribing? *Drugs* **66**: 893-901.
- SHLAES, D.M., PROJAN, S.J. AND EDWARDS, J.E. 2004. Antibiotic discovery: state of the state. *ASM News* **70**: 275-281.
- TYLER, V.E., BRADY, L.R. AND ROBBERS, J.E. 1988. *Pharmacognosy.* 9<sup>th</sup> ed. Lea and Febiger. Philadelphia pp. 247-248.
- YOUSAF, M. 1997. *Studies on the cultural conditions for the production of antibiotic bacitracin by B. licheniformis.* PhD Thesis, Islamia University, Bahawalpur, India.

Manuscript received on 17.02.2013; Accepted on 20.09.2013

*The Chittagong University Journal of Biological Sciences, Vol. 8 (1 & 2). Page No. 01-10*