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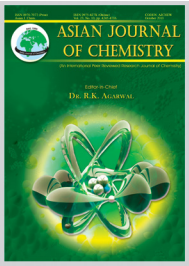

Production Parameters and Taxonomical Studies of *Pseudomonas aeruginosa* (MTCC No. 10620) Isolated From Marine Sponge for The Production of Antimicrobial Metabolites

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The culture conditions were optimizing for the production of antimicrobial metabolites by *Pseudomonas aeruginosa*. The metabolite production was started after 24 h of incubation and reached its maximum levels after 72 h and decreased gradually. The pH was adjusted to 7.0 and temperature to 35 ºC supported the production of antimicrobial metabolites. The best proved carbon and nitrogen sources were sucrose and peptone, respectively as basal medium. The other aim of study was to identify and characterize by its morphological, physiological and biochemical studies and deposited to Microbial Type Culture Collection and Gene bank, IMTECH, Chandigarh, India. *Pseudomonas aeruginosa* is also mesophilic, alkaliphilic and moderate salt tolerance in nature. It utilize carbon sources like dextrose, galactose, mannitol, xylose, mannose and showed positive results to citrate utilization, casein hydrolysis, cytrochrome oxidase, catalase test, gelatin hydrolysis and arginine dihydrolase. The results indicated that the natural marine environment is also good sources for isolation of novel varieties of antagonistic bacteria.

Key Words: *Pseudomonas aeruginosa*, Optimization, Taxonomy, Antimicrobial metabolites, Marine sponges.

INTRODUCTION

Pseudomonas aeruginosa is a gram-positive bacterium, rods with high mol % A + T content. These are circular in configuration, motile, surface moist and arranged in pairs. They are taxonomically diverse, biologically active and colonize all marine habitats, from deep oceans to the shallow west estuaries[1]. Among the population from marine sponges, *Pseudomonas* species are reported to be most abundant forms. *Pseudomonas* strains is of major interest in bacteriocin research since this genus produces a diverse array of antimicrobial peptides with several different basis chemical structures[2,3]. They are the producers of most of the known bioactive metabolites. They include numerous potentially useful compounds providing the widest range and most promising array of pharmacologically and agriculturally active compounds. There are wide spread in nature and can be found in greater or less frequency in most ecological niche[4]. They are widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics[5].

The nutritional source like carbon, nitrogen and minerals, the environmental factors such as time, temperature and pH are found to have profound influence on antibiotic production by *Pseudomonas*. Optimization of the culture conditions is essential to get high yields of the metabolites. Hence, an attempt was made to optimize the nutritional levels as well as pH and temperature requirements of *Pseudomonas aeruginosa* for the production of antimicrobial metabolites. Competition among microbes for space and nutrient in marine environment is a powerful selection pressure that endows marine micro-organisms to produce marine natural products possessing medical and industrial values[6]. Experimental designs are excellent techniques for optimization of culture conditions to achieve optimal production[7].

In the present study, we have designed an optimization strategy to study the influence of the physical and chemical conditions on the culture medium upon biosynthesis of bioactive molecules are reported.

It is perhaps not surprising that novel marine bacteria are providing to be such a valuable source of new bioactive compounds[8] as bacteria systematic is providing a taxonomic road map to genes hence products, including the discovery of first-in-class drug candidates[9,10].

The main aim of this present study was to isolation, identification and characterization potent antagonistic alkaliphilic marine sponge bacteria from Visakhapatnam coast of Bay of Bengal and to screen for their antagonistic activity against selective human pathogenic microorganisms. The strains was identified and deposited at Microbial Type Culture Collection and Gene bank, IMTECH, Chandigarh, India.

EXPERIMENTAL

Pseudomonas strain was isolated from the marine sponge of Bay of Bengal and the culture was identified as *Pseudomonas aeruginosa* (MTCC No. 10620) that closely related to many *Pseudomonas* species by 16S rRNA analysis. Pure culture of the strain was maintained on nutrient agar medium.

Time of incubation (0-144 h), pH (5, 6, 7, 8, 9), temperature (15-45 ºC) and carbon and nitrogen concentration (1-5 %) in the production medium were optimized for maximum production of antibiotic by *P. aeruginosa*. The strain was incubated at 30 ºC in an orbital shaker at 150 rpm and the samples were taken after every 24 h. Cell free supernatant was tested against the previously chosen indicator organisms using agar well diffusion technique aiming to obtain the highest productivity[11].

Effect of incubation period: The fermentations were run in Shake-flasks containing nutrient broth and incubated at room temperature for optimum yields on rotator shaker operating at 110 rpm. At every 24 h interval, the flasks were harvested and centrifuged. The production was determined for the antimicrobial activity. The culture filtrate was extracted with ethyl acetate by using separating funnel and the extract was concentrated and tested for antimicrobial spectrum. The concentrated solvent extract (50 µL) was tested for antimicrobial activity by employing agar diffusion method against the test organisms like *Bacillus subtilis* MTCC (441), *Staphylococcus aureus* MTCC 3160, *Pseudomonas aeruginosa* MTCC 424, *Escherichia coli* MTCC 443, *Bacillus cereus* MTCC 430, *Proteus vulgaris* MTCC 429, *Candida albicans* MTCC 229, *Aspergillus flavus* MTCC 3396, *Aspergillus niger* MTCC 961, *Saccromyces cervecea* MTCC 170.

Impact of pH and temperature on the production of bioactive metabolites: The effect of pH and temperature on the antimicrobial metabolites production by the *Pseudomonas aeruginosa* was studied by inoculating 24 h old culture in nutrient broth. Effect of different ranges of pH (5-9) and temperature (15-45 ºC) on the production of antimicrobial metabolites were examined after 72 h of incubation.

Effect of carbon and nitrogen sources on antimicrobial metabolites production: Different carbon sources like arabinose, dextrose, fructose, galactose, glycerol, inosine, lactose, maltose, mannitol, mannose, sucrose and trehalose were added to the nutrient broth in 1 % concentration at pH 7. The growth in the presence of sodium chloride was determined[12] and the growth of 12 carbon sources was determined on carbon utilization agar (ISP Medium 9; Difco) as described[13]. Ammonium chloride, $NaNO_3$, KNO_3, L-aspargine, L-glutamine, tyrosine, casein, peptone, soybean meal yeast extract was studied by adding 0.2 % to the nutrient broth at pH 7.

Morphological and physiological characterization: The potential isolate *Pseudomonas* were carried out for gram staining type, shape and size by under light microscope[14] and cultural characteristics such as colony morphology like elevation, surface, density and pigment production was carried out[15]. Physiological characterization of both strains was carried out by performing the growth at different temperatures range from 4 to 65 ºC, pH range from 5 to 11 and the growth under anaerobic condition.

Sodium chloride tolerance: Sodium chloride tolerance level of both the strains was evaluated on nutrient agar medium supplemented with graded doses of sodium chloride (2.5, 5.0, 7.0, 8.5, 10.0 % w/v). Maximum sodium chloride concentration in the medium allowing any growth was recorded.

Biochemical characterization: Various biochemical tests were performed for the identification of the potential isolate *Pseudomonas aeruginosa*. These tests includes growth on MacConkey agar, indole test, methyl red test, voges proskauer test, citrate utilization, casein hydrolysis, starch hydrolysis, urea hydrolysis, nitrate reduction, H_2S production, cytochrome oxidase, oxidation/fermentation, gelatin hydrolysis, arginine dihydrolase and lysine decarboxylase. To determine the production of acids by utilizing the different sources of carbohydrates like adonitol, inulin, cellobiose, dextrose, dulcitol, fructose, galactose, inositol, lactose, maltose, mannitol, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, xylose and mannose were tested[16].

RESULTS AND DISCUSSION

Pseudomonas aeruginosa reached to maximum cell growth after 24 h. Antimicrbial microbial production by the strain was started after 24 h of incubation and reached to high levels after 72 h of incubation and thereafter gradually declined its production is represented in Fig. 1. The effect of pH and temperature on antimicrobial metabolite production by the strain is presented in the Figs. 2 and 3). The optimum pH for antibiotic production was 7. The strain showed high levels of antibiotic production when culture medium incubated at 30 ºC. The strain was found to be strictly mesophilic for secondary metabolites production; extreme pH and temperature were unfavourable for antibiotic production. Temperature is also an important regulator of the growth rate of microorganisms. A shift in temperature can alter the utilization rate of one component as compared to another, thus unbalancing the medium with respect to growth[17].

The impact of different carbon sources on antibiotic production by the strain is presented in Fig. 4. Among all the carbon sources, sucrose amended basal medium proved to be

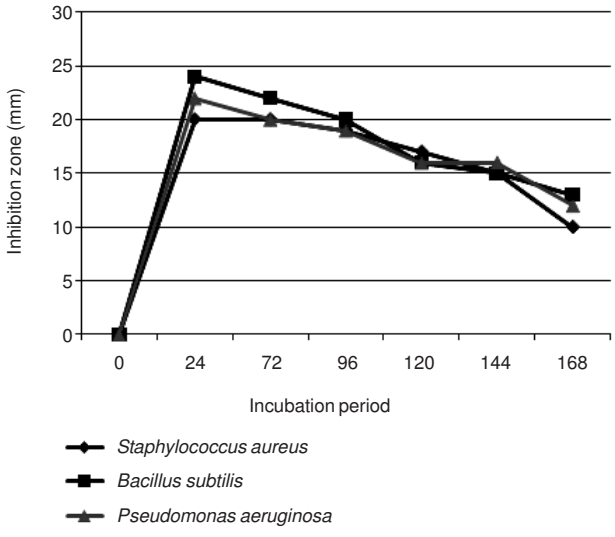

Fig. 1. Effect of incubation period on antibiotic production by *Pseudomonas aeruginosa*

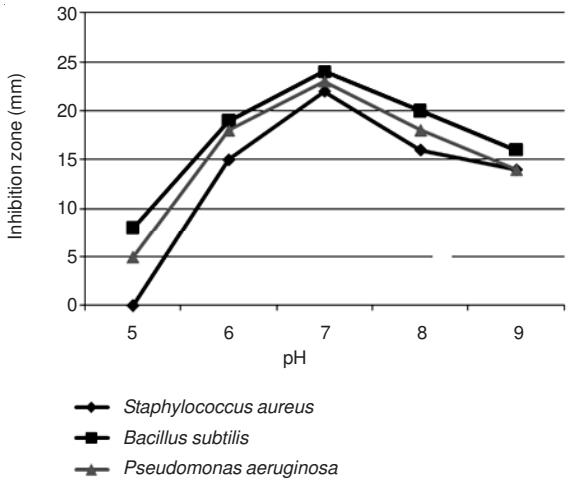

Fig. 2. Impact of pH on antimicrobial metabolites production by *Pseudomonas aeruginosa*

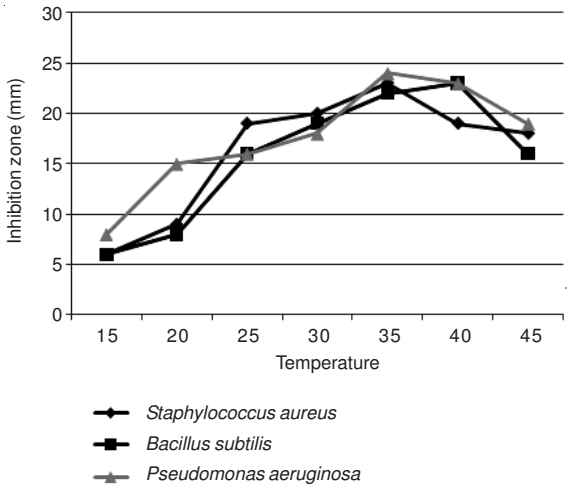

Fig. 3. Effect of temperature on antimicrobial metabolites production by *Pseudomonas aeruginosa*

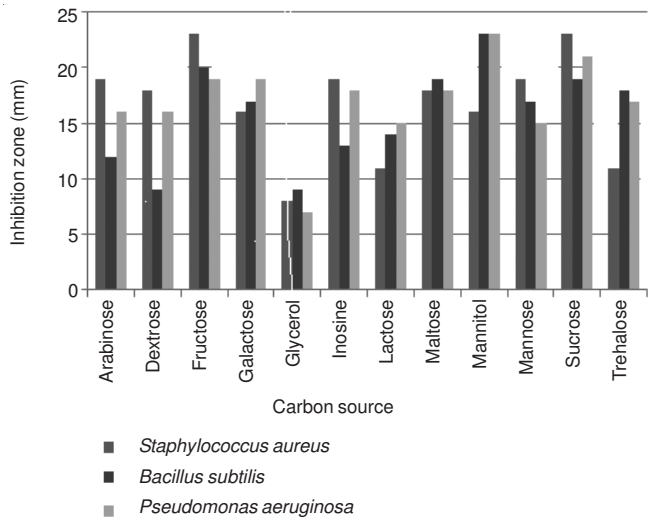

Fig. 4. Role of different carbon sources on antibiotic production by *Pseudomonas aeruginosa*

TABLE-1
INFLUENCE OF DIFFERENT NITROGEN SOURCES ON ANTIBIOTIC PRODUCTION BY *Pseudomonas aeruginosa*

Nitrogen sources (0.2 %)	Diameter of growth inhibition zone (mm)		
	Staphylococcus aureus (MTCC 3160)	*Pseudomonas aeruginosa* (MTCC 424)	*Bacillus cereus* (MTCC 430)
$(NH_4)_2SO_4$	14	12	16
NH_4Cl	10	12	17
$NaNO_3$	15	19	20
KNO_3	20	23	24
L-Aspargine	17	15	20
L-Glutamine	14	17	16
Tyrosine	15	18	14
Casein	16	14	19
Peptone	22	24	21
Soyabean meal	19	21	18
Yeast extract	23	19	16

the best for cell growth as well as antibiotic production by the strain followed by fructose, galactose, inosine. Carbon sources like lactose, maltose, trehalose were found to be moderately supported the antibiotic production by the strain.

Antibiotic production was totally absent in the medium supplemented with dextrose, glycerol, mannitol and mannose as sole carbon source. Carbohydrates such as glycerol, maltose, mannose, sucrose and xylose have been reported to interfere with the production of secondary metabolites[18]. In the present study, the strain was found to produce high levels of antimicrobial metabolites in the medium supplemented with sucrose as sole carbon source.

Data on the effect of nitrogen sources on antimicrobial metabolite production by the strain is given in Table-1. Organic nitrogen sources were the best nitrogen sources for the antibiotic production by the strain than inorganic nitrogen source. Medium supplemented with peptone was found to be suitable for maximum antimicrobial metabolite production followed by yeast extract, aspargine, $NaNO_3$ and casein. The yeast extract is favourable for growth but not the antibiotic production[19]. The greatest similarity between the predicted (100 % suppression) and the observed results (98.2 %) proves the accuracy of the model and its application validity as previously revealed by other workers[20].

Marine organisms are a rich source of structurally novel and biologically active metabolites[21]. Although, marine microorganisms have been increasingly of interest as a source of new bioactive molecules, a great percentage of them have not been described[22]. To discover novel by-products from marine environments, maintanence of not simply abundant but diverse microorganisms is necessary[23].

Pseudomonas species are widely distributed in nature and have remarkable ability to survive strong environmental stresses[24]. Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry[25]. Moreover, members of the genus *Pseudomonas* can be easily isolated from aquatic habitats and marine ecosystems[26]. To date, many chemically unique compounds of marine origin with various biological activities have been isolated and some of them are under investigation and are being used to develop new pharmaceuticals[27]. Bacteria belonging to the genus *Pseudomonas* have a long and distinguished history in the field of biotechnology[28]. *Pseudomonas* species produce many kinds of antibiotics which share a full range of antimicrobial activity such as bacitracin, pumulin and gramicidin[29]. Bacitracin production by *Pseudomonas aeruginosa* is pH dependent and that the inhibitory effect of glucose is

TABLE-2
IDENTIFICATION RESULTS FOR MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL TESTS BY MTCC:

Test	Results
Colony morphology	*Pseudomonas aeruginosa*
Configuration	Circular
Surface	Moist
Pigments	Greenish
Shape	Rods
Size	Short
Gram's reaction	–
Spore	–
Motility	+
Temperature (ºC)	15-37
pH	5-9
NaCl (%)	2.5-5.0
Bio-chemical tests	
Growth on Mac-conkey agar	+
Indole test	–
Methyl red test	–
Voges proskauer test	–
Citrate utilization test	+
Casein hydrolysis	+
Starch hydrolysis	–
Urea hydrolysis	–
Nitrate reduction	–
H_2S production	–
Cytochrome oxidase	+
Catalase test	+
Oxidation/fermentation	O
Gelatin hydrolysis	+
Arginine dihydrolase	–
Lysine decarboxylase	–

Acid production from carbohydrates:			
Adonitol	–	Melibiose	+
Inulin	–	Raffinose	–
Cellobiose	–	Rhamnose	–
Dextrose	+	Salicin	–
Dulcitol	–	Sorbitol	–
Fructose	–	Sucrose	–
Galactose	+	Trehalose	–
Inositol	–	Xylose	+
Lactose	–	Mannose	+
Maltose	–		

+ = Positive reaction; – Negative reaction

due to acidification as a result of the accumulation of organic acids reported[30]. This will proof that the use of this optimization strategy was powerful for achieving our goal.

Morphological and physiological characterization: *Pseudomonas aeruginosa* was isolated on nutrient agar medium. *Pseudomonas aeruginosa* is gram-negative, short, rod shaped, produces greenish pigmentation. It exhibited optimum growth under aerobic conditions at temperature 37 ºC and pH at 7. It was mesophilic and alkaliphilic in nature which showed growth range from 15 to 42 ºC.

Sodium chloride tolerance: Optimum growth was observed at 2.5 % (w/v) sodium chloride, but maximum tolerance of sodium chloride concentration was exhibited growth upto to 5.0 % (w/v) for *Pseudomonas*, indicating it was indigenous to marine environment and moderate salt tolerance in nature.

Biochemical characterization: *Pseudomonas aeruginosa* could utilize, dextrose, mannitol, melibiose, xylose, mannose as the carbon source along with acid production; however, adonitol, inulin, cellobiose, dulcitol, fructose, inositol, lactose, maltose, raffinose, rhamnose, salicin, sorbitol, sucrose and trehalose were not utilized by the organism and there is growth occured on MacConkey agar. The biochemical tests like citrate utilization, casein hydrolysis, cytochrome oxidase, catalase test, gelatin hydrolysis and Arginine dihydrolase were positive, but indole test, methyl red test, voges proskauer test, starch hydrolysis, urea hydrolysis, nitrate reduction, H_2S production, lysine decarboxylase were negative.

It is concluded that the marine bacteria *Pseudomonas aeruginosa* have the potentiality to produce highly effective bioactive compounds which must be applied in the production of pharmaceutical agents.

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