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Optimization of Marine Sponge Isolated Bacterium *Bacillus subtilis* (MTCC No. 10619) for the Production of Antimicrobial Metabolites

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The culture conditions were optimizing for the production of antimicrobial metabolites by *Bacillus subtilis*. 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.

Key Words: *Bacillus subtilis*, Optimization, Antimicrobial metabolites, Marine sponges.

INTRODUCTION

Bacillus subtilis 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, *Bacillus* species are reported to be most abundant forms. *Bacillus* 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 micro-organisms 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 *Bacillus*. 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 *Bacillus subtilis* 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 medicinal and industrial values[6]. Experimental designs are excellent techniques for optimization of culture conditions to achieve optimal production[7]. The production of 167 peptide antibiotics from *Bacillus subtilis* and *Bacillus brevis* was reported by Berdy[8]. Of this total, 66 different peptide antibiotics are elaborated by strains of *Bacillus subtilis* and 23 are products of *Bacillus brevis*.

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.

EXPERIMENTAL

Bacillus strain was isolated from the marine sponge of Bay of Bengal and the culture was identified as *Bacillus subtilis* (MTCC No. 10619) that closely related to *Bacillus amyloliqueficans* by 16S rRNA analysis. Pure culture of the strain was maintained on nutrient agar medium.

Time of incubation (0-144 h), pH (5-9), temperature (15-45 ºC) and carbon and nitrogen concentration (1-5 %) in the production medium were optimized for maximum production of antibiotic by *B. subtilis*. 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[9].

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 (15 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.

Effect of pH and temperature on the production of bioactive metabolites: The effect of pH and temperature on the antimicrobial metabolites production by the *Bacillus subtilis* 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 7.0. The growth in presence of sodium chloride was determined[10] and the growth of 12 carbon sources was determined on carbon utilization agar (ISP medium 9; Difco) as described by Williams[11]. 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 7.0.

RESULTS AND DISCUSSION

Bacillus subtilis 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 Figs. 2 and 3. The optimum pH for antibiotic production was 7.0. 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[12].

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 the best for cell growth as well as antibiotic production by the strain followed by fructose, galactose and 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

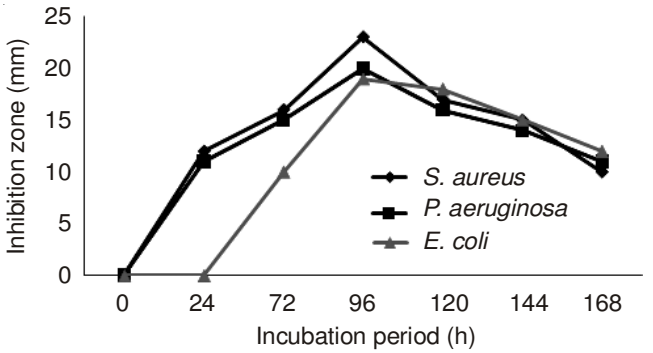

Fig. 1. Effect of incubation period on antibiotic production by *Bacillus subtilis*

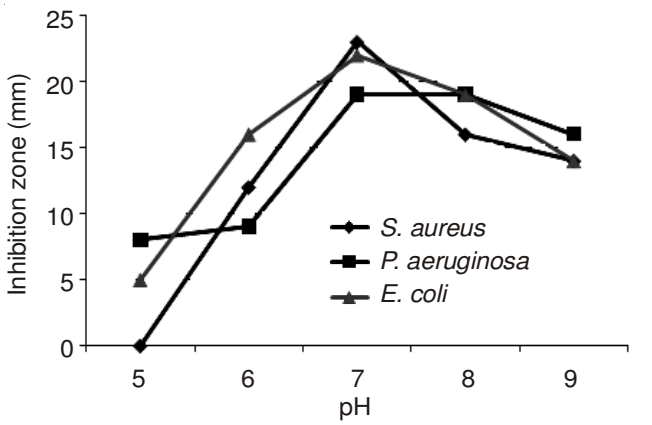

Fig. 2. Effect of pH on antimicrobial metabolites production by *Bacillus subtilis*

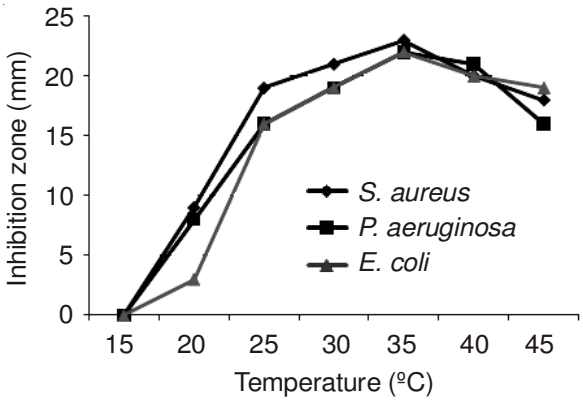

Fig. 3. Effect of temperature on antimicrobial metabolites production by *Bacillus subtilis*

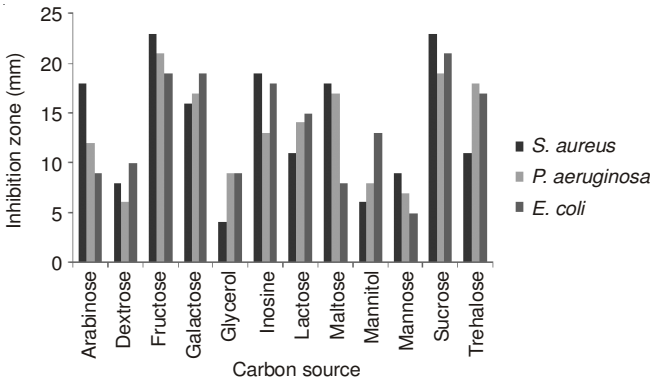

Fig. 4. Role of different carbon sources on antibiotic production by *Bacillus subtilis*

as sole carbon source. Carbohydrates such as glycerol, maltose, mannose, sucrose and xylose have been reported to interfere with the production of secondary metabolites[13]. In 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[14]. 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 revealed by previous workers[15].

TABLE-1
INFLUENCE OF DIFFERENT NITROGEN SOURCES ON ANTIBIOTIC PRODUCTION BY *Bacillus subtilis*

Nitrogen source (0.2 %)	Diameter of growth inhibition zone (mm)		
	S. aureus	*P. aeruginosa*	*E. coli*
$(NH_4)_2SO_4$	4	9	8
NH_4Cl	9	12	7
$NaNO_3$	12	11	16
KNO_3	2	6	8
L-aspargine	11	15	14
L-glutamine	4	7	6
Tyrosine	5	8	4
Casein	16	14	12
Peptone	23	22	21
Soyabean meal	9	6	8
Yeast extract	23	19	16

Marine organisms are a rich source of structurally novel and biologically active metabolites[16]. Although, marine microorganisms have been increasingly of interest as a source of new bioactive molecules, a great percentage of them have not been described[17]. To discover novel by-products from marine environments, maintanence of not simply abundant but diverse microorganisms is necessary[18].

Bacillus species are widely distributed in nature and have remarkable ability to survive strong environmental stresses[19]. Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry[20]. Moreover, members of the genus *Bacillus* can be easily isolated from aquatic habitats and marine ecosystems[21]. 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[22]. Bacteria belonging to the genus *Bacillus* have a long and distinguished history in the field of biotechnology[23]. *Bacillus* species produce many kinds of antibiotics which share a full range of antimicrobial activity such as bacitracin, pumulin and gramicidin[24]. Bacitracin production by *Bacillus subtilis* is pH dependent and that the inhibitory effect of glucose is due to acidification as a result of the accumulation of organic acids reported[25]. This will proof that the use of this optimization strategy was powerful for achieving our goal.

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

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