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OPTIMIZATION OF PHYSICAL AND NUTRIENT FACTORS FOR SECONDARY METABOLITES PRODUCTION BY A MARINE PSEUDOALTEROMONAS SP. AND ITS BIOLOGICAL ACTIVITY AGAINST ENTEROBACTER SP.

Microbiology

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ABSTRACT

Antibiotic resistance in bacteria has become a serious problem worldwide. Hence the search for novel antibiotic is significant in drug discovery. The aim of the present study was to screen secondary metabolites producing bacteria from marine origin and to optimize the process parameter for the potent bacterial isolate. The most active bacterial isolate which showed high zone of inhibition was identified as *Pseudoalteromonas* sp. The process parameters such as, carbon source, nitrogen source, pH, temperature and fermentation period was optimized by one-variable-at- a-time-approach. It was found that secondary metabolite production by this bacterial isolate was significantly influenced by nutritive and physical factors. The optimum carbon source was fructose, whereas, yeast extract was found to be the optimum nitrogen source. The secondary metabolite production was optimum at pH 7.0, at 35 ° C, and secondary metabolite production was high after 72 h of incubation.

KEYWORDS

Bacteria, *Pseudoalteromonas* sp., secondary metabolites; optimization

INTRODUCTION

The genus *Pseudoalteromonas* sp. are heterotrophic, Gram-negative and aerobic bacteria with a flagellum (polar) and has high GC content (38% to 50%)[1]. *Pseudoalteromonas* sp. belongs to the order Alteromonadales and the strains require seawater base for growth and metabolism and are therefore true marine bacteria. More than 41 species and over 3772 *Pseudoalteromonas* strains are currently explored and listed in the NCBI browser. *Pseudoalteromonas* strains are also associated with algae or healthy animals. However, few strains of *Pseudoalteromonas are known as* opportunistic or pathogenic. *Flavobacterium piscicida* was reclassified as *P. piscicida*[2] and involved in flavobacteriosis in fishes in intensive farming[3].

In marine microorganisms, secondary metabolites are mainly comprised of isoprene-derived (13%), acetate- (30%) and nitrogenated molecules (56%)[4]. On the basis of their pathways, they are classified as peptides or alkaloids, terpenoids and polyketides[5]. In *Pseudoalteromonas, a*lkaloids are biosynthesized from amino acids. The alkaloids are mainly made of heterocyclic incorporated basic amine nitrogen. Mainly derived from α-amino acids such as Phe, Lys, Tyr and Trp, the biosynthesis pathways of alkaloids remain poorly understood[6]. Bacteriocin-Like Inhibitory Substances (BLIS) and Bacteriocins are proteinaceous antibacterial compounds. These compounds are ribosomally synthesized and exhibit an antibacterial activity, generally limited to very closely related strains. In *Pseudoalteromonas*, most of the antibacterial compounds are large proteins (MW > 100 kDa).

Various *Pseudoalteromonas* strains have shown an antimicrobial activity and little information is available on the structure of the bioactive compounds[7]. There are about 41 *Pseudoalteromonas* species were described and 39% of organisms have been shown to produce antimicrobial/ secondary metabolites. The antimicrobial metabolites produced by *Pseudoalteromonas* sp. protect their host against various pathogens[8]. Recently, novel antibiotics were isolated from five marine bacteria including genera of *Pseudoalteromonas, Marinospora*, *Bacillus, Streptomyces and Pesudomonas*[9]. Considering the importance of secondary metabolite from the marine source, an attempt was made to screen antibiotic producing bacterial isolate.

MATERIALS AND METHODS

Bacterial strain

Five bacterial strains were isolated from the marine environment previously for the production of hydrolytic enzymes. These organisms were screened for the production of secondary metabolites[10]. Among the screened five bacterial isolates, *Psudoalteromonas* sp. was selected for further optimization. The 612 bp 16S rDNA sequence of this bacterial isolate has been submitted previously to the GenBank and assigned accession number Kf683956.

Screening of isolates for antibacterial activity

All five bacterial isolates from marine environment were screened for the production of antimicrobial secondary metabolites. Initially pure colonies were inoculated individually in marine broth medium and incubated at 30 ± 2 °C for 3 to 4 days. Then the culture medium was centrifuged at 10000 rpm for 15 min at 4 °C. The cell free culture supernatant was filtered (0.22 µm pore size) and antibacterial activity was assayed. Antibacterial activity of the culture supernatant was assayed by well diffusion test against *Enterobacter* sp. *Vancomycin was used as the standard antibiotic (30 µg).* Tested bacteria were cultured in nutrient broth (Himedia, Mumbai, India) at 37 °C for 18 h. 100 µl of this bacterial suspension was inoculated on Mueller Hinton Agar plates as cotton swab. The well was cut in these plates and 20 µl of culture supernatant of the bacterial isolate was placed. All plates were incubated at 37 °C for 24 h and the zone of inhibition around each well was measured.

Optimization of secondary metabolite production

The present study was conducted to evaluate the most suitable medium for secondary metabolite production by *Pseudoalteromonas* sp. Many process parameters were optimized for the maximum production of secondary metabolites. The factors such as, carbon sources, nitrogen source, pH, temperature and incubation period were optimized. The yield of metabolites by the strains was evaluated. The medium in which the selected bacterial isolate exhibits optimum level of metabolites was fixed for further studies.

Effect of carbon source on secondary metabolite production

To study the influence of carbon sources on the production of bioactive metabolites of the strain, the basal medium was supplemented with different carbon sources such as glycerol, glucose, galactose, dextrose, fructose and starch at a level of 1% (w/v) by keeping the other ingredients constant. The culture medium without any of the above carbon sources was treated as control. After 96 h, the yield of secondary metabolites by the *Pseudoalteromonas* sp. was tested from the culture supernatant.

Effect of nitrogen source on secondary metabolite production

To study the influence of nitrogen sources on cell growth and the production of bioactive metabolites of the strain, the basal medium was supplemented with different nitrogen sources such as peptone, yeast extract, casein, tryptone and beef extract at a level of 1% (w/v) by keeping the other ingredients constant. The culture medium without any of the above nitrogen sources was treated as control. After 96 h, the yield of secondary metabolites by *Pseudoalteromonas* sp. was tested from the culture supernatant.

Effect of pH on secondary metabolite production

To study the influence of pH on the production of secondary metabolites of the *Pseudoalteromonas* sp. it was cultured at various pH values (pH 5.0, 6.0, 7.0, 8.0 and 9.0) by keeping the other factors constant. After 96 h, the yield of secondary metabolites by *Pseudoalteromonas* sp. was tested from the culture supernatant.

Effect of temperature on secondary metabolite production

To study the influence of temperature on the production of secondary metabolite of *Pseudoalteromonas* sp., it was cultured at various temperatures (20 to 45 °C) by keeping the other factors constant. After 96 h, the yield of secondary metabolites by the strain was tested from the culture supernatant.

Effect of fermentation period on secondary metabolites production

To study the influence of fermentation period on the production of secondary metabolites of *Pseudoalteromonas* sp. This organism was inoculated into the medium and was incubated at various fermentation periods (24, 48, 72 and 96 h) by keeping the other factors constant. After every 24 h, the yield of secondary metabolites by the *Pseudoalteromonas* sp., was tested from the culture supernatant.

RESULTS AND DISCUSSION

In bacteria various process parameters significantly influence the production of secondary metabolites. Both environmental and nutritional parameters like carbon sources, nitrogen sources, pH, temperature and incubation period, have significant effect on the production of secondary metabolites. Also the combination of media components influences metabolite production[11]. Hence, to increase the yield of secondary metabolite, one prerequisite is to design and optimize the fermentation process which also helps to reduce the cost of fermentation process. Compared with terrestrial organisms, the secondary metabolites from marine bacteria have novel and unique structures owing to the complex living circumstance and diversity, and the bioactivities are much effective[12]. In the present study five bacterial isolates from marine sources were screened for secondary metabolite production. Among various bacteria, *Pseudoalteromonas* sp. showed maximum secondary metabolite production. It showed 25 mm zone of inhibition against *Enterobacter* sp. This zone of inhibition was found to be high than other tested bacterial isolates (Fig. 1). Hence this organism was used for optimization studies.



Figure 1 - Screening of secondary metabolites production by the marine bacterial isolates against *Enterobacter* sp. Among five bacterial strain *Pseudoalteromonas* sp., showed good activity (well 3) with 25 mm zone of inhibition against *Enterobacter* sp.

Carbon sources

In bacteria secondary metabolite production is regulated by nutrients, including carbon sources. Among the nutrients, the effect of carbon sources on secondary metabolite production has been the important subject of continuous study for research and industry groups, not only from fermentation point of view, but also from molecular and biochemical point of view. The carbon sources are the important constituents to be considered which are reported widely to have influenced on the secondary metabolite production by various bacteria[13]. It has been stated that secondary metabolite production by bacteria is often stimulated by slowly assimilated highly complex carbon sources like polysaccharides. The effect of carbon sources on the production of secondary metabolites was measured by supplementing various sources. Among the carbon sources tested, fructose (24 mm) significantly influenced on secondary metabolite production, followed by glycerol (18 mm) (Fig. 2). In fermentation process, optimal production has been achieved by cultivating bacteria in media containing slowly utilized nutrient sources. Starch was found to be the best carbon source for secondary metabolite production and was reported previously[14].

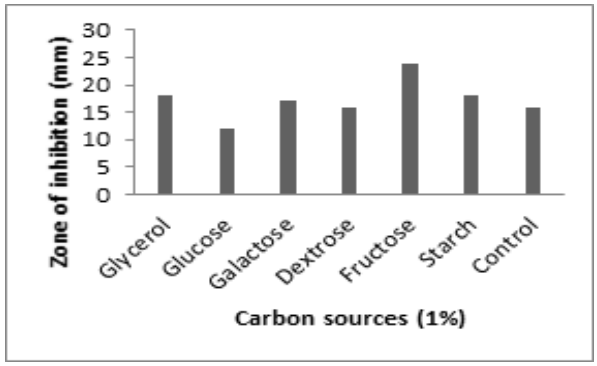

Figure 2 - Effect of carbon sources on secondary metabolite production by *Pseudoalteromonas* sp.

Nitrogen sources

The selection of suitable nitrogen source and its optimum concentration in the culture media play a critical role in secondary metabolite production. The bacteria can utilize both organic and/ or inorganic sources of nitrogen. The use of specific amino acids can increase the productivity in some cases and it was unsuitable in some cases and may inhibit the synthesis of secondary metabolites[15]. Bacterial growth and production of secondary metabolites are influenced by various nitrogen sources. Culture medium with various nitrogen sources was found to stimulate significant amount of secondary metabolite production. The results of the present study clearly indicated that the significant level of secondary metabolites was found with yeast extract (25 mm) which is much higher than other selected nitrogen sources (Fig. 3). Singh et al. [16] optimized actinomycin V production from *Streptomyces triostinicus* and found that production of actinomycin V involves tryptophan pathway and addition of tryptophan to the medium enhances the production of secondary metabolites. It was previously reported that the addition of complex nitrogen sources resulted in a significant increase in secondary metabolite production [17]. Pavl and Banerjee 18 concluded that the organic nitrogen sources produce fair amounts of antibiotic showing very little variation in quantity but the biomass production showed a wide variability. It was also stated that the increased production of bioactive metabolite by *Streptomyces* VITSVK 9 sp. [19] and *Streptomyces cheonanensis* VUK-A[20], when the medium containing peptone as the nitrogen source. Production of secondary metabolites by submerged fermentation can be significantly affected by various physical factors, including pH and temperature[21].

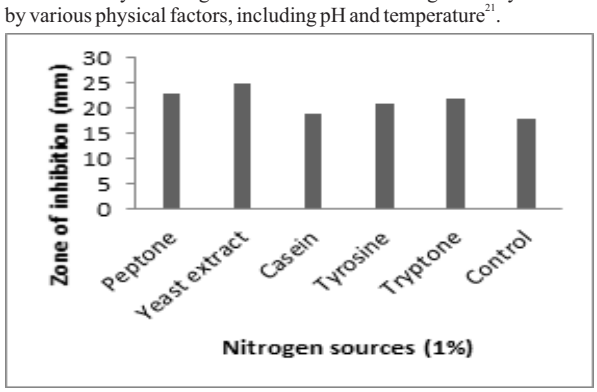

Figure 3 - Effect of nitrogen sources on secondary metabolite production by *Pseudoalteromonas* sp.

Effect of pH

In general, the pH of a culture medium is usually not similar throughout the fermentation bioprocess and the changes of pH occur during the production of secondary metabolites. To elucidate the optimum pH for maximum production of secondary metabolites, *Pseudoalteromonas* sp. was grown in the medium containing various pH values. The optimum pH value for secondary metabolite production by *Pseudoalteromonas* sp. varied widely and was found to

be optimum at pH 7.0 (27 mm zone of inhibition) (Fig. 4). It was earlier reported that the changes in culture pH significantly affect many cellular processes like the regulation of the biosynthesis of secondary metabolites among bacterial species[17]. Chang *et al.* [22] reported that the optimum for metabolite yield from *B. licheniformis* was obtained with culture medium containing pH 7.0. It was earlier reported that the bioprocess factors such as, pH and temperature significantly affect secondary metabolite production[23]. The pH of the culture medium significantly affects the activity of cellular enzymes, ionization of nutrient, and transporting across the cell membranes, subsequently its influence on the bacterial growth rate and secondary metabolites production. The present findings were in agreement with previous works[24, 35] which reported the optimum for antibiotic production from *Streptomyces hygroscopic*, *Streptomyces* species; was found to be pH 7 or between 6.5 and 7.5. In the case of *Streptomyces* isolates, the optimum pH for its growth and production of secondary metabolite was between pH 7.0 and 8.0[26].

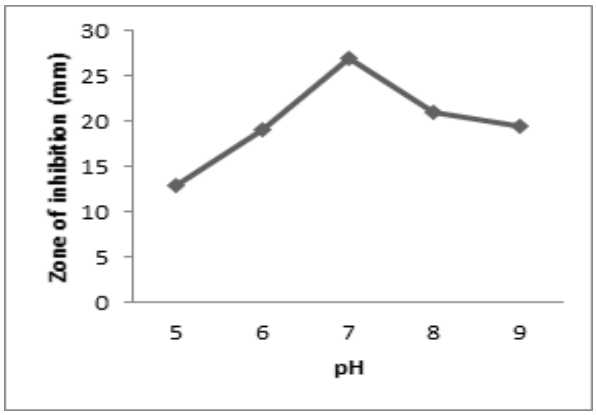

Figure 4 - Effect of pH on secondary metabolite production by *Pseudoalteromonas* sp.

Temperature

To evaluate the optimum temperature for the production of secondary metabolites, a wide range of temperature (25 °C to 45 °C) was used in the present study. This is the important process parameter which directly influences the growth and production of secondary metabolites. The incubation temperature 35 °C was found to be optimum for secondary metabolite production and the zone of inhibition was 29 mm (Fig. 5). Awais *et al*. [27] reported that maximum inhibition was observed by and *Bacillus pumilus* and *Bacillus subtilis* strains at 30ºC against *S. aureus* and *M. luteus*. Saha *et al*. [28] evaluated the optimum conditions of antimicrobial secondary metabolite production from S*treptomyces* sp. MNK7 and the reported optimum temperature was 35°C.

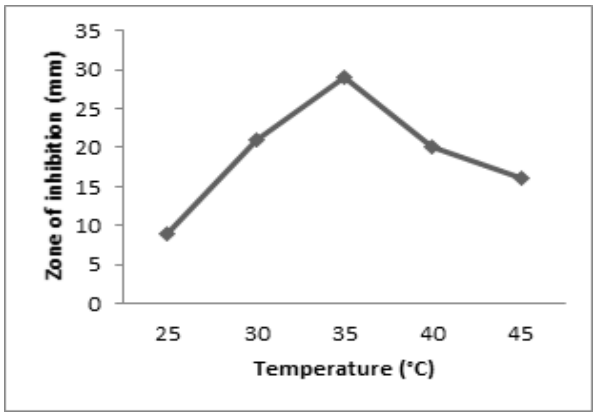

Figure 5 - Effect of temperature on secondary metabolite production by *Pseudoalteromonas* sp.

Fermentation period

Fermentation period determination for any new strain is very important for maximum production of the secondary metabolites because the incubation period required for growth of microorganism and secondary metabolite production. In this study, fermentation process was carried out upto 96 h and was found to be optimum at 72 h of incubation (Fig. 6). This kind of results were reported previously with other bacterial species. In *B. cereus* Bc7, secondary metabolite production was maximum at the early stationary phase[29]. In the case of *B. subtilis* 14B, the production of secondary metabolite started after 24-h of incubation then increased exponentially and reached its maximum level within 96 h in submerged fermentation[30].

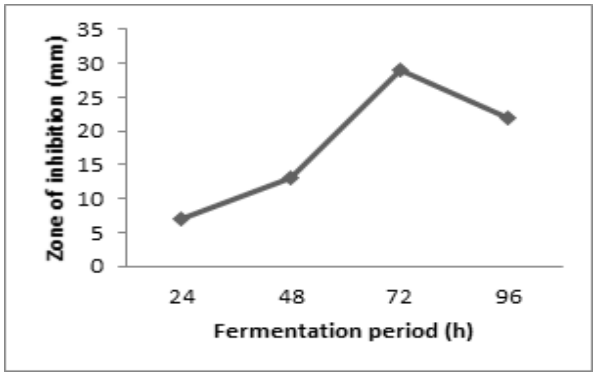

Figure 6 - Effect of fermentation period on secondary metabolite production by *Pseudoalteromonas* sp.

CONCLUSION

It was concluded that, the isolate *Pseudoalteromonas* sp. showed potent antibacterial secondary metabolite. Secondary metabolite production which was increased by optimizing the various environmental and nutritional parameters. The antibacterial metabolite produced by this bacterium could be characterized and used as an antibacterial substance for disease management.

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