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INFLUENCE OF CULTURAL CONDITIONS ON THE PRODUCTION OF ANTIBACTERIAL COMPOUNDS BY MARINE BACTERIA *BACILLUS LICHENIFORMIS* AND *PSEUDOMONAS AEUROGINOSA*

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Abstract: Present study was undertaken to investigate the influence of culture conditions and media components on production of antibacterial compounds by *Bacillus licheniformis* (*B. licheniformis*) and *Pseudomonas aeuroginosa* (*P. aeuroginosa*), which were isolated from marine waters of Chennai and Kothapattanam coastal regions, India. Morphological, biochemical and molecular characteristics suggested that the isolates were a candidate of *B. licheniformis* and *P. aeuroginosa*. These isolates showed strong antimicrobial activity against both Gram-positive and Gram-negative bacteria. *B. licheniformis* and *P. aeuroginosa* showed optimum production of antibacterial compounds at temperature 30°C and pH 7.0. Highest antibacterial activity was obtained when sodium chloride (3%), peptone (1%) and lactose concentration (2%) were used as salt, nitrogen and carbon sources respectively. Separation on silica gel by TLC using ethyl acetate: methanol (6:4 ratio) and direct bioautography showed the presence of two active substances: the more active one with Rf 0.8 than the other with Rf 0.7. In conclusion, the results suggested different culture conditions have a significant impact on the types of secondary metabolites produced by the bacteria.

Keywords: Marine bacteria, culture conditions, optimization, coastal areas and bioactive metabolite.

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INTRODUCTION

Infectious diseases are leading health problems with high morbidity and mortality in developing countries. Emergence of pathogens with multiple drug resistant properties, notable examples being methicillin resistance Staphylococcus aureus (MRSA) and vancomycin resistant Staphylococcus aureus (VRSA) have warranted the need of further research to discover new bioactive compounds to combat these multidrug resistance pathogens. The diversity of marine organisms' species and their complex living environment enable them to produce novel and unique secondary metabolites with much stronger bioactivities than their terrestrial counterparts[1]. Consequently, the past two decades have witnessed intense efforts aimed at discovering novel drugs from the marine species[2]. On this view point, attempts have been made to develop novel drugs against infectious diseases for the mitigation of suffering of the vast masses of humanity[3].

According to Leone *et al*., (2007), the main focus of research interests in marine bacteria is due to their ability to produce several biologically active molecules, such as antibiotics, toxins and antitoxins, antitumor and antimicrobial agents. The marine environment comprises a large reservoir of pristine resources for the discovery of bioactive compounds. Due to highly harsh conditions with regards to physical and chemical composition in marine environment, the organisms produce a variety of molecules with unique structural features and exhibit various biological activities[5].

Numerous antibiotics have been obtained from various microbes isolated from the marine environment played a significant role in the discovery of antimetabolites[6]. Production of the secondary metabolites by the microbes differs in quality and quantity based on the types of strain and also species used[7]. Biosynthesis of the secondary metabolites is influenced by numerous environmental factors including nutrients (nitrogen, phosphorous and carbon sources), growth rate, feedback control, and other physical conditions (oxygen supply, temperature and pH)[8, 9 & 10]. Therefore, the influence of both growth and environmental conditions play important role in the production of the secondary metabolites.

The objective of the present study is to design an appropriate culture medium and also optimize the cultural conditions of *B. licheniformis* and *P. aeruginosa* strains in order to reduce the cost of fermentation process and optimize the yield of antimicrobial compounds.

MATERIALS AND METHODS

Isolation of Bacteria

B. licheniformis and *P. aeuroginosa* were isolated from marine waters of south coastal areas of Andhra Pradesh, India using serial dilution plate technique and further maintained at 4°C. The

16S rRNA sequences of the strains *B. licheniformis* and *P. aeuroginosa* were submitted to the Genbank (Accession numbers were KC736854 and KC736855).

Optimization of culture conditions for the production of antibacterial compounds

For antibacterial metabolite production from *B. licheniformis* and *P. aeruginosa*, the culture conditions were optimized targeting different parameters such as pH, temperature, sources of carbon, nitrogen and concentration of NaCl. For the culture conditions after incubation of the *B. licheniformis* and *P. aeuroginosa* broth was filtered through the Whattman No.1 filter paper and filtrate was kept for evaporation. After evaporation biomass was weighed for production of antibiotic at concentration of mg/ml. Zone of inhibition around the disc was also measured.

Effect of pH and temperature on biomass and antibacterial production

To find out the influence of initial pH on growth and antibacterial production, *B. licheniformis* and *P.aeruginosa* were cultured in the media with different initial pH, ranging from 4.0 to 10.0 and at different starting temperatures, from 20 to 60°C. The biomass and antibacterial production were estimated to determine optimal pH and temperature conditions which were used in this study[11 & 12].

Effect of carbon and nitrogen sources on biomass and antibacterial production

To determine the effect of carbon sources on biomass and antibacterial production of the *B. licheniformis* and *P. aeruginosa*, different carbon sources like glucose, lactose, fructose, sucrose, dextrose and starch (each at a concentration of 1%) were added individually to the optimized media. Following the conclusion of the best, the effect of varying concentrations of the best carbon source (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0%) on antibacterial production was also determined. Similarly, the influence of various nitrogen sources on antibacterial production was evaluated by supplementing different nitrogen sources such as sodium nitrate, peptone, yeast-extract, glutamine and tryptone (each at a concentration of 0.5%) to the optimized media containing an optimal amount of the superior carbon source as determined above [13]. Furthermore, the impact of varying concentrations of optimized nitrogen source (0.1, 0.3, 0.5, 1.0, 1.5 and 2.0%) was studied to standardize the maximum antibacterial production.

Effect of NaCl concentration on biomass and antibacterial production

Effect of NaCl concentration was also reflected on biomass and antibacterial production[13]. The effect of salinity on antibacterial production was studied in varying concentrations of NaCl ranging from 1% to 10%.

Thin layer chromatography

Silica gel plates, 10x20 cm, 1mm thick, were prepared and were activated at 150°C for half an hour. Ten micro liters of methanol fractions and reference antibiotics were applied on the

plates and the chromatogram was developed using ethyl acetate: methanol (4:1 ratio) as solvent system. While the plates were run in duplicate; one set was used as the reference chromatogram and the other was used for bioautography. The spots in the chromatogram were visualized in the iodine vapor chamber and UV chamber. Muller Hinton agar (MHA) inoculated with bacterial culture was poured over the other chromatogram and the plate was incubated overnight at 37°C in sterile condition. The next day, the inhibition zones were noted and the Rf values of the antimicrobials were determined.

RESULTS

To attain the maximum yield of antibacterial production, optimization of cultural conditions plays a vital role. Hence, in the present study, cultural conditions like pH, temperature, carbon, nitrogen sources and salt (NaCl) concentration were subjected to optimization as they influence growth of microbes, biomass and antibiotic production.

Effect of Temperature on biomass and antibiotic production

Both biomass and the production of antibacterial metabolites from both *B. licheniformis* (Fig. 1) and *P. aeuroginosa* (Fig. 2) were maximum at 30°C. Both biomass and the metabolite production showed a decline with increasing temperature from 30°C to 60°C for both the bacteria studied.

Based on the zones of inhibition for antimicrobial function, both *B. licheniformis* and *P. aeuroginosa* exhibited similar order of preference among the three species studied with maximum for *S. aureus* (21.3 mm, 20.7 mm) > *K. pneumoniae* (18.3 mm, 17.6 mm) > *E. faecalis* (14.7 mm, 15.0 mm).

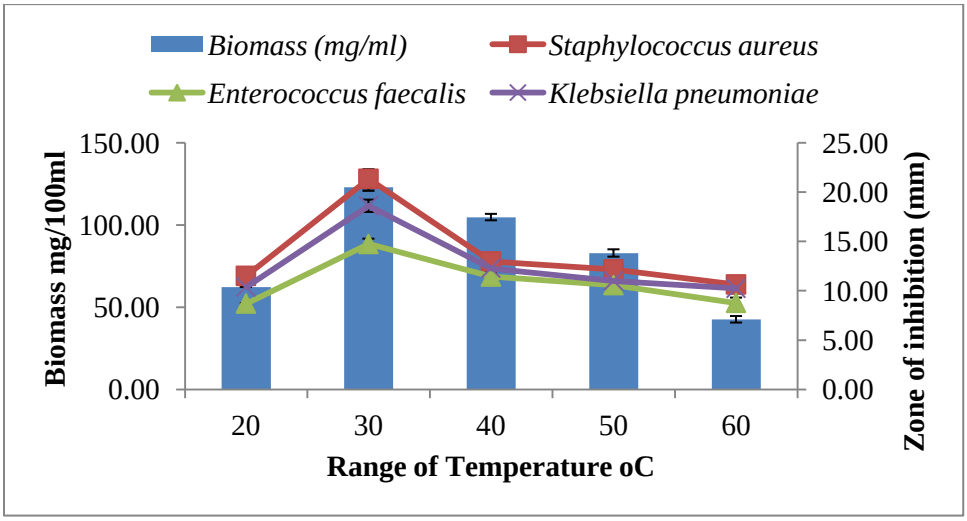

Figure 1. Effect of temperature on biomass and bioactive metabolite production by *B. licheniformis*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

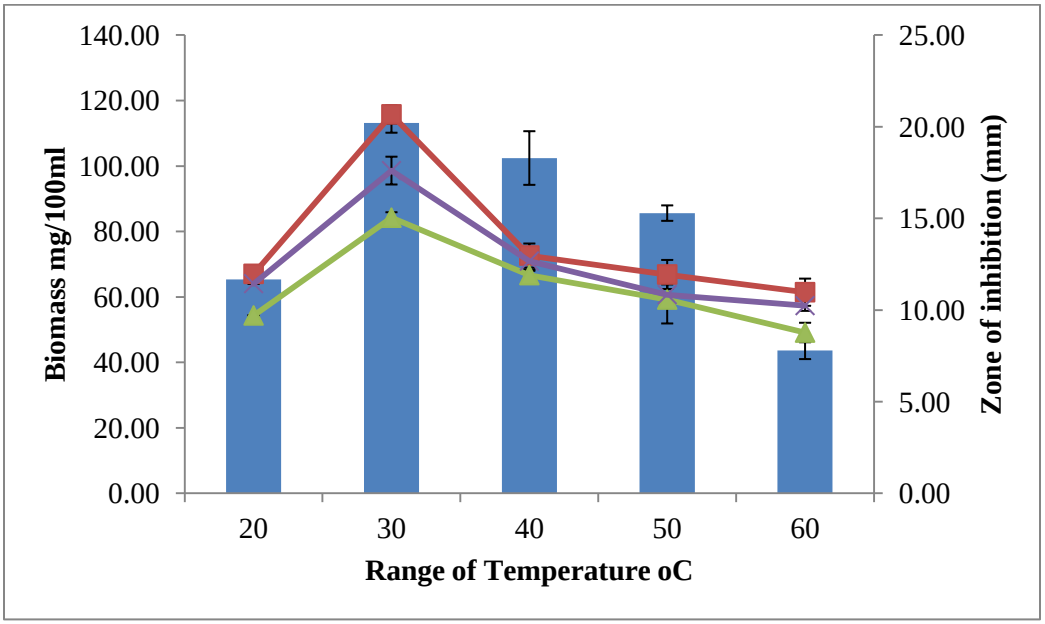

Figure 2. Effect of temperature on biomass and bioactive metabolite production by *P. aeuroginosa.* Each bar represents the Mean ± SEM of three independent values. P< 0.05

Effect of pH on biomass and antibiotic production

With a gradual increase from pH 4.0, both biomass and the production of antibacterial metabolites, reached maximum at pH 7.0 for both *B. licheniformis* (Fig. 3) and *P. aeuroginosa* (Fig. 4). Both biomass and the metabolite production showed a decline there after with increasing pH up to 10.0 for both the bacteria.

Based on the zones of inhibition for antimicrobial metabolite function, both *B. licheniformis* and *P. aeuroginosa* exhibited similar order of preference among the three species studied with maximum for *S. aureus* (20.3 mm, 19.7 mm) > *K. pneumoniae* (18.3 mm, 17.7 mm) > *E. faecalis* (14.7 mm, 14.3 mm).

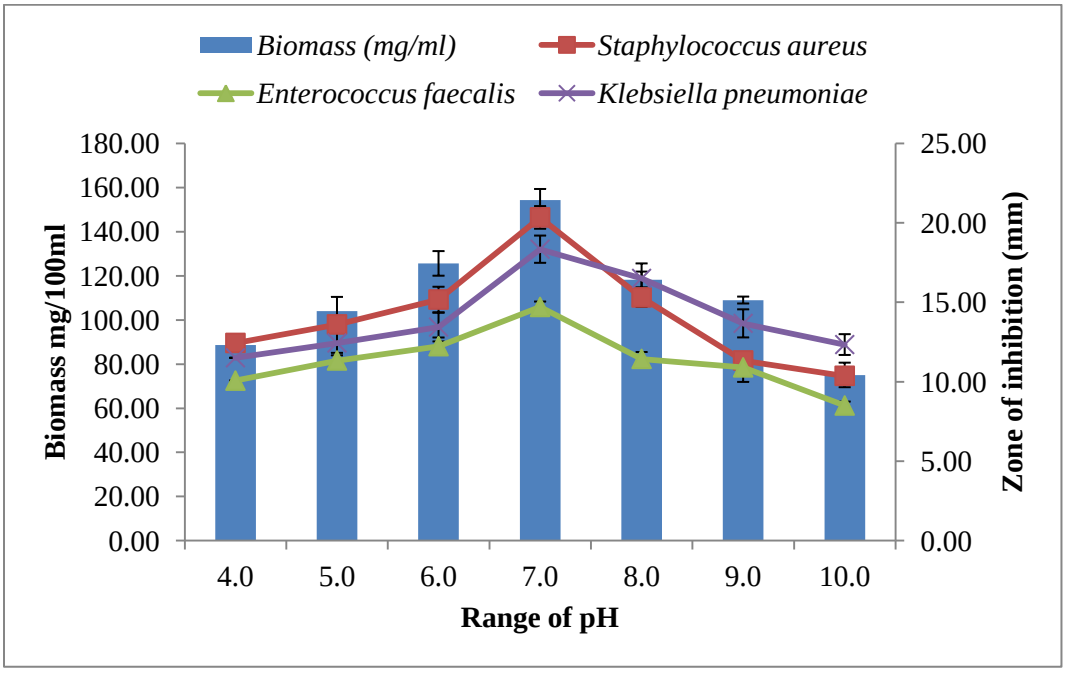

Figure 3. Effect of pH on biomass and bioactive metabolite production by *B. licheniformis*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

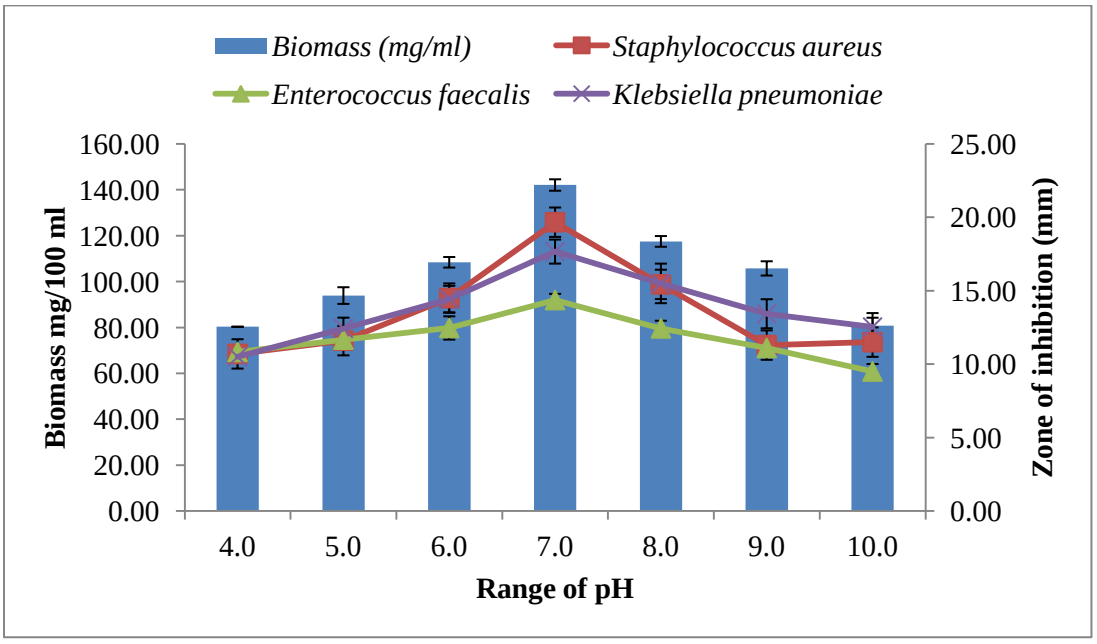

Figure 4. Effect of pH on biomass and bioactive metabolite production by *P. aeuroginosa*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

Effect of Carbon source on biomass and antibiotic production

Effect of carbon source on the production of biomass and antibacterial metabolite for *B. licheniformis* (Fig. 5) and *P. aeuroginosa* (Fig. 6) showed maximum production of bioactive metabolite was obtained in lactose amended media followed by fructose, sucrose, dextrose, glucose and starch. Also, both *B. licheniformis* and *P. aeuroginosa* showed similar order of preference with reference to the bactericidal activity with maximum for *S. aureus* (20.3 mm, 19.7 mm) > *K. pneumoniae* (18.3 mm, 17.7 mm) > *E. faecalis* (14.7 mm, 14.3 mm).

As lactose emerged as the most preferred carbon source for antibiotic production by the isolates, varying concentrations of lactose (0.5-5%) was tested to determine its optimal concentration. As shown in fig. 7 and 8, lactose at level of 1% showed optimal yield of bioactive metabolites of both *B. licheniformis* and *P. aeuroginosa*. As lactose emerged out to be the most preferred carbon source for antibiotic production by the isolates, a dose response of lactose (0.5-5%) was tested to determine its optimal concentration. While 1% lactose showed maximum yields of bioactive metabolites (may or may not be statistically significant from that of 2 % lactose of both *B. licheniformis* (Fig. 7) and *P. aeuroginosa* (Fig. 8), 2% lactose resulted in the optimal biomass production.

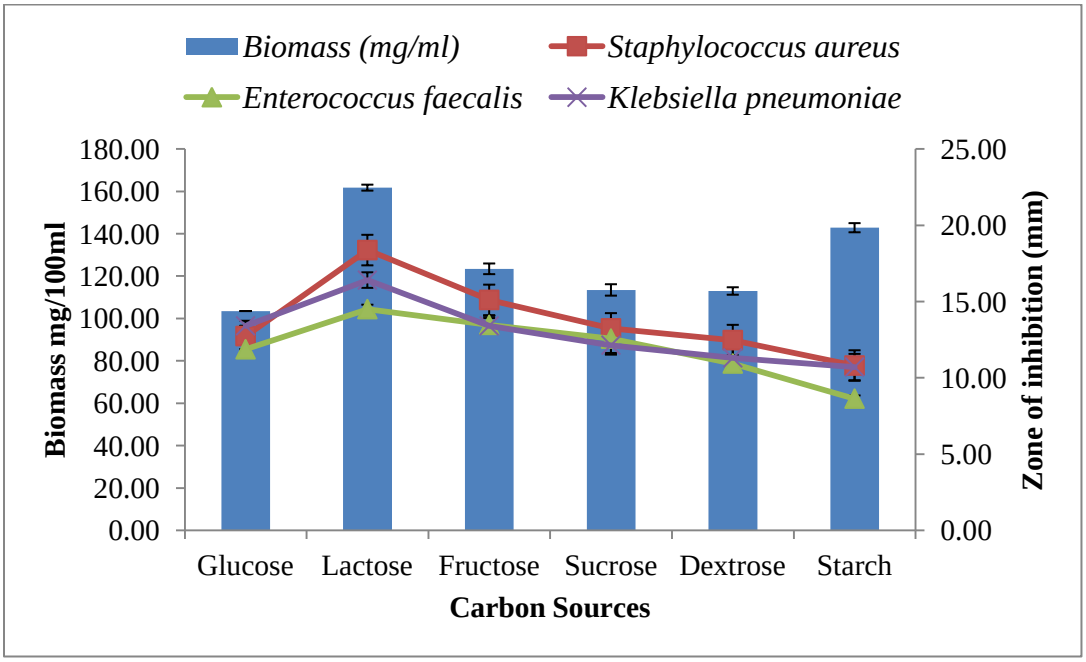

Figure 5. Effect of different carbon sources on biomass and bioactive metabolite production by *B. licheniformis*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

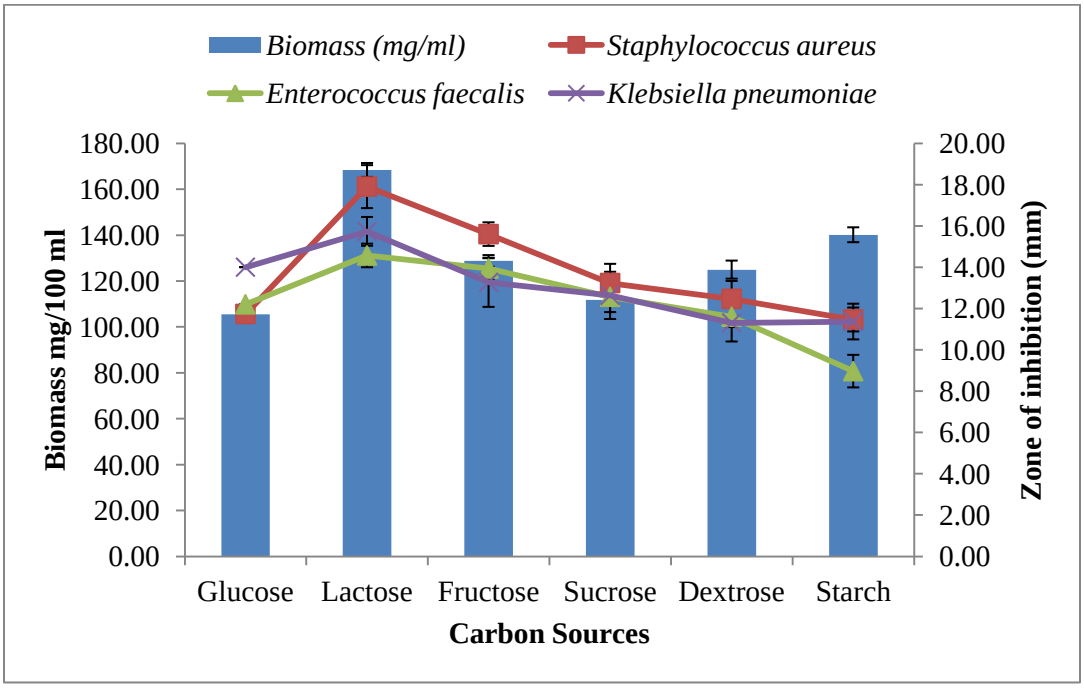

Figure 6. Effect of different carbon sources on biomass and bioactive metabolite production by *P. aeuroginosa*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

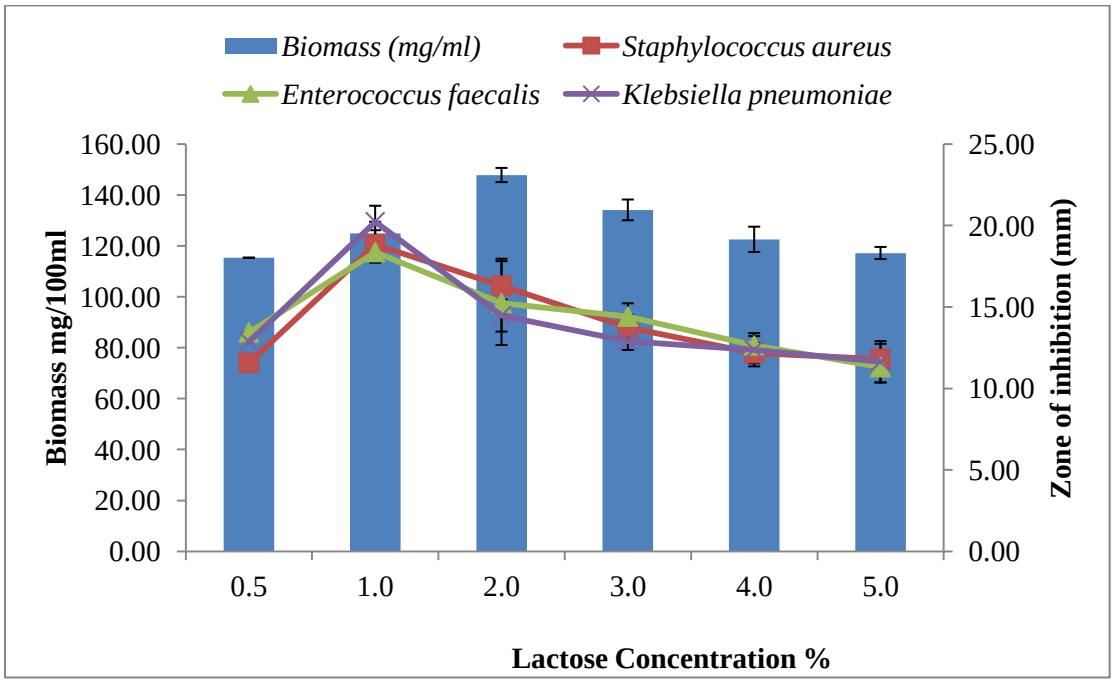

Figure 7. Effect of different concentrations of lactose on biomass and bioactive metabolite production by *B. licheniformis*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

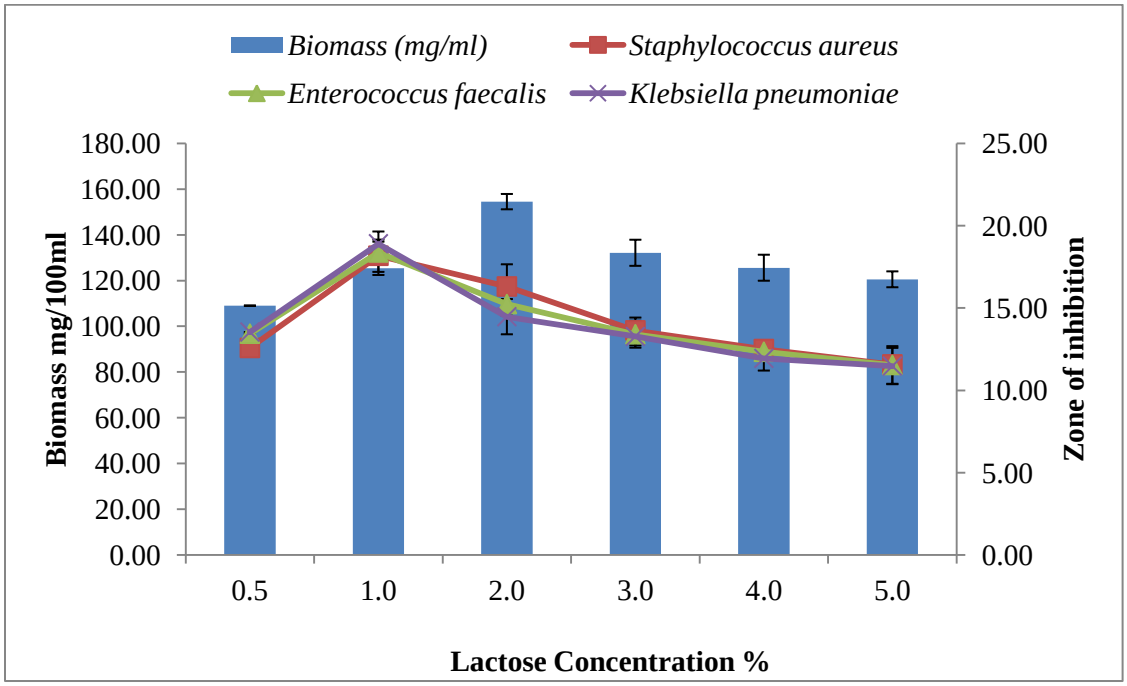

Figure 8. Effect of different concentrations of lactose on biomass and bioactive metabolite production by *P. aeuroginosa*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

Effect of Nitrogen source on biomass and antibiotic production

In order to develop effective composition of growth medium, the role of different sources of nitrogen was evaluated for their influence on growth and antibacterial production by the isolates of *B. licheniformis* (Fig. 9) and *P. aeuroginosa* (Fig. 10). Of all the nitrogen sources examined, bacteriological peptone was found to be the best source of nitrogen for growth of bacteria (biomass) as well as antibacterial metabolite production. It should be noted that tryptone, as a source of nitrogen, also favored good growth but the antibacterial compound yield was less in comparison to peptone (12.3 mm, 11.8 mm and 9.4 mm).

Inorganic nitrogen sources like ammonium sulfate, ammonium oxalate, sodium nitrate and some organic nitrogen sources like yeast extract did not show significant effect on antibiotic production by the *B. licheniformis* and *P. aeuroginosa* Peptone enhanced the biomass as well as antibiotic production by isolates. When tested for the dose response, peptone (Figs. 9 and 10) at a concentration of 0.5% and 1%exhibited optimal production of biomass and antibacterial production, respectively (Figs. 11 and 12).

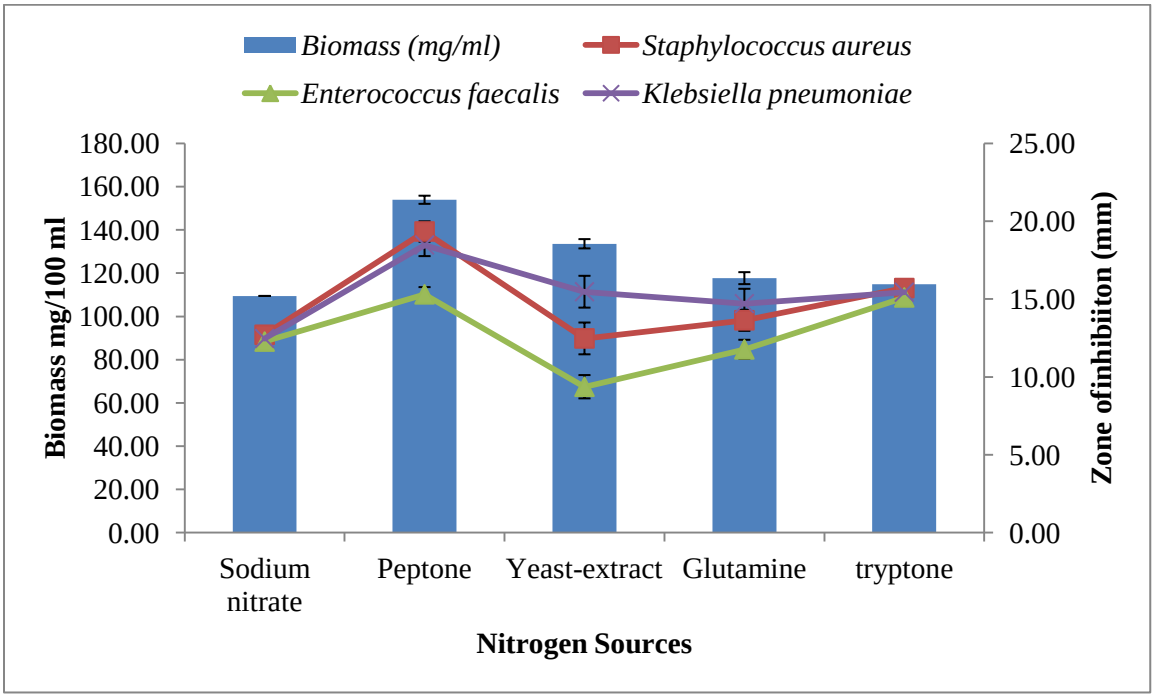

Figure 9. Effect of different nitrogen sources on biomass and bioactive metabolite production by *B. licheniformis*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

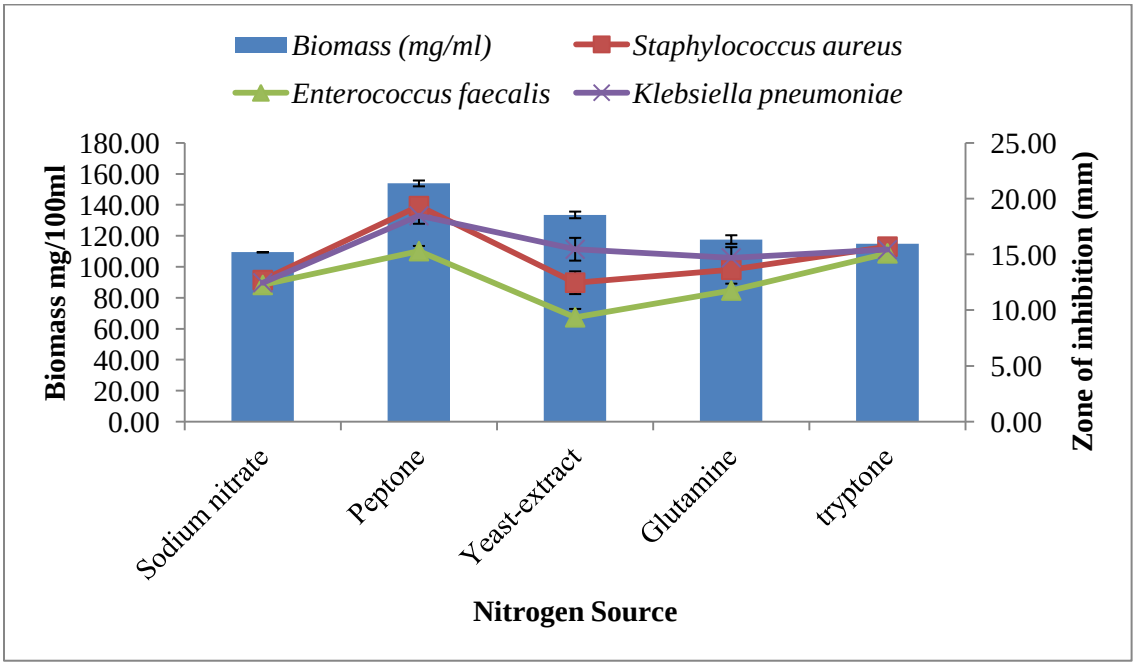

Figure 10. Effect of different nitrogen sources on biomass and bioactive metabolite production by *P. aeuroginosa*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

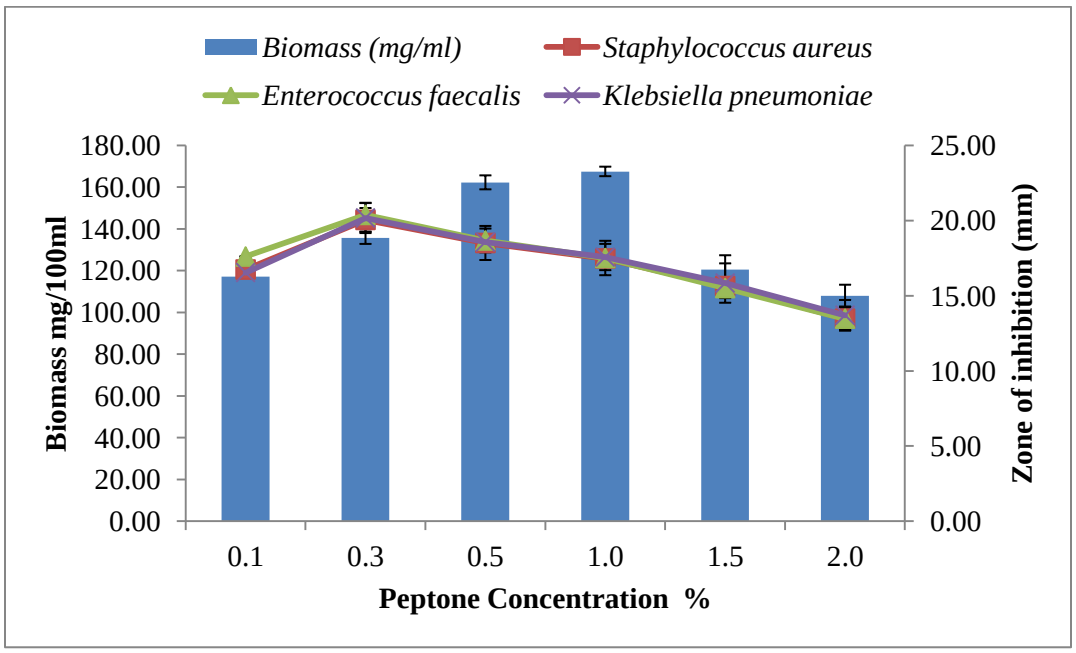

Figure 11. Effect of different concentrations of peptone on biomass and bioactive metabolite production by *B. licheniformis*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

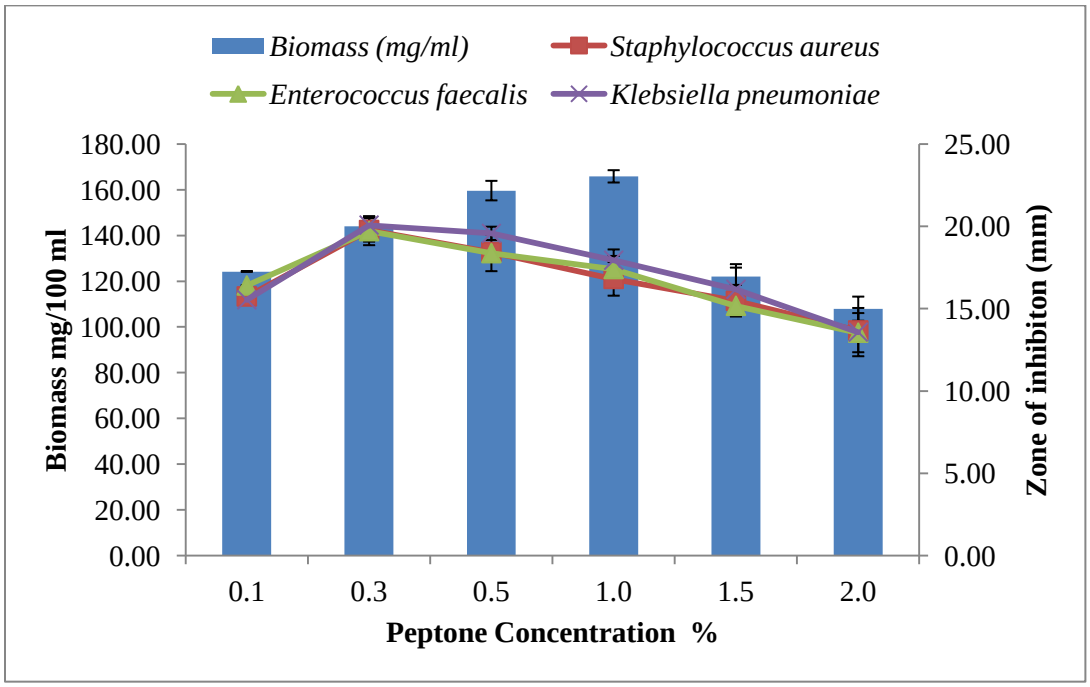

Figure 12. Effect of different concentrations of peptone on biomass and bioactive metabolite production by *P. aeuroginosa*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

Effect of NaCl concentration

Effect of NaCl concentration on production of biomass and antibiotic production of *B. licheniformis* (Fig. 13) and *P. aeuroginosa* (Fig. 14) showed significant production of bioactive metabolite was obtained in NaCl (3%) amended media by 4% and 6% NaCl concentration. However, the production of biomass was maximum in NaCl (3%), was and decreased with increasing concentration of NaCl.

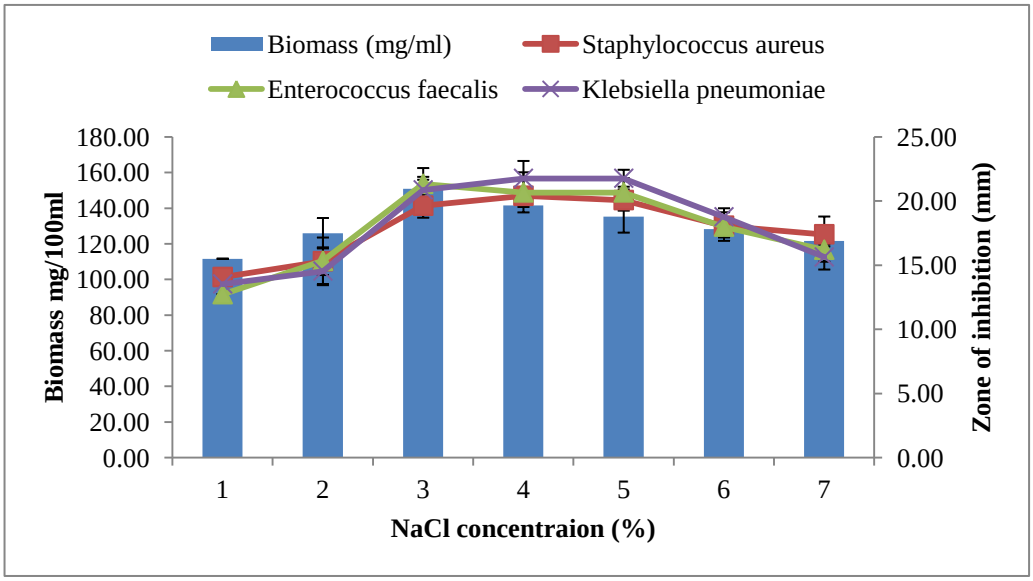

Figure 13. Effect of different concentrations of NaCl on biomass and bioactive metabolite production by *B. licheniformis*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

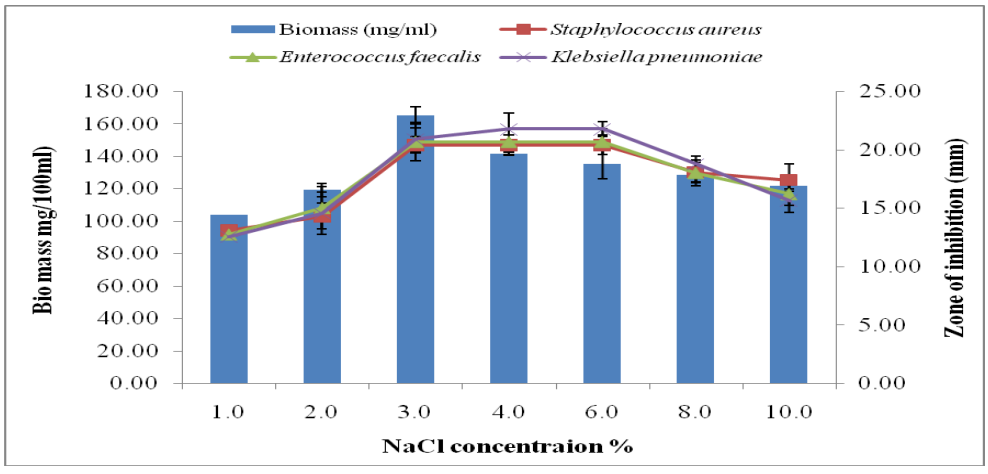

Figure 14. Effect of different concentrations of NaCl on biomass and bioactive metabolite production by *P. aeuroginosa*. Each bar represents the Mean ± SEM of three independent values. P< 0.05

Table 1: Antimicrobial activity of *B. licheniformis* and *P. aeuroginosa* before and after optimization.

Name of the isolate	Diameter of zone inhibition (mm)					
	Before optimization			After optimization		
	S.aureus	*E.faecalis*	*K.Pneumoniae*	*S.aureus*	*E.faecalis*	*K.Pneumoniae*
B. licheniformis	12.0	8.9	15.0	23.0	14.2	19.2
P. aeuroginosa	12.4	9.2	13.3	22.0	15.1	19.5

Thin Layer Chromatography

Ethyl acetate extract was subjected to TLC analysis using three mobile phases. Of the three mobile phases, the plate developed with ethyl acetate: methanol (6:4 ratio) gave the best separation. Direct bioautography was carried out to localize antibacterial substances. The study showed the presence of 2 active substances. CMB- 3 compound with Rf 0.8 showed more activity than the one with Rf 0.7 of KMB-1.

DISCUSSION

New and effective antibiotics are crucial in this current surge of multi-drug resistant bacterial infections which have rendered many of the currently available antibiotics totally ineffective. Natural products have served and continue to provide useful lead compounds for development into chemotherapeutic agents. Aquatic microorganisms have emerged as a source of diverse chemical compounds which have not been adequately studied for chemotherapeutic application. Our results have revealed two antibiotic producing microorganisms out of 24 isolates recovered from marine water sources in India. Many reports have been made of such studies elsewhere. For example, Ivanova *et al.* (1998) reported that out of the 491 bacteria isolated from different marine sources, 26% of the isolates were active for antibiotic function. Zheng *et al.* (2005) also reported that 8 out of 29 strains, representing 28% of the isolates considered in their study produced antimicrobial activity.

Antibiotic producers recorded in our study can be partly attributed to the nature of our water bodies: they are usually highly polluted with all kinds of waste materials; from domestic and industrial wastewater discharges, mining runoff, agro-chemicals and other sources[16, 17, 18 & 19]. To survive and maintain their niche under these harsh conditions therefore, the aquatic microorganisms need defense mechanisms and for some, antimicrobially active metabolite production could be one of such mechanisms. The differences among the detection rates

reported in literature strongly depend on the isolation and assay procedures, test organisms, type of media used, as well as the sources of bacterial isolates[20].

Optimization of cultural conditions may play prominent role in the growth of microorganisms and as well as antibiotic production. Among the physical factors, temperature is one of the most critical parameters that could affect the bioprocessing. *B. licheniformis* and *P. aeuroginosa* showed optimum growth at a temperature of 30^{o}C which was found to be optimum even for antibiotic production. Antibacterial activity was significantly more in case of *S. aureus* and low in *E. faecalis*. Generally intracellular pH of most microorganisms is maintained near neutrality regardless of the pH in the outside medium[21]. However as the proton gradient across the cytoplasmic membrane increases, the cells commit more of their resources towards maintaining the desired intracellular pH, thus changes in external pH affect many cellular processes such as growth and the regulation of the biosynthesis of secondary metabolites. The highest activity of the antibacterial metabolite by the strain was at pH 7.0. The antibacterial compound produced by the isolates had a moderate effect on the *K. pnuemoniae*. This results correlate with a study carried out by Charyulu and Gnanamani (2010) who reported maximum production of metabolite by P. aeruginosa MTCC 5210 at pH 7.0.

Several other nutritional factors also influence production of secondary metabolites by microorganisms[23]. Sole *et al.,* (1997) noted that glucose can be used as a source for bacterial growth while repressing the production of secondary metabolites. *B. licheniformis* and *P. aeuroginosa* utilized lactose and glucose best for maximum production of the antibacterial metabolites.

Nitrogen is very vital in the synthesis of enzymes involved in primary and secondary metabolism[25]. Shapiro (1989) noted that the type of nitrogen source (organic or inorganic) plays a role in the synthesis of secondary metabolites. Among the nitrogen sources tested maximum antibiotic production was obtained with 0.25% peptone. In comparison with inorganic nitrogen sources, organic nitrogen sources gave relatively higher antimicrobial agent production by *B. licheniformis* and *P. aeuroginosa*. This is inconformity with the findings of Vahidi *et al.,* (2004) which showed that the organic nitrogen sources are better for the production of antimicrobial agents. He stated that the nature of carbon and nitrogen sources strongly effect antibiotic production in different organisms.

CONCLUSION

Our study suggests that antibiotic production was greatly influenced by the media components. In the present study, the antibiotics produced by *B. licheniformis* and *P. aeuroginosa* grown under optimized conditions exhibited good antibacterial activity against gram positive, gram negative bacteria. Hence, further studies regarding the purification, characterization and identification of antibiotics produced by *B. licheniformis* and *P. aeuroginosa*.

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