Influence of Culture conditions and Nutritional composition on the production of Antibacterial metabolite by *Pseudomonas aeruginosa* VUR 102 isolated from Lake Hussain Sagar, Hyderabad, India

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Abstract
Microorganisms are one of the most diverse group among all the living organisms and the metabolic products of microbes are even more diverse. Among the microbes, bacteria are very versatile in having less generation time and ease of culturing, strain improvement and downstream processing of the metabolite. In addition, some of the metabolites are formed as a result of the chemical and biological stress that bacterial isolate undergoes, which might have led to the production of metabolic products that are chemically altered. Simple alteration even in the side chains of active molecules has great significance in their biological activities. These metabolic products possessing diverse biological properties, can aid in combating emerging infectious diseases and can thereby prevent antibiotic resistance. The present work is focussed on optimization of the bacterial isolate, Pseudomonas aeruginosa VUR 102, which is a potent producer of antibacterial metabolite, isolated from the lake Hussain Sagar, Hyderabad, India. The isolate, Pseudomonas aeruginosa VUR 102 exhibited an increased zone of inhibition against some selected Gram positive and Gram negative test bacteria.

The present study concludes that the bacterial isolate Pseudomonas aeruginosa VUR 102 produces greater amounts of potent antibacterial metabolite when optimal culture conditions and nutritional composition are provided. Optimal culture conditions are: 120 hours of incubation period at 35^{o}C temperature with pH 8.0 and 1% NaCl. Nutritional composition includes the addition of glycerol and peptone as carbon and nitrogen sources, respectively.

Keywords: *Pseudomonas aeruginosa*, growth, antibacterial activity, culture conditions, nutritional composition, optimization.

Introduction

Bacteria are one among the great reservoirs to produce natural, biologically active chemical compounds. These biologically active compounds exhibit diverse activities in agriculture, medicine, pharmaceutical industry etc[25]. Different kinds of bioactive compounds are produced by microbes against each other in search for food and space, manifesting their antagonistic properties[22,27]. *Pseudomonas* aeruginosa, a Gram-negative bacterium, is able to survive in different habitats including water, soil and on higher life forms[10]. Bacteria that harbour in contaminated site have diverse activities like production of bioactive metabolites[20,21,23].

Furthermore, they are able to degrade hydrocarbons[24]. They are found to synthesize metabolites to compete for food and space for their sustenance[28]. One of the metabolites of *Pseudomonas* is a pigment pyocyanin which exhibits antibacterial activity[2,11]. Apart from that, *Pseudomonas* is also reported to produce exopolysaccharide and rhamnolipid that helps the bacterium to survive in harsh climatic conditions and increases the growth rate even when cheap substrates are available[3,19,29]. These days, in spite of elevated development in medicine, newly emerging microbial infection is a serious problem for the mankind. Furthermore, concerning aspect is the increasing resistance to existing antibiotics, caused by their misuse while treating patients suffering from infectious bacterial diseases[26,37]. So, to effectively combat the appalling infectious bacteria, it is inevitable to search for new natural bioactive compounds possessing antibacterial activity against different bacterial pathogens.

Antibacterial metabolite production is of immense importance and the regulation of the bioactive compound production by microbes can be acquired by the availability of nutrients, enzyme induction and repression[34]. Large scale production of antibacterial compound from bacteria requires precise physical conditions and nutritional composition to obtain enhanced production of antibacterial metabolite. Several optimization studies reported that there is a great influence of physical and nutritional parameters on production of desired product in greater yields[6,13,14,31,36]. The scrupulous objective of present study is to optimize antibacterial metabolite production by *Pseudomonas aeruginosa* VUR 102 against test bacterial cultures by using different cultural conditions and different nutritional compositions.

Material and Methods

Isolation and identification: The bacterial isolate *Pseudomonas aeruginosa* VUR 102 used in the present work was isolated from water of Lake Hussain Sagar, Hyderabad. It was identified and confirmed by morphological, cultural,

biochemical tests[15] and 16S rRNA gene sequencing as *Pseudomonas aeruginosa* and was named as *Pseudomonas aeruginosa* VUR 102.

Inoculum preparation for optimization: The isolate was grown in nutrient broth medium until its OD value was 1 at 600nm, measured using UV-VIS Spectrophotometer[12]. Then the inoculum was maintained carefully and used for all the optimization parameters.

Test bacterial cultures used for antibacterial activity: Test bacterial cultures used include Gram positive bacteria (*Micrococcus luteus* MTCC 106, *Arthrobacter protophormiae* MTCC 2682, *Rhodococcus rhodochrous* MTCC 265, *Lactobacillus acidophilus* MTCC 10307, *Enterococcus faecalis* MTCC 439 and *Streptococcus mutans* MTCC 497) and Gram negative bacteria (*Alcaligenes faecalis* MTCC 126, *Enterobacter aerogenes* MTCC 10208 and *Proteus vulgaris* MTCC 426), procured from MTCC, IMTech Chandigarh, India.

Effect of incubation time on growth and antibacterial activity: One ml of *Pseudomonas aeruginosa* VUR 102 culture suspension, having optical density of 1 at 600 nm was aseptically inoculated into seven 250 ml Erlenmeyer flasks containing 100 ml of sterile nutrient broth medium and marked as 24, 48, 72, 96, 120, 144 and 168 hours. All the seven flasks were incubated at room temperature and each flask was removed after incubation period and the growth was measured in terms of optical density. Then the spent broth was centrifuged for obtaining cell free broth followed by solvent extraction using ethyl acetate. The crude extract obtained was used to test antibacterial activity in terms of zone of inhibition against the test bacteria.

Effect of temperature on growth and antibacterial activity: After inoculating 1ml of *Pseudomonas aeruginosa* VUR 102 culture suspension aseptically into five 250 ml Erlenmeyer flasks containing 100 ml of sterile nutrient broth medium, flasks were incubated at different temperatures, viz., 25°C, 30°C, 35°C, 40°C and 45°C for the period of optimized incubation time. Then, after measuring the growth in terms of optical density, the spent broth was centrifuged followed by solvent extraction using ethyl acetate. The crude extract obtained was used to test antibacterial activity in terms of zone of inhibition against the test bacteria.

Effect of pH on growth and antibacterial activity: The prepared nutrient broth medium in 100ml aliquots was dispensed into each of the six flasks. The pH of 6 flasks was adjusted to 5, 6, 7, 8, 9 and 10 by using 1N NaOH and/or 1N HCl as required and then sterilized. To each flask, 1ml of *Pseudomonas aeruginosa* VUR 102 culture was inoculated and incubated at optimized temperature for the optimized incubation period.

Then, the growth was measured in terms of optical density followed by centrifugation to remove cell mass and then solvent extraction using ethyl acetate. The crude extract obtained was used to test antibacterial activity in terms of zone of inhibition against the test bacteria.

Effect of NaCl on growth and antibacterial activity: To identify the optimum concentration of NaCl, the broth medium with optimized pH in different flasks was amended with different concentrations of NaCl viz. 1%, 2%, 3%, 4% and 5% and inoculated with 1ml of *Pseudomonas aeruginosa* VUR 102 culture. Then, the flasks were incubated at optimized incubation period, temperature and pH. After incubation, the growth was measured in terms of optical density. Then the bacterial culture was removed by centrifugation, followed by solvent extraction of the supernatant using ethyl acetate. The crude extract obtained was used to test antibacterial activity in terms of zone of inhibition against the test bacteria.

Influence of nutritional parameters on growth and antibacterial activity

Carbon Sources: To study the effect of different carbon sources on the growth and antibacterial metabolite production, 1% of 9 different carbon sources viz. glucose and fructose of monosaccharides, sucrose, lactose and maltose of disaccharides, cellulose and starch of polysaccharides and glycerol and mannitol of sugar alcohols were used. Control was maintained without adding any carbon source. Then, all the flasks were inoculated with 1ml of the *Pseudomonas aeruginosa* VUR 102 culture suspension aseptically and were incubated for 120 hours at 35°C with pH 8 and 1% NaCl. After incubation, bacterial growth was measured in terms of optical density and bacterial culture broth was centrifuged for obtaining cell free broth followed by solvent extraction using ethyl acetate. The crude extract obtained was used to test antibacterial activity in terms of zone of inhibition against the test bacteria.

Nitrogen sources: To assess the influence of different nitrogen sources on the growth and antibacterial metabolite, 1% of 3 different inorganic nitrogen sources viz. KNO_3, $NaNO_3$ and $(NH_4)_2SO_4$ and six different organic nitrogen sources namely urea, peptone, beef extract, yeast extract, casein and malt extract were used. These nitrogen sources were amended to the medium of different flasks containing 1% glycerol and 1ml of culture suspension was added and incubated at previously optimized physical conditions. After incubation, bacterial growth was measured in terms of optical density. Then, the spent broth was centrifuged for obtaining cell free broth, followed by solvent extraction using ethyl acetate. The crude extract obtained was used to test antibacterial activity in terms of zone of inhibition against the test bacteria.

Extraction of the bioactive compound: For each of the optimization parameter, the spent broth from different flasks was taken into different centrifuge tubes and centrifuged at 10,000 rpm for 10 minutes. The pellet was discarded and the supernatant was extracted with equal volume of ethyl

acetate, by vigorous shaking using a separating funnel. Then, the upper organic layer was retained and stored. This was repeated thrice to ensure the maximal extraction of the bioactive compound from the aqueous layer. Then the organic phase was concentrated using rotary evaporator and the residual concentrate was dried in a watch glass[1]. This was considered as the crude ethyl acetate extract of the bacterial isolate *Pseudomonas aeruginosa* VUR 102 for each parameter.

Antibacterial Assay: Antibacterial assay was performed by agar-well diffusion method[4]. The test bacterial culture suspension was added to the molten state nutrient agar medium, mixed well and poured aseptically into the sterile glass Petri plates. After solidification of the medium, wells were made using sterilized 6 mm cork borer. The crude ethyl acetate extract of *Pseudomonas aeruginosa* VUR 102 obtained from each of the parameters was dissolved individually in DMSO and 100µl of it was added into each well. After addition of the crude extract, the plates were incubated at 37ºC for 24 hours and the diameter of zone of inhibitions was noted. All the experiments were carried out in triplicate and the mean of triplicate values was given for all growth parameters. Mean of triplicate values and their standard deviation values were calculated using Microsoft Office-Excel for all antibacterial activity results.

Results and Discussion

Optimization of *Pseudomonas aeruginosa* VUR 102 isolate for growth and antibacterial metabolite: Considering all the optimization parameters, it can be consolidated that the optimal conditions for the better production of the antibacterial metabolites by *Pseudomonas aeruginosa* VUR 102 were found to be incubation for 120 hours at 35ºC temperature[12] and at pH 8.0[5], with 1% of NaCl in the medium. The isolate exhibited better growth as well as antibacterial activity at 35ºC and decrease or increase in the temperature has led to the decrease of bacterial growth as well as antibacterial activity.

This shows that the bacterial isolate is a mesophile, which is in concurrence with its natural habitat. Our results are in similarity with other reports showing the mesophilic behaviour of *Pseudomonas aeruginosa*[7,17].

Effect of incubation time on growth and antibacterial activity: Maximum growth of the isolate was observed after 72 hours of incubation as shown in figure 1 and maximum antibacterial activity against test bacteria was seen after 120 hours. *Lactobacillus acidophilus* (11.67±0.58 mm) was found to be more susceptible at 120 hours than other test bacteria whereas *Enterobacter aerogenes* (8.33±0.58 mm) was found to be less susceptible at 120 hours, evident by the zone of inhibition (figure 2).

Effect of temperature on growth and antibacterial activity: It was observed that the growth and antibacterial activity were optimum at 35ºC. The growth of the isolate increased up to 35ºC and as the temperature increased further, the growth of the isolate was found to be decreased (figure 3). Greater antibacterial activity was observed at 120 hours against *Lactobacillus acidophilus* (13±0 mm) and *Streptococcus mutans* (13±0 mm) followed by *Enterococcus faecalis* (12±1 mm). The least was recorded at 120 hours against *Alcaligens faecalis* (8.67±0.58 mm) which is evident from zones of inhibition (figure 4).

Effect of pH on growth and antibacterial activity: The growth of the isolate increased with the increase of pH till pH 8.0. It was found that the effect of pH was negative on the growth of the isolate beyond pH 8.0, as represented in figure 5. The antibacterial activity of the isolate was found to be maximum at pH 8.0. Highest zone of inhibition was observed against *Lactobacillus acidophilus* (13.67±0.58 mm) and *Alcaligenes faecalis* (13.67±0.58 mm), followed by *Enterobacter aerogenes* (13.67±0.58 mm). It was observed that zone of inhibition was also observed against *Micrococcus luteus* (12.67±0.58 mm), *Enterococcus faecalis* (12.67±0.58 mm) and *Streptococcus mutans* (12.5±0.71 mm). The least zone of inhibition was observed against *Rhodococcus rhodochrous* (11.67±0.58 mm) graphically presented in figure 6.

Effect of NaCl concentration on growth and antibacterial activity: It was observed that the increase in the percentage of NaCl beyond 1% has led to the decrease of the growth as well as the antibacterial activity. The optimum NaCl concentration for the best growth and antibacterial activity was observed as 1% shown in the figure 7. Maximum inhibition zone of 13.67±0.58 mm was observed against *Streptococcus mutans* and *Proteus vulgaris* followed by *Enterococcus faecalis* (12.67±0.58 mm). It was observed that *Arthrobacter protophormiae*, *Lactobacillus acidophilus* and *Alcaligenes faecalis* were not inhibited beyond 2% NaCl. The least zone of inhibition was recorded against *Alcaligens faecalis* (11±0 mm) when 1% NaCl was amended given in figure 8.

When medium was supplemented with NaCl beyond 3%, it has shown negative influence on growth of the isolate (Figure 7) which can be due to osmotic imbalance, as the isolate is not a halophile and it has negative impact on the production of antibacterial metabolite, evident from zone of inhibition as shown in the figure 8. It was also reported that high concentrations of NaCl had a negative effect on the bacterial growth and antibacterial activity[9].

Effect of carbon sources on growth and antibacterial activity: Among all the 9 different carbon sources used, glycerol (alcohol sugar) was found to be the best, followed by fructose that supported the optimal growth of the isolate. Lactose and starch as supplemented carbon sources, resulted in low growth of the isolate (figure 9). *Arthrobacter protophormiae* (21.33±0.58 mm) was found to be more susceptible to the antibacterial compound when glycerol was used as a carbon source (plate 1).

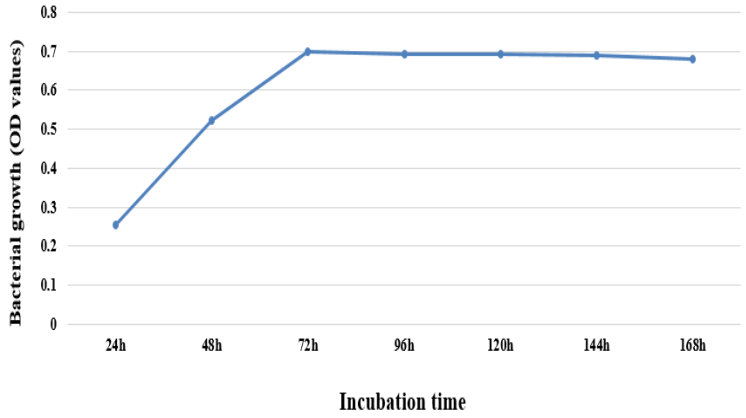

Fig. 1: Effect of incubation time on growth of *Pseudomonas aeruginosa* VUR 102

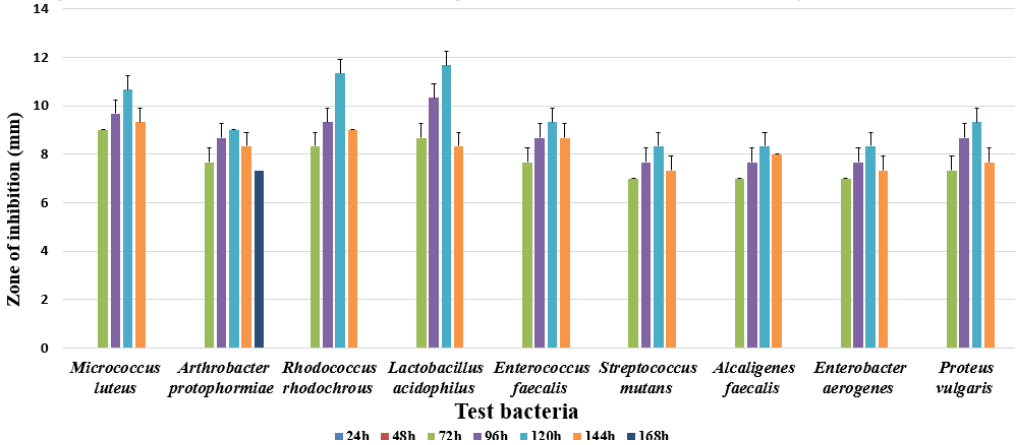

Fig. 2: Effect of incubation time on antibacterial activity of *Pseudomonas aeruginosa* VUR 102

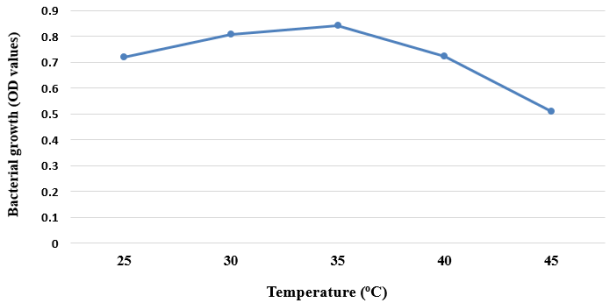

Fig. 3: Effect of temperature on growth of *Pseudomonas aeruginosa* VUR 102

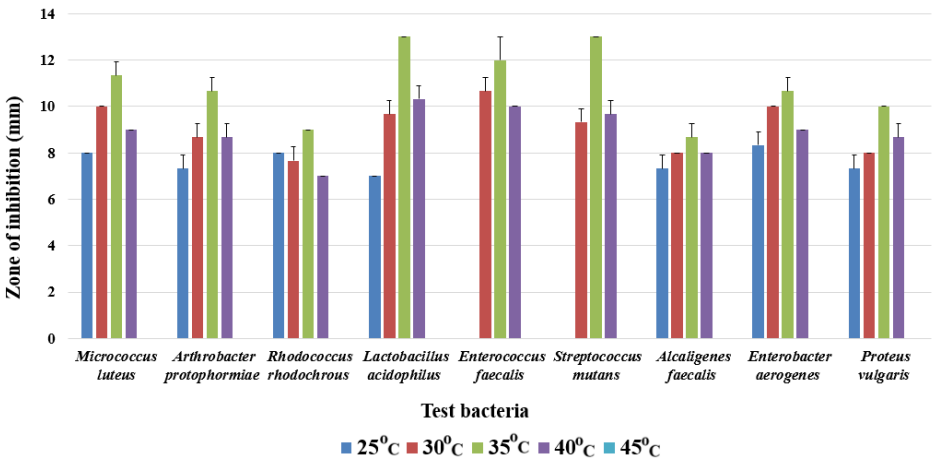

Fig. 4: Effect of temperature (°C) on antibacterial activity of *Pseudomonas aeruginosa* VUR 102

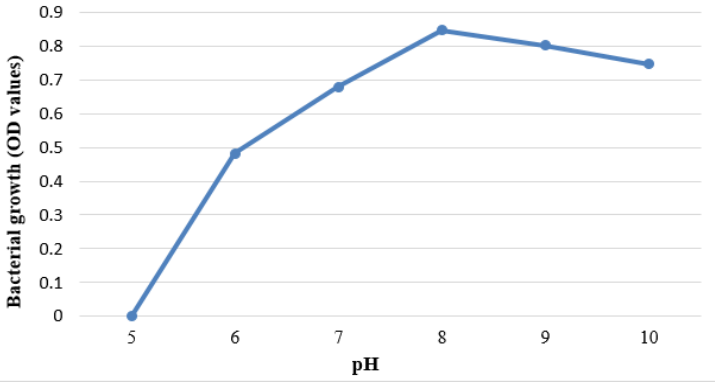

Fig. 5: Effect of pH on growth of *Pseudomonas aeruginosa* VUR 102

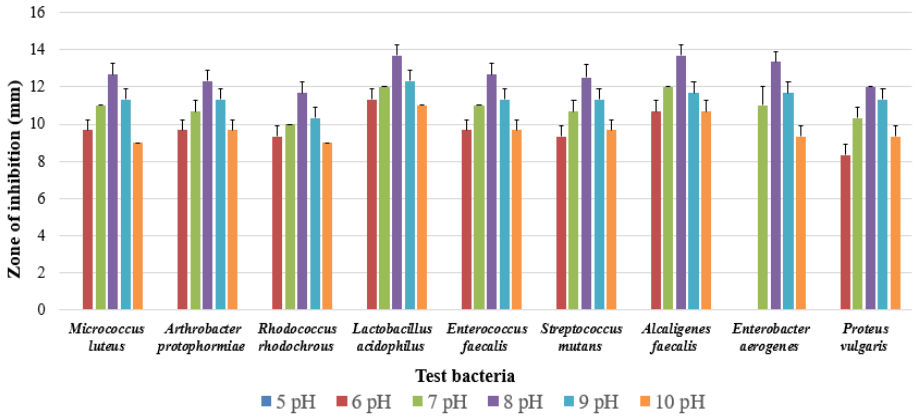

Fig. 6: Effect of pH on antibacterial activity of *Pseudomonas aeruginosa* VUR 102

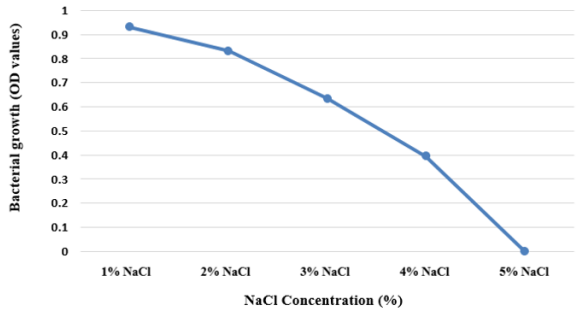

Fig. 7: Effect of NaCl concentration on growth of *Pseudomonas aeruginosa* VUR 102

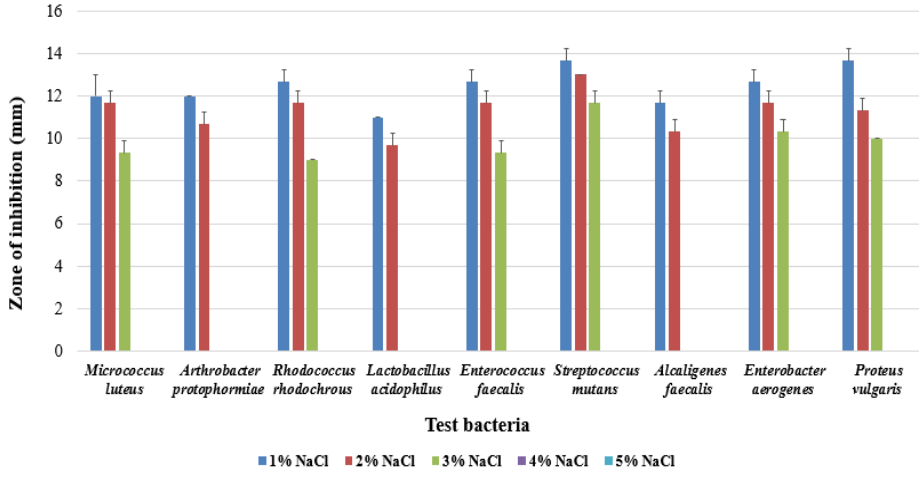

Fig. 8: Effect of NaCl concentration on antibacterial activity of *Pseudomonas aeruginosa* VUR 102

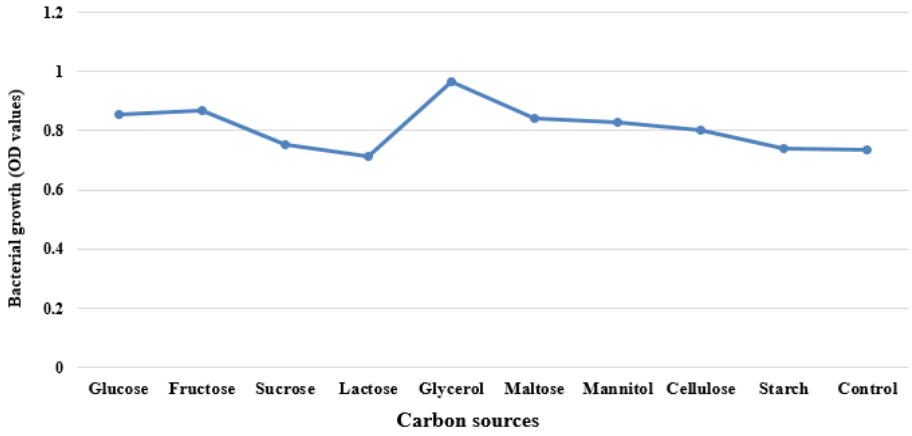

Fig. 9: Effect of carbon sources on growth of *Pseudomonas aeruginosa* VUR 102

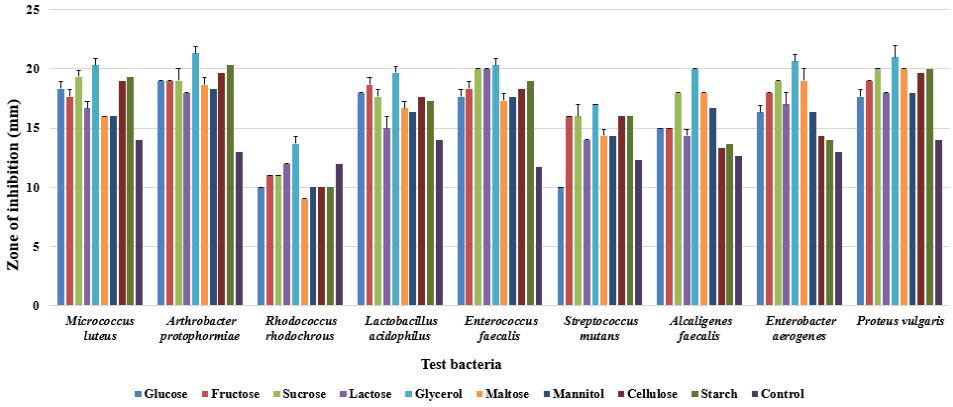

Fig. 10: Effect of carbon sources on antibacterial activity of *Pseudomonas aeruginosa* VUR 102

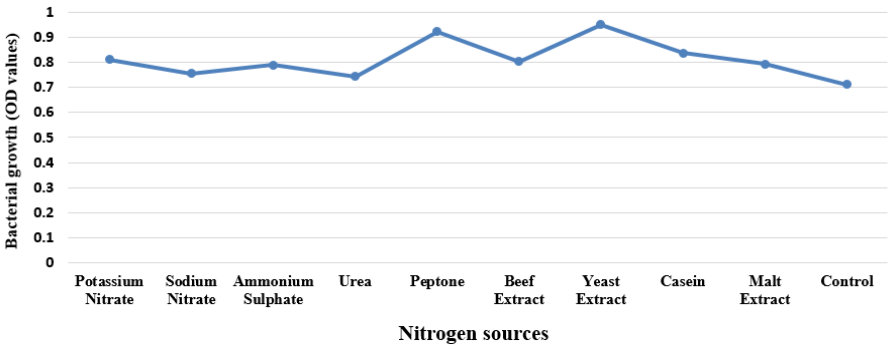

Fig. 11: Effect of nitrogen sources on growth of *Pseudomonas aeruginosa* VUR 102

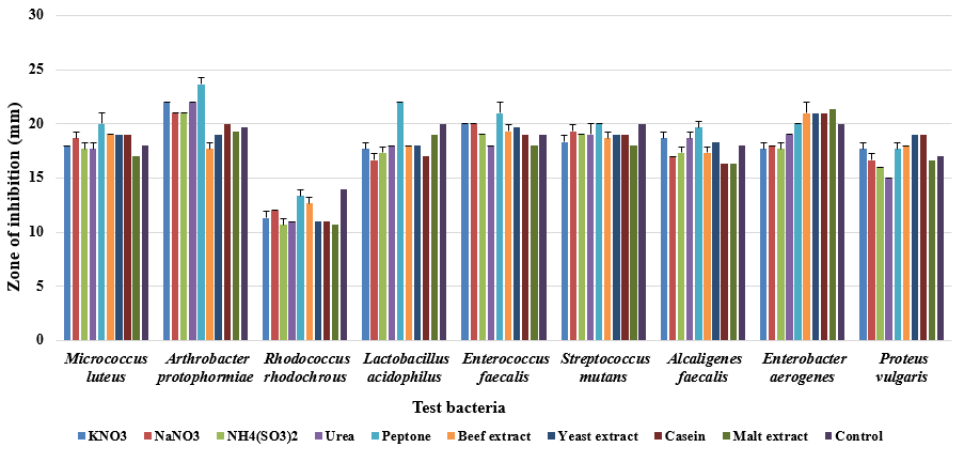

Fig. 12: Effect of nitrogen sources on antibacterial activity of *Pseudomonas aeruginosa* VUR 102

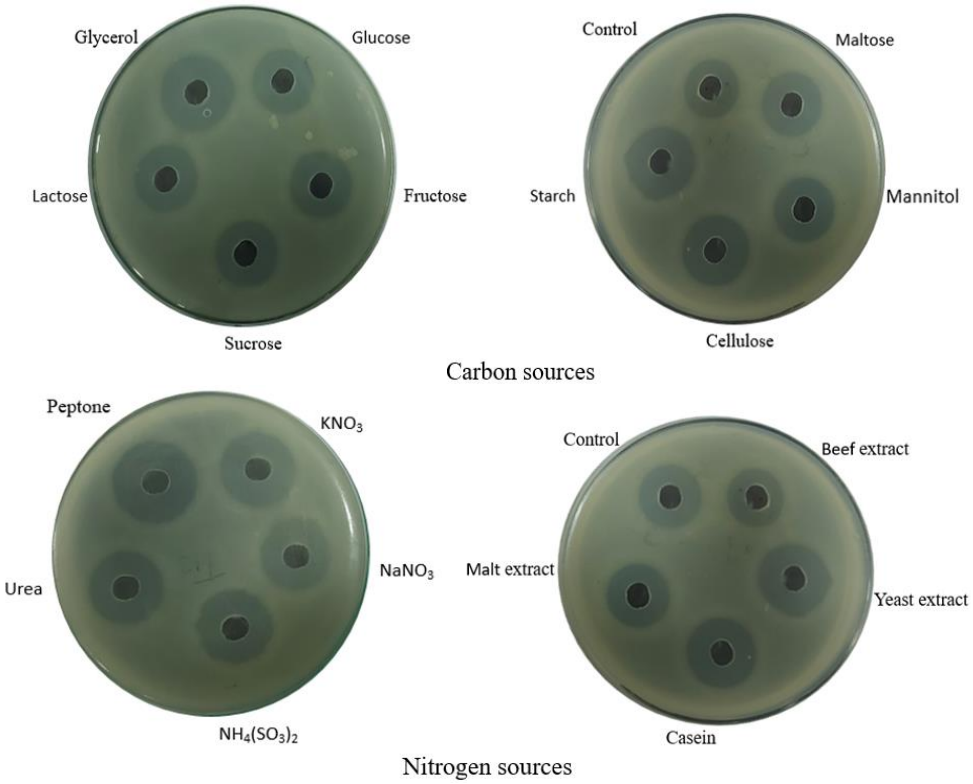

Plate 1: Pictures showing zone of inhibition of *Arthrobacter protophormiae* when different carbon and nitrogen sources were amended

Other test bacteria that were susceptible in the decreasing order when glycerol was amended, were *Proteus vulgaris* (21±1 mm) followed by *Enterobacter aerogenes* (20.67±0.58 mm), *Micrococcus luteus*, *Enterococcus faecalis* (20.33±0.58 mm) and *Alcaligenes faecalis* (20.0±0 mm).

Among all the carbon sources, glycerol was found to be the best source. Other carbon sources that supported the antibacterial activity in decreasing order were sucrose, fructose, starch, followed by maltose, cellulose, lactose, mannitol and glucose. It was observed that *Rhodococcus rhodochrous* (9.0±0 mm) was less susceptible to antibacterial compound when maltose was amended, when compared to other test bacterial cultures as shown in figure 10.

Effect of Nitrogen sources on growth and antibacterial activity: Of the 9 different nitrogen sources used, yeast extract was found to be the best, followed by peptone that supported maximal growth of the isolate and the least growth was recorded for urea (figure 11). When peptone was supplemented, it resulted in maximum antibacterial activity in terms of zone of inhibition against most of the test bacteria.

Among the inorganic nitrogen sources, potassium nitrate was the best followed by sodium nitrate to support the growth of the isolate. Among the organic nitrogen sources, peptone was the best followed by yeast extract and casein for better antibacterial activity. Maximum zone of inhibition was observed against *Arthrobacter protophormiae* (23.67±0.58 mm) and the zone of inhibition was found to be least against *Rhodococcus rhodochrous* (13.33±0.58 mm) when peptone was amended. It was found that urea and malt extract exhibited least antibacterial activity, as presented in the figure 12 and plate 1.

Glycerol (an alcohol sugar) and peptone can be supplemented as carbon and nitrogen sources respectively for better production of antibacterial metabolite. The ability of *Pseudomonas* to degrade different types of alcohols is reported by many researchers,[16,18]. The utilization of sugar alcohols like glycerol and mannitol by *Pseudomonas aeruginosa* have been reported by many authors[30,32,35] for the production of secondary metabolites. The present work shows that the isolate *Pseudomonas aeruginosa* VUR 102 is able to utilize glycerol.

It is remarkable that glycerol supplementation has resulted in highest antibacterial activity among all other sugars which were used. Though glucose is a simple sugar, easily utilized by many microorganisms, it was found that when glucose was amended, it resulted in the less antibacterial activity among all other sugars[8,29].

Peptone was recorded as the best source of nitrogen in the present study which enhanced the antibacterial activity of *Pseudomonas aeruginosa* VUR 102 against most of the test bacteria. Similarly, significant increase of antibacterial activity was obtained by researchers when peptone was used as the nitrogen source[33,38].

Conclusion

The present study reveals the optimal culture conditions and nutritional requirements for the isolate *Pseudomonas aeruginosa* VUR 102 viz. incubation for 120 hours, at 35ºC temperature, pH 8.0 and 1% NaCl, with addition of glycerol and peptone for the optimal production of the antibacterial metabolite that led to the significant increase in the antibacterial activity. Further work is to be carried out on large scale production, purification, characterization of the antibacterial principle and its applications.

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