

Enhanced production of a bioactive molecule from a symbiotic marine bacterium, *Paenibacillus macerans* SAM 9 isolated from the sea anemone, *Heteractis aurora*

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ABSTRACT

Pharmaceutical industries are in need of new bioactive molecules to precisely act against the newly developing infectious diseases. In view of this, an attempt was made in this study for the isolation of a potential bioactive molecule producing bacterium from the sea anemone, *Heteractis aurora*, collected from Mandapam, Ramanathapuram district, Tamil Nadu, India. Eleven symbiotic bacteria were totally isolated in which the isolate SAM 9 revealed promising antimicrobial activities against six clinically isolated human bacterial pathogens, namely, *Proteus mirabilis*, *Klebsiella oxytoca*, *Salmonella paratyphi*, *Salmonella typhi*, *Escherichia coli*, and *Staphylococcus aureus* with the growth inhibition activities of 54%, 49%, 47%, 43%, 39%, and 36%, respectively. The isolate SAM 9 was identified as *Paenibacillus macerans* using 16S rRNA molecular sequence method. During the media optimization, this isolate showed maximum bioactive molecule production at 96 h incubation with the other cultural conditions of pH 8, 35°C, maltose, and ammonium nitrate at the ratio of 2:1. Further, the bioactive molecule was purified using reversed-phase high-performance liquid chromatography and found one molecule responsible for the bioactivities. From the overall observations, the bioactive molecule produced by *P. macerans* SAM 9 showed the possibilities for future development as a promising therapeutical agent against many deadly infectious pathogens.

1. INTRODUCTION

There are certain molecules synthesized by some living organisms that carry out functions in the body of other organisms as well as modify metabolism, they are referred as bioactive molecules. When these bioactive molecules are known to be used for performing or regulating various biological activities of other organisms, they have been used for therapies and referred as therapeutic agents [1]. Since the beginning of civilizations, human being uses medical plants for the cure of many diseases in which they possess many bioactive properties, namely, antioxidant, antidiabetic, anti-inflammatory, antimicrobial, antiparasitic, anticancer activities, etc., [2].

Research on bioactive compounds has been a significant mission as long as we understood that therapeutical molecules are the solutions for different human diseases and disorders. In this decade, the progress in this field has seen much advancement both in science and enabling technologies that have been enterprising the pace of new identification

and production. Nature has diversified plant, animal as well as other living matter in which bioactive compounds are driven. In recent years, marine organisms are becoming appreciable sources for many biotechnological products, including bioactive molecules [3].

Ocean has covered nearly 70% of the earth atmosphere with unaccountable ecological, chemical, and biological diversity [4]. Different physicochemical parameters of the ocean have led the synthesis of diversified novel compounds in marine species which are unique with respect to structural, functional, and metabolic features when compared to the molecules originated from the terrestrial environment [5] and are also representing a hub of novel bioactive materials with great biomedical potential [6,7]. Marine organisms such as bacteria, cyanobacteria, sponges, bryozoans, tunicates, mollusks, seaweeds, and microalgae are presently being investigated for classifications of bioactive compounds aimed to the treatment of different diseases, namely, heart disease atherosclerosis, and asthma [8].

Till 2003, over 650 novel bioactive molecules were procured from marine organisms of green, brown, and red algae, phytoplankton, tunicates, bryozoans, sponges, mollusks, coelenterates, and echinoderms [9]. Among these sources, microbiota reveals an appreciable and endless resource for the development of novel drugs [10] with new targets, especially in the field of therapeutics to combat deadly infectious

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diseases and multiple drug-resistant pathogenic strains [11,12]. Despite the fact, marine microbes were poorly studied till date, especially in the field of therapeutics [13]. For this reason, we have studied the bioactive potential of a less reported symbiotic bacteria of a marine invertebrate specimen, *Heteractis aurora* and also investigated the possibilities on its enhanced fermentation production and purification.

2. MATERIALS AND METHODS

2.1. Collection and Processing of Sample

Sea anemone, *Heteractis aurora* was collected from Palk Bay of Mandapam, Ramanathapuram District, Tamil Nadu, India. The freshly collected specimen was transported and processed immediately in the laboratory. Initially, the specimen was gently washed thrice with pre-sterilized seawater (34 ppt and pH 8.4) prepared using synthetic sea salt (HiMedia, India) having the following composition: Calcium chloride H_2O (0.836 g/L), potassium chloride (0.435 g/L), strontium chloride H_2O (0.0007 g/L), sodium bicarbonate (0.1515 g/L), magnesium sulfate H_2O (3.8 g/L), magnesium chloride H_2O (2.94 g/L), borax (0.03 g/L), and sodium chloride (14.9 g/L) with a final pH (at 25°C) of 8.3 ± 0.2 . Adequate tentacles with a wet weight of 1 g were dissected using a sterile scissor which was homogenized using mortar and pestle and was serially diluted with the help of pre-sterilized synthetic seawater.

2.2. Isolation of Sea Anemone Associated Symbiotic Bacteria

The serially diluted samples were spread plated individually in freshly prepared Zobell marine agar plates (HiMedia, India) having the following composition: Peptone (5.0 g/L), yeast extract (1.0 g/L), ferric citrate (0.1 g/L), sodium chloride (19.45 g/L), magnesium chloride (8.8 g/L), sodium sulfate (3.24 g/L), calcium chloride (1.8 g/L), potassium chloride (0.55 g/L), sodium bicarbonate (0.16 g/L), potassium bromide (0.08 g/L), strontium chloride (0.034 g/L), boric acid (0.022 g/L), sodium silicate (0.004 g/L), sodium fluoride (0.0024 g/L), ammonium nitrate (0.0016 g/L), disodium phosphate (0.008 g/L), and agar (15.0 g/L) with the final pH (at 25°C) of 8.3 ± 0.2 . After 48 h incubation, bacterial colonies with distinct morphologies were isolated and repeatedly pure cultured using the quadrant strike plate method in the freshly prepared Zobell marine agar plates. The purity of the strains was concluded using distinct colony morphology under visual observation and Gram's staining procedure [14].

2.3. Screening of Bioactive Molecule Producing Marine Symbiotic Bacteria

2.3.1. Pathogenic bacteria

The production of bioactive molecules from isolated axenic marine bacteria was studied using ten clinically isolated human pathogenic bacterium, namely, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Salmonella typhi*, *Salmonella paratyphi*, *Proteus mirabilis*, *Escherichia coli*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* which were kindly gifted by Rajah Muthiah Medical College Hospital, Annamalai University, Tamil Nadu, India. These pathogenic bacteria were cultured on tryptone soy broth (HiMedia, India) at 37°C, which has the following composition: Tryptone (17.0 g/L), soya peptone (3.0 g/L), sodium chloride (5.0 g/L), dextrose (2.5 g/L), and dipotassium hydrogen phosphate (2.5 g/L) with the final pH 7.3 ± 0.2 at 25°C. The optical density of the broth cultures was standardized as 0.1 before inoculation according to the turbidity standard of McFarland.

2.3.2. Extracellular or intracellular production

All the isolates of this study were individually cultured in 30 ml screw cap tubes with 10 ml Zobell marine broth volume which has the same composition of Zobell marine agar except agar. After 96 h incubation, all the cultured broths were individual centrifuged under $1008 \times g$ for 15 min; cell pellet and cell-free supernatant were separately collected. The collected cell pellet was dissolved using 50 ml of phosphate buffer; further, it was sonicated under 20 KHz for 45 s using an ultrasonicator (Hielscher, USA). The cell debris formed during the sonication process was removed using the same applied centrifugal conditions as mentioned above and the supernatant was used for bioactivity, whereas the cell-free supernatant from the cultured broth was directly applied for the bioactivity study. This study confirms whether the test bacterial strains produced intracellular or extracellular bioactive molecules or found at both cellular locations.

2.3.3. Bioactivity assay

The production of bioactive molecules by the isolated axenic strains was evaluated by the microtiter plate-based assay method [15]. The procedure was conducted in 96-well flat-bottom polystyrene microtiter plates along with lids (Tarsons, India). In this assay, well plates were filled with 100 μL of fresh tryptone soy broth along with 10 μL of bioactivity testing samples and 10 μL different pathogenic cultures. All the ten pathogenic strains were individually tested for their percentage growth inhibition against all the eleven bacterial samples of both cell-free and lysed cell supernatants independently. The growth control well plates were done for all the individual pathogenic cultures added with 100 μL of tryptone soy broth, 10 μL different pathogenic cultures, and 10 μL Zobell marine control broth (uninoculated broth). The susceptibility control well plate was also carried out for all the individual pathogens added with 100 μL of tryptone soy broth, 10 μL of different pathogenic cultures, and 10 μL of 4mg/ml streptomycin. Finally, the well plates were incubated at 37°C after covered with lids. After 48 h incubation, the absorbance of each well broth was evaluated at 600 nm with the help of microplate reader (Biotek Elx808, WI, USA) and the percentage growth inhibition was estimated as follows:

$$\% \text{ Growth inhibition} = ([1 - (As/Ac)]) \times 100$$

Where As denotes the absorbance of the well having test samples and Ac denotes the absorbance of the control well (without any added bioactive sample).

2.4. Molecular Identification

The potential bioactive molecule producing marine bacteria was molecular identified with the help of 16S rRNA gene sequence analysis based on Eubac primer set of 27F (5'-AGAG TTTG ATCM TGGC TCAG-3') and 1492R (5'-GGTT ACCT TGTT ACGA CTT-3'). Purification was done on polymerase chain reaction (PCR) product with the Qiagen PCR kit and the sequence was performed using ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA). The resulting 16S rRNA partial gene sequence was compared with the available sequences of the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using BLAST version 2.5.0 for their pair-wise identities. The evolutionary distance was estimated using the maximum-likelihood method [16] and MEGA 7 was utilized for evaluating the evolutionary relationships [17].

2.5. Enhanced Production and Purification of Bioactive Molecule

2.5.1. Basal fermentation conditions

The promising bacterium was optimized for the enhanced bioactive molecule production by adopting a search method utilizing single

parameter at each time and the finalized conditions were used for the next level evaluations. The optimization process was evaluated in a 1000 mL conical flask with 400 ml Zobell marine broth volume as the basal fermentation conditions along with the other conditions of 37°C temperature, pH 8.3, and 150 rpm agitation. The log growth phase of the promising bacterium was used for inoculum preparation which was cultured under the same basal fermentation conditions, where the optical density of the inoculum evaluated at 620 nm was standardized as 0.1 according to the turbidity standard of McFarland. Further, the activity of the bioactive molecule was estimated using the microtiter plate-based assay method as described earlier using the most susceptible pathogen, *P. mirabilis* recorded during the screening studies. All the evaluations were expressed as mean \pm standard deviation after triplicate estimation.

2.5.2. Growth kinetics profile as a function of time on bioactive molecule production

The promising bacterium was studied for their optimal time of maximum bioactive molecule synthesis with reference to the bacteria biomass formation at 12 h regular time intervals from 0 h to 144 h. These estimations were performed using a portion of cultured broth after separation of the cell biomass and cell-free supernatant under $1008 \times g$ centrifugation for 15 min. Bacterial growth was estimated based on the dry weight of bacterial cell biomass resulted after hot air oven-dried cell pellets at 50°C for 30 min and bioactive molecule production was estimated directly from the cell-free supernatant.

2.5.3. Recapitulation of pH, temperature, carbon, nitrogen, and carbon/nitrogen ratio

Optimizing parameters such as pH, temperature, carbon, nitrogen, and carbon:nitrogen ratio plays a significant factor in the enhanced production of any microbial metabolites, including bioactive molecules. The influence of various pH parameters between pH 6 and 10 was evaluated for the enhanced bioactive molecule production; similarly, temperature conditions from 20 to 50°C were used in this study for the estimation of maximum production. Further, various carbon sources such as glucose, fructose, maltose, lactose, starch, and cellulose were utilized at 1% dry weight as well as nitrogen substrates such as peptone, yeast extract, malt extract, beef extract, ammonium sulfate, and ammonium nitrate were used at 0.5% dry weight in the optimized conditions for the evaluation of enhanced production. Likewise, different carbon and nitrogen ratio were also studied for the maximum production of bioactive molecules.

2.5.4. Bioactive molecule purification

Using the optimized conditions, production of the bioactive molecule was freshly carried out. During the standardized incubation time, the bioactive molecule was extracted from the cell-free supernatant using ethyl acetate. After overnight incubation, the organic phase containing the bioactive molecules was separated and rotary vacuum evaporated to a dried form. The dried crude form was dissolved in 3:2 ratio of 5 ml acetonitrile and methanol and passed through a 0.2 μm syringe filter. The purification was done by reverse phase (RP) – C₁₈ silica gel (230–400 mesh) column at 30°C temperature. The solvent system consisted of acetonitrile (solvent A) and methanol (solvent B) and the elutions were made at 0.5 ml/min flow rate using the stepwise gradient initiated from the ratio of 60:40, vol/vol (A:B) to the finalized ratio of 100:0, vol/vol (A:B). Forty-one fractions were totally collected and were individually dried under rotary vacuum evaporation and screened for bioactivity as described in the section 2.5.1. The purity of bioactive fraction was identified using normal followed by RP thin-layer chromatography (TLC) plates. The active fraction was initially separated on normal phase TLC plate (Silica gel 60G F₂₅₄, Merck, Germany) using n-hexane:

Dichloromethane (5:3) as the mobile phase and the fraction evidenced a single active spot was scrubbed, concentrated, and separated again using a RP TLC plate (C₁₈ 60RP–18 F₂₅₄, Merck, Germany) with acetonitrile:water (4:2) as the mobile phase. Iodine vapor was used for the identification for the purified active spots.

3. RESULTS AND DISCUSSION

3.1. Isolation and Screening of Potential Bioactive Molecule Producing Marine Symbiotic Bacterium

Symbioses are close physical relationships which fall within two or more living organisms [18]. Diversified symbiotic relationships are known from marine basin; especially, many invertebrates live in symbiosis with microbial communities [19]. These relationships count nutritional support as well as physical and biochemical protection to each other [20]. Symbiotic microbial communities can also provide defense mechanisms to the host, where some molecules produced by the symbiotic microbes for protecting themselves as well as the host from pathogens and predators [21]. Many reports are available on marine sponges for their symbiotic relationships with bioactive molecules producing bacterial species [22]; till date, other marine invertebrates are less studied, especially sea anemone for the investigation of bacteria symbionts producing bioactive compounds, hence the present study.

Sea anemone, *H. aurora*, [Figure 1] was investigated for the isolation of symbiotic bacteria from its tentacles. During this study, 11 morphologically distinct bacteria were isolated after 96 h incubation period. These axenic isolates were initially named as SAM 1–11 and were identified for the bioactive molecule production. All the isolates were examined for the production of extracellular and intracellular bioactive molecules in which SAM 3, 7, and 9 showed extracellular production [Table 1], whereas SAM 1 and 6 revealed intracellular production of bioactive molecules [Table 2].

Among the total isolates, extracellular bioactive molecule production of the isolate, SAM 7 evidenced promising activities against the human pathogens, *P. mirabilis*, *K. oxytoca*, *S. paratyphi*, *S. typhi*, *E. coli*, and *S. aureus* with the growth inhibition rate of $54 \pm 2.4\%$, $49 \pm 2.3\%$, $47 \pm 2.1\%$, $43 \pm 1.9\%$, $39 \pm 1.6\%$, and $36 \pm 1.6\%$, respectively, followed by the isolate, SAM 3 which was the next potential bacterium of this study with the extracellular bioactive molecule production against *P. mirabilis*, *S. typhi*, and *K. oxytoca* with the $31 \pm 1.3\%$, $27 \pm 1.2\%$, and $16 \pm 0.6\%$ of growth inhibition, respectively [Table 1]. The intracellular



Figure 1: *Heteractis aurora* collected from Palk Bay, Mandapam, South India.

Table 1: Screening of bioactive molecules from the cell-free supernatant of axenic marine bacteria against a panel of ten different human pathogenic bacteria, all the experimental values are expressed as mean±standard deviation, (n=3).

Marine bacteria	Growth inhibition percentage of different human pathogenic bacteria									
	<i>Escherichia coli</i>	<i>Salmonella typhi</i>	<i>Salmonella paratyphi</i>	<i>Klebsiella pneumoniae</i>	<i>Klebsiella oxytoca</i>	<i>Vibrio cholerae</i>	<i>Vibrio parahaemolyticus</i>	<i>Proteus mirabilis</i>	<i>Streptococcus pneumoniae</i>	<i>Staphylococcus aureus</i>
SAM 1	-	-	-	-	-	-	-	-	-	-
SAM 2	-	-	-	-	-	-	-	-	-	-
SAM 3	-	27±1.2	-	-	16±0.6	-	-	31±1.3	-	-
SAM 4	-	-	-	-	-	-	-	-	-	-
SAM 5	-	-	-	-	-	-	-	-	-	-
SAM 6	-	-	-	-	-	-	-	-	-	-
SAM 7	21±0.9	-	-	30±1.2	-	-	-	-	19±0.9	-
SAM 8	-	-	-	-	-	-	-	-	-	-
SAM 9	39±1.6	43±1.9	47±2.1	-	49±2.3	-	-	54±2.4	-	36±1.6
SAM 10	-	-	-	-	-	-	-	-	-	-
SAM 11	-	-	-	-	-	-	-	-	-	-

Table 2: Screening of bioactive molecules from the lysed cell supernatant of axenic marine bacteria against a panel of ten different human pathogenic bacteria, all the experimental values are expressed as mean±standard deviation, (n=3).

Marine bacteria	Growth inhibitory percentage of different human pathogenic bacteria									
	<i>Escherichia coli</i>	<i>Salmonella typhi</i>	<i>Salmonella paratyphi</i>	<i>Klebsiella pneumoniae</i>	<i>Klebsiella oxytoca</i>	<i>Vibrio cholerae</i>	<i>Vibrio parahaemolyticus</i>	<i>Proteus mirabilis</i>	<i>Streptococcus pneumoniae</i>	<i>Staphylococcus aureus</i>
SAM 1	27±1.1	-	-	-	-	-	-	-	22±0.9	18±0.7
SAM 2	-	-	-	-	-	-	-	-	-	-
SAM 3	-	-	-	-	-	-	-	-	-	-
SAM 4	-	-	-	-	-	-	-	-	-	-
SAM 5	-	-	-	-	-	-	-	-	-	-
SAM 6	-	19±0.7	18±0.8	-	-	-	-	-	-	-
SAM 7	-	-	-	-	-	-	-	-	-	-
SAM 8	-	-	-	-	-	-	-	-	-	-
SAM 9	-	-	-	-	-	-	-	-	-	-
SAM 10	-	-	-	-	-	-	-	-	-	-
SAM 11	-	-	-	-	-	-	-	-	-	-

bioactive molecules produced by the isolates, SAM 1 and 6, were less efficient against the pathogen used in this study when compared to the isolate, SAM 3, 7, and 9, which have revealed extracellular production.

Further, the isolate SAM 7 was chosen for the detailed study based on its promising activities against the maximum number of pathogens used in this investigation. Similar to this study, Devi *et al.* [23] isolated 21 bacteria from coastal waters of Thiruchendur, Thoothukudi, and Kanyakumari, Tamil Nadu, India, which were identified for the production of the bioactive molecule with the help of agar diffusion procedure. Among the isolated bacteria, *Alteromonas* sp. and *Rhodospseudomonas* sp. revealed appreciable growth inhibition activities against *S. typhi*, *Streptococcus mutans*, *Staphylococcus epidermidis*, *S. aureus* *Enterobacter* sp., and *Pseudomonas* sp. Likewise, two marine sponges, *Fasciospongia cavernosa* doc var. brown (dark brown) and *F. cavernosa* doc var. yellow (yellow), procured from the Bay of Bengal coast, Visakhapatnam, India which were studied for the isolation of symbiotic microbes and their potential production of bioactive compounds. Totally, 178 microorganisms were isolated in which two strains, B4 and B6, showed appreciable bioactive molecules production [24].

3.2. Molecular Identification of the Potential Marine Symbiotic Bacterium

The molecular identification of the potential bacterium SAM 7 was carried out based on amplifying the region of 16S rRNA gene sequence. The total sequence length of the PCR amplified product was 1420 bp [Figure 2] and the BLASTn homology search of the amplified region with the available nucleotide database of the NCBI GenBank sequence revealed 99.79% sequence similarity with a type strain, *Paenibacillus macerans* NBRC 3490 (accession number AB680091.1). Based on this closest gene homology comparison, the isolate SAM 7 was identified as *P. macerans* and the sequence was deposited in NCBI GenBank with the accession number MT941031.1.

The genus *Paenibacillus* included in the family Paenibacillaceae and the phylum Firmicutes. The phylogenetic tree of *P. macerans* SAM 9 was plotted against the highest 12 homology sequences resulted from the NCBI nucleotide collection [Figure 3]. The same molecular sequence method was followed for the identification of *Bacillus pumilus* produced a bioactive molecule named as Pumilacidin which

was active against a clinical pathogen, *S. aureus* [25]. Similarly to the above, a marine bacterium produced a bioactive glycolipid molecule which was identified as *Staphylococcus saprophyticus* SBPS 15 based on 16S rRNA molecular sequencing method [10].

3.3. Growth Kinetic Profile with Reference to Bioactive Molecule Production

The growth kinetics profile of *P. macerans* SAM 9 with reference to the bioactive molecule production as a function of the incubation period was estimated from lag to decline growth phase. The presence

of bioactive molecules revealed during the end phase of exponential growth to till the studied end of the decline growth phase of *P. macerans* SAM 9. Further, the production of the bioactive molecule recorded its peak at the end of the stationary growth phase (96th h) [Figure 4]. Further, this strain revealed a continuous increase in production during the stationary growth phase and recorded a continuous decrease in production during the decline growth phase.

This pattern of production evidenced that the bioactive molecule is a secondary metabolite. In support to this investigation, a marine actinobacterium, *Marinispora* NPS12745, isolated from

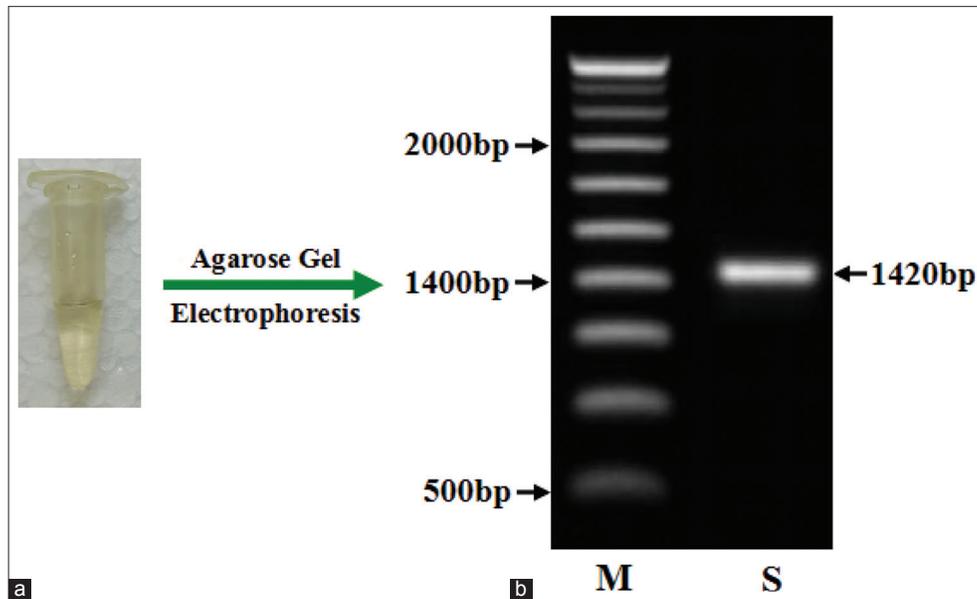


Figure 2: (a) Extracted and polymerase chain reaction (PCR) amplified DNA product of the 16S rRNA gene primers and (b) Agarose gel electrophoresis of the PCR amplified DNA where “M” stands for the lane of DNA marker and “S” stands for the lane of DNA sample extracted and amplified using PCR for the molecular identification of the marine *Paenibacillus macerans* SAM 9.

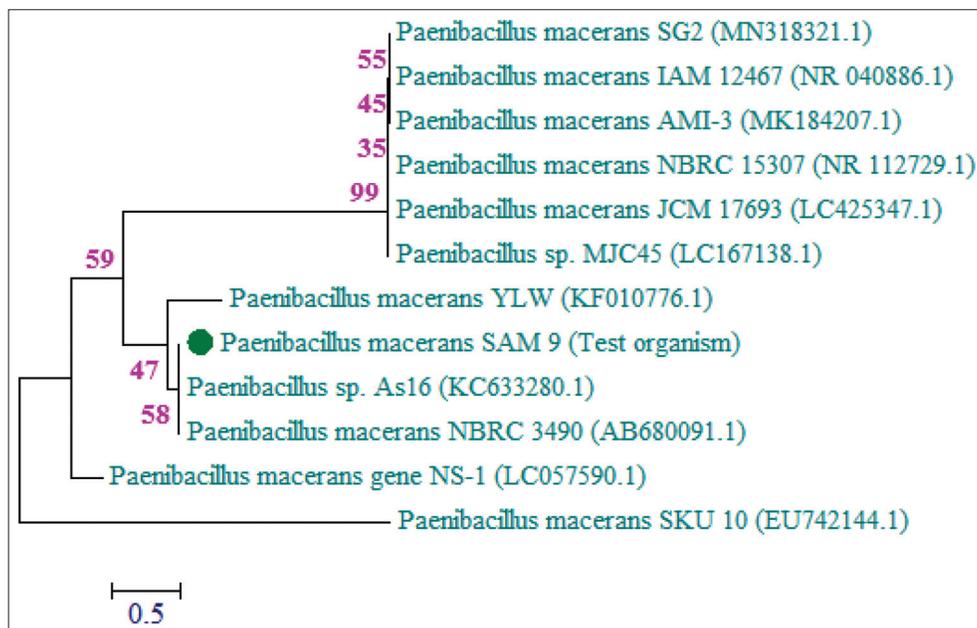


Figure 3: The evolutionary history was understood with the Neighbor-Joining procedure [26]. The standardized tree with the branch length of 6.32510527 is shown here. The percentage of 1000 replicated trees in which the associated taxa clustered together in the bootstrap method was evidenced next to the branches [27]. The evolutionary distances were analyzed based on the Maximum Composite Likelihood procedure [16]. This estimation utilized 12 nucleotide sequences with a total of 1421 base pairs in the final dataset. Evolutionary distance was analyzed using MEGA7 [17].

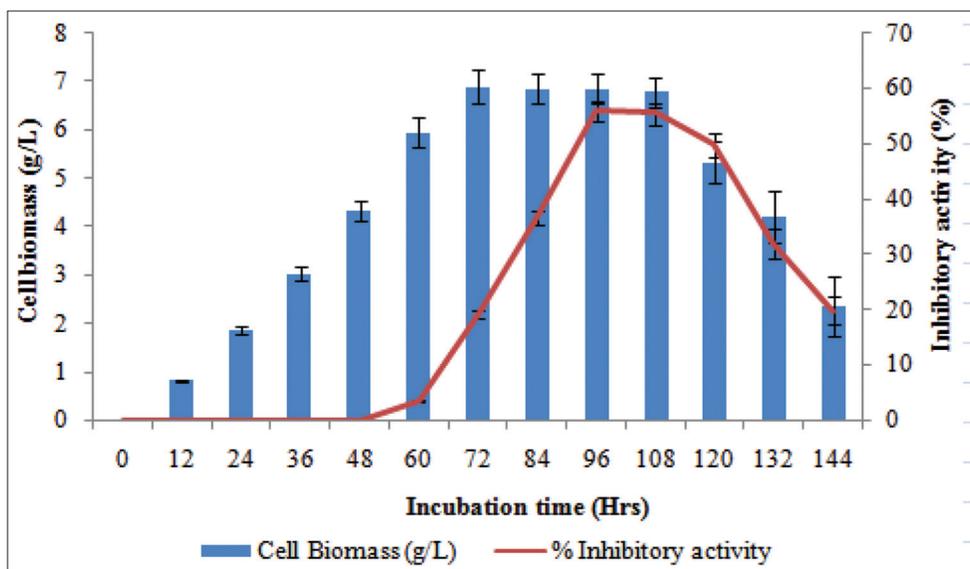


Figure 4: Growth kinetics profile as a function of time on bioactive molecule production using the marine *Paenibacillus macerans* SAM 9.

marine sediment of San Diego coast, California, yielded novel bioactive secondary metabolites which were active against both Gram-positive and Gram-negative bacteria [28]. According to Bhatnagar and Kim [29], most of the bioactive molecules were produced as secondary metabolites by diversified microorganisms of both terrestrial as well as marine origin.

3.4. Recapitulation of pH, Temperature, Carbon, Nitrogen, and Carbon/Nitrogen Ratio for the Enhanced Bioactive Molecule Production and its Purification

Optimization of physicochemical conditions plays a key factor in the production as well as the industrialization of any product [30]. Since every individual microbial strain has their unique biochemical, metabolic, and functional properties, this study standardized different physicochemical parameters aimed for the enhanced production of the bioactive molecule using *P. macerans* SAM 9. Among the studied various pH conditions, this strain showed maximum production at pH 8 with 59.7% growth inhibition followed by pH 8.5 with 55.9% growth inhibition [Figure 5]. Further, SAM 9 revealed a strong association on hydrogen ion concentration for the bioactive molecule production. Regarding temperature, SAM 9 showed maximum production at 35°C with 63.1% growth inhibition; further, the least value was recorded at 20°C with 20.2% growth inhibition [Figure 6].

Using different carbon sources at 1% concentration, the maximum production was evidenced in maltose with 68.5% growth inhibition followed by glucose and fructose with 64.4% and 59.4% growth inhibition activities. Further, poor active molecule production was recorded with polysaccharide substrates tested in this study, namely, cellulose and starch with 41.5% and 44.3% activities [Figure 7]. Similarly, when studied with various nitrogen substrates at 0.5% concentration, ammonium nitrate revealed maximum production of this bioactive molecule with 74.3% growth inhibition followed by beef extract (65.7% growth inhibition) and least activity of 41.2% growth inhibition was recorded with ammonium sulfate [Figure 8].

The carbon and nitrogen ratio was also examined for the enhanced bioactive molecule production using *P. macerans* SAM 9. This strain showed a strong dependence on the respective carbon and nitrogen ratio studied for the bioactive molecule production; further,

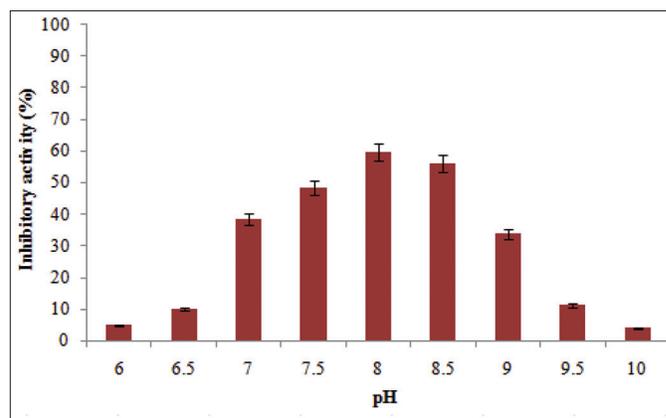


Figure 5: Influence of various pH conditions on the bioactive molecule synthesis using the marine *Paenibacillus macerans* SAM 9.

the maximum production was observed in 2:1 carbon and nitrogen ration with 74.4% growth inhibition and the lowest growth inhibition of 44.5% was revealed in 1:4 carbon and nitrogen ratio [Figure 9]. Moreover, the potential strain showed low production of the bioactive molecule during the increased concentration of nitrogen source.

Similarly, Kavitha and Vijayalakshmi [31] isolated an actinobacterium, *Nocardia levis* from Guntur, Andhra Pradesh, India, recorded its highest yield of bioactive metabolites using sucrose and tryptone at 96 h incubation along with the cultural conditions of 30°C temperature and pH 6.5, respectively. Likewise, an endophytic *Fusarium* sp. collected from Arunachal Pradesh, India, showed an appreciable production of the bioactive agent under the optimized conditions of 0.1% dextrose and yeast extract as carbon and nitrogen sources at pH 6 and 25°C temperature after 144 h incubation [32].

After 96 h incubation time, the bioactive molecule was extracted with ethyl acetate and purified with RP-C₁₈ silica gel chromatography. From the total collected fractions, the fraction eluted at 80:20 ratio of acetonitrile and methanol revealed bioactivity. Further, only one spot was observed on both the normal and RP TLC plates at the *r_f* values of 0.78 and 0.19 which confirmed the presence of a single bioactive molecule produced by the potential bacterium, *P. macerans* SAM 9, of this study

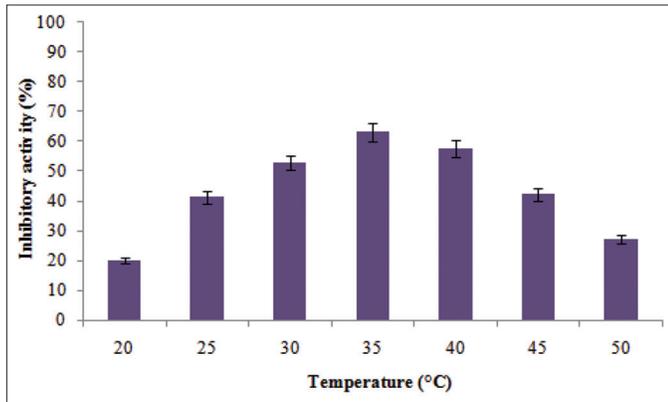


Figure 6: Influence of various temperature on the bioactive molecule synthesis using the marine *Paenibacillus macerans* SAM 9.

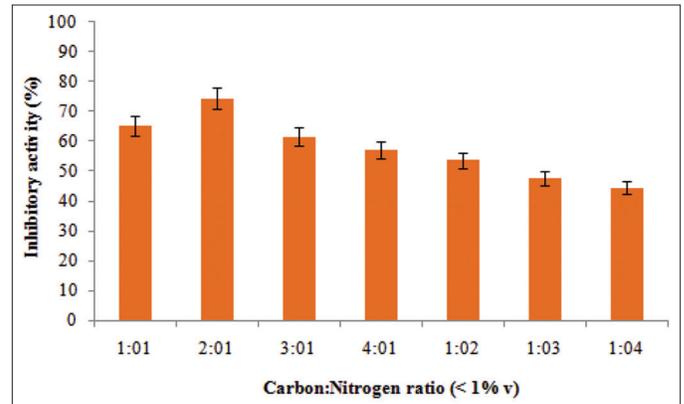


Figure 9: Effect of various carbon/nitrogen ratio on the bioactive molecule production using the marine *Paenibacillus macerans* SAM 9.

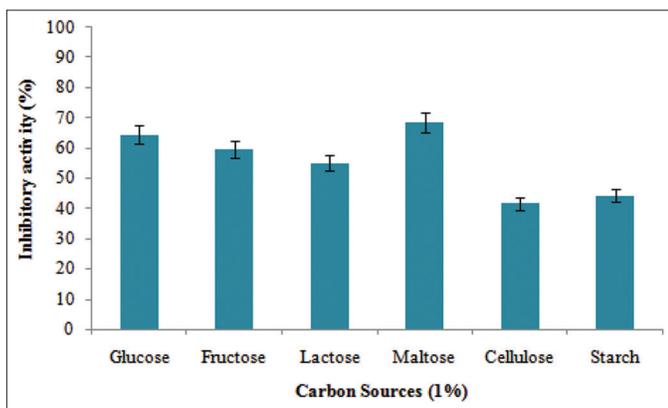


Figure 7: Study of various carbon sources on the bioactive molecule production using the marine *Paenibacillus macerans* SAM 9.

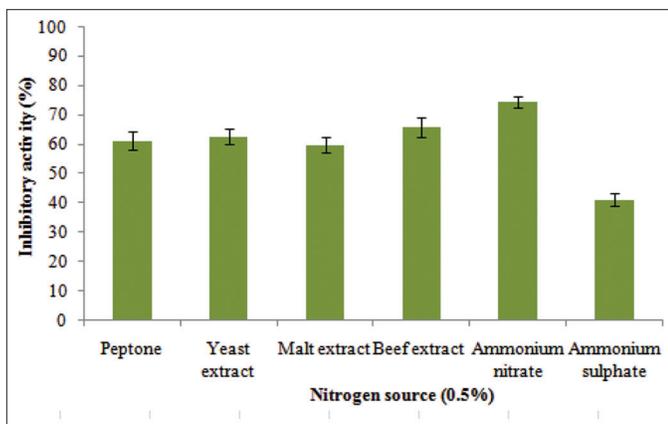


Figure 8: Study of different nitrogen sources on the bioactive molecule production using the marine *Paenibacillus macerans* SAM 9.

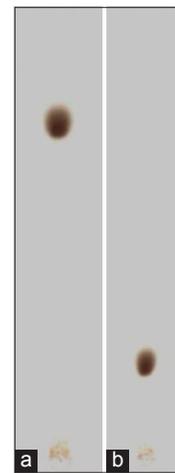


Figure 10: (a) normal and (b) reverse phase thin-layer chromatography plates showed single spot on the purified bioactive fraction using iodine vapor which proved the presence of one bioactive molecule.

[Figure 10]. Similar solvent extraction and the purification using RP-C₁₈ silica gel chromatography as well as the confirmation of purified molecule with 2D TLC were followed in many previous investigations [4,5,10,33], which proved the applicability of this used procedure.

4. CONCLUSION

The symbiotic marine bacteria, *P. macerans* SAM 9, isolated from the tentacles of sea anemone, *H. aurora* evidenced appreciable synthesis

of an extracellular bioactive molecule. The bioactive molecule showed promising antimicrobial activity against six clinically isolated human bacterial pathogens, namely, *P. mirabilis*, *K. oxytoca*, *S. paratyphi*, *S. typhi*, *E. coli*, and *S. aureus*. Further, this study achieved enhanced production of this bioactive molecule using the easily lab consumable nutrition medium and the purification evidenced the presence of one molecule responsible for these biological activities which suggesting its feasibilities for bioindustrial production. Based on these significant properties, it can further be investigated for the possible application of antibiotic drug development against the feasible deadly multi-drug resistant bacterial infections and newly emerging bacterial pathogens.

5. CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest on the publication of this article.

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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9. ETHICAL APPROVALS

This study does not involve the use of animals or human subjects.

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