

Novel Compounds From Marine Bacteria Of Ap And Tamilnadu Coastal Regions – A Review

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Running Title: Novel substances produced by A.P. marine bacteria

ABSTRACT

Marine bacteria are promising candidates for production of a wide range of unique substances due to the specific environment they survive in. Hence an extensive study has been made to explore the medical and environmental importance of these bacteria isolated from from different coastal regions. In the present study, two marine microorganisms namely *Bacillus licheniformis* and *Pseudomonas aeruginosa* have been proved to produce new antibiotics at 30°C and pH 7 with optimum yield at NaCl 3%, peptone 1% and 2% lactose concentration. Further, an unidentified bacterial isolate from Puducherry seacoast is a potent agent of anticancer enzyme L-Glutaminase (1160 units) at Ph 8 and 40°C. *Bacillus amyloliquefaciens* isolated from Nellore coastal region and another marine bacteria cultured from Chirala coast showed positive reaction for biodegradable plastic, polyhydroxy butyrate.

Key words: Marine bacteria- glutaminase enzyme- antibiotic -PHB production.

INTRODUCTION

Marine microbes especially actinobacteria represent a potential source for commercially important bioactive compounds and their bioremediation capabilities are also remarkable (Gulve and Deshmukh, 2011). Marine environment represent a largely untapped source for potentially new microflora and they have been a source of numerous useful products including pharmaceuticals, agrochemicals, enzymes, etc (Mudryk and Podgorska, 2006).

Many of the earlier studies made in this emerging field were pertained to temperate regions only and focus on tropical environment gained significance in the last two decades (Zheng et.al., 2005). In India, out of 9 maritime states, four have been extensively covered namely Maharashtra, Goa, Andaman Nicobar islands, Kerala. Especially in the southern parts, more investigation has been done in Visakapatnam and Chennai coastal parts (Raghunathan and Umadevi, 2012) and no comprehensive data is available on remaining coastal regions of Andhra Pradesh and Tamil Nadu

Hence the present attempt has been made to explore the marine microbial diversity in Nellore, Prakasham and Guntur coastal regions and also to reveal their antimicrobial potential, therapeutic enzyme production and ecofriendly PHB production.

MATERIALS AND METHODS

Collection of marine samples: Ten samples (both sea water and sediment) were collected 20 feet away from seashore covering all the three seasons in the intertidal zone at three coastal locations of Andhra Pradesh (Chirala and Ongole of Prakasham District; Vakadu - Nellore District)

and two from Puducherry and Chennai Beach. The samples were kept in sterile screw cap tubes under sterile conditions in the lab and labeled. Physicochemical nature of the samples was analyzed. They were stored at a temperature of 10°C until further use.

Isolation of marine bacteria: 100µl of sea water samples were spread on surface of Marine Agar medium prepared in sterile sea water (Himedia 2216; pH 7.6). After incubation at 30°C for seven days, all colonies with different pigmentation and morphology were chosen for bacterial characterization.

Preparation of bacterial crude extracts: Marine bacteria isolated were cultured in 250ml marine broth dissolved in 1L seawater for the production of secondary metabolites. Flasks were incubated on a rotary shaker at 220 rpm for 7 days at 25°C. Broth was centrifuged at 5000xg for 30 mins to remove cells, and then extracted 3 times with 100ml ethyl acetate. After removal of the solvent at reduced pressure, the extracts were used as crude samples for bioactivity assay (Siva Kumar et.al., 2007).

TLC autobiography assay: Two crude samples of marine bacteria showing antimicrobial activity were dissolved in ethyl acetate and made up to a concentration of 100µg/ml. Two µl of solution was plated on silica gel plate using dichloromethane, ethyl acetate and methanol in the ratio of 5:5:1 as mobile phase. The developed TLC plates were UV sterilized for 30 mins and then enched in nutrient agar containing test microorganisms. After 10 hr diffusion at 8°C, plates were incubated at 37°C for 24hrs and the agar plate was sprayed with 5mg.ml⁻¹. Inhibition zones were observed as clear spots against a purple background and their R_f values were calculated (Zheng et.al., 2005).

Screening of marine bacteria for glutaminase production: Marine agar medium incorporated with 0.5%

glutamine and phenol red was inoculated with the marine samples and incubated for 48hrs at room temperature. Appearance of bright pink colour of the medium is an indication of glutaminase positive cultures (Prashanth Kumar et.al., 2009).

Screening for polyhydroxybutyrate production by marine isolates: Microbial isolates were stained with Sudan black B (0.3%, w/v) for 5 to 15 minutes and counter stained with safranin (0.5%, w/v) for 30 seconds. PHB granules appeared as deep blue droplets in the pink coloured cytoplasmic part of micro organisms under oil immersion objective lens (Mirac et.al., 2005).

Fermentation conditions for optimum yield of glutaminase, antimicrobial compounds and PHB (Saurav et.al., 2010)

Carbon source: To determine the effect of carbon sources on production of antibiotic, glutaminase and PHB, different carbon sources namely glucose, lactose, fructose, sucrose and starch at 1% concentration was added separately to the medium.

Nitrogen source: Influence of various nitrogen sources on bioactive metabolite production, enzymatic activity and PHB was evaluated by supplementing individually sodium nitrate, peptone, yeast extract and glutamine to the fermentation medium.

C/N ratio: After estimating the suitable carbon and nitrogen sources, the effect of varying concentrations of the best carbon(0.5-5%) and nitrogen sources(0.1-2%) was analyzed..

NaCl concentration: NaCl at 1-5% concentration was used to estimate the optimum level favouring marine bacterial growth and products yield.

Substrate concentration: Glutaminase activity was assayed at different substrate concentrations of 0.3-0.8 M to assess its optimum amount yielding maximum enzymatic activity.

Temperature: In order to know the effect of environmental factors on the above three parameters, selected isolates were subjected to different temperatures ranging from 20-70°C.

Ph: Optimum pH for the three pharmaceutical products was determined by growing the marine isolates at various initial pH starting from 4 - 10.

Identification of the potent marine bacterial isolates: Marine cultures efficient in biometabolite, glutaminase and PHB production were first identified to the genus level by observing their morphology, cultural and biochemical fetures based on Bergey's Manual of classification. Species level identification was made by PCR amplification of the 16S r RNA gene, BLAST analysis and comparison with sequences in the Gen Bank nucleotide database(Valli et.al., 2012).

RESULTS AND DISCUSSION

Physico chemical characteristics of marine samples

Marine water samples were examined for color, odour and texture at the sampling site. pH, temperature, salinity and electrical conductivity of the samples are

estimated following standard procedures as described earlier (Krishna kumara et.al., 2013). pH of the three samples at different sites was found to be towards alkaline range, whereas the temperature indicate its mesophilic nature. Comparatively, electrical conductivity and salinity was more in Chennai marina beach water sample.

Isolation of marine bacteria from marine water

The marine bacteria were isolated from the marine water sample by serial dilution method. The marine water sample was aseptically added to the saline and serial dilution was made up to 10⁻⁵. A total of 24 isolates were obtained from the marine water samples. The numbers of samples and isolates in each sample were presented in Table. 1. Twenty four isolates which are present in the three marine samples include fifteen gram positive and nine gram negative organisms. Isolates were observed for different colony morphology, gram nature, size and arrangement of cells. Out of the 24 isolates, five cultures i.e. CMB-1 (Chennai Marine Bacteria), CMB-2, CMB-3, KMB-1 (Kothapattanam Marine Bacteria), KMB-2, CHMB-1 (Chirala marine bacteria) NVMB-1 (Nellore Vakadu Marine bacteria) and NVMB-2 were the seven strains selected for further analysis, since they showed distinct colony morphology.

Table. 1: Isolation of marine bacteria from marine water samples.

S. No	Geographic Location	Sampli ng spot	Type of sample	No of isolat es
1.	Chennai	Sea Shore	Water	10
2.	Ongole (Kothapattan am)	Sea Shore	Water	6
3.	Nellore- Vakadu	Sea Shore	Water	8
4	Chirala(Vada revu)	Sea Shore	Water	1
5	Puducherry	Sea Shore	Water	3

Analysis of antibiotic production by marine isolates

Thin layer chromatography

The concentrated 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) gave the best separation (Fig.1).

Direct bioautography was carried out to localize antibacterial substances.

The study showed the presence of 2 active substances (Fig.1). CMB-3 compound with Rf 0.8 showed more activity than



the one with Rf 0.7 of KMB-1. Similarly, Zheng et.al., (2005) reported Rf value for NJ5-1 isolate as 0.7 and 0.8 for QD1-2

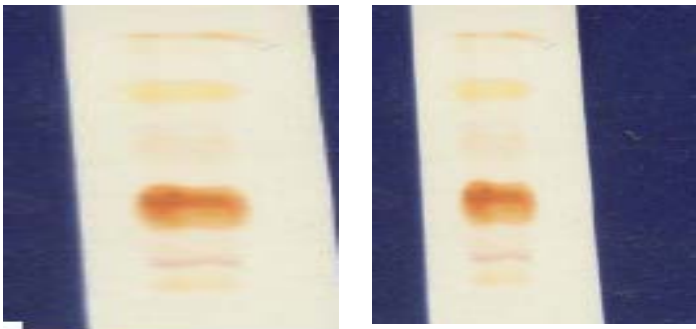


Fig 1: Thin layer autography of marine isolates CMB-3 and KMB-1 Glutaminase production by Puducherry marine bacteria (PMB 1 and 2):

Glutaminase enzyme activity was measured in terms of ammonium liberated at 459 nm. PMB1 positive isolate showed maximum enzyme activity of 1160units /ml, whereas 560 units/ml of activity was observed in PMB 2 (Table 2). But a study made by Rinehart(2000) showed that marine *Vibrio costicola* produced only 232U/ml of glutaminase activity in solid state fermentation. Mayiliraj et.al., 2006 also made a similar statement with actinobacteria isolated from Himalayas.

Table 2: Glutaminase activity by marine isolates

Marine isolate	Enzyme activity (U/ml)
PMB1	1160
PMB2	560
PMB3	550
PMB4	369
PMB5	210

PHB production: Among the 8 different marine isolates, two isolates named as NVMB1and NVMB2 obtained from Vakadu and CHMB from Chirala seacoast were identified positive for presence of lipophilic PHB granules.PHB granules were observed as dark blue coloured droplets in the pink vegetative cells by using sudan black B staining method (Fig 2).

Fig 2: Microscopic observation of potent isolate

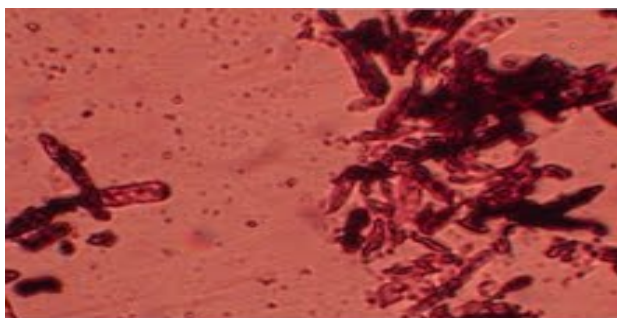


Fig 3: Observation of PHB granules on Phase contrast microscope

The above photographs illustrates that the formation of PHB granules change the cell shape from rod to oval to accommodate the size of growing granules. Similar to this, Mirac(2005) also observed PHB production from marine isolates of Egypt. This was further confirmed by Tohiyama et.al., (2002).

Fermentative conditions for product yield: Effect of temperature :

Zone of inhibition of CMB-3 was 21.3 mm on *S. aureus*, 14.7 mm on *E. faecalis*, 18.3 mm on *K. pneumonia* and KMB-1 was 20.7 mm on *S. aureus*, 15.0 mm on *E. faecalis*, 17.6 mm on *K. pneumoniae*. Further increase in temperature (above 30°C) resulted in the decreased growth rate and decline in the production of bioactive metabolite (Fig. 4 and 5). In terms of its optimum temperature for growth, the organism appeared to be mesophilic in nature.

Zone of inhibition was significantly more at the optimum temperature of 30°C in *S. aureus* with respect to the remaining test pathogens.

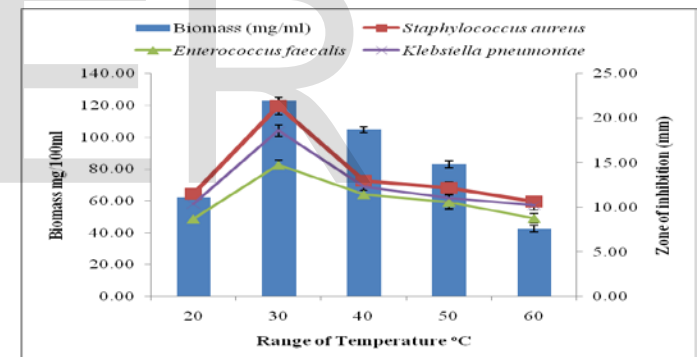


Fig 4. Effect of temperature on biomass and bioactive metabolite production by CMB-3. Each bar represents the Mean ± SEM of three independent values. P< 0.05

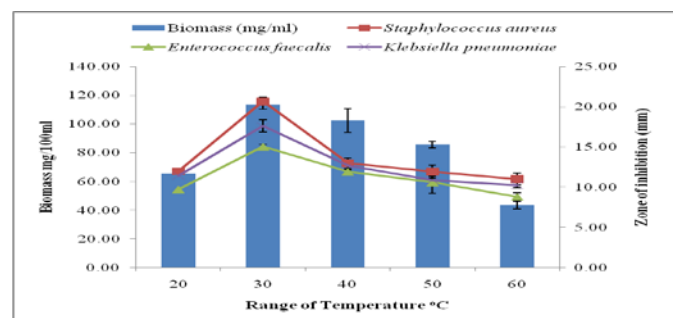


Fig 5. Effect of temperature on biomass and bioactive metabolite production by KMB-1. Each bar represents the Mean ± SEM of three independent values. P< 0.05

Vastrad, and Neelagund,(2011) made a report that among the physical factors, temperature is one of the most critical parameters that could affect the bioprocessing. Several studies revealed that temperature was found to influence extracellular metabolites secretion, possibly by changing the physical properties of the cell membrane (Rahman *et al.*, 2005).

Glutaminase activity: Enzyme activity was maximum at 40°C. However, it retained its activity to certain extent upto 60°C. Whereas at 70°C, there was a sharp decline in the activity (Table 3) coinciding with the experimental evidence of Prabhu and Chandrasekharan(1999).Accordingly, 85% of the activity was retained over a temperature range of 25-45°C. Similar observation was made by other workers also.

Table 3: Effect of temperature on glutaminase activity

Temperature(°C)	Relative enzyme activity
30	94
40	96
50	63
60	42
70	21

Effect of pH on antibiotic production:

The intracellular pH of most microorganisms is maintained near neutrality irrespective of the pH in the outside medium. 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 antimicrobial metabolite by the strain was at pH 7.0(Figs 6&7). The antimicrobial compound produced by the isolates had a moderate effect on the *K. pneumoniae*. This result agrees 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

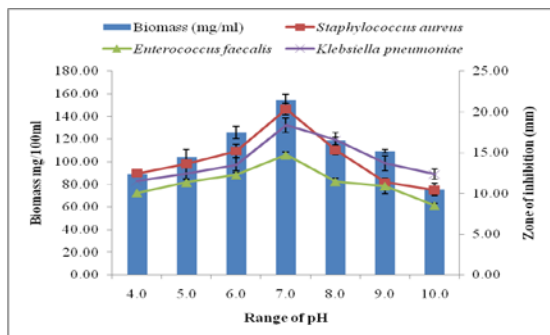


Fig.6. Effect of pH on biomass and bioactive metabolite production by CMB-3. Each bar represents the Mean ± SEM of three independent values. P < 0.05

Fig 7. Effect of pH on biomass and bioactive metabolite production by KMB-1. Each bar represents the Mean ± SEM of three independent values. P < 0.05

Glutaminase activity: As per the experimental data given in Table 4, glutaminase enzyme was active over a pH range of 4-8.Optimum enzyme activity was noticed at pH 8. The activity considerably decreased at both low and high pH. Similar values were obtained in the two isolates. Basha *et al.*, (2009) stated that another anti cancer enzyme asparaginase produced by actinomycetes exhibited maximum activity at Ph 7.5which is close to blood Ph. Whereas, bacterial asparaginase had optimum Ph of 8-8.5.

Table 4: Effect of pH on glutaminase activity

pH	Relative activity
4	24
6	67
8	96
10	21

Effect of carbon source on antibiotic production : The results indicated that antibiotic production is greatly influenced by medium constituents. It was found that the bioactive metabolite production by CMB-3 and KMB-1 was positively influenced by the carbohydrates and nitrogen sources. Sole *et al.*, new (1997) noted that lactose and glucose can be used as a source for bacterial growth while repressing the production of secondary metabolites. The results suggest that antibiotic production was higher in medium having 1% lactose as carbon source (Figs 8&9). Simple carbohydrates like lactose, glucose and dextrose through metabolic pathway affect on production of intermediates leading to primary as well as secondary metabolites in addition to CO₂, water and energy. Addition of lactose and glucose resulted highest growth of the microorganisms, but significantly in many fermentation processes higher concentration of carbon source has a suppressive effect on production of bioactive metabolites

(Gogoi *et al.*, 2008). The bioactive metabolite production got reduced with increase or decrease of lactose concentration.

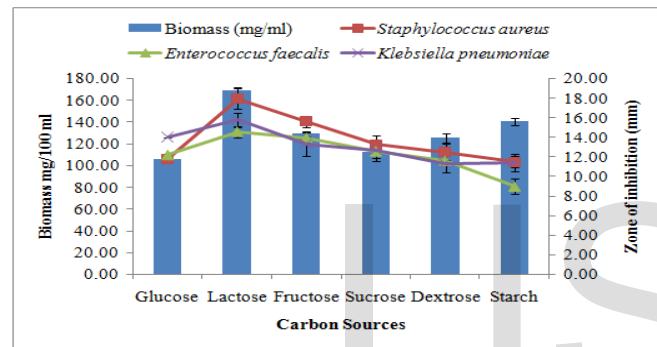
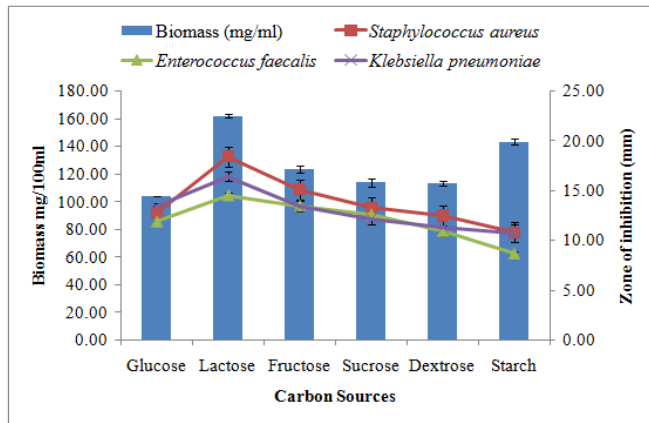


Fig. 8. Effect of different carbon sources on biomass and bioactive metabolite production by CMB-3. Each bar represents the Mean \pm SEM of three independent values. $P < 0.05$

Fig. 9. Effect of different carbon sources on biomass and bioactive metabolite production by KMB-1. Each bar represents the Mean \pm SEM of three independent values. $P < 0.05$

PHB production: PHB yield was determined using different carbon sources like glucose, sucrose, fructose, cellulose respectively. Interestingly, sucrose is found to be most suitable for PHB production, followed by glucose and least in cellulose (Table 5). The present experimental results were supported by Rajendran (2010). In contrast to this, Chien *et al.*, (2007) reported that glucose stimulated to the maximum extent. Similar to this, Singh *et al.* (2013) also stated that maximum yield is given by glucose.

Table 5: Effect of carbon source on PHB production:

Carbon Source	PHB yield
Glucose	120
Fructose	98
Sucrose	181
Cellulose	67

Effect of nitrogen source on antibiotic production : Nitrogen is very vital in the synthesis of enzymes involved in primary and secondary metabolism. Therefore depending on the biosynthetic pathways involved, nitrogen sources may affect antibiotic formation. Shapiro (1989) noted that the type of nitrogen source (organic or inorganic) plays a role in the synthesis of secondary metabolites. Charyulu and Gnanamani (2010) reported that *P. aeruginosa* MTCC 5210 utilized organic nitrogen source for better yield of antimicrobial metabolites than the inorganic sources.

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 CMB-3 and KMB-1 (Figs 10 & 11). 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. In the present study, the metabolites produced by CMB-3 and KMB-1 grown under optimized conditions like carbon and nitrogen sources exhibited good antimicrobial activity against gram positive and gram negative bacteria.

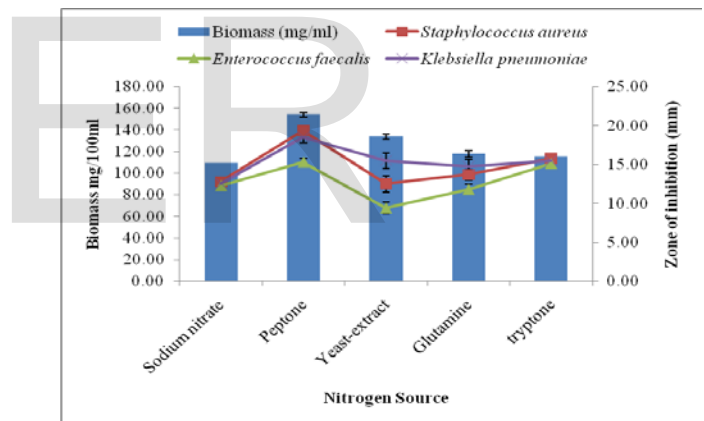


Fig. 10. Effect of different nitrogen sources on biomass and bioactive metabolite production by CMB-3. Each bar represents the Mean \pm SEM of three independent values. $P < 0.05$

Fig. 11. Effect of different nitrogen sources on biomass and bioactive metabolite production by KMB-1. Each bar represents the Mean \pm SEM of three independent values. $P < 0.05$

Effect of C/N ratio on antibiotic production:

As lactose emerged as the most preferred carbon source for bioactive metabolite production by the isolates, varying concentrations of lactose (0.5-5%) was tested to determine its optimal concentration. As shown in Figs 12 and 13, lactose at levels of 2% showed optimal yield of bioactive metabolites. (CMB-3 147.8 mg/100ml and 124.9

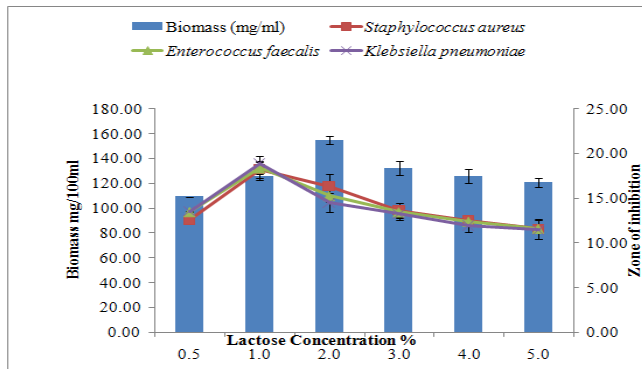


Fig. 12. Effect of different concentrations of lactose on biomass and bioactive metabolite production by CMB-3. Each bar represents the Mean \pm SEM of three independent values. $P < 0.05$

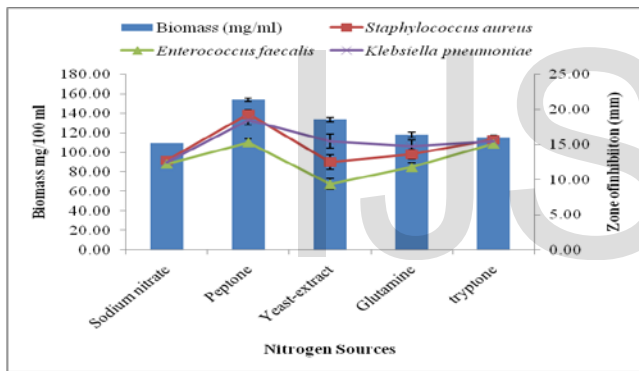


Fig. 13. Effect of different concentrations of lactose on biomass and bioactive metabolite production by KMB-1. Each bar represents the Mean \pm SEM of three independent values. $P < 0.05$

Effect of different concentrations of peptone on the production of bioactive metabolite production has been shown in Fig. 14 and 15. It should be noted that peptone at a concentration of 1% and 0.5% exhibited optimal production of biomass (CMB-3 was 167.4 mg/100ml and 162.2 mg/100ml, KMB-1 was 165.8 mg/100ml and 159.6 mg/100ml; zone of inhibition 19.33 mm and 18.5 mm) and antibacterial compound, respectively (Figs. 14 and 15).

Fig. 14 Effect of different concentrations of peptone on biomass and bioactive metabolite production by CMB-3. Each bar represents the Mean \pm SEM of three independent values. $P < 0.05$

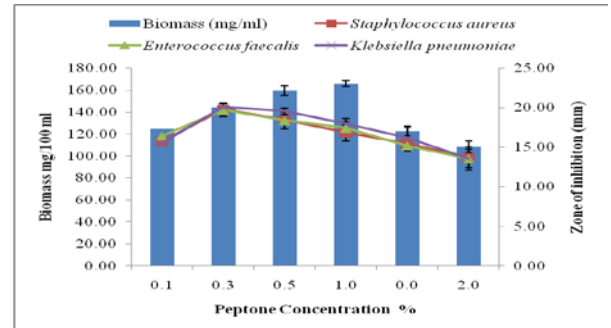


Fig. 15. Effect of different concentrations of peptone on biomass and bioactive metabolite production by KMB-1. Each bar represents the Mean \pm SEM of three independent values. $P < 0.05$

Effect of NaCl on antibiotic production

The details of the effect of NaCl concentration on production of biomass and bioactive metabolites by CMB-3 and KMB-1 were shown in Figs 16 and 17. Significant production of bioactive metabolite was obtained in NaCl (3%) amended media by 4% and 6% NaCl concentration. Similarly, the production of biomass was high in NaCl (3%), biomass was decreased with increasing concentration of NaCl. Biomass production of CMB-3 was 150 mg/100ml and KMB-1 was 165 mg/100ml in NaCl (3% concentration). The zone of inhibition was 19.63 mm on *S. aureus*, 21.33 mm on *E. faecalis* and 20.8 mm on *K. pneumoniae* on CMB-3 at NaCl (3%)

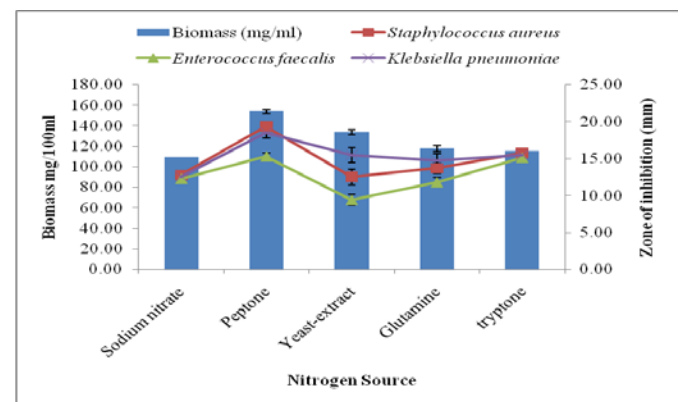


Fig. 16. Effect of different concentrations of NaCl on biomass and bioactive metabolite production by CMB-3. Each bar represents the Mean \pm SEM of three independent values. $P < 0.05$

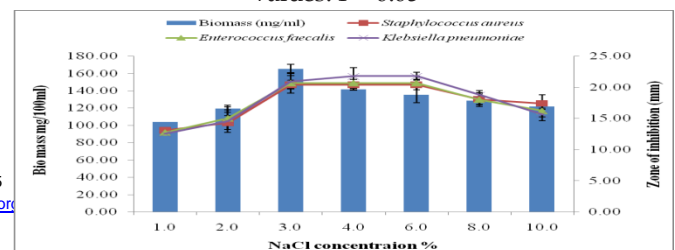
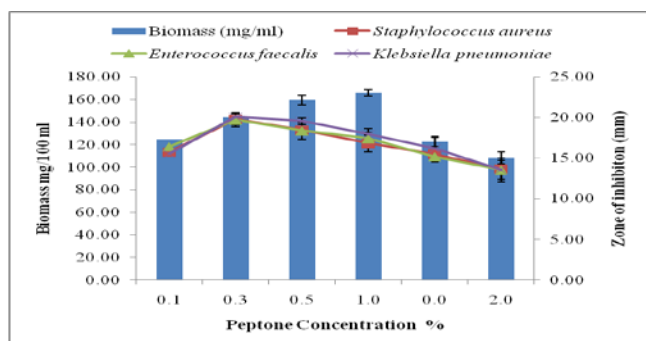


Fig 17. Effect of different concentrations of NaCl on biomass and bioactive metabolite production by KMB-1. Each bar represents the Mean \pm SEM of three independent values. $P < 0.05$

Effect of substrate concentration on glutaminase enzyme activity:

Enzyme activity was observed at different concentrations of glutamine substrate. Ninety four percent (94%) of activity was observed in presence of 0.8M concentration of substrate. At 0.6M, moderate amount (83%) of enzyme activity was observed (Table 6). Experimental findings of Prabhu and Chandrasekharan(1999) reveal substrate concentration of 0.04M was optimum for glutaminase which contrasts with the present experimental result.

Table 6: Effect of substrate concentration on glutaminase enzyme activity

Substrate concentration	Enzyme activity(%)
0.4	52
0.6	83
0.8	94
1.0	65

Identification of bacterial isolates:Antibiotic production:

Morphological and cultural characteristics of the isolates are illustrated in .figs 18,19&20. Details of the data obtained from the two isolates was also given in tables 7 and 8.



Chennai marine sample
Kothapattanam marine sample



Chennai marine bacteria
Kothapatnam marine bacteria



Nellore marine sample

Figure.18. Bacterial colonies isolated from marine water
Morphological and cultural characteristics of the isolated strains

Among the seven isolates, three grown overnight in marine broth at 37°C (CMB-1, CMB-2 and CMB-3) were found to be aerobic, gram positive, motile, rod-shaped, and endospore-forming bacteria. Colony morphology was also examined on the plates.

After incubation for 16 h at 37°C

Morphological characteristics	Isolates						
	CMB-1	CMB-2	CMB-3	KMB-1	KMB-2	NMB-1	NMB-2
Gram stain	+	+	+	-	-	-	-
Straight rods	+	+	+	+	+	+	+
Arrangements	Single	Single	Clusters	Single	Clusters	Single	Single
Spore	+	+	+	+	+	+	+
Motility	+	+	+	-	-	-	-

on marine agar medium, the bacterial colonies observed were creamy white or white-colored, irregular to round, and flat in appearance. But two isolates (KMB-1 and KMB-2) are large, irregular and pale colour appearance. They were found to be gram negative rod shaped motile and endospore-forming bacteria. NMB-1 and NMB-2 were found to be Gram negative coccobacilli. On the agar medium, the colonies were irregular, large, non motile and grey in appearance. Cultural and morphological characteristics of the five potential isolates were analyzed and represented in the Tables.7 and 8

CMB-1 isolate

KMB-1 isolate



KMB-2 isolate

Fig. 19. Colony morphology of the different marine bacterial isolates

Table. 7: Cultural characteristics of marine bacterial isolates

Cultural characteristics	Isolates						
	CMB-1	CMB-2	CMB-3	KMB-1	KMB-2	NMB-1	NMB-2
Configuration	Irregular	Round	Irregular	Irregular	Irregular	Round	irregular
Size	Small	Medium	Small	Large	Large	Large	Small
Colour	Creamy	White	White	Pale	Pale	Grey	Grey
Consistency	Viscous	Moist	Moist	Viscous	Moist	Viscous	Viscous
Margin	Wavy	Wavy	Entire	Wavy	Wavy	Entire	Entire
Elevation	Convex	Convex	Flat	Convex	Convex	Convex	Convex
Growth in broth	Turbidity	Turbidity	Forms pellicle	Turbidity	Turbidity	Turbidity	Turbidity

Table. 8: Morphological characteristics of marine isolates

Morphology of antibiotic producing isolates

The morphology of antibiotic producing isolates was examined under 100X oil immersion objective. As shown in Fig.20 the marine isolates CMB-3 was gram positive bacilli and KMB-1 was observed as gram negative

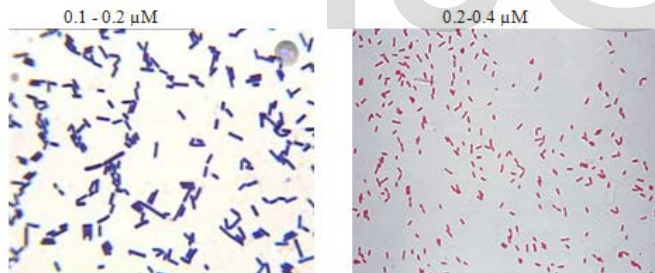
rod. The size of the isolates was measured by using micrometry. CMB-3 is 0.1 - 0.2 μM and KMB-1 is of 0.2-0.4 μM. Relatively CMB-3 is smaller than KMB-1.

Fig.20. Marine isolates gram staining reaction

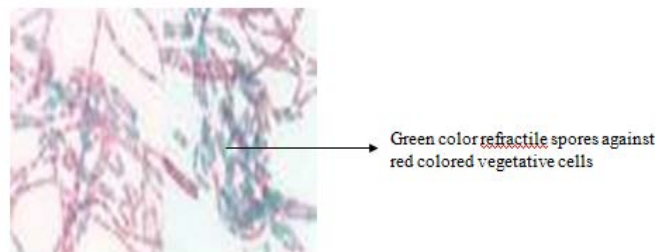
Biochemical characterization of antibiotic producing marine bacteria isolates CMB-3 and KMB-1

Isolate CMB-3 isolate showed a positive reaction on catalase, oxidase, urease and citrate utilization, nitrate reduction and a negative reaction of indole, methyl red and VP (Table 9; Fig.21). CMB-3 showed negative reaction for sugar fermentation with glucose and sucrose and positive reaction for lactose (Fig.21). KMB-1 isolate showed positive reaction for nitrate reduction, catalase test and citrate utilization. Negative reaction in indole, methyl red, VP, urease and oxidase. Lactose fermentation was also observed in KMB-1. Both acid and gas production was observed. Biochemical characteristics of the isolates were listed in Table.9. In contrast to KMB-1, CMB-3 showed positive results for urease. Based on the cultural, morphological and biochemical nature of the isolates and with reference to Bergey’s manual, the organisms were tentatively identified as species of *Bacillus* and *Pseudomonas*.

Table.9. Biochemical characterization of selected marine bacterial isolates.

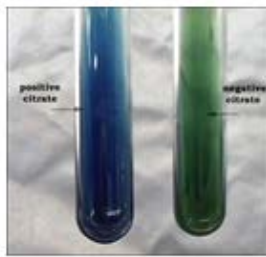


KMB-1 (Gram negative rods)

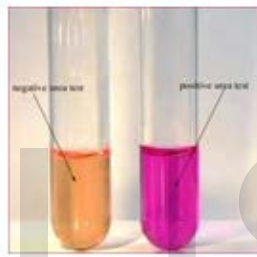


Endospore staining

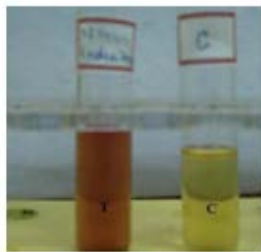
Biochemical tests	CMB-3	KMB-1
Indole production	-	-
Methyl red test	-	-
Voges Proskeaur	-	-
Citrate utilization test	+	+
Urease production	+	-
Nitrate reduction	+	+
Aerobic growth	+	+
Lactose fermentation	+	+
Glucose, Sucrose fermentation	-	-
Catalase test	+	+
Oxidase test	+	-
Production of fluorescent pigment	-	-
Identity	<i>Bacillus</i>	<i>Pseudomonas</i>



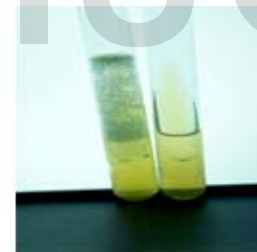
Citrate utilization (CMB-3 and KMB-1)



Urease test (CMB-3)



Nitrate reduction (CMB-3 and KMB-1)



Oxygen test (CMB-3 and KMB-1)



Oxidase test (CMB-3)



Lactose fermentation (CMB-3 & KMB-1)

+ : indicates positive reaction
- : means negative reaction

identified as *Bacillus sp.* and *Pseudomonas sp.* Partial sequencing of 16S rRNA gene was utilized at BioAxis, Hyderabad to report the organism up to species level. As mentioned in the methodology universal primers were used for the amplification of which results in a product of 546 bp for *Bacillus* and 639 bp for *Pseudomonas*. The BLAST search analysis of the 16S rRNA gene sequence of the isolates was done against the *Bacillus sp.* and *Pseudomonas sp.*

The sequence was multiple aligned with closely related homologous species with CLUSTAL W. Phylogenetic tree was constructed with PHYLIP analysis by using neighbor joining method. Results revealed CMB-3 had 98% similarity with *B. licheniformis* (Fig.22 and 23) and KMB-1 possess 100% similarity with *P. aeruginosa*. The 16S rRNA sequence was found to be similar with to *B. licheniformis* and *P. aeruginosa*, and its sequences were deposited in the GenBank nucleotide sequences database under accession numbers KC736854 and KC736855

Sample CMB-3

```
>AGGGCCTTTAGCTTTCCTTTAGTGTGTCAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACTGCCTGTAAGAC
TGGGATAACTCCGGGAAACCGGGGCTAATACCGGAT
GCTTGATTGAACCGCATGGCCATTCTAAAAAGGGGG
TTTTAACCTCCCCTTTCGAAGGGCCCCCGGCCCTT
ACTTATGGGGAAGGTACGGCTTCCCCAGGGCAACA
AGGCTTACCCAACCTAAAAGGGGAACCGGCCACCTG
AATGGAACCCCGGCCAAATCCTTACGGAAGGAACC
ATAAGGAATTCCTCCCATGGAACAAAGTTTGAAGG
AACCACCCCTTAATTGAGAAAGGTTTCCGGACCT
AAAACCTTCGGTGGTTAGAAAAACAATTCCGGTCCAA
AAAGGGGGCACCTTGCCGTAACCTACCAAGAAACCA
CCGCCTACTTAGTGCCAACCGCGGCGTTATACGTAG
GTGCAAGGGTTGTGCGAATTATTGGGCGTAAAGGGC
GCACGCGGTTTCTTAAGTCTGATGTGAAACCCCGGC
TCACCGGGGAGGGTCATGTAACCTGGGAACCTTGATC
CAGAGAGGAAGGTATTCCACTGTACGCTGAATGCTA
AGATCGACGACACGGCCAGGCATCTCTGTCTGCACT
CACCTGAGCTGAACCTGAGGATCCTCGATAGAGCTG
TATTCGCGCATCAACAATGCAATGTAACGATCCTCTT
TATGCAGATCAACGATTACTGTCTGCTGGAGACGGCT
ATCAATTATTGATTGCGGTTCCGCAATACGTATGTCG
TTATTCGTAGGACCAGAATTTTCGCTGCACTTCAGTA
ACGATAGAGGTACATAAGAGTACAGTTAGGACAGA
GTGTTCTTACGGCCGTGACCGCACTTC
```

The amplified product when sequenced was identified as *Bacillus licheniformis*

partial 16S rRNA gene Score = 678 bits (367)

Fig.21. Biochemical testsMolecular characterization of the marine bacterial isolates (CMB-3 & KMB-1)

Based on the morphological, physiological and biochemical tests of the isolates, CMB-3 and KMB-1 were

The amplified product when sequenced was identified as *Pseudomonas aeruginosa*. W214 16S ribosomal RNA gene, partial sequence Score = 1181 bits (639)

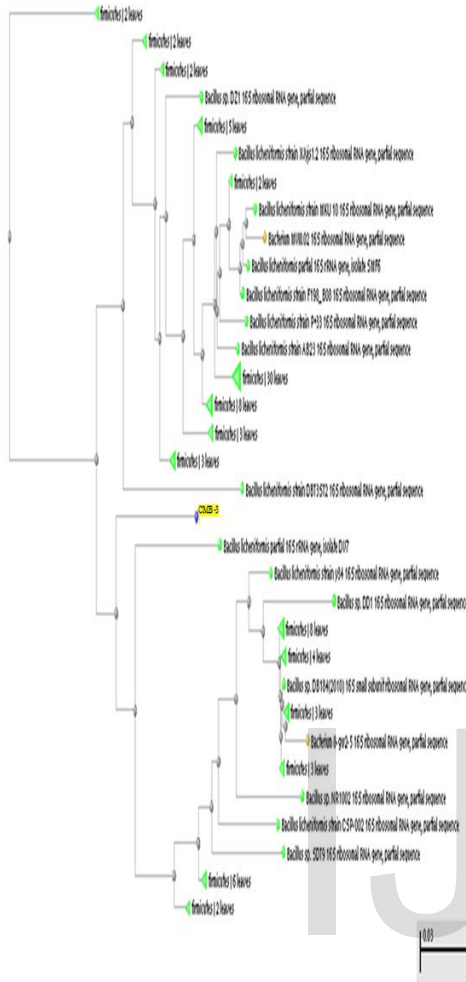


Figure.22. Phylogenetic tree of CMB-3 based on 16s rRNA sequences

Sample KMB-1

```
>CGGGTGAGTAACGCGTGGGAAATTGCCAGTAGTG
GGGGACAACATTCGGAACGGATGCTAATACCGCAT
ACGCCCTACGGGGAAAGCAGGGGATCTTCGGACCT
TGCGCTACTGGATATGCCCGTCGGATTAGCTAGTTGG
TGAGGTAATGGCTACCAAGGCGACGATCCGTATCT
GGTCTGAGAGGATGATCAGCCACACTGGGACTGAGA
CACGGCCAGACTCCTACGGGAGGCAGCAGTGGGG
AAATGGACAATGGGCGCAAGCCTGATCCAGCCATGC
CGGTGTGTGAAGAAGGCCCTAGGGTTGAAAGCAC
TTTACAGCAGGGAGGAAGGCCTTAAAGTTAATACCTT
GAGGATTGACGTTACCTGCGAGAAGCACC GGCTAAC
TCCGTGCCAGCAGCCGCGTAATACGGAGGGTGCAA
GCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTA
GGCGTTAGTTAAGCTGGATGTGAAAGCCCTGGGCT
CACC GGAACTGCATT CAGA A CTGGCTGGCTAGAGT
ACGAGAGAGGGTAGTGAATTCTCTGTGTAGCCGGTG
AAATGCGTAGATATAGGAAGGAACATCAGTGGCGA
AGGCGACTGCCTGGCTCGAT
```



Figure. 23. Phylogenetic tree of KMB-1 based on 16s rRNA sequences

Identification of glutaminase producing marine bacteria:

Morphological and cultural based identification of the potent isolate is done up to genus level following Bergeys manual of bacterial classification. Accordingly, the positive isolate is found to be gram positive bacilli. On the culture medium, the colony appeared as small, round, orange colored, with smooth surface. Biochemically, it is positive for indole production, methyl red and citrate tests. Hydrolysed casein and showed positive reaction for enzymatic activities namely catalase and oxidase. Based on the above data, it is tentatively identified as sp. of *Bacillus*.

Characterization of potent marine isolates:

Potent PHB producing bacteria were subjected to morphological and biochemical characterization by performing various tests. The potent isolates MM1 and MM2 appeared as gram positive and non motile rods under microscope. Biochemical properties of potent isolates are listed below in table 10 and fig. 24.

Table 10: Biochemical properties

S.No	Name of the test	MM ₁	MM ₂
1.	Indole Production Test	-ve	-ve
2.	Methyl Red Test	-ve	-ve
3.	Voges Proskauer Test	+ve	+ve
4.	Citrate utilisation Test	+ve	+ve
5.	Oxidase Test	-ve	-ve
6.	Catalase Test	+ve	+ve
7.	Urease Test	+ve	+ve
8.	Carbohydrate Fermentation Test	-ve	-ve
9.	Oxidative Fermentation Test	-ve	-ve
10.	Nitrate Reduction	+ve	+ve



Fig. 24. Catalase test Citrate utilisation Test Urease Test

Molecular characterization is done for the most efficient PHB producer MM₁ only at Cledora Life Sciences, Bangalore. Phylogenetically, it has 99% similarity with *Bacillus amyloliquefaciens* (Fig. 25). The bacterial sequence is submitted to Gen Bank for accession number.

CONCLUSIONS

The four marine isolates obtained from Chennai, Kottapatnam, Puducherry and Vakadu seacoasts are proved to be potent antibiotic, glutaminase and PHB producers.

The selected bacterial isolates are active and stable over a wide range of Ph and temperature and also salt tolerant indicating their suitability in industrial sector.

Polyhydroxy butyrate produced by *Bacillus amyloliquefaciens* is a biodegradable product that can be used in various fields

Based on the above results, it is confirmed that these marine bacteria have immense potential to produce ecofriendly products like PHB and have a prominent role in medical and pharmaceutical industries.

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