ISOLATION AND IDENTIFICATION OF ANTIMICROBIAL PRODUCING BACTERIA FROM MARINE SEDIMENT SAMPLE

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Abstract: Searching for novel antimicrobial agents with novel target or mechanism of action become a global demand as the bacterial resistance against antibiotics is in constant increase and this has a great effect on the mortality and increase the cost of treatments associated with control of bacterial infection. Marine natural products gained greet attentions as they have shown many interesting activities such as: anti-microbial, anticancer, anti-coagulant, anti-inflammatory, anti-diabetic, cytotoxic effect and other pharmacological activities. Marine microorganisms are able to produce unusual bioactive metabolites as they live in biologically competitive environment and they have to adapt to extreme conditions such as: pH, temperature, high pressure and salinity. In this work, marine sediment sample was collected from Red Sea, Egypt. From this sample, bacteria were isolated by pour plate method using Zobell 1/4 strength media at two different temperatures, 37°C and 28°C. All the 25 different bacterial isolates were screened for antimicrobial material production using disc diffusion method. The most potent antimicrobial agent was identified by the means of biochemical tests and molecular identification using 16S rRNA sequence analysis. The isolate was found to be belonging to *Pseudomonas* genera**.**

1.Introduction

As nature remains the most versatile and the richest source for new antibiotics and as terrestrial environment had become a drained source of new metabolites, the marine environment considers as an enormous pool of structurally diverse and biologically active products [1]. Marine sediments are well known to possess diverse microbes especially bacterial diversity which are the prolific producers of novel natural bioactive compounds that haven't been explored properly [2, 3]. The diversity of these bioactive chemical compounds is a result of competition between microorganisms for resources in the marine habitat [4]. Many bacterial strain isolates from marine habitats have been shown to produce secondary metabolites that display antibacterial properties whereas the most important bacterial genera used as antimicrobial agents are *Bacillus* and *Pseudomonas* [5].

Genus *Pseudomonas* is one of the most ecological significant group of known bacteria that found in fresh water, soil, and marine environments. It's gram negative, rod, non-endospore forming, obligate aerobic and motile bacteria [6, 7]. Some species of genus *Pseudomonas* are considered to be important phyto-pathogens and agents of human infections, while its other strains and species have activities of bioremediation and biocontrol [8]. *Pseudomonads* are well known as biological agents especially the fluorescent *Pseudomonas* group, that include *Pseudomonas fluorescens, P.aeruginosa, P. putida, P.pyrocinia* and *P.aerofaciens,* that produce secondary metabolites against various pathogens such as pyocyanin, pyrrolnitrin, and pseudomonic acid [9-11]. 50 different antibiotic substances from pseudomonads have been discovered yet. Only two of these, pyocyanin

and pyrrolnitrin, have been produced on a commercial basis [12].

Some strains of *Pseudomonas aeruginosa* can produce many of phenazines compounds, including 1-hydroxy-5-methylphenazine (pyocyanin), phenazine-1- carboxamide and phenazine-1-carboxylic acid. *Pseudomonas aeruginosa* gain a great attention due to its color and an extracellular phenazine pigment production (pyocyanin) which is the main phenazine compound associated with this organism and can be produced from about 90-95% of all *Pseudomonas aeruginosa* isolates [13].

The main target of this research is to isolate bacteria from marine environment and study the ability of the isolates to produce antimicrobial materials with distinctive characteristics.

2.Materials and Methods

1. Sample collection

Deep sea core soil sediment sample was collected from Red Sea by a digger at 1800 m depth and was transferred to the laboratory in sterile and clean plastic bag [2].

2. Isolation of bacteria from sediment sample

One gram of the collected sea sediment was diluted up to 10^{-9} dilutions in 9ml Zobell 1/4 strength broth media containing (g/l): 1.29 yeast extract, 3.75 peptone, 18 NaCl, 2 $MgCl_2$, 0.525 KCl, 0.075 $CaCl_2$ and the pH was adjusted to 7.5 by using 1N NaOH. 1ml from each dilution was plated in Zobell 1/4 strength agar plates. The plates were incubated at two different temperatures (28°C and 37°C) for two days. The developed bacterial colonies were purified by compound streak method, sub cultured in agar slants and subsequently stocked in glycerol 25% at -20 for further utilization [14, 15].

3. Screening of bacteria for antimicrobial activities

3.1. Primary screening of the most potent antimicrobial agent producer

The marine bacterial isolates were inoculated onto 50 ml of Zobell 1/4 strength broth media separately and incubated in a shaker incubator at 150 rpm for 3-5 days at 37°C, centrifuged and cell free supernatants

were then impregnated onto sterile 6 mm discs and antibacterial activity was determined following the disc diffusion assay where 50µl, of 24hrs old culture, of the tested pathogenic microbes (*Bacillus subtilus*, *Streptococcus pyogenes*, *Staphylococcus epidermidis* and *Staphylococcus aureus* as Gram positive bacteria, *Eriwinia carotovora*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Shigella dysenteriae* as Gram negative bacteria and *Candida albicans* as a yeast) were spread on the Luria Bertani agar media containing (g/l): 5 yeast extract, 10 peptone, 10 NaCl and 20 agar. The saturated disc paper of 6 mm was placed on the surface of the medium. Petri dishes were incubated for 24 h at 37°C, then the diameter of the clear zones were measured. The same steps were done for bacterial isolates incubated at 28°C [16].

3.2. Secondary screening of the most potent antimicrobial agent producer

For the most potent antimicrobial agent producer, seed cultures were prepared by inoculating bacterial colonies into 50 ml Zobell1/4 strength broth and incubating at 37°C in shaker at 150 rpm for 24h individually. 1ml of these seed cultures was used to inoculate 50 ml of 3 different media individually: Zobell 1/4 strength, LB and Marine broth containing (g/l): 1.29 yeast extract, 3.75 peptone and media was raised to total volume by adding sea water. These cultures were incubated at 37°C in a shaker for 3 days and then cultures were centrifuged at 5000 rpm for 20 min to remove bacterial cells. Sterile 6 mm discs were soaked onto cell free supernatant and antimicrobial activity was determined by the disc diffusion assay [17, 18].

3.3. Statistical analysis

The statistical analysis was done using the SPSS V21 programme. To determine the significant differences between means, one – way ANOVA was performed followed by Tukey multiple range. All the data were presented as mean ± SE and differences were regarded statistically significant when $p < 0.05$.

4. Characterization and identification of the bacterial isolate I_9

4.1 Morphological identification of the isolate I_9

Colony characteristics including pigment production that was determined on LB media and Gram stain of the bacterial isolate were examined as described by **Sepehri, Mahmoodi [19].**

4.2 Molecular identification of the isolate I_9

Gene Jet genomic DNA purification Kit (Thermo K0721) was used for genomic DNA extraction from the strain under investigation. The extracted genomic DNA was used as template for amplification of the 16S rRNA gene through PCR reaction that carried out using Maxima Hot Start PCR Master Mix (Thermo K1051). PCR reaction contains: 25µl master mix, 1µl forward primer: AGAGTTTGATCCTGGCTCAG, 1µl reverse primer: GGTTACCTTGTTACGACTT, 18µl of nuclease free water and 5µl template DNA [20]. The condition of thermal cycler was programmed as follow: initial denaturation of DNA at 95°C for 10 min, followed by 30 cycle for DNA denaturation at 95°C for 30s, primer annealing at 65°C for 1 min and primer extension at 72°C for 1.30 min. At the end, the reaction mixture was kept at 72°C for 10 min. The PCR product was cleaned up using Gene JET™ PCR Purification Kit (Thermo K0701) then the purified DNA was stored at -20°C for subsequent analysis. The PCR products was sequenced by use ABI 3730xl DNA sequencer by using forward and reverse primers.

4.3 Biochemical identification

The strain was biochemically identified by using of API 20E identification system (API 20 E, Biomerieux); the strain was identified using the API database [6].

3. Results and Discussion

1. Isolation of bacteria from sediment sample

A total of 25 different bacterial isolates were isolated from Red Sea deep core soil sediment on Zobell 1/4 strength media. The isolates were collected, separated and named differently depending on morphological variations. 12 of the isolates were collected from plates previously incubated at 37°C; while 13 isolates were collected from plates previously incubated at 28°C.

2. Screening for antimicrobial activities

2.1. Primary screening

The antimicrobial activity of the secondary metabolites was tested against groups of Gram positive and Gram negative bacteria in addition to *Candida albicans* as a representative of eukaryotic pathogen. In the present study, at 37°C, 11 (out of twelve) bacterial isolates had shown a notable antimicrobial activity against pathogens. The result presented in Table 1 showed that, all isolates except I_2 & I_{10} showed broad spectrum activities against pathogens including, *Bacillus subtilis*, *Streptococcus pyogenes*, *Staphylococcus epidermidis, Erwinia carotovora* and *Candida albicans* but they didn't have any activities against *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Shigella dysenteriae.* The isolate I_9 showed the highest antimicrobial spectrum.

Regarding the microbes isolated at 28°C; two bacterial isolates B_1 & B_2 showed antimicrobial activity against pathogenic bacteria as shown in Table 1.

From Table 1: It was found that isolates I_3, I_4, I_5, I_6, I_7, I_9 and I_{11} had shown the largest clear zone and the most broad-spectrum activity against gram positive, gram negative bacteria and yeast, so they were introduced to secondary screening.

2.2 Secondary screening of the most potent antimicrobial agent producer

Eight bacterial strains, that showed notable antimicrobial activities in the first round of screening, were introduced to a second screening round for antimicrobial activities using 3 different types of media: Zobell 1/4 strength, Luria-Bertani (LB) and Marine broth (MB) media. The result in Table 2 shows that; the best antimicrobial activities against Gram positive, Gram negative bacteria and *Candida albicans* was produced by isolate I_9 in LB broth media (data displayed **in bold**).

From Table 2, the isolate I_9 showed inhibition zones in the range between 13-17 mm against different Gram positive, Gram negative and *Candida*. This isolate was chosen for further work and investigation.

Table 1: Screening of bacterial isolates for antimicrobial activities using disc diffusion method against tested pathogens.

| Isolate code	Zone of inhibition (mm)										
	Gram positive				Gram negative						Yeast
	B. subtilis	S. pyogenes	S. aureus	S. epidermidis	E. carotovora	E. cloacae	P. aeruginosa	E-coli	K. pneumoniae	S. dysenteriae	C. albicans
	Isolates grow at 37°C										
I_1	10.07±(0.47) $^{c\,d}$	12.07±(0.23) ^{c}	0.00	0.00 ^{a}	0.00 ^{a}	0.00	0.00	0.00	0.00	0.00	0.00 ^{a}
I_2	0.00 ^{a}	0.00 ^{a}	0.00	0.00 ^{a}	0.00 ^{a}	0.00	0.00	0.00	0.00	0.00	0.00 ^{a}
I_3	11.07±(0.23) ^{d}	0.00 ^{a}	0.00	9.00±(0.29) ^{bc}	10.07±(0.47) ^{bc}	0.00	0.00	0.00	0.00	0.00	9.00(±0.00) ^{d}
I_4	14.00±(0.29) ^{ef}	0.00 ^{a}	0.00	8.00(±0.00) ^{b}	11.07±(0.23) ^{cd}	0.00	0.00	0.00	0.00	0.00	10.00±(0.29) ^{e}
I_5	8.93±(0.33) ^{bc}	0.00 ^{a}	0.00	7.90±(0.30) ^{b}	13.90±(0.30) ^{fg}	0.00	0.00	0.00	0.00	0.00	11.93±(0.18) ^{g}
I_6	15.00(±0.0) ^{f}	0.00 ^{a}	0.00	12.00(±0.00) ^{cd}	12.93(0.33) ^{e}	0.00	0.00	0.00	0.00	0.00	13.3±(0.13) ^{h}
I_7	14.73±(0.43) ^{f}	0.00 ^{a}	0.00	11.00(±0.00) ^{b}	12.00(0.58) ^{de}	0.00	0.00	0.00	0.00	0.00	11.00(±0.00) ^{f}
I_8	10.00±(0.29) ^{cd}	0.00 ^{a}	0.00	11.87±(0.37) ^{cd}	9.00(±0.00) ^{b}	0.00	0.00	0.00	0.00	0.00	9.00(±0.00) ^{d}
I_9	11(±00.0) ^{d}	15.10±(0.10) ^{d}	0.00	14.00±(0.29) ^{d}	15.00±(0.50) ^{g}	0.00	0.00	0.00	0.00	0.00	14.17±(0.30) ^{h}
I_{10}	0.00 ^{a}	9.00(±0.00) ^{b}	0.00	0.00 ^{a}	0.00 ^{a}	0.00	0.00	0.00	0.00	0.00	0.00 ^{a}
I_{11}	12.90±(0.30) ^{e}	0.00 ^{a}	0.00	0.00 ^{a}	9.00(±0.00) ^{b}	0.00	0.00	0.00	0.00	0.00	9.90±(0.30) ^{e}
I_{12}	7.93±(0.23) ^{b}	0.00 ^{a}	0.00	0.00 ^{a}	0.00 ^{a}	0.00	0.00	7.00(±0.00)	0.00	0.00	7.00(±0.00) ^{b}
	Isolates grow at 28°C										
B1	8.00(±0.00) ^{b}	9.33(±0.33) ^{b}	0.00	0.00 ^{a}	0.00 ^{a}	0.00	0.00	0.00	0.00	0.00	0.00 ^{a}
B2	0.00 ^{a}	0.00 ^{a}	0.00	0.00 ^{a}	13.00(±0.00) ^{ef}	0.00	0.00	0.00	0.00	0.00	8.00(±0.00) ^{c}
B3-B13	0.00 ^{a}	0.00 ^{a}	0.00	0.00 ^{a}	0.00 ^{a}	0.00	0.00	0.00	0.00	0.00	0.00 ^{a}
S_{10}	24.3±(0.67) ^{g}	12.0±(0.00) ^{c}	20.0±(0.00)	20.0±(0.00) ^{e}	19.3±(0.7) ^{h}	14.0±(0.00)	0.00	0.00	0.00	0.00	21±(0.6) ^{i}
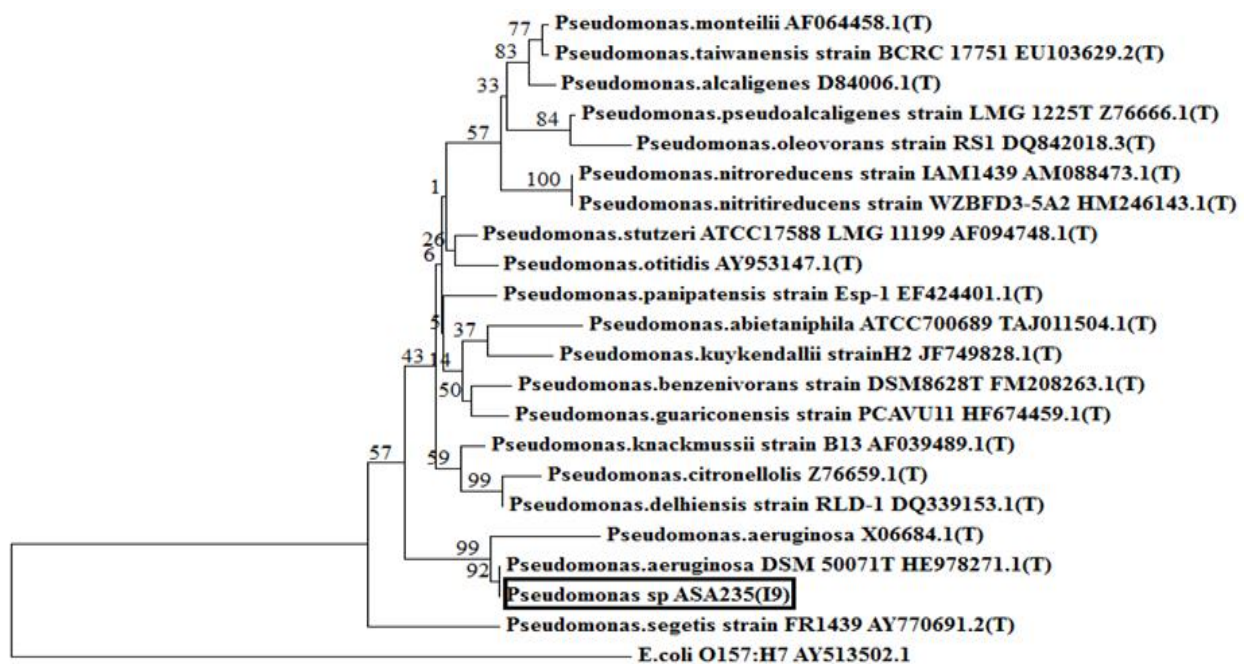
Figure 2: Phylogenetic dendrogram of the I_9 based on 16S rRNA gene sequence displaying its position to the closely related species of the genus *Pseudomonas*. The tree was constructed based on the neighbor-joining method. *E.coli*_O157:H7_AY5135021 was used as external reference.

Table 2: Inhibition zone (mm) for crude extracts produced by the tested isolates when growing on 3 different media (Zobell, LB, Marine broth) by using disc diffusion method

| Isolate code | Media | Gram positive | | | | Gram negative | | | | | | Yeast |
		B.subtilis	S. pyogenes	S. aureus	S. epidermidis	E. carotovora	E. cloacae	P. aeruginosa	E-coli	K. pneumoniae	S. dysenteriae	C. albicans
I$_3$	Zobell	10.83±(0.17)^{cd}	0.00^a	0.00	9.00±(0.00)^c	0.07±(0.47)^b	0.00	0.00	0.00	0.00	0.00	8.93±(0.23)^b
	LB	10.00±(0.00)^{bc}	0.00^a	0.00	16.13±(0.33)^i	11.07±(0.23)^c	0.00	0.00	0.00	0.00	0.00	14.07±(0.03)$_f$
	MB	10.83±(0.17)^{cd}	0.00^a	0.00	12.07±(0.23)^f	0.00^a	0.00	0.00	0.00	0.00	0.00	8.00±(0.00)
I$_4$	Zobell	13.50±(0.29)^{fg}	0.00^a	0.00	8.00±(0.00)^b	11.17±(0.13)^c	0.00	0.00	0.00	0.00	0.00	10.00±(0.00)$_{cd}$
	LB	10.00±(0.00)^{bc}	0.00^a	0.00	11.17±(0.13)^e	0.00^a	0.00	0.00	0.00	0.00	0.00	11.00±(0.00)$_{de}$
	MB	12.23±(0.15)^e	0.00^a	0.00	12.10±(0.10)^f	0.00^a	0.00	0.00	0.00	0.00	0.00	14.00±(0.29)$_f$
I$_5$	Zobell	9.00±(0.00)^b	0.00^a	0.00	8.00±(0.00)^b	14.00±(0.29)^d	0.00	0.00	0.00	0.00	0.00	12.10±(0.10)$_e$
	LB	12.27±(0.15)^e	0.00^a	0.00	14.20±(0.10)^g	15.10±(0.10)^c	0.00	0.00	0.00	0.00	0.00	15.10±(0.10)$_f$
	MB	11.00±(0.1)^{cd}	0.00^a	0.00	10.00±(0.00)^d	0.00^a	0.00	0.00	0.00	0.00	0.00	12.07±(0.23)$_e$
I$_6$	Zobell	15.17±(0.44)^{hji}	0.00^a	0.00	12.07±(0.23)^f	12.90±(0.30)^c	0.00	0.00	0.00	0.00	0.00	14.00±(0.29)$_f$
	LB	14.57±(0.32)^{ghi}	0.00^a	0.00	15.00±(0.00)^h	15.00±(0.50)^e	0.00	0.00	0.00	0.00	0.00	14.03±(0.26)$_f$
	MB	0.00^a	0.00^a	0.00	12.13±(0.09)^f	0.00^a	0.00	0.00	0.00	0.00	0.00	8.97±(0.20)^b
I$_7$	Zobell	15.30±(0.3)^{ji}	0.00^a	0.00	11.17±(0.07)^e	11.00±(0.00)^c	0.00	0.00	0.00	0.00	0.00	11.07±(0.23)$_{de}$
	LB	12.00±(0.00)^{de}	0.00^a	0.00	0.00^a	15.00±(0.00)^e	0.00	0.00	0.00	0.00	0.00	15.00±(0.50)$_f$
	MB	0.00^a	0.00^a	0.00	0.00^a	0.00^a	0.00	0.00	0.00	0.00	0.00	0.00^a
I$_9$	Zobell	11.00±(0.00)^{dc}	5.20±(0.12)^c	0.00	14.13±(0.09)^g	15.10±(0.10)^c	0.00	0.00	0.00	0.00	0.00	14.00±(0.29)$_f$
	LB	13.07±(0.12)^{ef}	5.70±(0.10)^d	0.00	15.00(±0.00)^h	16.97±(0.17)^f	0.00	0.00	0.00	0.00	0.00	14.03±(0.26)$_f$
	MB	14.07±(0.12)^{fgh}	0.00^a	0.00	8.07±(0.03)^b	0.00^a	0.00	0.00	0.00	0.00	0.00	12.00±(0.00)$_e$
I$_{11}$	Zobell	12.90±(0.06)^{ef}	0.00^a	0.00	0.00^a	9.00±(0.31)^b	0.00	0.00	0.00	0.00	0.00	10.00±(0.00)$_{cd}$
	LB	16.13±(0.07)^j	0.00^a	0.00	0.00^a	14.87±(0.13)^e	0.00	0.00	0.00	0.00	0.00	16.90±(0.06)$_g$
	MB	10.83±(0.23)^{cd}	0.00^a	0.00	0.00^a	0.00^a	0.00	0.00	0.00	0.00	0.00	11.03±(0.27)$_{de}$
S$_{10}$		24.3±(0.67)^k	12.0±(0.00)$_b$	20.0±(0.00)	20.0±(0.00)^j	19.3±(0.7)^g	19.3±(0.7)	0.00	0.00	0.00	0.00	21±(0.6)^h

Values listed are the mean values of three replicates ± SE. Values followed with the same letter and values without any letters in the same column are not significantly different at p≤0.05

The nucleotide sequence of 16S rRNA gene was used in building phylogenetic tree using Mega7 software as shown in Figure 2. To determine the closest type strain of the isolate, BLAST search was applied. The sequence showed that the isolated strain is most closely related to the bacterium *Pseudomonas aeruginosa* **DSM_50071T_HE978271.1^T** type strain (100%). The 16s rRNA sequence was submitted to gene bank and assigned an **accession number of MH580294.**

3. Characterization and identification of the isolate I$_9$

3.1 Morphological characterization of the isolate I$_9$

Morphological features of the isolate I$_9$ were determined by growing bacteria on LB agar media where the colony is circular and produce water soluble pigment that spread through the media. By applying Gram stain, the strain was found to be Gram negative, short, rod shaped.

3.2. Molecular identification of the bacterial isolate I$_9$

The extracted genomic DNA of the isolated bacteria was purified and used as a template for the amplification of 16S rRNA gene via PCR reaction. The PCR product was collected, purified and sequenced by ABI 3730xl DNA sequencer. The sequence file was visualized

using Finch TV program as shown in Figure 1, followed by basic local alignment search tool (BLAST) and Seq. Match analysis

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ATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC

GGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCT

AGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCG

CATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATG

AGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGA

TCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCC

AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCT

GATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTA

AGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACA

GAATAAGCACCGGCTAACTTCGTGCCAGCAG
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Figure 1: Nucleotide sequence of 16S rRNA gene of *Pseudomonas* sp strain ASA235 (I_9)

3.3. Biochemical identification of the isolate I_9

Multiple biochemical tests were performed to characterize the isolated strain. Biochemical identification of the isolate was done using API 20E strip. The Biochemical characterization is outlined in Table 3.

Biochemically, the isolate I_9 was found to be capable of using of arginine, citrate, gelatin and urea (25%), fermenting glucose and arabinose (25%) and found to be positive in oxidase test. The isolate was found to be negative for beta-galactosidase, lysine decarboxylase, ornithine decarboxylase, and incapable of production of H_2S, tryptophan deaminase, Indole, voges-proskauer, also it is negative for mannitol, inositol, sorbitol, rhamnose, sacchrose, melibiose, amygdaline fermentation.

According to the API identification system, the profile for this combination of reactions is 2216006, whereas the isolate was identified as *P. aeruginosa* (excellent identification).

Table 3: Biochemical characterization of the isolate I_9

Test	Result	Test	Result
Beta-Galactosidase (ONPG)	-	Glucose fermentation (GLU)	+
Arginine dehydrolase (ADH)	+	Mannitol fermentation (MAN)	-
Lysin decarboxylase (LDC)	-	Inositol fermentation (INO)	-
Ornithine decarboxylase (ODC)	-	Sorbitol fermentation (SOR)	-
Citrate utilization (CIT)	+	Rhamnose fermentation (RHA)	-
H_2S production	-	Sacchrose fermentation (SAC)	-
Urease (URE)	+	Melibiose fermentation (MEL)	-
Trptophan deaminase (TDA)	-	Amygdaline fermentation (AMY)	-
Indole production (IND)	-	Arabinose fermentation (ARA)	+
Voges-Proskauer (VP)	-	Oxidase (OX)	+
Gelatin hydrolyse (GEL)	+		

(-) is a symbol representing negative result and (+) representing positive results

Discussion

Searching for new antibiotics is a global demand as development of multi resistance bacteria to the conventional antibiotics is in continuous increasing [21]. This work was attempted to isolate antimicrobial producing marine bacteria from sediment of red sea with characteristic features.

As ocean covers 70% of surface area of earth, marine bacteria believed to be untapped source of versatile bioactive secondary metabolites especially after the discovery of antibiotic producing marine bacteria done by Rosenfeld and Zobell in 1947 as reported by Chandra Sekhar, Krishna [22]. Several studies have assured that the most antimicrobial producers in sea water are Gram negative [23] and 36% of the strains were Gram-negative rods as reported by Anand, Bhat [24]. In our study, the most active isolate is gram negative bacteria *(Pseudomonas aeruginosa)*.

Among the isolated bacteria, *Pseudomonas aeruginosa* ASA235 (I_9) shows a potent antimicrobial activity against selected

pathogenic microorganisms. The results revealed that, *P.aeruginosa* has antibacterial effect against Gram positive bacteria more than Gram negative ones. Darabpour, Ardakani [25] mentioned many reasons explain this difference, this may be due to possession of Gram negative bacteria to cell wall with multilayer structure which effect on permeability of substances or due to breaks down of any foreign molecules comes from outsides by the means of enzymes found in periplasmic space or due to membrane accumulation mechanism.

Variation in the growing media can have effects on the diversity and quantity of secondary metabolites. Such media optimization becomes challenging in a drug-discovery situation [26]. The isolates were screened for antimicrobial material production using three different media where as LB media was found to be the most suitable media for production as shown in the results (Table 2).

Identification of the isolate I_9 was done based on morphological, biochemical characters and also via sequencing and analysis of 16S rRNA gene. Cells of the isolate I_9 is circular and produce bluish green water soluble pigment. Based on Gram stain cells are Gram negative and short rods. Furthermore, the isolate can unitlize citrate, liquify gelatin, ferment glucose, produce oxidase and urease enzyme, in addition it can't produce indole, and it give negative result with voges-Proskauer test which agreed with Ningthoujam and Shovarani [27].

Upon the construction of the phylogenetic tree, the isolate was found to be closely related to *Pseudomonas aeruginosa* DSM_50071T_HE978271.1(T) with 100 % similarity. The isolate is known to produce phenazines compounds, including 1-hydroxy-5-methylphenazine (pyocyanin) which has reported to have antimicrobial effect [28].

4.Refrences

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