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PURIFICATION, CHARACTERIZATION AND *IN VITRO* ANTI MICROBIAL ACTIVITY OF PROTEINS FROM MARINE BACTERIUM -*Bacillus* sp

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ABSTRACT

Three purified proteins G-I, G-II and G-III were obtained from *Bacillus* sp using the techniques of DEAE Sepharose Fast Flow Chromatography, filtration chromatography. Anion exchange chromatography molecular determination by SDS page gel electrophoresis (SDS-PAGE). The purity of G-I, G-II, and G-III was measured by DEAE Sepharose Fast Flow Chromatography. G-I, G-II and G-III were measured by SDS-PAGE to have molecular weights of 14.4 k Da and 94.4 k Da, and three bands appear in the molecular weights of 46 k Da band 35 k Da and 32 k Da band respectively. The amino acid analysis of purified compound G-III was determined because the greater antimicrobial activity of G-III compound. The amino acid analysis using the technique of automatic amino acid analyzer (Shimatzu-High-performance liquid chromatography LC 4A) 20 µl of the purified sample was injected into the single column and analyzed using sodium buffer system. G-I, G-II, and G-III inhibited the clinical pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa.* By antimicrobial activity of Agar well diffusion assay. Test the clinical pathogens shoe the greater activity of G-III and Zone of inhibition various concentration of (25 µl, 50 µl, 75 µl, 100 µl). G-III shows the maximum results of 18 mm and 15 mm against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

KEYWORDS

Bacillus sp, Protein, Purification and *In vitro* antimicrobial activity.

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INTRODUCTON

The world's oceans, covering more than 70% of the earth's surface, represent an enormous resource for the discovery of potential chemotherapeutic agents. Taking higher Taxonomic levels as an estimate of biodiversity, more phyla are found in the oceans than on land. Of the thirty-three known phyla of extant animals, only one is exclusive of land, while as many as twenty-one phyla are exclusive of the sea.

Marine biotechnology is the science in which marine organisms are used in full or partially to make or modify products, to improve plants or animals or to develop microorganisms for specific uses. With the help of different molecular and biotechnological techniques, humans have been able to elucidate many biological methods applicable to both aquatic and terrestrial organisms. According to 10% of over 25,000 plants have been investigated for biological activity.

The marine environment may contain over 80% of world's plant and animal species. In recent years, many bioactive compounds have been extracted from various marine animals like tunicates, sponges, soft corals, sea hares, nudibranchs, bryozoans, sea slugs and marine organisms.

Marine microorganisms which are salt-tolerant provide an interesting alternative for therapeutic purposes. Marine microorganisms have a diverse range of enzymatic activity and are capable of catalyzing various biochemical reactions with novel enzymes. Especially, halophilic microorganisms possess many hydrolytic enzymes and are capable of functioning under conditions that lead to precipitation of denaturation of most proteins. Further, it is believed that seawater, which is saline in nature and chemically closer to the human blood plasma, could provide microbial products, in particular, the enzymes, that could be safer having no or less toxicity or side effects when used for therapeutic applications to humans.

Screening the culture broth of marine bacteria collected at Yap (Micronesia), Palau (Belau), and Okinawa (the southwest islands of Japan) for antimicrobial algal activity, 37 out of 2,594 bacterial isolates tested were found to produce anti cyanobacteria substances against *Oscillatoriaamphibia*. (Kazuhiro Yoshikawa *et al*., 2000)[1].

Screened bacteria from seawater, sediment, marine invertebrates and seaweeds collected from different coastal areas of the China sea. The antimicrobial activities of these bacteria were investigated. Ethyl acetate extracts of marine bacterial fermentation were screened for antimicrobial activities using the method of agar diffusion. The results showed that 42 strains of the isolates have antimicrobial activity. The proportion of active bacteria associated with marine invertebrates (20%) and seaweeds (11%) is higher than that isolated from seawater (7%) and sediment (5%). The active marine bacteria were assigned to the genera *Alteromonas sp, Pseudomonas sp, Bacillus sp* and *Flavobacterium sp*. (Li zheng*et al.*, 2005)[2].

Pseudomonas sp PS-102 recovered from Muttukkadu brackish water lagoon, situated south of Chennai, showed significant activity against a number of shrimp pathogenic *Vibrio.* Out of the 112 isolates of bacterial pathogens comprising *Vibrio harveyi, Vibrio vulnificus*, *Vibrioparahaemolyticus, Vibrio alginolyticus, Vibrio fluvialis,* and *Aeromonas* sp, 73% were inhibited in vitro by the cell-free culture supernatant of Pseudomonas sp PS-102 isolate. (Vijayan *et al*., 2006)[3].

MATERIAL AND METHODS[4-10]

Collection of sample

The marine water sample was collected from the Kilakarai deep sea and the sample was transferred to the lab aseptically, the collected sample was stored in the air tight container.

Isolation of bacterial strains

Serial dilution plate method was used for the Isolation of bacteria for dilution plate method. The 9.0ml of sterile distilled sea water was added to each test tube aseptically. The 1ml of water samples was added into the first dilution blank of 9.0ml of distilled sea water. Shake the tube vigorously for few minutes. Allow the large particle to settle. Pipette 1.0ml from the first dilution blank (10^{-1}) to the second dilution blank (10^{-2}). Then drawn up and release the fluid. Several times to wash out the pipettes. Shake the test tubes vigorously for two minutes. Reported this kind of serial dilution from on to next tube till the last tube (10^{-9} dilution). The same was carried out for the other samples. 1ml of sterile serially diluted seawater was spread on the marine agar medium plates and incubated at 25°C for 20 days. Single colonies with different morphologies were picked and purified using streak plate and spread plate method.

Identification of Microorganism[11-30]

Microscopic examination

Gram staining

This test was performed to find out whether the organism is gram positive or gram negative. It is the

ability of gram-positive bacteria to take up crystal violet (CV) after mordanting with iodine (I) to retain crystal violet-iodine (CV-I) complex. Following the extraction with alcohol, bacteria which loose (CV-I) complex after alcohol treatment are gram-negative. Safranin was used as a counter stain. Gram-positive will appear as violet color and gram-negative as purple color. Isolated unknown colonies were smeared in clean glass slide, and the gram staining procedure was performed.

Motility test

Place a drop of bacterial suspension on the center of the coverslip, apply wax or soft paraffin over the corners of the coverslip. Put a glass slide gently over the coverslip and hold it upside down. It should be in such a manner that bacterial suspension should be hanging between the coverslip and glass slide. Examine under the microscope, first under 10x, then under 40x.

Biochemical analysis IMVIC

Indole test

A loop full of test organism was inoculated into sterile tryptone broth and incubated at 37^{o}c for 24 hours. After incubation, the production of indole was tested by the addition of kovac's reagent and the positive results indicated cherry-red ring and results were observed.

Methyl red test

The test organism was inoculated into the sterile MR-VP broth and they were incubated at 37^{o}c for 24 hours. After incubation the methyl red indicator was added. The presence of red color indicated the positive result and the results were observed.

Voges- proskauer test

The test cultures were inoculated into the sterile MR-VP broth and they were incubated at 37^{o}c for 24 hours. After incubation Barrits reagent A and B were added and gently mixed, allow standing for 15 minutes. The formation of red or pink color indicated the positive result then the results were observed.

Citrate utilization test

The test cultures were inoculated into sterile simmon's citrate agar slants and incubated at 37^{o}c for 24 hours. After incubation, the color change of the medium from green to blue indicated the positive result and the results were observed.

Catalase test

The test cultures were placed on a clean glass slide and 3% of hydrogen peroxide (H_2O_2) solution was pipette out it. The results were observed for the presence or absence of air bubbles.

Nitrate reduction test

The test cultures were inoculated into sterile nitrate broth and incubated at 37^{o}c for 24 hours. After incubation add nitrate reagent A&B (containing sulphanilamide and α-naphthylamine) red color appearance indicates a positive result, and results were observed.

Fermentation test

The test cultures were inoculated into sterile carbohydrate fermentation broth with different carbon sources. (glucose, lactose, sucrose, mannitol) along with Durham's tube and incubated at 37^{o}c for 24 hours. The color of the medium changes and gas accumulate in durham's tube indicates positive results. The results were observed.

Hydrolysis test

Starch hydrolysis

The test cultures were streaked on the sterile starch agar medium and incubate at 37^{o}c for 48 hours. After incubation pours iodine solution in the plates, colony area gets clear background and formed blue-black color indicate a positive result.

Gelatin hydrolysis

Prepare gelatin agar plates and streak with suitable culture. Allow the microbes to grow at 37°C for 24 to 48 hours. Flooded the plates with trichloroacetic acid.

Extraction of total proteins

Isolated bacteria as above were cultured in 300 ml Marine FePO4 0.1 g, dissolved in seawater, pH 7.2–7.6) for the production of bioactive compounds in 500 ml Erlenmeyer flasks. Flasks were incubated on a rotatory shaker at 220 revs/min at 25°C. After 7 days of cultivation. The culture was centrifuged. After centrifugation (16,000 rpm, 20 min) at 4°C, the supernatant was collected and the crude extract obtained. The crude extract of bacteria was fractionated by salting out with increasing concentrations of ammonium sulfate. Solid ammonium sulfate was slowly added to the above crude extract with gentle stirring, up to 35% saturation in 20 min. After the crude extract was left

at 4 °C with the ammonium sulfate under vortexing for another 40 minutes, the protein precipitate was collected by centrifugation (16,000 rpm, 20 min) at 4°C. The supernatant was transferred to another beaker, and solid ammonium sulfate was added to it up to 70% saturation. The mixture was treated as above. Likewise, another protein precipitate was obtained at 70%-100% saturation of ammonium sulfate. Each of the three protein pellets was suspended in 10 ml of ice-cold PBS (10 mM, pH 8.0), and dialyzed against a large volume (3 L) of distilled water for 24 h at 4 °C. Dialysis bags were employed. During this process, the dialysate was changed three times to completely remove any residual ammonium sulfate.

Antimicrobial activity by agar well diffusion assay

The agar well diffusion method was used for the inhibitory effects of crude extract of marine Bacillus protein the clinical pathogens such as *Staphylococcus aureus*, *pseudomonas aeruginosa,* extract was loaded on the Muller Hinton agar plates. This was swabbed with clinical pathogen such as *Staphylococcus aureus, pseudomonas aeruginosa,* Five wells (6 mm in diameter) were made equidistance in each of the plates using a sterile cork borer. Up to 25µl to 100 µl of each concentration of the extract were respectively introduced into the wells using sterile automatic pipettes, with the stock in one well. It was allowed to diffuse at room temperature for 2 hrs and the plates were incubated at 37°C for 24 hrs. Diameters of the inhibition zones were measured. The antibacterial activity was expressed as the mean zone of inhibition diameters (mm) produced by marine microbes. After purification named G-1, G-2, G-3 were subjected to antimicrobial activity against clinical pathogens by Agar diffusion method.

Purification of proteins

Anion-exchange chromatography

The Fraction-I, Fraction-II, and Fraction-III were obtained at the ammonium sulfate saturation of 0-35%, 35-70% and70-100%, Fraction III were active in the antimicrobial activity. Fraction III was obtained from the crude extract at 70%−100% saturation of ammonium sulfate was dialyzed against 10mM Tris-HCl, pH 7.46 for 5 h and the dialyzed solution was subsequently injected into a DEAE Sepharose Fast Flow column, which was pre-equilibrated with the mentioned Tris-HCl buffer. The column was washed with the same buffer until the baseline returned to zero and remained stable. The column was then eluted with increasing concentration of NaCl prepared in 10mM Tris-HCl buffer, pH 7.46 at 4°C. Aliquots of 5 ml/tube were collected at a flow rate of 1.2 ml/min and the absorbance was measured at 280 nm. Three A280 nm peak fractions, named G-1, G-2, and G-3 were collected respectively.

Molecular determination by SDS PAGE

Gel electrophoresis (SDS-PAGE)

The Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed. (Fraction III and G1)

Analysis of amino acid components

The qualitative and quantitative estimation of amino acids was done using an automatic amino acid analyzer (Shimatzu-High-performance liquid chromatography LC 4A) 20 µl of the purified sample was injected into the single column and analyzed using sodium buffer system.

RESULTS

Collection and transport of sample

The marine water samples were collected from the kilakarai deep sea, and the sample was transferred to the lab aseptically, the collected sample was stored in the airtight container.

Isolation of marine microorganism

Isolation using differential media. The water sample was serially diluted and spread into the marine agar medium. After the incubation period the colonies were counted and the dominated colonies were picked out and their morphology morphological characterization was studied. The dominated colonies were streaked and the pure cultures were stored for further studies. The isolated colony results as *Bacillus* sp.

Morphological and Biochemical characterization

Finally, the morphological and biochemical Characterization indicated that the suspected organisms were *Bacillus* sp. (Table No.1).

Crude extraction

The Fraction-I, Fraction-II, and Fraction-III was obtained at the ammonium sulfate saturation of 0-35%, 35-70%, and 70-100%.

Antimicrobial activity by agar well diffusion assay

The antimicrobial potential of the marine microbes the Fraction I, Fraction II, Fraction III shows the greater effect when tested with the clinical pathogens. *Staphylococcus aureus, Pseudomonas aeruginosa*. Among them, Fraction III shows a greater zone of inhibition in various concentration of (25 µl, 50 µl, 75 µl, 100 µl). The higher concentration of 100 µl of the sample shows the greater results. The high concentration of the 100 µl of the crude sample exhibit higher zone of inhibition against the *Staphylococcus* sp (15mm), *Pseudomonas* sp (12mm). Which is higher than the standard antibiotic streptomycin. The results suggested that the fraction-III warranted further purification in order to unveil the active components of *Pseudomonas* sp. (Table No.2).

Similar to G I, G II, G III shows the greater effect when tested with the clinical pathogens *Staphylococcus aureus, Pseudomonas* sp G III shows a greater zone of inhibition in various concentration of (25 µl, 50 µl, 75 µl, 100 µl). G I, G II, G III had an inhibition zone diameter of 13 - 15mm. which is higher than to a standard antibiotic streptomycin hence it was suggested their effectiveness as antimicrobials from the protein. Among that G-3 shows the maximum results of 18 mm and 19 mm against *Staphylococcus aureus, Pseudomonas* sp respectively. G III had a better inhibition zone then the Fraction III. (Table No.3)

Elution in DEAE Sepharose Fast Flow Chromatography

Column specification: 1.6 × 30 cm; Equilibrate liquid: buffer C (Tris-HCl, pH 7.46, 10mM); Sample: Fraction-III; Detection wavelength: UV 280 nm; Flow rate: 1.2 mL/min; Collection rate: 5 mL/tube. Aliquots of 5 ml/tube were collected at a flow rate of 1.2 ml/min and the absorbance was measured at 280 nm. Three A280 nm peak fractions, named G-1, G-2, G-3 were collected respectively. (Table No.4).

Characterization of Purified Proteins

To estimate the molecular weight of G-3, Molecular weight marker (range from 14.4kDa to 97.4 kDa). Three bands appear, the molecular weight of band 46kDa, band 35kDa and b and 32kDa. This clearly indicated that different type of proteins presents in the G III sample.

Estimation of Amino Acids

Estimation of amino acids was done using an automatic amino acid analyzer for the two fractions of F III and G3. The amino acids present in these two samples were shown in the (Table No.5 and 6). F III and G3 showed almost similar amino acids. Serine was present only in G3. This result suggests that all extracts possess compound with antimicrobial properties which can be used as antimicrobial agents in new drugs for therapy of infectious disease in human.

DISCUSSION

Marine bacteria have been recognized as an important and untapped resource for the novel bioactive compound. Development of marine biotechnology is expected to produce novel compounds that may contribute significantly towards drug development over the next decade.

In the present study, the antimicrobial potential of the marine microbes the Fraction I, Fraction II, Fraction III shows the greater effect when against with the clinical pathogens. *Staphylococcus aureus, Pseudomonas* sp, Fraction III shows a greater zone of inhibition. The results suggested that the fraction-III warranted further purification in order to unveil the active components of Pseudomonas sp. From the curve of elution in DEAE Sepharose Fast Flow Chromatography Three A280 nm peak fractions, named G-1, G-2, G-3 were collected respectively. In the previous studies of the Liyan Song isolated seven fractions from the protein of invertebrates.

The antimicrobial potential of the marine microbes the-1, G-2, G-3 shows the greater effect when against with the clinical pathogens. *Staphylococcus aureus, Pseudomonas* sp. Among that G-3 shows the maximum results of 18 mm and 19mm against *Staphylococcus aureus, Pseudomonas aeruginosa* respectively.

Table No.1: Biochemical characterization of bacterial isolates

S.No	TESTS	S1
1	Gram's Staining	+
2	Motility test	+
3	Indole Test	-
4	Methyl red Test	-
5	VP Test	-
6	Citrate Utilization Test	+
7	Starch hydrolysis	+
8	Gelatin Hydrolysis	+
9	Nitrate reduction Test	+
10	Catalase Test	+
11	Glucose Test	A
12	Lactose Test	A
13	Sucrose Test	A
14	Mannitol Test	A

(+ Positive, - Negative, A-Acid Production, NA-No Gas production, W- Weak, G-Gas production

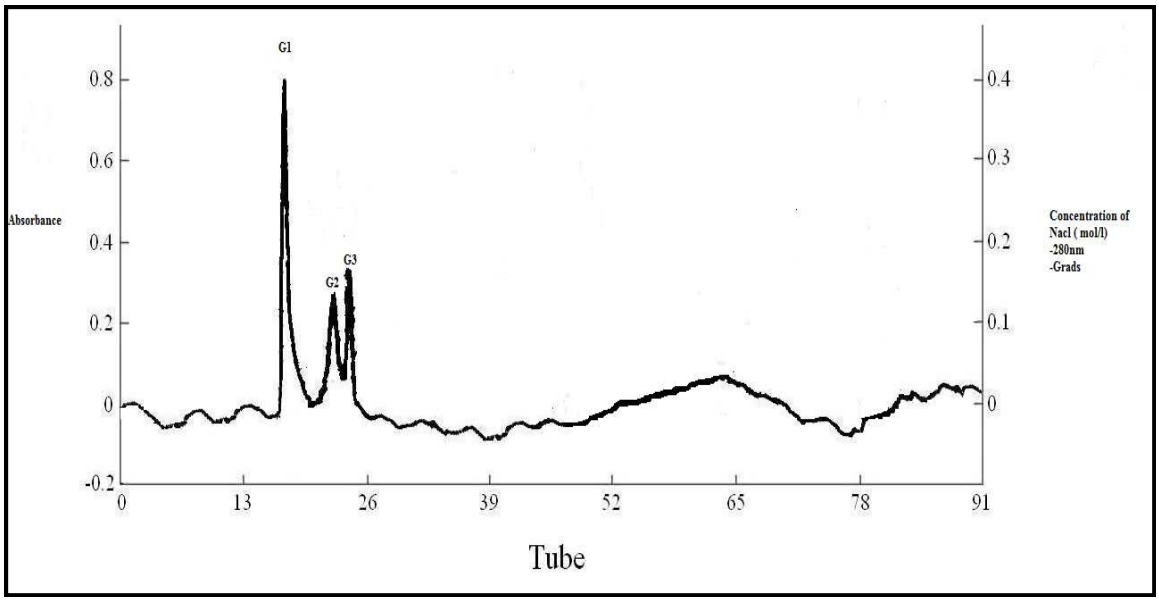
The identified biochemical and morphological of organism S1-*Bacillus* sp

Table No.2: Antimicrobial activity of the crude extract against clinical pathogens

S.No	Name of the organisms	Crude extract exhibit zone of inhibition in mm				
		25 µl	**50 µl**	**75 µl**	**100 µl**	**Antibiotic streptomycin 100 µl**
Fraction I						
1	*Staphylococcus aureus*	8	9	11	12	10
2	*Pseudomonas aeruginosa*	8	10	12	14	11
Fraction II						
3	*Staphylococcus aureus*	10	10	11	12	10
4	*Pseudomonas aeruginosa*	9	10	12	14	11
Fraction III						
5	*Staphylococcus aureus*	10	12	12	15	10
6	*Pseudomonas aeruginosa*	9	9	10	12	11

Table No.3: Antimicrobial Activity of the Purified Sample against Clinical Pathogens

S.No	Name of the organisms	Purified sample exhibit zone of inhibition in mm				
		25 µl	**50 µl**	**75 µl**	**100 µl**	**Antibiotic streptomycin 100 µl**
GI						
1	*Staphylococcus aureus*	10	12	14	17	11
2	*Pseudomonas aeruginosa*	16	16	16	18.	12
GII						
3	*Staphylococcus aureus*	10	11	12	14	11
4	*Pseudomonas aeruginosa*	11	14	15	15	12
GIII						
5	*Staphylococcus aureus*	10	12	14	18	11
6	*Pseudomonas aeruginosa*	11	14	15	19	12

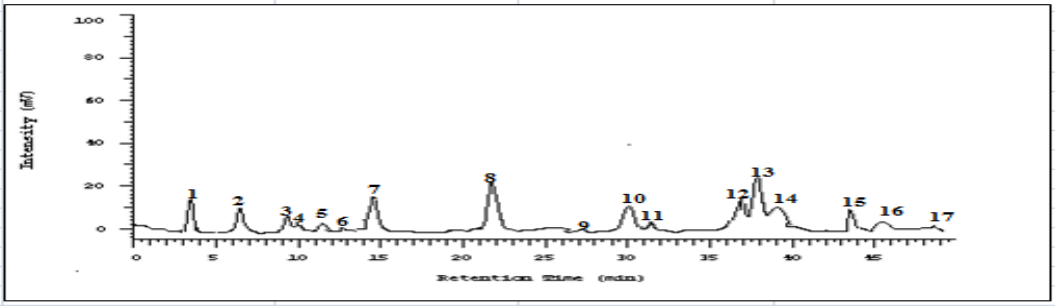


Column specification: 1.6 × 30 cm; Equilibrate liquid: buffer C (Tris-HCl, pH 7.46, 10 mM); Sample: Fraction-III; Detection wavelength: UV 280 nm; Flow rate: 1.2 mL/min; Collection rate: 5 mL/tube

Figure No.1: DEAE Sepharose Fast Flow Chromatography

D-7000 HPLC System Manager Report

System Name:	LACHROM L-7000	Series:	AMINO ACIDS
Proc. Method:	Atoz	Sample Name:	FIII

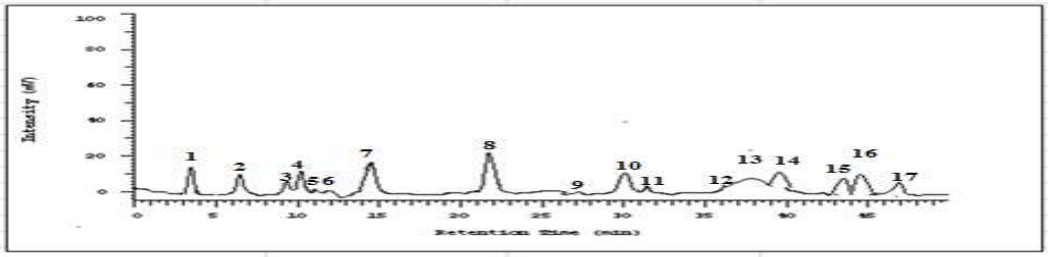


No.	COMPONENT NAME	R.T.	AREA	AREA %
1	ASPARTIC ACID	4.06	10123	0.113
2	GLUTAMIC ACID	6.49	454	0.0343
3	ASPARAGINE	9.21	116	0.0011
4	GLYCINE	13.43	147	0.0054
5	THREONINE	14.9	3465	0.201
6	ARGININE	18.09	2604	0.109
7	ALANINE	19.55	134	0.005
8	CYSTINE	21.66	3423	0.2007
9	TYROSINE	24.78	121	0.008
10	HISTIDINE	27.44	324	0.0456
11	VALINE	29.91	1343	0.073
12	ISO-LEUCINE	36.65	343	0.024
13	PHENYL ALANINE	37.95	6545	0.298
14	LEUCINE	39.36	4067	0.189
15	LYSINE	43.41	6232	0.785
16	PROLINE	45.12	121	0.005
17	TRYPTOPHAN	46.13	878	0.078
				2.1850

Figure No.2: Analysis of Aminoacids

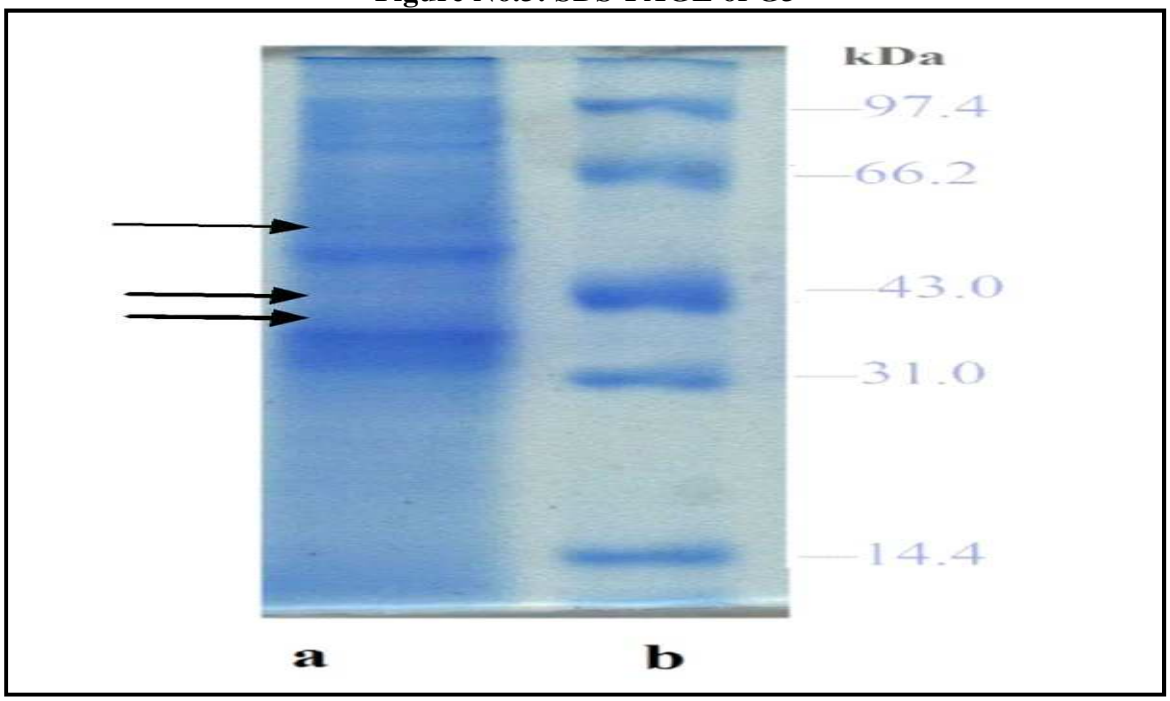
D-7000 HPLC System Manager Report

System Name: LACHROM L-7000 Series: AMINO ACIDS
Proc. Method: Atoz Sample Name: G3



No.	COMPONENT NAME	R.T.	AREA	AREA %
1	ASPARTIC ACID	4.03	129124	9.972
1	GLUTAMIC ACID	6.49	90531	8.35
2	ASPARAGINE	9.24	67525	1.549
3	SERINE	10.22	1301	0.072
4	GLYCINE	13.45	2012	0.201
5	THREONINE	14.89	121210	7.543
6	ARGININE	18.08	119	0.063
7	ALANINE	19.53	115	0.004
8	CYSTINE	21.67	305237	22.100
9	TYROSINE	24.76	115	0.004
10	HISTIDINE	27.25	10741	0.537
11	VALINE	29.90	10521	0.425
12	ISO-LEUCINE	36.62	12012	3.874
13	PHENYL ALANINE	37.93	16530	5.325
14	LEUCINE	39.34	18491	1.370
15	LYSINE	43.39	138124	13.771
16	PROLINE	45.11	209671	20.652
17	TRYPTOPHAN	46.12	475	0.321
			1133866	96.133

Figure No.3: SDS-PAGE of G3



Molecular weight marker (range from 14.4kDa to 97.4 kDa).
Three bands appear, the molecular weight of band
46kDa, band 35kDa and b and 32kDa.

Figure No.4: Antimicrobial activity of the crude extracts (Fraction I, Fraction II, Fraction III) against *Pseudomonas aeruginosa*

Figure No.5: Antimicrobial activity of the crude extracts (Fraction I, Fraction II, Fraction III) against *Staphylococcus aureus*

Pseudomonas aeruginosa ***Staphylococcus aureus***

Figure No.6: Antimicrobial activity of the purified sample against clinical pathogens *Pseudomonas aeruginosin Staphylococcus aureus*

CONCLUSION

The purification method consists of Ammonium Sulphate precipitation, Dialysis, and Fast flow Column chromatography. The Molecular mass was a determination by SDS PAGE. Then purified the protein using antibacterial activity two protein samples Fraction III and G3 from *Bacillus sp* shows the bioactivity-guided fractionation and purification. Important characteristics of proteins G3- were identified, showing three bands and the molecular weights to be 46kDa, band 35kDa, and band 32kDa. Additionally, our present study reveals that the studies of amino acids pharmaceutical agents were discovered by screening natural products from marine microorganisms

ACKWOLEDGEMENT

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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