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DETERMINATION OF FATTY ACIDS AND PROTEINS FROM THE FRESH WATER ALGA *CHLAMYDOMONAS REINHARDTII* CC 2137 AND ITS ANTAGONISM AGAINST AQUATIC BACTERIA

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Abstract

Chlamydomonas reinhardtii, a wild-type algal strain (CC 2137) was grown on tris acetate phosphate agar (TAP) medium. The growth curve values showed 2.0 logs till 6 days and declined thereafter. Later, the cells were harvested and extracted for fat using chloroform and methanol (2:1). The extract was hydrolyzed, lyzed and found to have unsaturated fatty acids like linolenic, linoleic, oleic acid, palmitoleic acid and saturated fatty acids viz. myristic, palmitic and stearic acid,using gas chromatography as well as mass spectrometry (GC-MS). Cell wall bound iron containing fatty acid elongation (FAE1) proteins of CC2137 were extracted and their size (ranging from 25-250 KD) was detected by SDS PAGE. The FAE1 protein was inhibitory to *Bacillus cereus* as evidenced by "Agar Spot Assay". Organic extracts of CC2137 cells with hot and cold extraction were tested against marine bacteria. In cold extraction, methanolic extracts showed highest inhibition (11.0 mm) to V*ibrio harveyi* (MM30) and subsequently acetone and ethyl acetate extracts showed inhibition to *V. harveyi* (MM32). In hot extraction, highest inhibitions were observed by ethanol and ethyl acetate (13.0 mm) to MM30 followed by methanol, ethanol and ethyl acetate extracts (12.0 mm) to MM32. Extracts of ethyl acetate and acetic acid showed maximum and identical inhibitions (8.0 mm) against *Pseudomonas putida* (PP) and lowest by methanoles and chloroform (6.0 mm) in cold extraction. Under hot extraction, the highest inhibition was shown by ethanol extract to PP followed by methanol and acetone (11.0 mm) and lowest by chloroform and acetic acid extracts (6.0 mm).

Introduction

At the global level, algae are remarkably important in generating photosynthetic organic bio-molecules. As inhabitants of freshwater, brackishwater and marine environment, algae are the base for aquatic food webs, sustaining an immense abundance and diversity of animals. They have enormous potential for energy (Sharif –Hussain & Aisha, 2008) and are proficient to produce bio-fules, fatty acids, proteins and biologically significant energetic compounds. Marine algae have been proved to have curative properties and are a good source of lipids (Valeem &Shameel., 2009). Currently, bio-active compounds are becoming vital as a number of bacteria show resistance against the usual antibiotics (Karunasagar *et al*., 1994). Antibiotics are not universally established as bio-preservative as they cause objectionable attributes to the consumers. Copious algae have been used as medicines in Asia. With that reason many biological compounds have been screened from algae. Antibacterial activity of algae such as diatoms, phytoplankton has been reported (Reichelt & Borowitska 1984; Viso & Baby, 1987). Marine algae represent a source of natural products, including antiviral agents. The potential of antiviral activities of algal polysaccharides were first described by Gerber *et al*, (1958) who observed that polysaccharide extracted from *Gelidium cartiliginium* protected embryonic eggs against the influenza B and mump viruses. Majority of the antibacterial activities have been tested against human pathogens and the active mechanistic compounds have rarely been purified. Numerous studies have been documented about the antibacterial compounds from marine algae (Padmakumar & Ayyak Kannuz, 1997; Naviner *et al*., 1999) but not much from algae of fresh water origin (Moore *et al.,* 1996). Therefore, the present study was carried out to screen protein and fatty acids from *Chlamydomonas reinhardtii*, wild-type strain (CC 2137) and to test their antagonistic efficacy against aquatic bacteria.

Materials and Methods

Algal strain and medium: *Chlamydomonas reinhardtii* wild-type strain (CC 2137) was maintained in tris acetate phosphate agar (TAP) at 25°C (Harris, 1989). Individual colonies were inoculated into TAP liquid shake cultures (175 rpm) under continuous white light (75 Kmol photons m-2s-1) with 100mg/mL of ampicillin and grown for 5 days to a cell density (measured at OD 750) greater than 1.2. The TAP-grown algal cells were pelleted by centrifugation at 4,000 g for 10 min at 20°C.The *V. harveyi* strains BB120 (wild type) MM30, MM32 were generously provided by Dr. Bonnie L. Bassler (Princeton University-USA). *Escherichia coli, Staphylococcus aureus, S. epidermis, Micrococcus luteus, Corynebacterium glutamicum, Bacillus cereus, B. megatricum* were isolated from fish and confirmed in using various bio-chemical tests (Tatini *et al*., 1984). These strains again re-confirmed with standard type strains obtained from American Type Culture Collection Centre (ATCC). Bacterial cultures were re-generated in Luria Bertani (LB) broth (Fisher-USA) and incubated at 37°C for 18 h.

Revival of *Chlamydomonas reinhardtii*: One hundred μL of CC 2137 was inoculated into 100 mL of TAP medium and incubated at 28°C with the same level of light in a shaker incubator for 3 days to get an OD of 0.67. The cells of 50mL each were transferred into 1L flasks of two numbers containing TAP medium. The cells in the flask were incubated for 3 days with the same incubation condition or to get the OD of 0.7 (Allen *et al*., 2007). The cells were then transferred into 250mL poly propylene tube and harvested using RC5C Sorval

refrigerated centrifuge (Dupont) at 7, 980g at 4°C, for 10 min. The harvested cells were washed once with sterile TAP medium and made up with 15mL of TAP medium. The cells were then dried at 40°C in a vacuum drier for a day.1g of dried CC 2137 cells were obtained from the CC2137 grown in 5L of TAP medium (Pyle *et al*., 2008).

Extraction of cell wall bound protein: One hundred µL of CC 2137 was inoculated into 100mL of TAP medium and grown in light for 3 days at 25°C to a cell density 1.2 OD. The TAP-grown CC 2137 was pelleted by centrifugation as described earlier. The cells were washed in 2mL wash buffer containing 25 Mm sodium phosphate and 50 Mm sodium chloride (pH 7.0) and treated thrice with liquid nitrogen for 5 min each and the supernatant collected through centrifugation at 4,000 g for 5 min at 20°C (Sheoran *et al*., 2009). The supernatant was tested for bio-inhibition against bacteria through well diffusion assay (Kannappan & Manja, 2004).

Solvent extraction: Chloroform, methanol, acetic acid, acetone and ethyl acetate were used for fat extraction. Two types of extraction, i.e cold and hot extraction were carried out. Five samples were prepared by adding CC2137 cells in wet weight (1 g each) with 5 mL of each solvent. They were incubated at 37°C/24 h, called cold extraction. Similarly five samples were prepared by adding dry CC2137. Thereafter, the samples were kept for 48 h in a hot water bath at 60°C, called hot extraction (Das *et al*., 2005).

Total fat extraction: Two mL of CC2137 (OD: 1.2) was inoculated with 1000mL of TAP medium, grown in a shaker incubator under light for 3 days (1.2 OD). The cells were harvested at 4000 g/15 min at 5°C and cell mass was estimated as 4 g/L (wet weight basis). The cells were mixed with chloroform and methanol (2:1 ratio (V/V) and kept for 3 h in a shaker incubator at 250 rpm. The mixture was incubated overnight at room temperature and centrifuged at 3000 g for 15 min/10°C. The filtrate was drained into a beaker (7 mL), evaporated at 80°C and the total fat determined (Bligh & Dyer 1959). For fat hydrolysis, the total fat mixture was mixed with 1mL of HCl (3N) and 2 mL of methanol. This was incubated at 80°C /90 min and later mixed with 2 mL of 0.9 % NaCl and 800 µL hexane (Pyle *et al*., 2008). This mixture was vortexed and centrifuged at 4000g/20 min. The upper layer was collected and analyzed for fatty acids.

Fatty acid standards: The following unsaturated and saturated fatty acids were procured from Supelco-Bellefonte, PA (USA). Unsaturated fatty acids were Docosahexaenoic acid (C22: 6), Palmitoleic acid (C16: 1), Oleic acid (C18: 1), Linoleic acid (C18: 2), Linolenic acid (C18: 3), Erucic acid (C22: 1), Cis-5, 8, 11, 14-Eicosatetraenoic acid (C20: 4), Elaidic acid (C18: 1), Nervonic acid (C24: 1) and Petroselinic acid (C18: 1). Saturated fatty acids were Myristic acid (C14: 0), Palmitic acid (C16: 0) and Stearic acid (C18: 0). For standards, 1.0 mg of each fatty acid was dissolved in 1.0 mL of solvent (1µg/µL concentration) and fat hydrolysis was followed.

Description of GCMS & fatty acids analysis: GC-MS: Model No: Thermo Finnigan, Type of Chromatograph : TRACE 2000 GC, TRACE MS, Type of detector: Electron impact ionization, Type of column: Restek RTX-SMS, Carrier gas: He, Linear velocity: 1 mL/min constant flow rate, Column split ratio: samples 1: 10 and Standards: 1: 300, Injection port temperature: 250°C, Detection port temperature: 250°C (MS interface), The temperature profile of column: 100°C ramped 2°C /min 300°C, hold 300°C for 2 min (Ms: Peak width: 4 (Sec), minimum scans per peak: 10, Ms window: 45-465 m/z). The sample of 5 µL was injected into the GC-MS for analysis.

Extraction of FAE1 protein from CC2137: The cells were centrifuged at 8000g for 10 min at 4°C (Sorval GSA rotor). The supernatant was passed through a 0.2 µm filter, kept on ice and ammonium sulphate added for 80% saturation (516 g/L) slowly in the cold room stirring slowly for an hour (Michael *et al*., 2007). The precipitated proteins were collected by centrifugation at 30,000g for 90 min at 4°C. The pellet was re-suspended in 5mL of Buffer A (50 Mm Tris-HCl, 10 mM EDTA, 1 mM PMSF, 1 mM ε-amino-n-caproic acid, pH 8.0). The sample was re-precipated by adding 100% cold TCA to 10% (w/v) final concentration. The protein was collected by centrifugation (19,000g for 30 min at 4°C: Sorval SS-34 rotor) and re-suspended in buffer B (0.1 M sodium carbonate and 0.1 M DTT) and tested against *V. harveyi* for antagonism.

Antimicrobial assay by well diffusion method: Fifty µL of *Vibrio* cells (10^7cfu / mL / 18h old) of BB120, MM32 and MM30 were inoculated into sterile petri dishes separately. Thirty five mL of LB agar was poured into the plates and allowed to solidify at room temperature for 1h. Two wells of 6 mm dia were made on the LB agar plates using the sterile steel borer. The wells were sealed at the bottom using 10 µL of 1% soft agar and filled with 200µL of crude filtrate. Various solvents were used alone as control. The plates were incubated at 37°C/48 h. The zones of inhibition of bacteria around the well, excluding the well and average of three determinations were measured (Das *et al.*, 2005). The antibacterial activities of CC 2137 extracts were compared with inhibition zones around three commercial antibacterial discs, i.e., Ampicillin (A) Chloramphenicol (C) and Cephaloxin (Cp) that were used as references (Sigma, USA).

Statistical analysis: Screening and estimations were performed in average of three replications, where as hot and cold extractions were performed in average of 5 replications and the results were expressed as means and standard errors.

Results

The growth of CC2137 increased up to fourth day then declined (Fig. 1). No antagonism was observed against *V. harveyi,* when both the cell wall bound and Fae1 proteins of CC2137 were tested. But cell wall bound protein of CC 2137 inhibited *B. cereus* (8.0 mm). The cell wall bound protein profiles were ascertained in SDS PAGE. The protein size ranged from 25 to 250 KD (Fig. 2). CC 2137 was observed to have unsaturated fatty acids like linolenic, linoleic, oleic & palmitoleic acid and saturated fatty acids such as palmitic, stearic, myristic acid. GC-MS was unable to detect petroselinic and elaidic acids (Figs. 3 & 4).

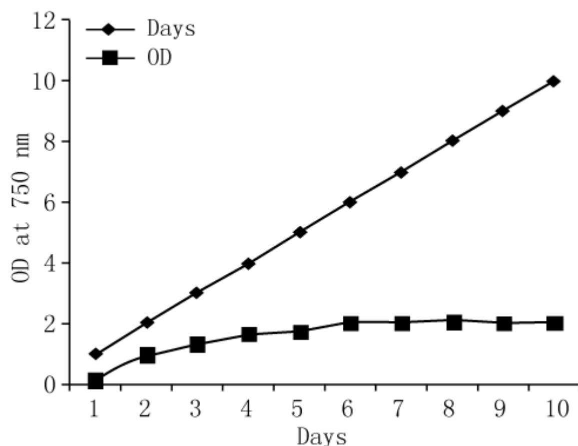

Fig. 1. Growth curve of CC 2137 at 25°C.

In cold extraction, methanolic extract showed highest inhibition (11.0 mm) against MM30 whereas acetone and ethyl acetate extracts showed identical inhibition against (9.0 mm each) MM30 and MM32. However, in the case of hot extraction, highest inhibitions were observed by ethanol and ethyl acetate extract (13.0 mm) to MM30 followed by methanol, ethanol and ethyl acetate extracts (12.0 mm) to MM32. The lowest inhibition (5.0 mm) was observed to MM32 by chloroform extract in cold extraction (Table 1). While comparing the extracts under cold and hot extraction, it was found that chloroform extract produced a greater zone (14.0 mm) of inhibition against *B. cereus* under cold extraction followed by identical inhibitions produced by extracts of other solvents against *B. cereus*. The ethanolic extract produced a higher zone of inhibition (12.0 mm) against *B. cereus* followed by methanol (11.0 mm) and acetone (9.0 mm) respectively. Ethyl acetate and acetic acid extracts showed highest (8.0 mm) and identical inhibitions (7.0 mm) towards *P. putida* (PP) and lowest inhibitions showed by methanolic and chloroform extracts (6.0 mm) under cold extraction. In hot extraction, the highest inhibition was exhibited by ethanol extract (Table 1) towards PP (12.0 mm) followed by methanolic and acetone extracts (11.0 mm).

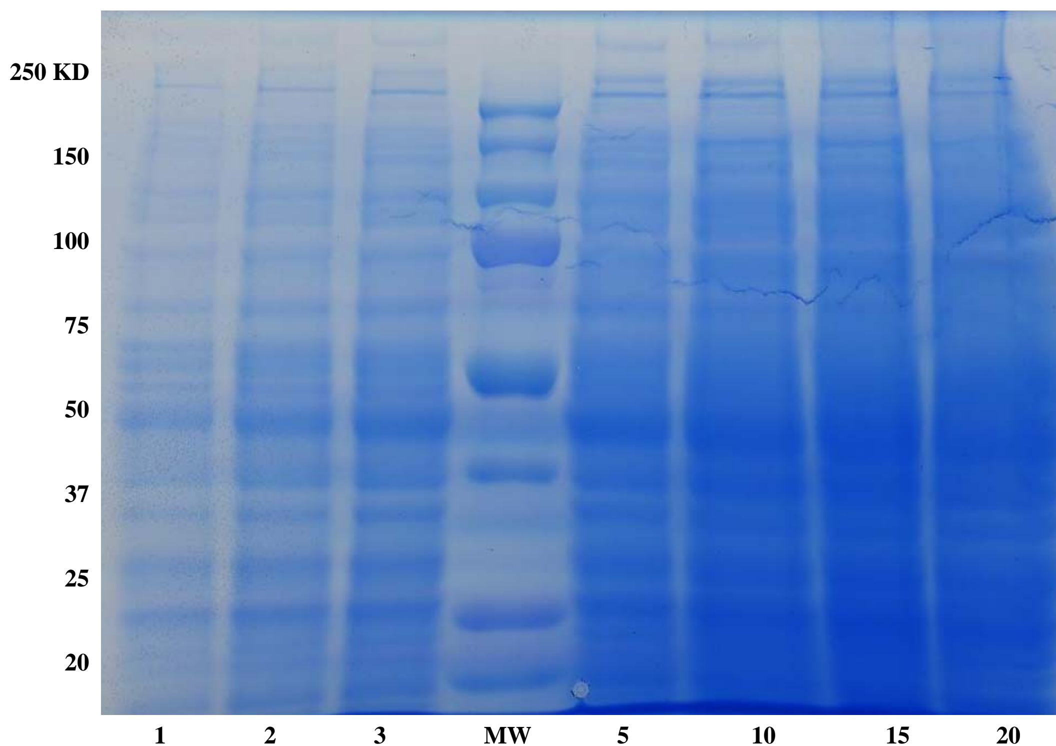

Fig. 2. SDS PAGE showing the cell wall bound protein profile of CC2137.

In cold extraction, ethyl acetate and acetic acid extracts showed highest inhibition zone (8.0 mm) to E. coli followed by ethanol and acetone extracts (7.0 mm). Lowest zone of inhibition was exhibited by methanol and chloroform extracts (6.0 mm). In hot extraction, highest zone of inhibition was exhibited by ethyl acetate extract (12.0 mm) to E. coli followed by acetone (11.0 mm) and lowest by acetone and acetic acid extracts (6.0 mm) respectively. Under cold extraction, B. cereus showed highest zone of inhibition (14.0 mm) by chloroform extract followed by other extracts and lowest by methanolic extract. In contrast, under hot extraction, highest zone of inhibition was exhibited by ethanolic extract (12.0 mm) followed by methanolic extract (11.0 mm). There was not much difference between the zones of organic extracts as compared with conventional antibiotics. Out of seven CC2137 extracts, tried in cold extraction for antibacterial activity against V. harveyi, methanol extract showed highest inhibition zone (11.0 mm) to MM30 followed by acetone (9.0 mm). In the hot

extraction, ethanol, ethyl acetate extracts showed highest (13.0 mm) and identical inhibitions to MM30 followed by ethanol extract (12.0 mm) respectively. Out of seven extract of CC2137, tried for antibacterial activity against non-vibrios, chloroform extract showed highest (14.0 mm) inhibition zone to B.cereus followed by acetone, ethyl acetate, acetic acid extracts. In hot extraction, ethanol showed highest inhibition zone (12.0 mm) to P. putida and B.cereus followed by methanol, acetone extracts (Table 1).

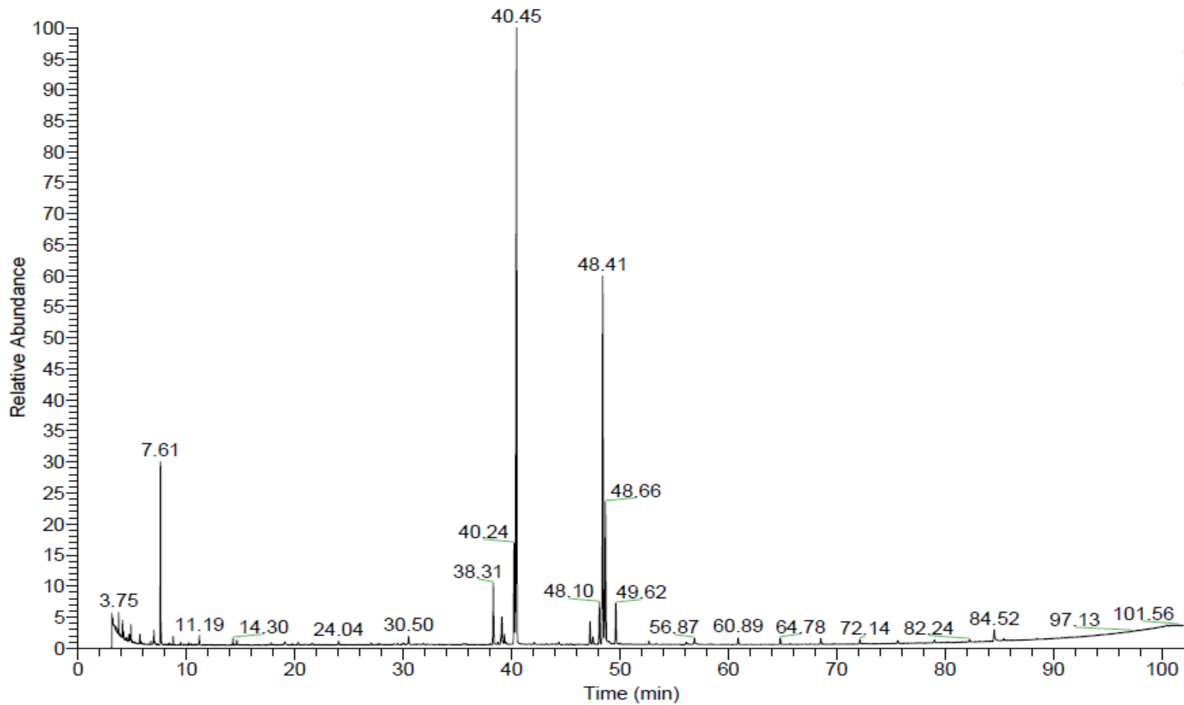

Fig. 3. Various fatty acids detected using GCMS from CC2137, 48.45: Palmitic acid, 48.41: Linolenic acid, 48.66: Oleic acid, 49.62: Stearic acid, 48.10: Linoleic acid, 40.24: Palmitoleic acid, 30.50: Myristic acid, others are unknown.

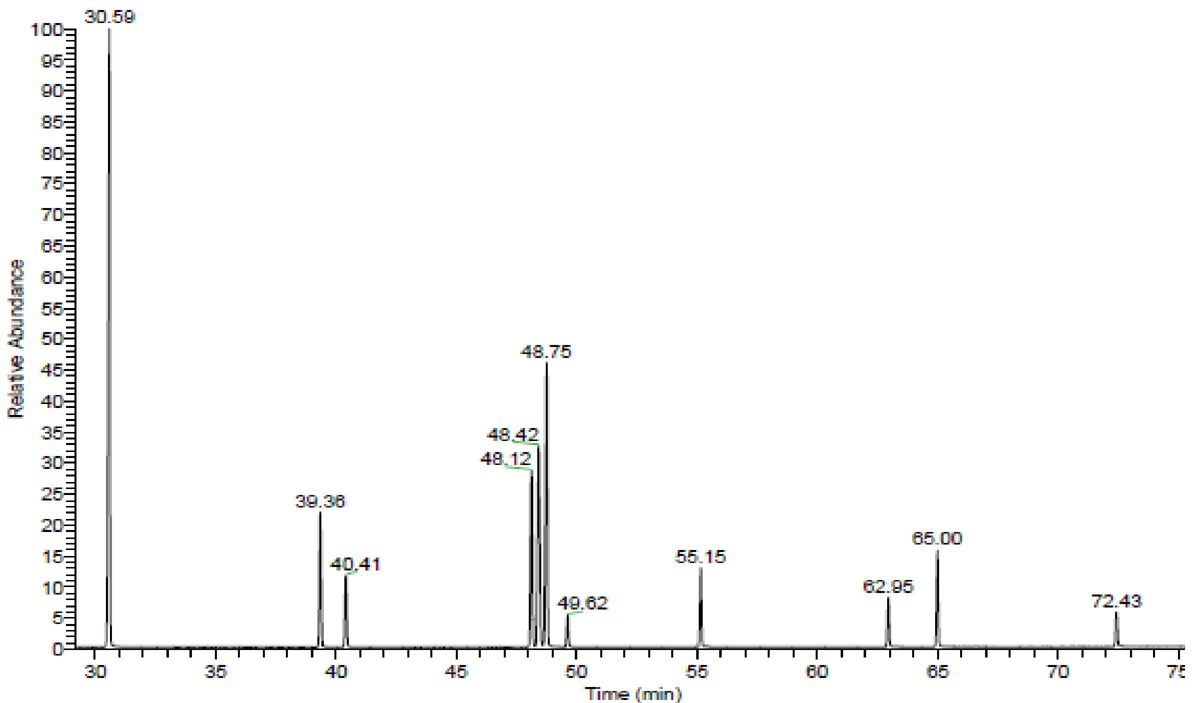

Fig. 4. Standards of various saturated and unsaturated fatty acid detected in GCMS, (0.59): Myristic acid, 48.75: Oleic acid, 48.42: Linolenic acid, 48.12: Linoleic acid, 39.36: Palmitoleic acid, 40.41: Palmitic acid, 49.62: Stearic acid 55.15: ETA, 62.95: DHA, 65.0: Eruic acid, 72.43: Nervonic acid (Peteroselinic acid and Elaidic acids are not detected by GCMS).

Table 1. Antibacterial activity of various solvents treated *C. reinhardtii* (Mean ± SE).

Different extracts	*V. harveyi* (BB120)	*V. harveyi* (MM32)	*V. harveyi* (MM30)	*P. putida*	*E. coli*	*B. cereus*
			Cold extraction			
Methanol	6.01 ± 0.10	6.01 ± 0.05	11.01 ± 0.01	6.02 ± 0.02	6.01 ± 0.02	8.02 ± 0.10
Ethanol	6.01 ± 0.10	7.02 ± 0.01	7.01 ± 0.02	7.03 ± 0.01	7.01 ± 0.01	9.02 ± 0.03
Chloroform	7.02 ± 0.10	5.02 ± 0.01	7.02 ± 0.01	6.01 ± 0.01	6.02 ± 0.01	14.02 ± 0.2
Acetone	9.03 ± 0.2 0	6 .01 ± 0.05	9.01 ± 0.02	7.02 ± 0.03	7.02 ± 0.03	9.01 ± 0.01
Ethyl acetate	8.01 ± 0.10	9.01 ± 0.02	7.01 ± 0.01	8.02 ± 0.02	8.01 ± 0.02	9.01 ± 0.06
Acetic Acid	7.01 ± 0.10	6.01 ± 0.01	8.02 ± 0.01	8.02 ± 0.02	8.02 ± 0.01	9.02 ± 0.02
Cephalaxin (Cp) 30mcg	10.01 ± 0.10	9.02 ± 0.10	8.02 ± 0.20	9.01 ± 0.10	10.01± 0.20	9.02 ± 0.01
Ampicillin (A) 10mcg	11.02 ± 0.10	9.01 ± 0.20	10.01± 0.20	8.01 ± 0.10	8.01 ± 0.20	11.01 ± 0.02
Chloramphenicol (C) 10mcg	10.01± 0.20	9.02 ± 0.10	9.02 ± 0.10	9.01 ± 0.10	7.01 ± 0.20	10.01 ± 0.01
			Hot extraction			
Methanol	11.01 ± 0.10	12.02 ± 0.01	11.01± 0.01	11.03 ± 0.02	9.50 ± 0.10	11.02 ± 0.10
Ethanol	12.02 ± 0.20	11.01 ± 0.1	13.02 ± 0.20	12.03 ± 0.03	10.01 ± 0.02	12.02 ± 0.02
Chloroform	6.01 ± 0.03	6.02 ± 0.02	6.01 ± 0.20	6.01 ± 0.23	6.03 ± 0.30	6.01 ± 0.30
Acetone	11.01± 0.02	11.01 ± 0.03	12.01 ± 0.01	11.01 ± 0.01	11.02 ± 0.10	9.02 ± 0.20
Ethyl acetate	10.01± 0.01	12.01 ± 0.02	13.02 ± 0.20	10.02 ± 0.02	12.02 ± 0.20	8.01 ± 0.20
Acetic acid	6.01 ± 0.01	6.01 ± 0.01	6.01 ± 0.30	6.02 ± 0.30	6.02 ± 0.30	6.02 ± 0.20
Cephalaxin (Cp) 30mcg	9.02 ± 0.10	10.02 ± 0.10	8.02 ± 0.20	10.01 ± 0.10	11.02 ± 0.20	9.01 ± 0.10
Ampicillin (A) 10mcg	8.01 ± 0.10	9.01 ± 0.20	7.02 ± 0.20	11.01 ± 0.1	9.01 ± 0.2	9.03 ± 0.20
Chloramphenicol (C) 10mcg	9.03 ± 0.20	8.01 ± 0.10	7.01 ± 0.10	10.01 ± 0.1	9.01 ± 0.2	8.03 ± 0.10

Values are average of 5 determinations, represented in mm, Zones are excluding the spots

Discussion

Under intense photosynthesis, higher level of unsaturated fatty acids has been reported in CC2137 by Poerschmann *et al.,* (2004). Out of 10 unsaturated fatty acids screened, 5 were reported in this study. However all the three saturated fatty acids tried were also reported (Figs. 3 & 4). The results of this study established that the crude fatty acid extracts of CC 2137 showed antibacterial properties. The cell wall bound protein of CC2137 did not show any activity on *V. harveyi* but showed against *B. cereus*. This may be due to the change in the culture condition of bacteria. It was noticed more predominantly in hot extraction process, where ethanol extract showed highest inhibition to *Vibrios* and lowest by acetic acid extract. Cold extraction process gave less inhibition to *Vibrios* as compared with hot extraction. Under cold extraction, mild inhibition zone was shown by chloroform and ethanolic extracts to *Vibrios*. Acetone and ethyl acetate extracts showed identical inhibitions in cold extraction to *Vibrios* (Table 1).

Many authors have found that antibacterial activities of micro algae are due to fatty acids (Kellam *et al*., 1988; Das *et al.,* 2005). Antibacterial activity of unsaturated and saturated long chain fatty acids of chain length more than 10 carbon atoms induced lysis of bacterial protoplasts. The antibacterial activities of crude fatty acid extracts of CC 2137 were very encouraging against all marine *Vibrios, P. putida, E. coli* and *B. cereus* (Table 1). Naviner *et al*., (1999) reported inhibition of various marine bacteria using fatty acids of algae such as, *Skeletonema costatum*. The organic fractions of the *Skeletonema costatum* inhibited *Vibrio mytili, Vibrio* sp. S322, *Vibrio* sp. VRP and *Listonella anguillarum.* However, the growth of *Aeromonas hydrophila, A. salmonicida, Serratia liquefasciens, Vibrio alginolyticus* and *Yersinia ruckeri* were not inhibited by the extract of *Skeletonema costatum.* Alternatively, in this study aqueous extracts of CC2137 showed inhibitory potential to *V. harveyi.* Therefore, CC2137 extract could possibly be used as an antimicrobial agent against bacteria that resist conventional antibiotics (Karunasager *et al*., 1994) and there appears to be a significant role for *V. harveyi* in the control of fish and shrimp larval diseases.

Various solvents under hot extraction process exhibited highest inhibition against marine *Vibrios* as compared with solvents in cold extraction. In cold extraction, extracts such as chloroform, ethyl acetate and ethanol showed highest inhibition to *B. cereus, E. coli, P. putida* than hot extraction process (Table 1). *P.*

putida causes infection (Smolowitz *et al.,* 1998) to the fish *Opsanus tau* (Oyster toad). The inhibitory mechanism to bacteria may be based on the nature of solvents, test bacteria used and the conditions of extraction process. Due to the increase of therapeutic resistance to conventional antibiotics (Aoki 1992; Nash *et al*., 1992) against marine aquatic pathogenic bacteria such as *V. harveyi* and related species, organic micro algal based fatty extract could be used to control aquatic pathogens. Unlike other micro algae possessing antibacterial properties, *C. reinhardtii* also had the same property and easy to handle with its s fast growing in nature (Rupprecht 2009).

We could not compare the results of the present study as similar investigations on antibacterial activity of *C. reinhardtii* on aquatic pathogens are scarce. The present study showed enough data to develop potential algal fatty acids and proteins for using as antibacterial agents in bio-therapeutic, industrial, aquaculture and health applications. There may also be a possibility that fatty acids produced by *C. reinhardtii* would contribute as nutritional supplement in fish feed and to prevent fish diseases caused by bacteria present in the aquaculture. Economically feasible procedures for accomplishing generally recognized as safe (GRAS) status may be developed in preparation and implementation of the algal extracts in large scale as bio-therapeutic agents.

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