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EFFECT OF ACULEACIN A IN FATTY ACIDS METABOLISM IN *CYMBELLA* SPECIES

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ABSTRACT

The marine diatom C*ymbella* sp. isolated from the coastal water of Mahabalipuram Beach, Chennai, and Tamilnadu was made as unialgal and investigated for growth. The culture is grown aseptically in F/2 medium under constant temperature and light. Before carrying out the studies, the fatty acid, carbohydrates, cellular dry mass and pigments of the mother culture were analyzed. The scope of this study was "The effect of Aculeacin A in fatty acid synthesis of marine diatom C*ymbella* sp." Different concentrations of Aculeacin A with respect to time towards cellular growth, dry mass and fatty acids were studied. The experimental evidence shows, the control of metabolic pathways during the various phases of Aculeacin A treatment. This review focuses on the contribution of inhibitor or compound, in our understanding of the mechanism by which Aculeacin A induces the metabolic pathway of marine diatom C*ymbella* sp.

KEYWORDS: **Algae, Cymbella, Aculeacin A, Fatty acids, Diatom.**

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INTRODUCTION

Diatom C*ymbella* sp is one of the most common types of phytoplankton and are a major group of algae. A unique characteristics feature of diatom cells is that they are encased within unique cell wall made of silica. Most diatoms are unicellular, although they can exist as colonies. Diatoms are a widespread group and can be found in oceans, fresh water and in soils. Diatoms belong to a large group of algae called the heterokonts that includes both autotrophs and heterotrophs. Diatoms yellowish brown chloroplasts are typical of heterokonts, with four membranes and containing pigments such as the carotenoids and fucoxanthine[1]. Diatoms can be grown both on agar gels and in liquid. Many species are easy to grow and that the same time it easily contaminated with other algae. Before isolation, it may be necessary to grow a mixed culture from materials collected from nature in order to obtain a large number of cells or it may be possible to pick out a small number of cells from natural population and grow them in a defined culture medium. The initial inoculums can be streaked on agar plates [1-2% made up in culture medium]. The individual colonies can then be removed with a wire loop. The cultures can be maintained unialgal in various light/dark cycles and at various temperatures. Aeration will be required, if a large volume of cultures is maintained. Bacterial contaminants can be eliminated by the addition of appropriate antibiotics of the media[2]. Diatoms store carbon in carbohydrate and lipid form. By the process of photosynthesis, algae cells assimilate carbon. There are many metabolic pathways through which the excess carbon can enter, resulting in synthesis of many compound required by the cell. These pathways consist of sequence of enzymes, each of which catalyses a specific reaction. Key enzymes involved in lipid synthesis pathway, whose level of activity in the cell influences the rate at which oils are produced. These enzymes act as valves controlling the flow of carbon down the pathway. Higher enzyme activity gives higher rates of oil production. Algae cells increase the activity of metabolic active enzymes, and are the opening gate to allow greater flow of carbon for oil production. Instead of over expressing enzymes involved in fatty acid metabolism, identify the enzymes involved in the synthesis of storage carbohydrates and inactive them. By this shutting off flow of carbon to carbohydrates and it would force carbon to flow down the lipid metabolic pathway. Increasing the proportion of algal lipid is to limit the flow of newly assimilated carbon into other cellular pathways. Many diatoms including *Cymbella* can produce a significant amount of a storage carbohydrate called chrysolaminarin, a β-[1-3]-linked glucan. The biochemical pathways of chrysolaminarin were not known. In C*ymbella* sp., chrysolaminarin can make up 40% of cell dry weight, and thus chrysolaminarin synthesis pathways presumably compete for newly fixed carbon with the pathways for lipid biosynthesis. Inhibiting chrysolaminarin production by inhibiting one or more enzymes in the carbohydrate synthesis pathway could result in the flow of more carbon into lipid production[3]. Aculeacin A is a cyclopeptide-containing long-chain fatty acid, representing a new class of antibiotics. It has a relatively narrow antifungal spectrum in vitro and is highly active against some groups of yeasts[2]. Aculeacin group antibiotics consist of aculeacin-Aα, -Aγ, -Dα and -Dγ which are produced by culturing *Aspergillus aculeatus* having strong anti-fungal activity, showing the peak of ultraviolet absorption at 278 nm and containing threonine as amino acid component[4]. Since the Aculeacin groups of antibiotics are peptide antibiotics, hereinafter these groups of antibiotics will be designated as Aculeacins. Aculeacins are produced by inoculating a strain of *Aspergillus aculeatus* in a suitable nutrient medium. The cultivation of the microorganism can be carried out in a number of different ways such as liquid culture or solid culture. The culturing temperature for production of Aculeacins may be selected within the range of temperature in which the microorganism can grow and Aculeacins can be produced, preferably at 25°-28° C. Aculeacins are impossible to titrate due to decomposition at an alkaline pH, and due to

slight solubility Aculeacins are immobile upon electrophoresis. Aculeacins is highly soluble in lower alcohols, slightly soluble in ethyl acetate and almost insoluble in acetone, chloroform, n-hexane, petroleum ether and water. Aculeacin A, an amphophilic antibiotic, inhibits the biosynthesis of glucan by selective blockage of glucan synthase[5]. By advantage of the above property Aculeacin A were used to block the enzymes involved in carbohydrate metabolism to enhance the flow of carbon towards the fatty acid metabolic pathway in diatom C*ymbella* sp.

MATERIALS AND METHODS

The algae C*ymbella* sp. upon which work has been carried out was isolated in Department of Biotechnology, Dichemz Biotech, Chennai, from the coastal water of Mahabalipuram Beach, Chennai, and Tamilnadu. The algae C*ymbella* was identified and isolated from the sample and grown in a suitable culture medium. Isolation in pure culture is an important preliminary to the study the effect of Aculeacin A in fatty acid metabolism in C*ymbella* sp. Since it is possible that different physiological races of a species of algae may exist, it is desirable that the cultures used should have originated from a single individual. Such unialgal cultures of C*ymbella* sp. were obtained by isolating an individual with a sterile serial dilution followed by the agar streaking method and C*ymbella* colonies were isolated. The culture was examine under the low power microscope and inoculated into sterile f/2 culture medium. Cultures obtained by above method were still contaminated with bacteria. To obtain C*ymbella* culture free from bacteria has been difficult and this was achieved by sub culturing on agar plates. A small portion of algae culture was immersed in chlorine water of a concentration of 25mg per 100 ml for 5 minutes and centrifuges the sample at 10,000 rpm at 30°C for 5 minutes in cooling centrifuge. After centrifuge the supernatant was discarded and the pellet washed in sterile water and the pellet was transfer in freshly prepared culture medium. All the cultures used in the subsequent work were grown from this culture. The chemicals used were of analytical quality. The medium was sterilized at 120°C for 15 minutes in the autoclave and allow standing at least 3 hours before inoculation and its pH was checked for 7.8. Conical flask of 1000ml capacity, plugged with cotton wool, was used throughout this work. The flasks were cleaned with chromic acid and rinsed with distilled water before use. Culture chamber was maintaining a constant temperature below 30°C. The temperature remained at 21°C for most of the time. Illumination was for a period of about 12 hours per day. Stock cultures were maintained on agar slants in test tubes. Before using a culture for inoculums, it was thoroughly examined for contaminants. A portion of material from the young stock culture was shaken with sterile medium in a sterile flask closed with a tightly fitting cotton plug for 15 minutes. The heavier materials were allowed to settle and after decantation portions of 50ml of the suspension were used as inoculums. The suspension prepared in this manner was found to be sufficiently uniform for all ordinary purposes. For the contents of a culture flask were to analyzed, the alga was first detached from the sides of the flask by means of vigorous shaking then the algae and medium were separated by centrifugation at 10,000 rpm. The algae was washed with distilled water and the pellet was added to the medium. The bio mass was dried for analysis by evaporation in hot plate at 75°C.

ACULEACIN A TREATMENT

Aculeacin A from sigma Aldrich was used for this study. Since Aculeacin A is insoluble in water, 0.05% of Aculeacin A reagent is prepared for this experiment by dissolving in few drops of ethanol and then make up with water. Ten number of 1000ml conical flask with cotton plug with sterile f/2 medium with Aculeacin A were prepared and 50 ml of stock culture were inoculated to the entire flask mixed well. Maintain the culture flasks with controlled temperature and light. Algae samples were collected from flasks for every day and centrifuge the samples and dried for biomass. All biomass samples were analyzed for dry mass weight, fatty acids, carbohydrates and pigments.

BIOMASS ANALYSIS

Biomass derived from C*ymbella* sp. was analyzed for various parameters. The algal mass was harvested by centrifugation. 50 mg of dry mass is extracted with 30 ml acetone and then with 30 ml hexane for saturated and unsaturated fatty acids and concentrate in a distillation apparatus with cooling vacuum pump[6].

EFFECT OF pH

The effect of pH on the growth of the algae and hydrocarbon yields was studied using F/2 media in the pH range of 6.5, 7.0, 7.5, 8.0 ,8.5,9.0 and 9.5.The experiment was carried out in Erlenmeyer flasks (1000ml) containing 500ml F/2 medium and the pH of the medium was adjusted before autoclaving. Two weeks old *Cymbella* culture was inoculated uniformly to all the flasks. The culture flasks were incubated for 3 weeks at 21 °C temperature with 1.0 to 2.0 k lux and 12:12 hrs light, dark cycle, and the culture was harvested and analyzed for biomass and hydrocarbon yields[7].

BIOMASS ESTIMATION

The cultures were harvested and the cells were washed with distilled water after centrifugation at 5000 rpm. Then the pellet was freeze dried. The dry weight of algal biomass was determined gravimetrically and growth was expressed in terms of dry weight[8].

CHLOROPHYLL ESTIMATION

A 100 ml of culture was centrifuged (8000 rpm) for 10 min and the pellet was treated with 30 ml of methanol and kept in water bath for 30 min at 60°C. Absorbance of the pooled extracts was measured at 652 nm and chlorophyll a was estimated using Lichtenthaler equations[9].

ESTIMATION OF CAROTENOIDS

50mg of algal biomass was homogenized and extracted repeatedly with 30 ml acetone; concentrate in a distillation apparatus with cooling vacuum pump. 15mg of concentrate is dissolved in 250ml of cyclohexane and absorbance was calculated at 455nm and total carotenoid contents were quantified according to Lichtenthaler equations.

HYDROCARBON EXTRACTION

Hydrocarbon was extracted with hexane after homogenizing the dry biomass in a mortar in the presence of glass powder and the supernatant recovered after centrifugation was evaporated to complete dryness under the stream of nitrogen. Hydrocarbon content was measured gravimetrically and expressed as dry weight percentage[10].

TLC FOR HYDROCARBONS

Fatty acids are analyzed by TLC. Silica plates are used and the solvent system hexane: diethyl ether: acetic acid is prepared in the ratio 26:8:0.8 and 0.1% of potassium permanganate in water is used as staining solution.

HYDROCARBON ANALYSIS BY GC

Hydrocarbon hexane extract was purified by column chromatography on silica gel. The hydrocarbon samples were analyzed on innowax column and carrier gas as helium. 5mg of sample was dissolved in 10ml hexane and FID detector was used[11].

FATTY ACID ANALYSIS

Lipids were extracted with chloroform - methanol (2:1) and quantified gravimetrically. The fatty acid methyl esters (FAME) were prepared[12]. FAME was analyzed by innowax column and carrier gas as helium. 5mg of sample was dissolved in 10ml hexane and FID detector was used. The FAME was identified by comparing their fragmentation pattern with authentic standards.

HPLC ANALYSIS OF CAROTENOIDS

The acetone extract of the alga C*ymbella* was analyzed by HPLC using a supleco C-30 silica column with the solvent system Methanol: Acetonitrile: 0.2N Ammonium acetate: 2 – Proponol: HPLC water: Ethyl-di-iso-propanolamine: Butylated Hydro toluene, 500ml: 455ml: 20ml: 20ml: 5ml: 0.2ml: 0.05g. 10mg of sample were dissolved in 10 ml of acetone and 0.5µl of sample were injected. DAD was detected at 455 nm. Lutein, β-carotene, xanthine were identified using authentic standards[13].

RESULTS AND DISCUSSION

The effects of Aculeacin A in culture medium on growth kinetics and fatty acid production of C*ymbella* sp. were investigated. Two different concentrations (0.25 g/L and 0.5g/L) in culture medium were used. Results showed that induced an increase in cell concentration and enhanced accumulation of fatty acids in the cells. When cells were grown in a medium containing Aculeacin A, the production of fatty acids depended on the concentration of components. At a given Aculeacin A, an increase in fatty acid production was obtained by increasing the cell concentration. In contrast, an increase in fatty acid concentration caused a decrease in carbohydrate production when the Aculeacin A concentration was fixed as 0.5g/L. These results suggest that an appropriate concentration of Aculeacin A can enhance the accumulation of lipids of C*ymbella* sp. cells. When the culture was treated with 0.5g/L of Aculeacin A, the biomass is gradually increased from 0.015 – 1.09 g/L of culture. Growth studies and biomass production are represented in graph 1. Lipid content of mother culture is gradually increased along with biomass from 0.0037- 0.2720g/ L, whereas lipid content of the Aculeacin A treated *Cymbella* culture is gradually increased from 0.0037 -0.4900 g/L. Effect of Aculeacin A in lipids of *Cymbella* sp. are represented in Table 1 and Graph 2. The carbohydrate of mother culture is gradually increased from 0.0060– 0.4360g/L. In contrast the carbohydrate of the Aculeacin A treated *Cymbella* culture is gradually decreased from 0.0060–0.0054 g/L. Effect of Aculeacin A in carbohydrates of *Cymbella* sp content are represented in Table 2 and Graph 3. Triglycerides and fatty acids were analyzed using TLC, Carotene was estimated daily by UV spectrophotometer and it's produced from 0.09 – 2.12 g / kg of biomass. Xanthine, chlorophyll was also analyzed using HPLC and xanthine produced from 0.13 – 3.16g /kg of biomass. HPLC of *Cymbella* sp. pigments are represented in Graph 4. Extracted fatty acids were also transesterfied using acid reaction and fatty acid esters are also produced. Fatty acids and its esters were analyzed in gas chromatography. Gas chromatography of *Cymbella* sp. fatty acids and its esters are represented in Graph 5 and Graph 6. The fatty acid content was seen to be 25 % in their biomass where the carbohydrates content was 40%. Since our focus is to enhance the fatty acid content, the enzymes involved in carbohydrate metabolism is targeted. Glucan synthase is a glucosyltransferase enzyme involved in the generation of beta-glucan in carbohydrate metabolism in algae. An antifungal agent aculeacin A were used to inhibit the enzyme glucan synthase. Since this inhibits the enzyme, the flow of carbon in algae is directed towards lipid metabolism[14]. This is proved by the effect of Aculeacin A towards lipid and carbohydrate content in growth studies in *Cymbella* sp. By this experiment the fatty acid content is raised from 25% to 45% to enhance the biofuel production in *Cymbella* sp. Among the fatty acids, palmitic acid seen to be high in this species, that is confirmed by gas chromatography. Since our focus to enhance the fatty acid, particularly the oleic acid in C*ymbella* sp., due to the activation energy of oleic acid esters are high when compared to palmitic acid esters.

Table 1
Effect of Aculeacin A in Lipids of Cymbella sp. Lipids production of Cymbella sp in Mother Culture and Aculeacin A treated culture with respect to biomass.

Days	Dry mass g/L of culture	Lipids Mother culture g/L of culture	Lipids Culture with Aculeacin A g/ L of culture
0	0.015	0.0037	0.0037
5	0.019	0.0047	0.0057
10	0.20	0.0500	0.0740
15	0.89	0.2220	0.3827
20	1.09	0.2720	0.4900

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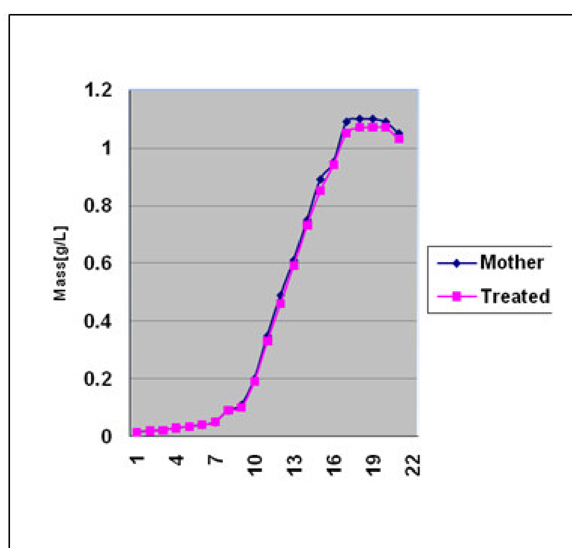
Table 2

Effect of Aculeacin A in Carbohydrates of Cymbella sp. Carbohydrate production of Cymbella sp in Mother Culture and Aculeacin A treated culture with respect to biomass

Days	Dry mass g/L of culture	Carbohydrates Mother culture g/ L of culture	Carbohydrates Culture with Aculeacin A g/ L of culture
0	0.015	0.0060	0.0060
5	0.019	0.0076	0.0076
10	0.20	0.0800	0.0600
15	0.89	0.3560	0.2220
20	1.09	0.4360	0.0054

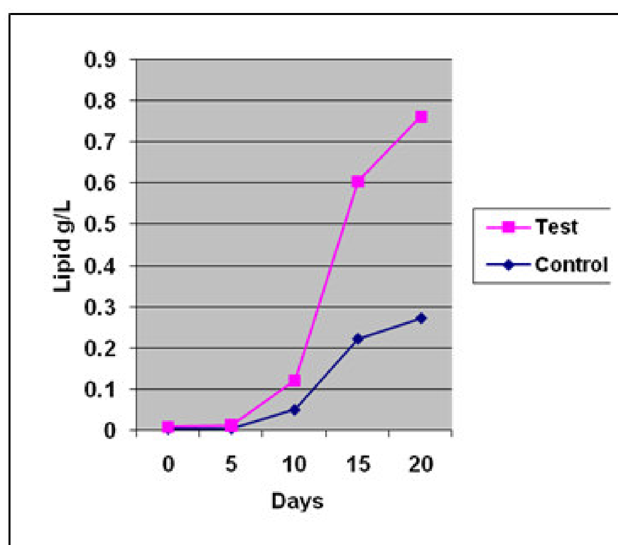
Graph 1

Growth studies of Cymbella sp Growth studies of Cymbella sp in Mother Culture and Aculeacin A treated culture with respect to biomass production



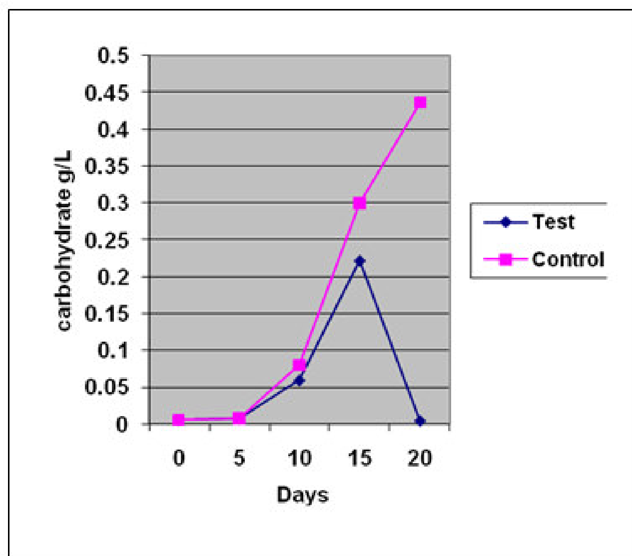
Graph 2

Effect of Aculeacin A in Lipids of Cymbella sp. Lipids production of Cymbella sp in (Control) Mother Culture and (Test) Aculeacin A treated culture with respect to biomass.



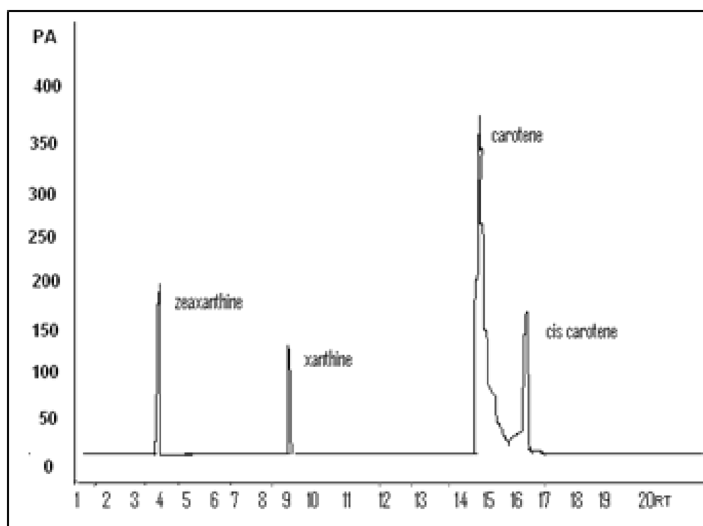
Graph 3

Effect of Aculeacin A in Carbohydrates of Cymbella sp. Carbohydrates production of Cymbella sp in (Control) Mother Culture and (Test) Aculeacin A treated culture with respect to biomass.

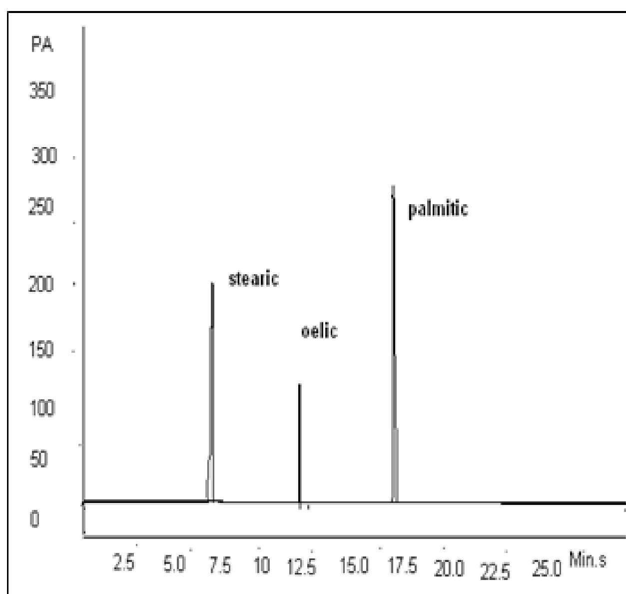


Graph 4

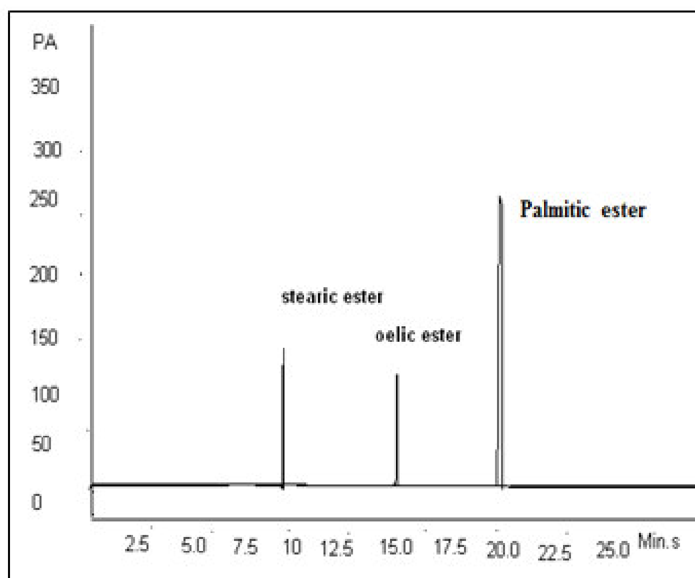
HPLC repot of Pigments in Cymbella sp using C-30 supelco silica column. Cymbella sp containing pigments like Zeaxanthine, Xanthine, Carotenoids. Carotenoids and its isomers were seen to be high.



Graph 5
***Fatty acids of Cymbella sp by using innowax column in G.C. Palmitic acid were high in Cymbella sp fatty acids when compare to Stearic acid and Oleic acid*.**



Graph 6
Fatty acid esters of Cymbella sp using carbowax column in G.C. Palmitic acid esters were high in Cymbella sp. fatty acids when compare to Stearic acid esters and Oleic acid esters.



CONCLUSION

From this study it was concluded that fatty acids in cymbella sp can enhanced by shutting off flow of carbon to carbohydrates and it would force carbon to flow down the lipid metabolic pathway by aculeacin A. Our future studies will aim to understand and alter the metabolism to enhance the oleic acid with high biomass production in C*ymbella* sp.

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