intensity of 1500 lux was provided for their growth at room temperature. They were sub cultured every 15 days once.

3.2 Molecular identification-

3.2.1 DNA Extraction

Cultures were pelleted by centrifuging at 10000rpm for 10 minutes. The pellet was suspended in 750 µl of suspending buffer and heated at 65°C for 45minutes.Then 650µl of lysis buffer was added and heated for 45 minutes at 65°C.This solution was then centrifuged at 10000rpm for 5minutes and the supernatant was collected in a column containing silica membrane. The columns were spun at 5X for 30-35 seconds twice. Then the membrane was washed to remove other debris and colour by using 500µl of wash buffer by spinning at 10X for 1min twice. This was followed by a dry spin at 10000rpm for 5minutes.The DNA was eluted by adding 20µl of elution buffer and heating for 2minutes followed by spinning at 10000pm for 2minutes twice.4µl of the collected DNA was mixed with 2µl of the gel loading dye and 1µl of the buffer and it was loaded onto the electrophoresis boat. The samples were run at 35volts for 25minutes and the concentration of the DNA obtained was noted [6].

3.2.2 PCR Amplification

5µl of the extracted DNA was collected in a 1ml vials. To this 10µl of buffer, 4µl of forward primer. 4 µl of reverse primer, 4µl of dNTP's and 72µl of double distilled water was added. Primers used were 16s rDNA and 18s rDNA. The PCR machine was set for 40 cycles out of which the first 5 cycles was set for a step up process of 1°C rise for every cycle. Denaturation was set for 5 minutes at 94°C; Annealing – 94°C for 30seconds, 50°C for 30seconds, 72°C for 5seconds; for 5 min at 72°C.After setting the instrument. 1 µl of taq polymerase enzyme was added to the reaction mixture and loaded onto the PCR instrument. After the completion of the reaction, the vials were removed after the temperature of the PCR reduced to 4°C.6µl of the sample was mixed with 2µl of the gel loading dye and 1µl of the buffer and electrophoresis was performed to check for amplified bands. Once the bands were observed, the remaining sample was run for further processing [7].

3.2.3 PCR Purification

Once the bands were obtained, they were cut and treated with sodium acetate and gel elution buffer and kept for heating till the gel melted. Then 200µl of iso propanol was added and left for 5minutes.This was then take into silicon columns and spun at 5X for 1minute twice. Then the debris and colouring was removed by spinning at 10X for 1minute twice by using 500µl of wash buffer. This was followed by a dry spin at 10000rpm for 5minutes.The DNA was eluted from the silica membrane using 2µl of elution buffer and heating for 2min followed by a spin at 10000rpm for 2 minutes. 4µl of this eluted DNA was mixed with 2µl of the gel loading dye and 1µl of the buffer and run in the electrophoresis tank for 30minutes at 35volts to check for the concentration of the DNA obtained.

3.2.4 Nucleotide sequence analysing using gene sequencer

The Applied Biosystems® 3130 Genetic Analyzer was used to analyze the sequence of the amplified samples in this step. This sequencer uses Sanger's Dideoxy method for identification of the bases. In the final step, the obtained sequences were compared with Gene Bank using Basic Local Alignment Search Tool (BLAST) to identify them up to genus and species level.

3.2.5 Comparison of obtained sequences with Gene Bank using BLAST

Once the chromatogram and the sequences were obtained from the gene sequencer, the sequence was copied onto the BLAST tool and the most likely species of the organism was identified and analyzed [8].

3.3 Lipid Extraction

The cultured organisms were subjected to three methods of lipid extraction.

3.3.1 METHOD 1: General Solvent Method

Two steps were followed in this protocol:

LYSIS: 600OD of cells were pelleted down and 1ml of YPR buffer was added. This was incubated on ice for 20minutes.Then 1gram glass beads were added and vigorously vortexed for 30minutes.The solution was then centrifuged at 1500g for 6minutes and the lysate was separately collected. To the pellet 1ml of YPR buffer was added and vortexed for 30minutes.This was then centrifuged at 1500g for 6minutes and the lysate was collected and pooled with the lysate collected after the first spin.

LIPID EXTARCTION: To 5mg of lysate chloroform: methanol: acidified water (1:2:1) was added. Lipids were extracted by vortexing (keeping on ice) for 45minutes.The organic phase (bottom phase) was collected separately and 400µlt of chloroform was added to the top layer and vortexed for 5 minutes. Then the organic phase was collected and they were pooled together. This phase was washed several times with acidified water till the milky interface (proteins) disappeared. The tubes were left overnight for the organic phase to evaporate.

3.3.2 METHOD 2: Hexane Solvent Method

[9] 40ml broth was centrifuged and 1ml of 1M sulphuric acid was added to the pellet and kept in the water bath for 30minutes at 90°C.Then 5M sodium hydroxide was added and kept in the boiling water bath for 30minutes at 90°C.Then this was cooled and the solution was centrifuged at 10000rpm for 10minutes.The supernatant was collected and 3ml 0.5M Sulphuric acid was added to it and centrifuged at 10000rpm for 10minutes.The suspended solids were collected and 5ml hexane was added to it and kept in the boiling water bath for 15minutes at 90°C then the vials were collected and the hexane phase was collected. The vials were left overnight for the solvent to evaporate.

3.3.3 METHOD 3: Bligh Dyer Method

Cells were pelleted by centrifugation at 10000rpm for 10minutes and 3.75ml of chloroform: methanol (2:1) was added to it. The solution was vortexed for 15-20 minutes. Then 1.25 ml of chloroform and 1.25 ml of distilled water was added and the top milky top layer was discarded and the bottom layer was left overnight for the solvent to evaporate [10].

3.4 Qualitative Analysis

Qualitative analysis was performed using Thin Layer Chromatography (TLC) [12]. The extracted lipids were dried overnight. They were then dissolved in 45µl of chloroform: methanol (2:1). 45ml of the solvent system [Petroleum ether: Diethyl ether: glacial acetic acid (70:30:1)] was used.TLC sheet of 7cm height and 4cm width was used. The standards used were linolenic acid and olive oil.2µl of the samples and the standards were spotted onto the TLC plate and put into the glass chamber concentrated with the solvent system. The plate was left in the chamber till the solvent reached three quarters of the sheet and then the plate was transferred into the developing chamber containing iodine crystals for development and comparison of the bands.

4. Results / Discussion

4.1 Results

4.1.1 MOLECULAR IDENTIFICATION

The given organism was isolated and identified the at molecular level using 16s rDNA technology as microalgae *Scenedesmus obliquus*

Forward:

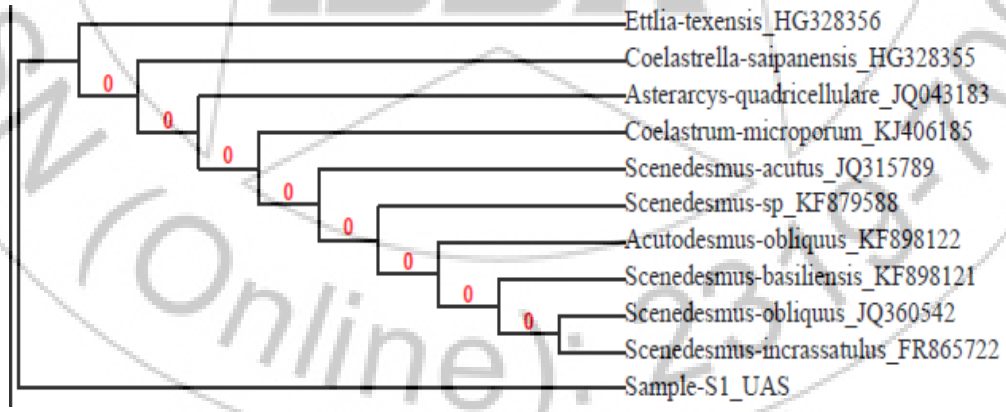
TGATTCATATATTGGGTCCGTCGGGGGCTTCGTATT
TCCATTGTCAGTAGGTGAATTCTTGGATTTATGAAA
GTACGAACTACTGCGAAAGCATTTGCCAAGGATGTT
TTCATTAATCAAGAACGAAAGTTGGGGGCTCGAAG
TCGATTAGATACCGTCGTAGTCTCAACCATAAACGA
TGCCGACTAGGGATTGGCGAATGTTTTTTTAATGAC
TTCGCCAGCACCTTATGAGAAATCAAAGTTTTTGGG
TTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTA
AAGGAATTGACGGAAGGGCACCACCAGGCGTGGAG
CCTGCGGCTTAATTTGACTCAACACGGGAAAACTTA
CCAGGTCCAGACATAGTGAGGATTGACAGATTGAG
AGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGC
CGTTCTTAGTTGGTGGGTTGCCTTGTCATTTGAGTAT
TTCCCGCGCAACCGGAATTTAAAGGCGGGGGGGGG
CGGGGCTTCCGTCTCATCTCTTATGGACTGTCTATAT
TTTCCGTGACTGGTCAATTTTGCGCGTGCTCACCCTT
GTGGCGCCGTCTCTCCTTCTGTAGCTTCCCCTGTTCA
CATACCCTTCCTTGCAATATCTTGACAAGATGGGGG
TGCAGGAAGGGAGTGAGATAACCTTTCTCATACAG
TTGCTCATCTGTTTCGGATCGAAAGTGATCGGATAT
TATTAAGACCGACTCACTCCTTTTTCGTTCAACTTAA
ATATTGAGTAAAGGCAAATACTCCTCGGAATGCTTT
TCATTCATACTTATGTCTGCTCCTCGGATTTATTTGC
GCAGCTCGCAGTGCATGGACCATTATCGGCCTTGGA
CACCGAAATG

Aligned Sequence: (628 bases)

TGATTCATATATTGGGTCCGTCGGGGGCTT
CGTATTTCCATTGTCAGTAGGTGAATTCTT
GGATTTATGAAAGTACGAACTACTGCGAA
AGCATTTGCCAAGGATGTTTTCATTAATCA
AGAACGAAAGTTGGGGGCTCGAAGTCGAT
TAGATACCGTCGTAGTCTCAACCATAAACG
ATGCCGACTAGGGATTGGCGAATGTTTTTT
TAATGACTTCGCCAGCACCTTATGAGAAAT
CAAAGTTTTTGGGTTCCGGGGGGAGTATGG
TCGCAAGGCTGAAACTTAAAGGAATTGAC
GGAAGGGCACCACCAGGCGTGGAGCCTGC
GGCTTAATTTGACTCAACACGGGAAAACTT
ACCAGGTCCAGACATAGTGAGGATTGACA
GATTGAGAGCTCTTTCTTGATTCTATGGGT
GGTGGTGCATGGCCGTTCTTAGTTGGTGGG
TTGCCTTGTCA

The above sequence is showing maximum similarity with *Scenedesmus* sp.

The cladogram was obtained for the sp. As given below:



The given organism was isolated and identified at the molecular level using 18s rDNA technology as cyanobacteria *Synechococcus elongatus*

Forward:

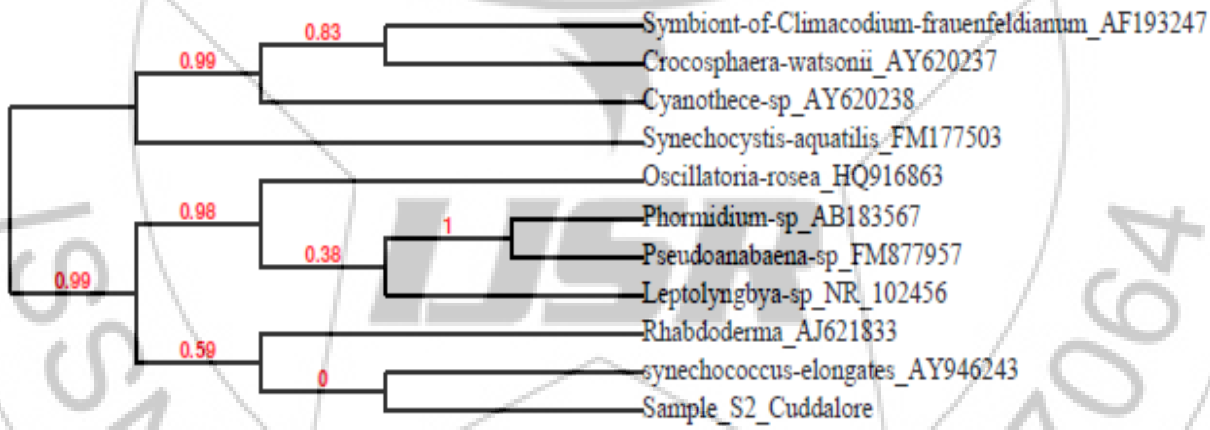
CAAATTTGGATCGGGGTACAAGTTGGAAA
CGAAGTGCTAATAACCCGATATGCCGAGA
GGTGAAATCTTTTTTGGCCTGAAGAGGAGC
TCGCGTCTGATTAGCTAGTTGGTGGTGTAA
GGGACCACCAAGGCGACGATCAGTAGCTG
GTCTGAGAGGAGGAGCAGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATTTTCCGCAATGGGC
GAAAGCCTGACGGAGCAATACCGCGTGAG
GGAGGAAGGTCTTTGGATTGTAAACCTCTT
TTCTCAGGGAAGAATATTGACGGTACCTGA
GGAATAAGCACCGGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCAAGCG

TTATCCGGAATTATTGGGCGTAAAGAGTCC
GTAGGTGGTCATGCAAGTCTGCTGTCAAAG
CCCACAGCTTAACTGTGGATCGGCGGTGG
AAACTGTGTGACTTGAGTACGGTAGGGGT
AGAGGGAATTCCCAGTGTAGCGGTGAAAT
GCGTAGATATTGGGAAGAACACCGGTGGC
GAAAGCGCTCTACTGGACCGTAACTGACA
CTGAGGGACGAAAGCTAAGGTAGCGAAAG
GGATTAGATACCCCCTTTTATTTCACAGGG
TCTCGCTCCGTCTCCCACCCGGATTTAAGA
GGTGCTAGTTAGTGTGTGTGCCTCTTATCA
GCAGATTTGCTGTGGACCCCCTAGCGACTC
TTTACGCCCACTATTCCAGATATCCTGCGC
CCCTCCGCATTTCACGCGTTGCGGCTCTGA
TGCAGCTGTGCTGATCTGCACGGACGTCTA
TAGTCTCCATGACAGATGAGCTTGACTCCA
ATGACTTACTCGTACGCTGCTATGATCGAC
AGACTAGCAGTCGTAAATACGATCGTGCG
TACGATAGGGATTCATGAAGAGTC

Aligned Sequence: (629 bases)

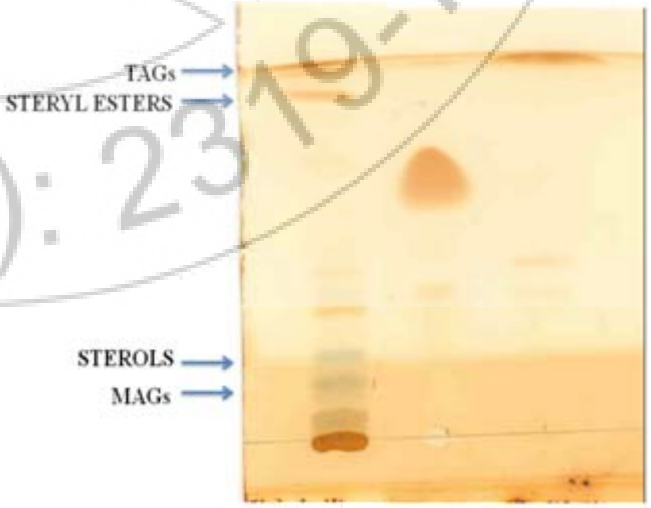
CAAATTTGGATCGGGGTACAAGTTGGAAA
CGAAGTGCTAATAACCCGATATGCCGAGA
GGTGAAATCTTTTTTGGCCTGAAGAGGAGC
TCGCGTCTGATTAGCTAGTTGGTGGTGTAA
GGGACCACCAAGGCGACGATCAGTAGCTG
GTCTGAGAGGAGGAGCAGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATTTTCCGCAATGGGC
GAAAGCCTGACGGAGCAATACCGCGTGAG
GGAGGAAGGTCTTTGGATTGTAAACCTCTT
TTCTCAGGGAAGAATATTGACGGTACCTGA
GGAATAAGCACCGGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCAAGCG
TTATCCGGAATTATTGGGCGTAAAGAGTCC
GTAGGTGGTCATGCAAGTCTGCTGTCAAAG
CCCACAGCTTAACTGTGGATCGGCGGTGG
AAACTGTGTGACTTGAGTACGGTAGGGGT
AGAGGGAATTCCCAGTGTAGCGGTGAAAT
GCGTAGATATTGGGAAGAACACCGGTGGC
GAAAGCGCTCTACTGGACCGTAACTGACA
CTGAGGGACGAAAGCTAAGGTAGCGAAAG
GGATTAGATACCCC

The above sequence is showing maximum similarity with *Synechococcus elongatus.*



4.1.2 TLC Results

The Thin Layer Chromatography was performed to qualitatively analyze the lipids extracted from the sample using respective protocols. After optimizing concentration of the standards, and the lipid extraction protocol, the lipids extracted from *Scenedesmus obliquus* and *Synechococcus elongatus* using the Bligh Dyer method were run on TLC. Clear band separation was observed. Bands corresponding to free fatty acids and TAGs were observed by using Linolenic acid (Dilution 2) and coconut oil (Dilution 2) as standards respectively.



Sample Preparation: Lipid extracted using Bligh Dyer method. Sample mixed with 45 µl of chloroform: methanol (2:1)
Lane 1: *Scenedesmus obliquus*
Lane 2: *Synechococcus elongatus*
Lane 3: Linolenic acid (10µl of dilution 1 in 90µl of methanol- dilution 2)
Lane 4: coconut oil (1µl in 90 µl of methanol)
Solvent system: Petroleum ether: Diethyl ether: glacial acetic acid (70:30:1)

4.2 Discussion

The organisms are best identified at the molecular level. The sample organisms were identified and their respective phylogenetic trees were obtained which showed the evolution taking time into consideration. The mid-point shows the divergence of the species with respect to evolution. The Bligh-Dyer method was optimized as the best lipid extracting method showing clear band separation of lipids. The quantitative analysis shows the presence of clear bands for free fatty acid and TAGs in *Scenedesmus obliquus* which is in line with the recent findings of Mallick et al (2012)[13].The TLC data clearly demonstrated the separation of the extracted lipids into different bands like Sterols, Mono acyl glycerides, Free Fatty acids, Steryl esters, Tri acyl glycerides. The neutral lipids that are useful for biodiesel production are Fatty acids and TAGs. TAGs are composed of Fatty acids. Palmitic acid (saturated) was present in maximum quantity among the different fatty acids present in *Scenedesmus* sp. But in the case of *Synechococcus* species very less free fatty acid content was observed in the TLC and Gas chromatography data which is not comparable with the findings of Atsumi etal (2013)[14]. Further medium optimzation experiments were required to understand the lipid profiling of *Synechococcus elongatus*.

5. Conclusion

Owing to the fast depleting fossil fuel and the pollution caused by it, interest in the alternate sources of fuel has grown enormously. Biodiesel from secondary generation fuel sources like biomass has been proved to be more efficient and advantageous on many levels. However, identifying the right oleaginous species is a very step which decides the success of the fuel production and yield. Identifying the right source which can be used as feedstock for biodiesel production will bring us one step closer to making large scale production practically feasible. It also will reduce unnecessary wastage of monetary and time investment.

Comparative study of two or more species will enable narrowing down the organism which can be more suitable as feedstock. Different species have different lipid metabolism. The lipid metabolism is modified according to the environmental conditions and the related changes. Growing different species under the same conditions and using the same protocol for lipid extraction resulted in different bands in the TLC run. Free fatty acids and TAGs are used for biodiesel. The presence of clear bands corresponding to Free fatty acids and TAGs refers to better source, the quantity being proportional to the yield.

From the TLC and GC data, fatty acid content in *Scenedesmus obliquus* was found to be more than in *Synechococcuselongatus*under the given lab conditions. Palmitic acid (saturated) was present in maximum quantity among the different fatty acids present in Scenedesmus. Also presence of higher amount of saturated fatty acids is an advantage since they are better for biodiesel production than unsaturated fatty acids due to their lesser nitrogen emission and higher thermal efficiency. Since fatty acid content is directly proportional to the Biodiesel yield, *Scenedesmusobliquus* a more suitable organism for Biodiesel production compared to *Synechococcuselongatus* given the experimental conditions are similar.

Further study and manipulation of this species will open new possibilities for low cost large scale production in the future.

6. Future Scope

Micro Algae are very sensitive to changes in the environment they grow in and the lipid metabolism is in turn dependent on it. Hence, optimizing the growth conditions for better lipid accumulation will help in better yield of biodiesel. Genetic modification to increase the expression of the gene responsible for production of DAG acyl transferase enzyme which is responsible for the conversion of DAG to TAG. Perform trans-esterification of the extracted lipids (free fatty acids) to convert it into methyl esters (biodiesel). Performance test to check the Engine efficiency of the biodiesel by mixing in varying ratios with diesel. Large scale production of bio-diesel to meet the ever increasing fuel demand. Cost cutting strategies should be developed for each step of production. Lipidomic analysis of the organisms with a similar sequence to the identified species which could also be used for bio diesel production.

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References

[1] Yusuf Chisti, Biodiesel from microalgae, Biotechnology Advances, Vol 25, May–June 2007, pp 294–306.
[2] Mary Mennes Allen,Simple conditions for growth of unicellular blue-green algae on plates, Journal of Phycology, Vol 25,Jan 2004, pp: 1–4.
[3] Nichols, H. W. (1973) Growth media – freshwater. In Stein, J. (Ed.) Handbook of Phycological Methods, Culture Methods and Growth Measurements, Camb. Univ. Press, Vol 24, Nov 2004 pp: 7-24.
[4] J. B. Waterbury, J. M. Willey,Isolation and growth of marine planktonic cyanobacteria, Methods in Enzymology, Vol167,June 1988 pp: 100–105.
[5] Markham, J.W., Hagmeier, E, Observatons on the effects of germanium dioxide on the growth of macro-algae and diatoms. Phycologia, Vol 21, Jan 1982 , pp:125-130.
[6] Nathaniel C. Cady, Scott Stelick,Carl A. Batt, Nucleic acid purification using microfabricated silicon

structures, Biosensors and Bioelectronics,Vol 19,Oct 2003, pp 59–66.
[7] Weisburg WG, Barns SM, Pelletier DA, Lane DJ (January 1991). "16S ribosomal DNA amplification for phylogenetic study". J Bacteriol, Vol173, July 2003 ,pp: 697–703.
[8] Scott mcginnis, Thomas L. Madden, BLAST: at the core of a powerful and diverse set of sequence analysis tools, Nucleic Acids Research, Vol. 32, April 2004,pp 203-210.
[9] Folch et al., A simple method for the isolation and purification of total lipids from animal tissues. J BiolChem, Vol 226, June 1957, 226, 497.
[10] Bligh.E.G., Dyer.W.J., A rapid method for total lipid extraction and purification. Can.J.Biochem.Physiol, Vol 37, April 1959, pp:911- 917.
[11] Ghosh A.K , N.Chauhan, S. Rajakumari, G. Daum, and R. Rajasekharan, At4g24160, aSolubleAcyl-CoenzymeA Dependent Lysophosphatidic Acid Acyltransferase, Plant Physiol, Vol 151, Oct 2009,pp: 869–881.
[12] Feng D, Chena Z, Xuea S, Zhang W. Increased lipid production of the marine oleaginous microalgae Isochrysis Zhangiangensis (Chrsophyta) by nitrogen supplement. Bioresource Technology 2011; 102:6710-6716.
[13] S. Mandal, S. Mallick, Biodiesel Production by the Green Microalga Scenedesmusobliquus in a Recirculatory Aquaculture System Appl Environ Microbiol, Vol 78, Aug 2012, pp: 5929–5934
[14] McEwen JT1, Machado IM,ConnorMR,Atsumi S, Engineering Synechococcuselongatus PCC 7942 for continuous growth under diurnal conditions, Appl Environ Microbiol, Vol 78, Aug 2012, pp:1668-1675.

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