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Research Article

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Transcriptome analysis of *Botryococcus braunii* Race A and Race B to identify the enzymes involved in biodiesel biosynthesis

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ABSTRACT

Biodiesel has turned into the need of the hour with the massively expanding transportation sector. With the shrinking scope of fossil fuel reliance newer options such as green plants were targeted as the alternative source. The first and second generation biofuel resources comprised the edible and non-edible plants. Since these plants were competing with the food and land availability the third generation biofuel source was selected meticulously to overcome these disadvantages. Thus algae were the ultimate choice as its growth requirements were limited to marine or freshwater, least fertile terrain and sunlight. Several algal species, including both macroalgae and microalgae are studied extensively for their hydrocarbon producing capacity. Sargassum, Chlorella vulgaris, Botryococcus braunii, Chlamydomonas and Desmococcus olivaceous are some of the algal species which are targeted for various reasons. Botryococcus braunii is a green, microalgae known for its high hydrocarbon content. In this study, we have attempted to analyze the transcriptome data of two races of B braunii. The transcriptome data was subject to pre-processing as it contains some low quality reads. After the quality check the obtained high quality reads were assembled using Trinity transcriptome assembler. The short reads were assembled into contigs. These contigs were further annotated using BlastX. The reads with the homologous hits were analyzed for the presence of enzymes involved in the hydrocarbon biosynthesis. Hence, in this study, we were able to map the transcripts corresponding to the enzymes involved in various pathways.

Keywords: Algae, Biodiesl, *Botryococcus braunii*, NGS, Transcriptome.

INTRODUCTION

Locomotion is vital for human survival, hence fuel consumption in the transport sector ranges up to 80% of total fuel usage. The demographic growth and fuel accessibility are continually being contrarily corresponding. In recent times fuel has become the symbol of economic threat as India imports 90% of crude oil from oil producing countries [1]. To handle this disparity the quest for alternative fuel resources have coordinated towards the green assets, the plants and green growth. The initial two generations of biodiesel sources were the edible and non-edible plants [2]. The sources were rivaling nourishment yields and land accessibility. Presently, the third generation biofuel resources are centered towards the microalgal biomass [3]. Green growth can develop effortlessly in less fertile terrains with the accessibility to daylight and water. This allowed researchers to characterize the algae and optimize methods for their growth.

Algae with a total of 139,704 species as reported in Algabase [url http://www.algaebase.org/] has been classified into macroalgae and microalgae. According to 2012 reports, researchers across the globe have started investigating

the following algal species for their role as a mass oil-producer, *Botryococcus braunii,Chlorella,Dunaliella tertiolecta,Gracilaria,Pleurochrysis carterae,* and *Sargassum.*

Microalgae can be grown in fresh, saline or marine water utilizing solar energy and CO_2 from the atmosphere. These simple needs of microalgae make it an option for biofuel. Several algal species have proven to produce hydrocarbon but the genetic information available about the species is restricted. The genomes of only a very few algae, including *Chlamydomonas reinhardtii, Chlorella variabilis, Volvox carteri* has been sequenced and annotated. Microalgae has the potential to produce 1,36,900 liters of oil per acre in contrast to *Jatropha* which can produce only 1,892 liters [1].

Botryococcus braunii is one such green microalga which is potential in hydrocarbon production. *B braunii* is a microscopic colonial alga belonging to the Chlorophyta division. Each single cell ranges in size from 7μm-11μm. The colony is embedded in a polymeric hydrocarbon matrix. *B braunii* is classified into three races A, B & L based on their hydrocarbon composition. Race A produces alkadienes and alkatrienes [4], race B produces mainly triterpenoids known as botryococcenes and methylated squalenes [5-6], and race L produces a tetraterpenoid known as lycopadiene [7]. Among the three races, A and B are known to produce higher hydrocarbon content. Lipid accumulation in the extracellular space is the unique feature of the algae as compared to other oleaginous microalgae, which stores lipids in the cytoplasm [8]. The majority of the hydrocarbon which is secreted outside the cell binds the colony of cells, which enables milking out the hydrocarbon content [9]. Non destructive oil extraction techniques including heptane treatement for chemical method and blotting i.e. applying gentle pressure for physical method [10] are suggested as efficient methods for extracellular oil extraction.

Race A produce alkadienes and alkatrienes which are straight, odd-number C_{27}, C_{29}, and C_{31} hydrocarbon chains. These are formed by the elongation of oleic acid (C18) followed by the loss of carbonyl carbon. Hydrocarbon is produced during the exponential and linear phases of cell growth. Lipid bodies aid in the accumulation of extracellular lipid. Lipid accumulation is found maximum just after the septum formation, this is regulated via the trans-Golgi networks, located near the apex [11].

Race B is known to produce triterpenes (C30-C37), in the form of methylated, oxidized, and cyclized botryococcenes, as well as methylated squalenes, intracellularly and transported to the extracellular matrix [7]. The oil produced is described as bio-crude as it is considered as petroleum replacement [9]. Botryococcene is the major triterpene found in the extracellular matrix [12]. Other terpenoids such as squalene derivative tetramethylsqualene [13] its epoxide [14], botryoxanthins [15] and braunii xanthins[16] as minor metabolites in the extracellular matrix. C_{30} botryococcene is the precursor of all botryococcenes which undergoes methylation with S-adenosylmethionine to produce other homologs upto C_{34} [17-19].

The transcriptome sequencing of *B braunii* strains BOT-88-2(Race A) and BOT-22(Race B) has been performed by the National Institute for Environmental Studies, Japan and the sequences are deposited in the DDBJ Read Archive. Since the genome has not been sequenced functional annotation was carried out using the transcriptome sequence. The annotation performed by M. Ioki *et al*. has revealed the presence of a few enzymes involved in hydrocarbon biosynthesis. But the studies have left some essential enzymes unidentified. The present study has taken efforts to predict the presence of some enzymes found in the fatty acid biosynthesis pathway of Race A and reductive pentose phosphate pathway of Race B with the aid of bioinformatics and statistical tools.

EXPERIMENTAL SECTION

- **NGS data collection**

The transcriptome data of *Botryococcus braunii* has been deposited in the DDBJ website and made publicly accessed through DRA Search option. The transcriptome was sequenced using pyrosequencing technique in the 454 GS FLX sequencer. The strain BOT-88-2 corresponding to Race A with 185,936 cDNA reads was retrieved from the accession DRR000585 and strain BOT-22 of Race B with 209,429 cDNA reads was retrieved from the accession DRR000584 from DDBJ-DRA [20] (http://trace.ddbj.nig.ac.jp/DRASearch/).

- **NGS data analysis:**

The sequence data was run through the DDBJ Read Annotation Pipeline, [21] which consists of the set of steps including pre-processing, mapping/*de novo* Assembly, Genome SNP and RNA seq analysis. The data was imported

using the option Import Public DRA. Upon data uploading the pre-processing was performed. The preprocessed data was subject to *de novo* assembly since the reference genome for the algae is not available. The assembly was done using Trinity tool.

- **Transcriptome Functional annotation:**

The trinity assembled contigs were subject to functional annotation. The data were run through BlastX algorithm, the sequences with the best hits were searched against the enzyme database PRIAM [22].

- **Phylogenetic analysis**

The phylogenetic tree was constructed using MEGA6 [25]. The tree was constructed to predict the evolutionary and functional relationship between the contigs corresponding to the enzymes in *B braunii* with that of the orthologous sequences from other algal species.

RESULTS

The two transcriptome sequences of *B braunii* were retrieved from the DDBJ-DRA database. Once the transcriptome data is downloaded the first step is to perform the quality check of the data.

Primary analysis:

The quality check of the data is performed in the pre-processing step.

The below mentioned parameters were set in the pre-processing step:

- The encoding type of the quality values for sequence was set to Phred+33.
- Base Trimming option was used to trim the low quality bases from 5'end and 3'end of each read.
- Minimum read length was set to 25bp.
- Quality threshold was set to 19.

Secondary analysis:

The trimmed reads were assembled into contigs using the Trinity assembler.

Quality statistics:

Table 1: **The assembly parameters of Race A and Race B of *B braunii* transcriptome**

Parameters	Race A	Race B
No: of reads	185,936	209,429
No: of contigs	15540	19592
Minimum sequence length	201	201
Maximum sequence length	3275	2674
Average sequence length	482.50	436.11
Median sequence length	391.00	360.00
N50 Length	559	498

Tertiary analysis:

The assembled contigs were run against the nr database for identifying the best hits. The contigs with the best hits were subjected to similarity search against the PRIAM database to map the contigs coding for the essential enzymes. M. Ioki *et al*. 2012 [23-24] performed the transcriptome sequencing and analysis of the *B braunii* Race A strain BOT-88-2 and Race B strain BOT-22. The annotation predicted the presence of a few essential enzymes involved in the targeted hydrocarbon biosynthesis pathway.

Race A

Table 2: List of newly predicted enzymes in Race A

Enzyme name	EC number	PRIAM ID	Pathway
Aldehyde dehydrogenase (NAD+)	1.2.1.3	PRI003762	Very Long Chain Fatty acid Synthesis
Enoyl-CoA hydratase 2	4.2.1.119	PRI014066	Very Long Chain Fatty acid Synthesis
Beta-ketoacyl-acyl-carrier-protein synthase II	2.3.1.179	PRI002169	Fatty acid elongation

Race B

Table 3: List of newly predicted enzymes in Race B

Enzyme name	EC number	PRIAM ID	Pathway
Glyceraldehyde-3-phosphate dehydrogenase (NAD(P)(+)) (phosphorylating)	1.2.1.59	PRI000978	Reductive pentose phosphate cycle in photosynthesis

The enzymes were predicted with the bit score of 50. The score was set in accordance with the M. Ioki *et al*. annotation.

Phylogenetic analysis

For the predicted enzymes phylogenetic tree was constructed to determine their relationship with the other known algal species.

Aldehyde dehydrogenase

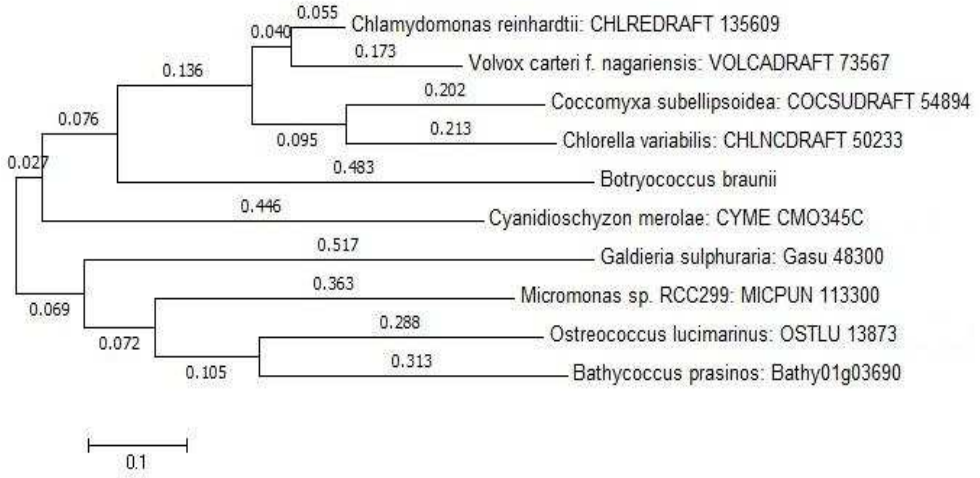

Figure1: Phylogenetic tree predicting the relationship of aldehyde dehydrogenase transcript of *B braunii* with other green and red microalgae

Beta-ketoacyl-acyl-carrier-protein synthase II

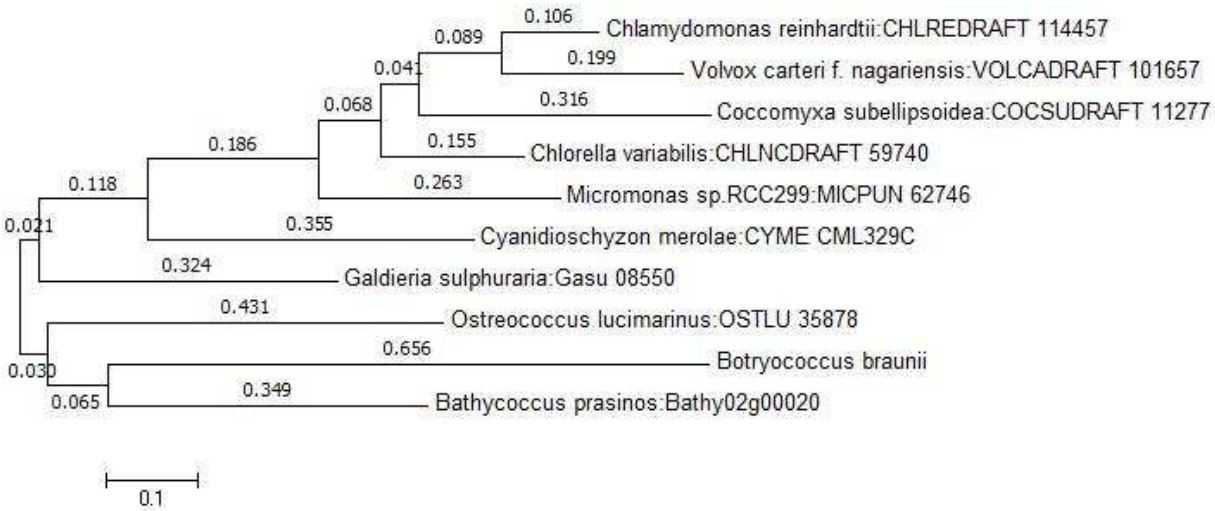

Figure2: Phylogenetic tree predicting the relationship of Beta-ketoacyl-acyl-carrier-protein synthase II transcript of *B braunii* with other green and red microalgae

From Figure 1 it can be inferred that the aldehyde dehydrogenase transcript of *B braunii* is closely related to that of *Chlamydomonas reinhardtii*, *Volvox carteri f.nagariensis*, *Coccomyxa subellipsoidea*, *Chlorella varaibilis*. Similarly, Figure 2 shows the close association of the predicted Beta-ketoacyl-acyl-carrier-protein of *B braunii* with that of *Bathycoccus prasinos*. The enzymes Enoyl-CoA hydratase 2 and Glyceraldehyde-3-phosphate dehydrogenase (NAD(P)(+)) (phosphorylating) had no significant orthologs from the other algal species.

DISCUSSION

The transcriptome sequence of *Botryococcus braunii*, race A strain, BOT-88-2 and race B strain BOT-22, is sequenced and deposited by M. Ioki *et al*. 2012 at DDBJ DRA. Race A which produces alkadienes and alkatrienes through the following pathways; fatty acid elongation in the form of acyl-ACP, fatty acid elongation in the form of acyl-CoA, fatty acid desaturation, and very long-chain fatty acid synthesis [23]. Similarly, botryococcenes and squalenes are synthesized via the reductive pentose phosphate cycle in photosynthesis, pyruvate synthesis, acetyl-CoA synthesis, mevalonate-independent pathway, and triterpene synthesis pathway [24] of *B braunii* race B. The first annotation reported a list of enzymes potentially associated with the biosynthesis of hydrocarbon. The enzymes including Aldehyde dehydrogenase (NAD+), Enoyl-CoA hydratase2, Beta-ketoacyl-acyl-carrier-protein synthase II in Race A and Glyceraldehyde-3-phosphate dehydrogenase (NAD(P)(+)) (phosphorylating) in Race B were left unpredicted in the annotation. The current study has predicted the presence of the contigs corresponding to the enzymes and also investigated the evolutionary relationship with other algal species.

High cost and energy required to cultivate and harvest the microalgae makes it unsuitable for the large scale production of biodiesel [10]. One of the biggest bottlenecks of the algae is marked by its slow growth rate. Addressing these bottlenecks using bioinformatics approaches shall improve the hydrocarbon yield. Investigating the genetic makeup and identifying the genes involved in hydrocarbon biosynthesis shall invite the usage of genetic engineering techniques to augment biodiesel production form microalgae [26].

CONCLUSION

B braunii is extensively studied for the high extracellular lipid accumulation that is directly linked to the hydrocarbon biosynthesis. Analyzing the genetic makeup without the availability of the genome is challenging. In the recent times the transcriptome data are being used for extracting information such as expression profile and signaling pathways of the organism. Functional annotation of the transcriptome has been effective in identifying the presence of the essential enzymes involved in the hydrocarbon biosynthesis. There are essentially several gaps as a few enzymes were left unpredicted. Further studies have to be performed to fill the gaps so as to proceed with the pathway reconstruction and flux balance analysis. These comprehensive analyses shall enhance the insight into the *B braunii* oil producing mechanism.

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