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Comparative transcriptomics reveals molecular components associated with differential lipid accumulation between microalgal sp., *Scenedesmus dimorphus* and *Scenedesmus quadricauda*

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ABSTRACT

Scenedesmus is considered as a potential oil-producing green microalgae having higher lipid content with suitable fatty acid profile (high oleic acid) for biodiesel production. Comparative transcriptome analysis of two *Scenedesmus* sp., viz. *Scenedesmus dimorphus* (26%) and *Scenedesmus quadricauda* (14%) having equivalent biomass and variable lipid content was performed to uncover molecular mechanisms controlling differential lipid production. 76,969 and 40,979 CDSs were predicted from the transcriptomes of *S. dimorphus* and *S. quadricauda* respectively, which were subsequently mapped to metabolic pathways. Overall up-regulation of metabolic pathways contributing precursors to storage lipid biosynthesis was observed in *S. dimorphus*. Glyceraldehyde 3-phosphate dehydrogenase, enolase, acetyl-CoA synthetase, pyruvate dehydrogenase, ATP citrate lyase, glycerol kinase, citrate synthase were identified as major regulators of high lipid content in *S. dimorphus*. Further, WRINKLED1 transcription factor significantly correlated with high lipid accumulation as revealed by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) in three growth stages of two species. This study provides a broad view of storage lipid production in *Scenedesmus* species with potential implications in designing suitable genetic interventions towards increase in lipid content vis-à-vis central carbon metabolism.

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1. Introduction

Biomass from different feedstocks is considered a major component for the production of biofuels such as biodiesel, bioethanol, biohydrogen, bio-oil, and biogas [1]. Each feedstock has its own advantages in terms of lipid content, biomass yield, fatty acid composition, and geographical distribution [2]. Microalgal feedstock has recently emerged as the only renewable approach to produce biofuels as it offers various advantages over other conventional sources for biofuel production, such as rapid growth rate, high lipid content and no competition with other agricultural crops for land [3]. Microalgal species with high biomass productivities and robust lipid yield are preferred for the production of biodiesel [4]. However, only few microalgal strains are available, which have the combination of traits of high biomass productivity with considerable lipid content [5]. Lipid content of microalgae is species/strain dependent as different strains and species have shown variation for oil content and fatty acid composition. Usually, microalgae synthesize low lipids under favourable conditions where lipid content gets increased up to several folds under stress conditions [2]. There are various reports in which molecular mechanisms behind the increase of lipid content under stress conditions were examined. However, the mechanism behind interspecies variation among microalgae is unknown. As storage lipid biosynthesis is regulated by multiple pathways, transcriptomic analysis aids in deciphering the regulatory mechanisms leading to high lipid production in microalgae [6]. Transcriptomic analysis is a powerful approach for the molecular dissection of phenotypic traits in contrasting conditions [7]. A number of transcriptomes have been sequenced and annotated for the model as well as oleaginous microalgae, which provided the broad view of different metabolic pathways involved in biofuel production from microalgae. However, comparative transcriptome analysis is required to gain preliminary information about the differentially expressed pathways and genes, that can be targeted for metabolic engineering of microalgae to have desired strains with enhanced lipid production [8].

Transcriptome of oleaginous microalga *Neochloris oleoabundans* was assembled where quantitative gene expression analysis under nitrogen replete and nitrogen limiting conditions demonstrated the up-regulation of pathways leading to storage lipid accumulation, such as fatty

Abbreviations: CDS, Coding Sequence; GO, Gene Ontology; KO, KAAS Orthology; KAAS, KEGG Automatic Annotation Server; KEGG, Kyoto Encyclopedia of Genes and Genomes; RSEM, RNA-Seq by Expectation Maximization; TPM, Transcript Per Million; FPKM, Fragments Per Kilobase per Million; SSR, Simple Sequence Repeat; MISA, Microsatellite Searching Tool; NCBI, National Centre of Biotechnology Information; EXP, Exponential Phase; ESP, Early Stationary Phase; LSP, Late Stationary Phase; TAG, Triacylglycerol; TF, Transcription Factor; RNA-seq, Ribose Nucleic Acid-sequencing; RT-qPCR, Reverse transcriptase-quantitative polymerase chain reaction; ATP, Adenosine Tri Phosphate.

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acid and TAG biosynthesis and the pentose phosphate pathway [6]. Also, differentially expressed genes under nitrogen depletion conditions were examined in the model organism *Chlamydomonas reinhardtii* using 454 and Illumina next-generation sequencing techniques which revealed repressed β-oxidation pathway and up-regulated TAG biosynthesis [9]. In another report, transcriptomes of *Dunaliella tertiolecta* under nitrogen repletion, nitrogen depletion, and high salinity conditions were analysed for identification of genes involved in starch and lipid biosynthesis [10]. Also, in model green microalga *Chlamydomonas reinhardtii*, time-course transcriptome sequencing was carried out during lipid biosynthesis and accumulation processes using Illumina RNA-seq platform, which subsequently revealed higher expression of triacylglycerol biosynthetic genes in lipid accumulating phase [11]. Further, differential transcriptome analysis of *Chlorella protothecoides* in contrasting growth conditions of nitrogen repletion and depletion showed up-regulation of genes in the lipid accumulating condition of nitrogen depletion [12]. In a recent study, transcriptome sequencing of halophilic green alga *Dunaliella parva* was performed in nitrogen limited and nitrogen sufficient conditions to identify important pathways and genes for biofuel production [13]. All of the previous transcriptome based studies were focused on the changes in metabolic flux due to stress conditions or during lipid accumulating growth phases, but no report exists as of today on the comparative transcriptomic analysis between species having contrasting lipid contents. It is, therefore, essential to reveal the pathways associated with high lipid content in microalgae.

Scenedesmus is a genus of algae belonging to Chlorophyceae class, which has gained attention for its use in biodiesel production due to its high biomass productivity with high lipid content and desired fatty acid profile among green algae [14–16]. Various studies have reported the optimization of biomass productivity as well as lipid content by varying the concentration of supplemental nutrients. *Scenedesmus* sp. has shown highest biomass (4 mg/ml) and lipid content (49%) in comparison to *Botryococcus* and *Chlorella* [17]. Also the fatty acid composition of *Scenedesmus* was found to be suitable with high percentage of oleic acid, which is a prerequisite for biodiesel production [18].

Variation in the lipid content has been reported among different species of *Scenedesmus* [19]. Different isolates of two *Scenedesmus* species, viz. *Scenedesmus dimorphus* and *Scenedesmus quadricauda* were collected from various geographical locations of Himachal Pradesh, India [20]. Also, it has been reported that an increase in the lipid content under stress conditions in microalgae is due to the reduction of biomass of the cell, rather than enhanced lipid accumulation [21]. In the current study, two isolates of *Scenedesmus* species, viz. *S. dimorphus* (26% lipid content) and *S. quadricauda* (14% lipid content) with equivalent biomass yields were taken. Fatty acid profiling of these two species revealed higher proportion of oleic acid in *S. dimorphus* [22]. Transcriptomes of the two species at lipid accumulating phase were sequenced and assembled de novo and further comparative analysis of the gene ontology categories, pathways and genes was performed. The present study not only provides molecular basis for the inter-species variation of lipid content, but also presents novel insights into metabolism and transcriptional regulation leading to differential lipid accumulation, which provided the molecular cues to enhance and regulate lipid content in microalgae through metabolic engineering approaches.

2. Materials and methods

2.1. Culturing of Scenedesmus species

Cultures of *S. dimorphus* and *S. quadricauda* were maintained in the glass house of the Jaypee University of Information Technology, Waknaghat, H.P., India, under optimal conditions of temperature and light and grown in the BG11 media. Axenic cultures of both the species were obtained by treating with antibiotics: penicillin, streptomycin and chloramphenicol following the protocol by Guillard [23]. These cultures were incubated in 250 ml flasks containing 100 ml BG11 media, at a light intensity of 42 μmol photons m^{-2} s^{-1} with a diurnal cycle of 16 h light and 8 h dark at temperature of 25 ± 2 °C. Cultures were continuously aerated with 0.22 μm filtered air through a mechanical pump. Cultures were harvested after 20 days and stored at −80 °C for further use.

2.2. Estimation of lipid content and biomass in S. dimorphus and S. quadricauda

Lipid extraction from microalgal strains was carried out as described by Bligh and Dyer with minor modifications [24]. Equal amount of biomass was freeze-dried using lyophilizer and the lyophilized cells were suspended in chloroform: methanol solution (1:2) and vortexed immediately. The mixture was sonicated for 5 min and kept on a shaker overnight. Next day, equal amount of chloroform: distilled water (1:1) was added to the mixture, vortexed and then centrifuged at 6000 ×*g* for 10 min. Lipids are soluble in chloroform, hence form a dense layer at the bottom of the centrifuge tube, while methanol and water create a uniform top layer, and cell debris creates a middle layer. Lipid with chloroform was taken with the help of micropipette by applying gentle, positive pressure and passed through a 2.5 cm thick layer of anhydrous sodium sulfate using Whatman filter paper in a funnel into a preweighed container suitable for rotary evaporation. The solvent was removed using a rotary evaporator under reduced pressure at 60 °C and weight of remaining lipids was recorded. Total lipid content was calculated as percentage of the total biomass (in % dry weight). Fatty acid methyl esters (FAMEs) were estimated for the determination of triacylglycerol [22].

Microalgal cells of both the species were stained with the fluorescent dye, BODIPY 505/515 (Invitrogen) according to the protocol described by Cooper et al. [25]. Fluorescence analysis of the green BODIPY stained micoalgal cells was determined by fluorescence microscope (Olympus BX53).

2.3. RNA extraction

Total RNA was isolated using RaFlex RNA isolation kit (GeNei™) following manufacturer's instructions. The quality of RNA was checked in 1% (w/v) ethidium bromide-stained denaturing agarose gel at 100 V for 30 mins. Further, total RNA was quantified through the absorbance spectrum at wavelengths 260 nm and 280 nm using NanoDrop 8000 spectrophotometer (Thermo scientific).

2.4. Library preparation for transcriptome analysis

Paired-end cDNA sequencing libraries were prepared using IlluminaTruSeq stranded total RNA library preparation kit according to the protocol described by the manufacturer. rRNA was depleted from total RNA followed by fragmentation. The fragmented rRNA depleted RNA was converted into first-strand cDNA, followed by second-strand cDNA synthesis, A-tailing, adapter ligation and finally ended by index PCR amplification of adaptor-ligated library. Library quantification and validation was performed using Qubit dsDNA HS kit and High Sensitivity Assay Kit, respectively.

2.5. De novo transcriptome assembly of S. dimorphus and S. quadricauda

The raw data of two microalgal species were generated on NextSeq. The raw reads generated were filtered using Trimmomatic (v 0.30) with quality value QV > 20 and other contaminants, such as adapters were also trimmed. Parameters considered for filtration were adapter trimming, sliding window, leading and trailing. Minimum length was taken as 40 bp.

QC passed reads of the two species were subjected to de novo assembly with Trinity and final assembled transcripts were generated. CD-HIT-EST was run on the assembled transcripts to get unigenes.

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