IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Metabolic Engineering of Microalgae for Enhanced Lipid Production

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Abstract

Increasing awareness in recombinant protein technologies for human and animal health applications has focused on microalgae as a platform with the potential to meet a large impending demand. Here we describe a microalgae protein expression system and compare the advantages and disadvantages to other platforms currently operating on a commercial level. High-value recombinant proteins that have been produced in microalgae are presented, and strategies for developing production strains with improved commercial properties are discussed.

Keywords; Microalgae, Protein, Biodiesel, Biofuel and Triacylglycerol

1. Introduction

The ecological footprint and economic performance of the current suite of biofuel production methods make them insufficient to displace fossil fuels and reduce their impact on the inventory of Green House Gas (GHG) in the global atmosphere. Algae metabolic engineering forms the basis for 4th generation biofuel production which can meet this need. The first generation biofuels are known to be made from agricultural products such as corn or sugarcane. The second generation biofuels use all forms of (lingo) cellulosic biomass. The third and fourth generation of biofuel production involves ‘‘algae-to-biofuels’’ technology: the former is basically processing of algae biomass for biofuel production, while the latter is about metabolic engineering of algae for producing biofuels from oxygenic photosynthetic microorganisms (Historical , Kagan 2010, Sreenikethanam et al. 2022).

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms which can grow rapidly and live in diverse environments due to their unicellular or simple multi-cellular structure. Their size range varies from a few micrometers (μm) to a few hundreds of micrometers. Examples of some typical microalgae currently being looked at as feedstocks for biofuels are: *Nannochloropsis salina, Chlorella sp., Tetraselmis sueica, Chlorella vulgaris* and *Botryococcus braumii* based on high productivities and high neutral lipid content (Raja et al. 2008). Microalgae have very high surface productivity, and can be cultivated on non-arable land. Because microalgae are photosynthetic organisms, their use provides green house gas mitigation benefits. Depending on the species, the microalgae can grow on fresh, brackish, sea or even waste water and can accumulate up to 60% oil per dry weight under stress conditions (Chisti 2007). Therefore, microalgae have attracted increasing attention for their potential as producers of biodiesel and other lipid-based biofuels (Ho et al. 2014, Scott et al. 2010).

1.1. Lipid biochemistry in algae

Lipid biochemistry processes are very important in extracting fuel from microalgae. Identification and target to increase fatty acid content in microalgae different enzymes involves in rate limiting steps of pathways. Though study related to fatty acid content is a forward step but still much more clarity is required. Therefore, biochemistry of lipid droplets is a crucial factor to be studied for enhancement of biofuel production.

The Triacylglycerol (TAG) accumulation in microalgae is usually correlated to environmental stresses, such as high light intensity, high temperature, nitrogen limitation, and salinity (Haslam et al. 2013, Misra and Panda 2013). TAG is produced from diacylglycerol (DAG) in microalgae through two major pathways: the Kennedy Pathway involving transfer of acyl-CoA units onto DAG, catalyzed by diacylglycerol acyltransferase (DGAT), and an acyl-CoA-independent pathway, in which acyl groups are transferred from phospholipids, catalyzed by phospholipid: diacylglycerol acyltransferase (PDAT**)** (Yoon et al. 2012).

1.2. Metabolic engineering of lipid catabolism in microalgae

Lipid engineering in microalgae can be achieved by conventional, genetic engineering and metabolic engineering approaches. **Conventional Methods** includes nutrient deprivation, physical stress like temperature, salt stress and heavy metal stresses etc. which are thought to increase the activity of several enzymes. Among different types of stress especially nitrogen stress are being highly reported to trigger the TAG accumulation in different class of microalgae. Nitrogen, phosphorus stresses are being responsible for activating acyltransferase's and variation in phosphorus transporter system respectively, which again triggers TAG accumulation in microalgae (Dubey et al. 2015, Khozin-Goldberg and Cohen 2006). Though, temperature will vary depending on microalgae (Tamiya 1957) but normally it has an optimal growth rate at 15– 26°C (Qiang et al. 1998). Thus, in the day time higher photosynthetic activity results in high growth rate and vice-versa in night. Similarly in case of pH, some can resist high pH owing to their higher adaptability. Higher CO_2 means higher biomass but this will also decrease the pH (Kumar et al. 2010). The actual reason for increasing lipid in other stresses like pH, heavy metal is still unknown. Besides the reporting of high cell density culture, some recent bio polymeric harvesting approach has also been reported (Banerjee et al. 2013, 2014). Stresses can become the constructive strategy for increasing the lipid droplets due to the inherent advantages like ease in handling method, requirement of no skilled labor. On other side it also lowers down photosynthetic activity resulting in lower growth rate (Li et al. 2008). Metabolic engineering strategy is defined as tuning of metabolic pathways in a cell to trigger the target metabolite production. Achieving such targets various strategies can be adopted which are listed below:

- Flux balance analysis
- Improving photosynthetic efficiency (Increasing light penetration/ decreasing cell shading)
- Engineering different enzymes toward lipid biogenesis
- Identifying rate limiting enzymes/committed step
- Carbon partitioning/capture
- Mathematical modeling
- Over expression of a gene/ multiple gene
- Transcription factor engineering

The following are the major favorable points toward production of lipid droplets in microalgae. Lipid biogenesis is governed by three steps namely Acetyl CoA carboxylation, Chain elongation followed by TAG formation. Furthermore, synthetic biology aspect requires preliminary information about the microalgae. Whole genome sequencing of model as well non model microalgae is required in order to reconstruct the metabolism. The reconstruction of metabolic fluxes using stoichiometric model i.e., S.v = 0; Where v is a vector of fluxes and S is matrix, and matrix is constructed by balancing the masses in each of the cell compartment of *Chlamydomonas reinhardtii* (Boyle and Morgan 2009). Kyoto Encyclopedia of Genes and Genomes (Ogata et al. 1999) and MetaCyc **(Caspi et al. 2006)** are the major key resource to trace the metabolic pathways. Gene expression data set or differentially expressed genes can also be put into the picture to draw metabolic construction using path Express (Goffard and Weiller 2007). Recently, fluxome study of *Pseudomonas fluorescens* (Lien et al. 2015)

regarding fructose metabolism in EMP, EDP, PPP, TCA cycle has also been performed. Nutrient limitation is a key player to increase lipid droplets and is widely reported. It is one of the expensive and easy scheme where redirecting of metabolic flow occurs toward lipid (TAG) formation. In this facet the major disadvantage are slow growth rate and low photosynthetic activity. Since lipid productivity is directly proportional to cell number therefore two stage cultivation approaches may be employed to circumvent the above stated problem but photosynthetic one still remains. Metabolic flux analysis using GC-MS and LC-MS/MS under photoautotrophic growth in *Synechocystis* sp. PCC6803 has also been depicted to locate the carbon distribution using INST-MFA algorithms with high accuracy (Roesler et al. 1997, Young et al. 2011). Cytosolic ACCase was transferred to *Brassica napus* from *Arabidopsis* in order to increase the fatty acid content **(Gu et al. 2011)**. Nevertheless, after transformation the fatty acid content increases to 6% which led to identification of some other limiting steps. ACCase is present as a multi domain enzyme in most eukaryote and the heteromeric four different subunit from *Jatropha curcas* was characterized using 5' RACE technique and was found maintain the conserved domain. A strain of *Escherichia coli* that yields anteiso-branched fatty acids to decrease the freezing point and escalate the fluidity (Haushalter et al. 2014). Analysis by qPCR was also done to evaluate the differential gene expression pattern which is directed toward pyruvate and acetyl-coA synthesis under nitrogen depriving condition (Li et al. 2008). Similarly, metabolic engineering for fatty acid synthase is also a challenging target due to its multi subunit structure and have a multipoint controls. Current progress in whole genome sequencing and its annotation will definitely pave the way toward lipid biogenesis. Recent genetic tools like multi gene approach, transcription factor like CRISPR/TALEN, reverse genetics are well reported. Manipulating genetic code will show a manipulation in metabolic pathway and its flux toward the target / desired compound. Though knockdown technology (RNAi), genome editing through modern tools have been described and is established in *Chlamydomonas reinhardtii* (Kim and Cerutti 2009) and *Dunaliella salina* (Jia et al. 2009). But still we are unable to establish a base line system where every microalgae can be manipulated. Recently, robust and nuclear expression of xylanase1 in *C. reinhardtii* with viral 2A peptide has been achieved. This technology involves less number of transformation steps. High quality transcriptomic reads to the tune of 45% were assembled and identified in case of *Dunaliella tertiolecta* for ascertaining lipid genesis and carbohydrate metabolism network (Rismani-Yazdi et al. 2011). Knock down gene expression by two micro RNAs in *C. reinhardtii* for *RBCS1*/2 and *MAA7* gene was also reported (Zhao et al. 2009). Similarly, over expression of CrDGTT4 (type-2 diacylglycerol acyl-CoA acyltransferase) from *C. reinhardtii* with SQD2 (sulfoquinovosyl diacylglycerol synthase 2) as a promoter will also increase TAG accumulation under phosphorus starvation (Iwai et al. 2015). ***In silico* metabolic engineering** another approach of metabolic engineering could be generated by designing the large-scale models which use various *in silico* tools to decipher the role of different metabolites, genes, transcripts and crucial enzymes responsible for metabolic fluxes (Patil et al. 2004, Wu et al. 2016)**.** There are enough reports which establish the role of different computational techniques which prove to be significant for understanding key components of lipid regulation and can be very crucial for researchers working in the area of biofuel. However various other reports also summarizes various metabolic network modeling and flux balance analysis which plays a vital role while designing some novel pathways or establishing an idea about enhanced recovery of lipids from microalgae (Schuhmann et al. 2012). As a whole, the availability of metabolic models and *in silico* tactics on identifying key residues of lipid metabolism can be the role establishing characteristics and give quite sufficient information. Additionally, the improvement of the available models on transcriptomics, proteomics and metabolomics based data will facilitate to obtain key components toward good quality biofuel. Certainly, such information generated through *in silico* metabolic engineering on microalgal lipid metabolism has to be appraised by wet lab experiments.

1.3. Research Gaps

Algal feedstock is the basis of the emerging algal biofuel industry. However, few algae found in nature have demonstrated the combination of high biomass buildup rate, robust oil yield and tolerance to environmental stresses. All complex traits that a large-scale, economically competitive manufacture scheme demands. Therefore, untangling the intricate sub-cellular networks underlying these complex traits, in one or a series of carefully chosen algal research models. These have become an imperative research mission, which can take benefit of the emerging model oleaginous microalgae that have already demonstrated small, simple and tackle able genomes and the potential for large-scale open-pond cultivation. The revolutions in whole-genome-based technologies, together with systems biology, metabolic engineering and synthetic biology approaches, would

enable the rational design and engineering of algal feedstock and help to fill the gaps between the technical and economical reality and the huge potential of algal biofuels (Gomaa et al. 2016, Lü et al. 2011, Niu Y. F. et al. 2016). In the previous report, authors successfully developed transgenic *P. tricornutum* with increased lipid accumulation by over-expressing type 2 diacylglycerol acyl transferase (DGAT) (Niu Ying-Fang et al. 2013). Similarly, over-expression of DGAT in *P. tricornutum* resulted in increased proportion of polyunsaturated fatty acids (Cui et al. 2013, Gong et al. 2013).

Methodology

The proposed research are focused on: (i) key factors for sample collection, isolation, and identification to obtain a pure microalgal species, (ii) Cloned rate limiting enzymes of lipid biosynthesis from the isolated microalgal species and characterized its biological functions. This study will enable the use of genetically improved microalgae strains for industrial biofuels production. To accomplish research, the following sub-objectives are being selected and are as follows:

3.1 Isolation of microalgal species

Microalgae had been isolated from aquatic bodies (freshwater, marine and brackish water systems). lakes, ocean, river , pond (Duong et al. 2012) and environmental conditions (Schlichting Jr 1974) (hot spring lakes, acid mine drainage and ice-lakes) by streak plate, spread plate, pour plate and Fluorescence Activated Cell Sorting (Acreman 1994).

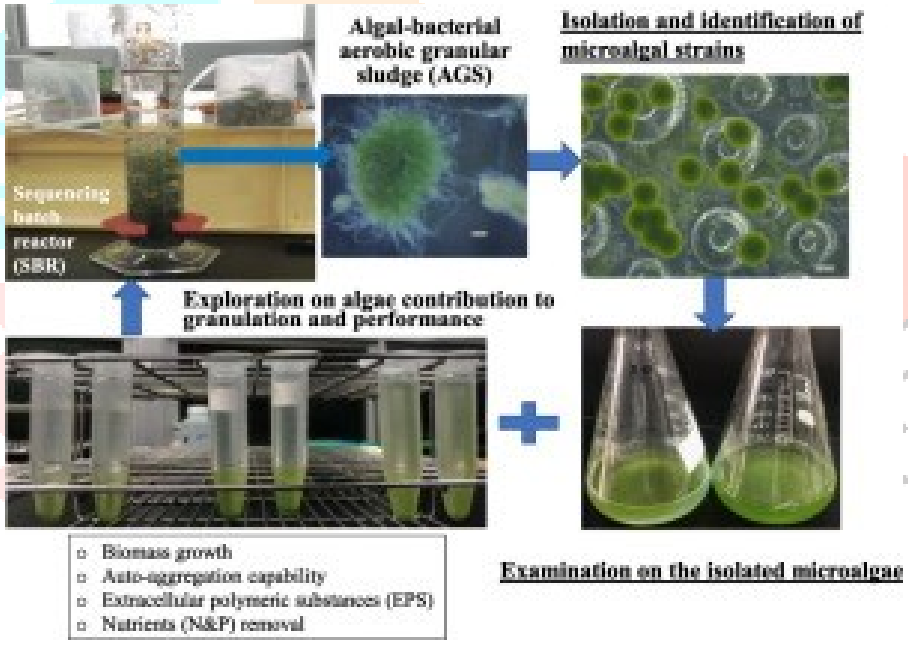

Figure 1: Isolation of microalgae

3.2 Identification of microalgal species

Microalgae isolates had been identified by Matrix-assisted laser desorption / ionization mass spectrometry (MALDI-MS) analysis (von Bergen et al. 2009), reverse dot blot hybridization (RDBH) (Chen et al. 2015) and 18S rDNA analysis (Minhas et al. 2016).

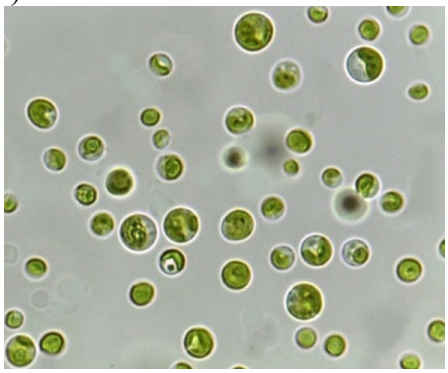

Figure 2 : Molecular Identification of Microalgae

3.3 Analysis of lipid biosynthesis enzymes and generation of transgenic microalgae

The full-length cDNA of the lipid biosynthesis enzymes had been amplified by PCR using respected primers. The lipid biosynthesis enzymes fragments are cloned into a transformation vector (Xue et al. 2015). Microalgae are transformed with vector containing transgene (lipid biosynthesis enzyme) by electroporation (Ladygin 2003), particle bombardment (Koop et al. 2007), agitation with glass beads (Kindle 1990) and silicon carbide whiskers (Wang et al. 1995).

3.4 Molecular analysis of transgenic microalgae

Transformed microalgae are screened by single cell PCR analysis. The expression level of lipid biosynthesis enzymes mRNA are determined by using quantitative real-time PCR (qPCR) and amplified with respected primers (Xue et al. 2015). To examine the expression level of lipid biosynthesis enzyme, the total protein are extracted from transformants and an untransformed wild type control, using a Protein Extraction Kit. Proteins are separated by SDS-PAGE gel and electro-transferred to a polyvinylidene fluoride membrane for Western blot analysis. Western blots using mouse 6X His primary anti-His tag antibody and goat anti-mouse horseradish peroxidase-conjugate secondary antibody are used to detect recombinant proteins (Trentacoste et al. 2013).

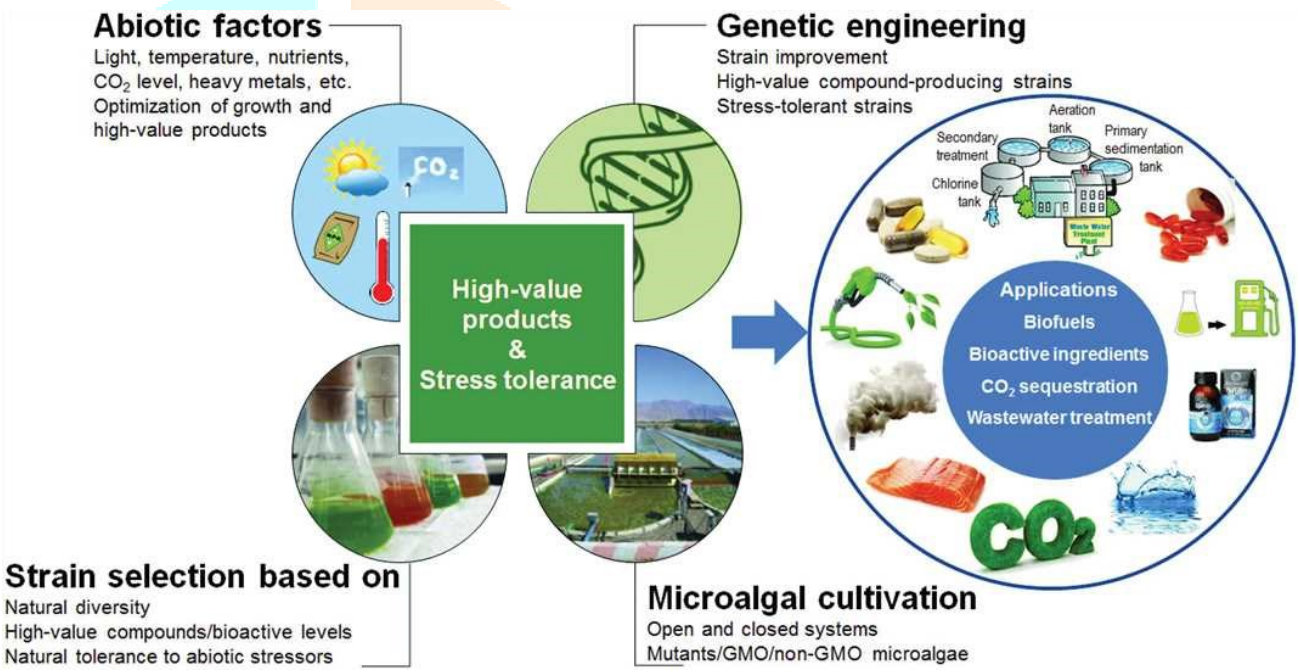

Figure 3 : Molecular analysis of transgenic microalgae

3.5 Lipid extraction and analysis

Lipid content in transgenic microalgae with nitrogen deprivation, and wild type are measured by Nile red staining (Yang et al. 2013) and by gravimetric determination (Phillips et al. 2008). The lipid composition had been analyzed by gas chromatography-mass spectrometry (GC–MS) (Yang et al. 2013). Fatty acids had been identified according to the equipped NBS spectrum library. The integrated peak areas had been determined and calculated by normalization to obtain the percent contents of fatty acid composition.

Conclusions of the research

Biodiesel production from microalgae is being widely developed at different scales as a potential source of renewable energy with both economic and environmental benefits. Although many microalgae species have been identified and isolated for lipid production, there is currently no consensus as to which species provide the highest productivity. Different species are expected to function best at different aquatic, geographical and climatic conditions. In addition, other value-added products are now being considered for commercial production which necessitates the selection of the most capable algae strains suitable for multiple-product algae biorefineries.

Metabolic engineering of lipogenic pathways represents a promising strategy to enhance the efficacy of microalgal oil production without compromising growth is an important aspect of advancing economic feasibility. Proposed research will demonstrate the isolation, identification of microalgae, cloning and overexpression of lipid biosynthesis enzymes from isolated microalgae. This report will illustrate the promising role of lipid biosynthesis enzymes in microalgal TAG biosynthesis and would also pave the way for metabolic engineering in photosynthetically driven cell factories for commercialization.

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