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Invited Review

# 바이오디젤용 지질 생산을 위한 미세조류 배양에서 환경 스트레스 조건의 활용 전략

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# Environmental Stress Strategies for Stimulating Lipid Production from Microalgae for Biodiesel

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**초 록**

미세조류는 통상적인 에너지 작물에 비하여 빠른 성장속도와 높은 오일함량으로 바이오디젤 생산의 원료로 관심을 받고 있다. 미세조류의 지질은 주로 트리글리세라이드인 중성지방으로 에스테르교환반응을 통하여 바이오디젤인 지방산 메틸에스테르로 전환할 수 있다. 본 논문에서는 영양분의 제한, 염도 및 금속 성분의 변화와 같이 미세조류의 지질 생산을 촉진할 수 있는 배양환경 스트레스 조건의 영향들을 비교 고찰하였다. 사용하는 미세조류 종에 따라 스트레스에 대응하여 지질의 양이 변하거나 구성하는 지방산의 조성이 변화될 수 있다. 비록 질소원 제한 조건이 가장 많이 사용되는 지질생산 촉진조건이긴 하지만, 미세조류로부터 바이오디젤 생산성의 향상을 위해서는 그 외에도 영양분 과잉 조건, 염도의 변화, pH, 온도, 금속 성분 농도 변화 등의 다른 조건들도 고려되어야 한다.

**Abstract**

Microalgae are a promising alternative feedstock for biodiesel production because their growth rates and oil contents are higher than those of conventional energy crops. Microalgal lipid is mainly triacylglyceride that can be converted to biodiesel as fatty acid methyl esters through trans-esterification. In this paper, the influence of several important lipid inducing factors such as nutrient limitation and changes in salinity and metallic components in microalgae and their potential strategies to be used for biodiesel production are reviewed. Depending upon strains/species that we use, microalgae react to stresses by producing different amount of triacylglyceride and/or by altering their fatty acids composition. Although the most widely applied method is the nitrogen starvation, other potential factors, including nutrient surplus conditions and changes in salinity, pH, temperature and metal concentrations, should be considered to increase biodiesel productivity.

***Keywords:*** *Microalgae, biodiesel, lipid, environmental stress*

## 1. Introduction

Biodiesel is defined as monoalkyl esters of fatty acids derived from trans-esterification of oil/fats with alcohol. Biodiesel is being recognized currently as an attractive alternative renewable fuel because it is sustainable under the condition of carbon-neutral. It can be blended with conventional petroleum based in any ratio, and can be used in existing diesel engines. It is also biodegradable and generates less emission of gaseous pollutants than petroleum diesel oil. Commercial biodiesel is currently produced mostly from energy crops such as soybean, rapeseed, palm oil or corn. However, the production of biodiesel from these crops is not sustainable because they not only occupy agriculture land but also many of them are food resources.

Microalgae are a promising alternative feedstock for biodiesel production because their growth rate and oil content are higher than those of conventional energy crops[1]. Microalgae basically grow photoautotrophically using inorganic carbons and solar energy, and some species are able to utilize organic carbons also mixotrophically or heterotrophically[2-4]. High-density cultivation of biomass up to several grams per liter is possible in a small space using appropriate bioreactors, and their photosynthesis efficiency is higher than that of terrestrial plants. Microalgal lipid is mainly triacylglyceride (TAG) that can be converted to biodiesel as fatty acid methyl esters (FAMEs) through trans-esterification[5,6].

Some strains of microalgae can accumulate large quantity of lipid in their cells that can be converted to biodiesel. The suitable spe-

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cies/strains for economically feasible biodiesel production are the ones with high lipid content and high cell growth at the same time. Unfortunately, microalgae with both high growth rate and high lipid content are rare and the strains used for lipid production can generally be divided into two groups : (i) one with high lipid content but low cell growth, such as *Botryococcus braunii* which has up to 70% lipid content but with low biomass productivity of several tens mg/L/d[7], (ii) the other with high cell growth but low lipid content, such as *Chlorella vulgaris* with short doubling time of 19 hour or less but with lower lipid content as 20～30%. The species with the high oil contents usually grow slowly resulting in lowered oil productivity[8].

Microalgae produce proteins, carbohydrates and lipids under normal conditions in a balanced manner, but it is known that, under certain environmental stress, algal carbon flux can be switched to lipid synthesis and more lipids are produced[9]. It is generally accepted that nitrogen depletion from the medium induces the accumulation of lipid in many microalgae species[10]. The reason for the accumulation of lipid under nutrient depletion is that the production rate of routine cell components is lowered, but oil production remains as it is in many cases. Therefore, environmental stresses like nitrogen depletion can result in inhibiting cell growth without slowing down the lipid production.

Since which stress factors trigger lipid synthesis induction is species/strain-specific, a variety of methods for lipid induction in microalgae by environmental stress, including nitrogen depletion and other factors, has been intensively studied to increase triacylglyceride (TAG) production in microalgae. There has been a wide range of studies carried out on the effects of nutrients stress including nitrogen and/or phosphorus starvation, changes in light irradiation, pH, temperature and medium components concentrations on the microalgae growth and lipid content. In this paper, the influence of several important lipid inducing factors such as nutrient limitation and changes in salinity and metallic components in microalgae and their potential strategies to be used for biodiesel production are reviewed.

## 2. Overview of Microlagal Biodiesel Production

Microalgae cells can be cultivated in open pond systems and in photobioreactors. In earlier studies with *Chlorella vulgaris*[11,12], the algal concentration for the harvesting stage is 0.6 g/L with a lipid content of 30 wt. %, in a 0.3 m-deep raceway pond with 0.2 m depth of water and the residence time of 4 days. Several grams per liter of cell concentration can be achieved using elaborated photobioreactors these days. Nutrients in forms of nitrate, ammonium or urea and inorganic phosphate are used as the nitrogen and phosphorous sources. For photoautotrophic cultivation, $CO_2$ is supplied at 2～10% (v/v) or a ratio of 2.2 kg/kg algae. It is beneficial if $CO_2$-containing emission is available from a nearby power plant. Natural gas emission would be more advantageous due to the low content of SOx which can act as an inhibitor for cell grow by reducing pH. NOx components in the emission gas can be used in algae cultivation since dissolved NOx is assimilated by microalgae as nitrogen source[13]. Some biodegradable organic carbons by algae can be used as carbon and energy sources together with $CO_2$[3,4].

The harvesting stage usually adopts centrifugation or coagulation-flocculation-settling scheme. Flotation or coagulant-assisted flotation can be used if specific gravity of the cells is small enough. For the case of *Chlorella vulgaris*, the dosage of coagulant alum was 0.74 mg/L[14] and the final biomass concentration and recovery yield were around 30 g/L and 97% on mass basis, respectively. For further dewatering, a self-discharged disc-stack centrifuge was used to obtain a final concentration of 150 g/L algal biomass with the recovery yield of 85%[12,14]. Dried biomass with moisture content of 12 wt% is obtained by using a thermal dryer without biomass loss. Because drying is a high energy-demanding process, an appropriate decision is important on which degree of dewatering is necessary, which depends on the extractability of lipids by the organic solvent[15].

Although techniques of organic solvent extraction and supercritical extraction are available, n-hexane is widely used for industrial-scale lipid/oil extraction from biomass. The requirement of the solvent is around at the mass ratio of 0.5 (hexane/biomass) and the spent solvent should be recovered and reused after distillation to meet economic feasibility. For *Chlorella vulgaris*, the lipid recovery yield is 80% and up to 98% of hexane can be recovered[12,14].

The extracted lipids are in the form of triacylglycerides (TAGs) or free fatty acids (FAs). For trans-esterification, TAGs and FAs are reacted with an alcohol in the presence of acid or base catalyst. Although any kind of low molecular weight alcohols can be used for esterification, methanol is generally used and thus FAMEs are produced as biodiesel. Sodium or potassium hydroxide, sodium methoxides and $CaCO_3$ are used as base catalysts, meanwhile sulfuric acids, phosphoric acid, p-toluenesulfonic acids and methylsulfonic acid as acid catalysts. In industrial esterification for biodiesel production, base catalysts such as NaOH or KOH are popularly used because base-catalyzed esterification rate is higher than acid-catalyzed one. For the trans-esterification of the extracted lipid, is used and the optimal molar ratio of methanol to oil is 6, catalyzed by 1.5 wt% of KOH or NaOH[15,16]. However, base catalysts have saponification problem in the presence of free fatty acids, which decreases catalysts activity and increases processing cost. Therefore, acid trans-esterification is recommended if the fraction of free fatty acids is too high in extracted lipid. Saponification of free fatty acids can be avoided because acid catalysts can convert free fatty acids as well as TAGs to FAMEs.

Products of trans-esterification are biodiesel, glycerol and unreacted alcohol where approximately 1 g of biodiesel and 0.11 g of glycerol are produced for 1 g of C18 (stearic acid) triglyceride[6,15]. Glycerol is separated by phase separation or centrifugation. The unreacted alcohol is mixed with glycerol and can be recovered through distillation or vacuum evaporation. For neutralization and clean-up washing of crude biodiesel, phosphoric acid and water are used. Final moisture is completely removed by drying.

## 3. Lipid Production by Environmental Stress

Lipids produced by microalgae can be either storage lipids or struc-

tural lipids. Storage lipids are mainly in the form of TAG made of saturated and unsaturated fatty acids (FAs), which can be the source of biodiesel production. In general, TAGs are mostly synthesized in the light, stored in cytosolic lipid bodies, and then some of them are converted to polar lipid in the dark[17]. Storage TAGs are catabolized to provide metabolic energy.

Some microalgae can change lipid metabolism in response to abnormal environmental conditions[8,10,18]. Under normal growth conditions, the assimilated carbons can go either to microalgae biomass or to TAGs, and lipid contents in their biomass are relatively low (5-20%). When they are exposed to unfavorable environment or stress conditions, microalgae can modify their lipid biosynthetic pathways towards more formation and accumulation of neutral lipids, enabling microalgae to endure adverse conditions.

### 3.1. Nutrient stress

Nutrient starvation has significant impact both on the growth and propagation of microalgae and on their lipid synthesis. Cell growth rate is generally declining under environmental stress condition when nutrients are limited, while fatty acids biosynthesis is maintained in some algae species if light and carbon source are sufficient for photosynthesis. Under normal growth conditions, the energy carrier and reducing power (ATP and NADPH) produced by photosynthesis are consumed in carbon fixation (resulting ADP and $NADP^+$ as electron acceptors) and in generating biomass. When major nutrients are limited, cell growth and proliferation becomes limited and the pool of the major electron acceptor for photosynthesis, $NADP^+$, will become depleted. Thus the surplus NADPH tends to be consumed in FA biosynthesis, which is stored in TAGs and replenishes the pool of $NADP^+$[17,19]. As a result, cell growth is slowed down and then fatty acids tend to be accumulated to TAGs.

Among the nutrients for microalgae cultivation, nitrogen is the first essential one, and its starvation is the most widely applied for lipid induction techniques in many species. Many of green microalgae and cyanobacteria showed the increase in lipid production under nitrogen starvation[9,20,21]. It is well known that green alga *Scenedesmus* and *Chlorella* species tend to increase lipid content under nitrogen-starved condition[9,22]. It was also found that the lipid composition of *Chlorella vulgaris* is changed from free FA-rich lipids to TAG in low nitrogen media[23].

Along with nitrogen, phosphorous is another major nutrient for algal proliferation. The effects of phosphorus limitation on lipid production in microalgae are contrasting depending upon strains/species. TAG synthesis is increased in *Chlorella* sp. under phosphate limitation, but decreased in *Nannochloris* and *Tetraselmis* sp.[24,25]. Sulfur is the next important nutrient after nitrogen and phosphorous. It was reported that sulfur limitation for *Chlorella* sp. and *Clamydomonas reinhardtii* resulted in increased lipid content[26].

One of the strategies to enhance economic feasibility of microalgal biodiesel production is to achieve high lipid productivity, which is the multiplication of cell growth rate and cellular lipid content. However, biomass productivity should sacrifice in order to maximize lipid accumulation when nutrient-limited cultivation is used because nutrient limitation reduces biomass productivity as mentioned above. Usually repeated supply of nitrogen source is necessary to maintain high cell growth rate and to achieve high cell density[27,28], where final lipid productivity is not high although high cell density is achieved at the end of cultivation. Therefore, a two-stage culture strategy can be an option to enhance lipid productivity[23,29,30]. At the first stage, the cultivation of normal nutrition is carried out with sufficient supply of required nutrients to obtain a maximized high cell density as quickly as possible and then, in the second stage, conditions are changed to trigger the induction of lipid production. Ghulam et al.[30] reported that lipid accumulation is maximized quickly within 1～3 days in the second stage of two-stage lipid production which is composed of the stage I for fast cell growth under nutrient repletion and the stage II of so-called post-cultural incubation (PCI) including N-starvation. The lipid synthesis was slowed down after the maximal point and thus a timely termination of the second stage is necessary to enhance lipid productivity.

Meanwhile, for some marine microalga, nitrogen surplus condition could be a stress leading to increased lipid production. Feng et al.[28] and Wan et al.[31] reported that *Nannochloropsis* and *Isochrysis* species showed increased lipid content responding to supplementing nitrogen. Marine microalga *Tetraselmis* sp. did not accumulate lipids under nitrogen-deficient condition, whereas the lipid content increased under nitrate-surplus condition compared to the normal F/2 medium[32]. Biomass production was also increased by supplementing high concentration of nitrate, resulting in enhanced lipid productivity (Figure 1).

Amongst all nutritional stress approaches, nitrogen starvation technique is most widely applied and studied in many microalgae species that can be considered for the production of biofuel. Nitrogen is the first essential growth-limiting factor for microalgae and many species tend to increase TAG production under nitrogen stress. However, as mentioned earlier, low nitrogen content sacrifices cell growth rate and thus it is not easy to achieve high lipid productivity. Therefore, screening the species/strains with lipid accumulating capability at high nitrogen condition would definitely be beneficial to enhance lipid productivity since nitrogen surplus condition can favor both cell growth and lipid production.

### 3.2. Salinity stress

Salinity is an important factor for the survival of both freshwater and marine microalgae. Lowered or increased salinity than that of inherent habitats of given microalgae can serve as a stress because they have to adjust themselves to the changed osmotic pressure environment. Freshwater microalgae may change lipid production behavior responding to high salinity condition and marine microalgae would be the opposite.

Even though the change in total lipid content is not significant, the composition of consisting FA can be varied responding to salinity change. Many *Dunaliella* species can tolerate high salt concentrations and are known to increase lipid content there. An increase of NaCl

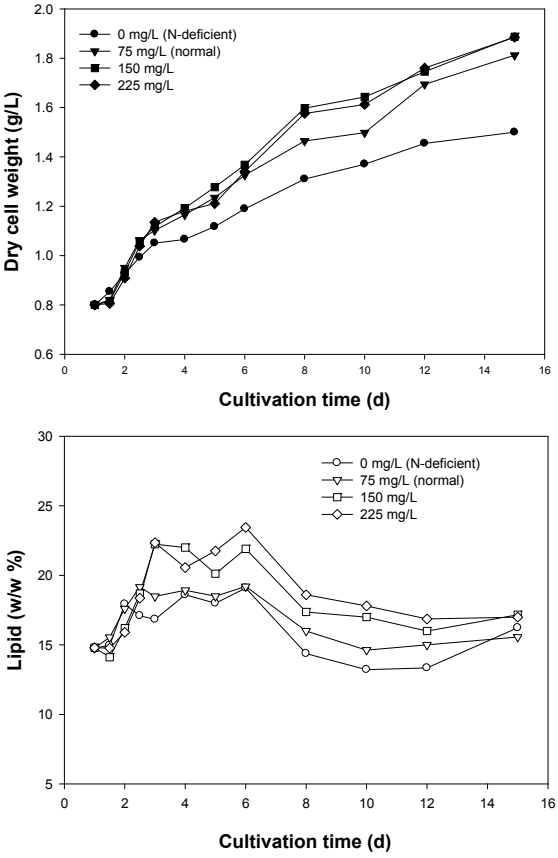


**Figure 1. Changes in cell growth and lipid content of *Tetraselmis* sp. at different nitrate concentration conditions. Normal f/2 medium has 75 mg/L as $NO_3$-N. Surplus N condition favors both cell growth and lipid production[32].**

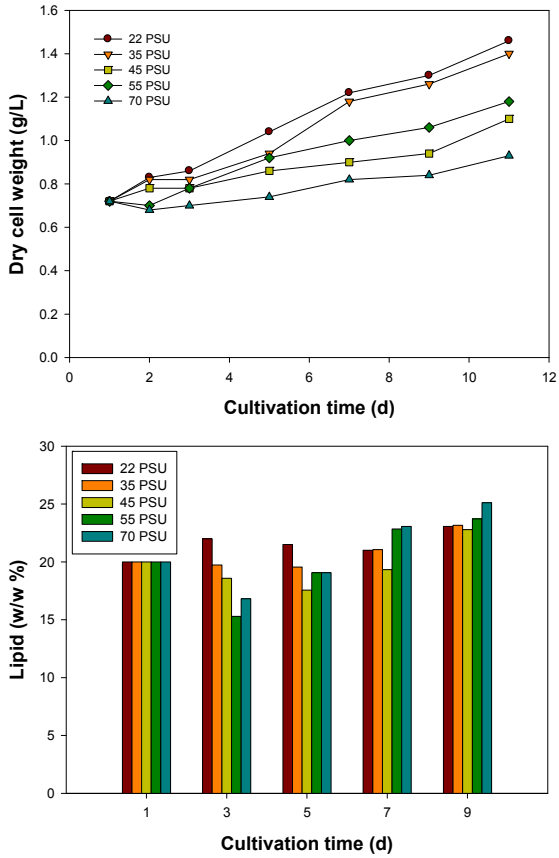


**Figure 2. Changes in cell growth and lipid content of *Tetraselmis* sp. at different salinity conditions. Low salinity of 22 PSU favors cell growth and lipid production compared to normal sea water salinity, 35 PSU[36].**

concentration during cultivation of *Dunaliella tertiolecta* resulted in an increase in lipid content and a higher percentage of TAG[33], in which the fractions of saturated fatty and monounsaturated fatty acids were increased, while the fraction of PUFA was decreased. For the diatom *Nitzschia laevis* the degree of FA unsaturation increased when salt concentrations increased[34]. On the other hand, *Schizochytrium limacinum* showed increased lipid content, especially saturated fatty acids, at lowered salinity conditions[35].

Marine microalga *Tetraselmis* sp. showed a high salinity tolerance under wide range of salinities 9 through 70 PSU. The highest lipid content was obtained under salinity 70 PSU with sufficient nitrate, but the overall lipid productivity was reduced due to the low biomass production. Whereas, relatively low salinities in the range of 9 to 22 PSU showed higher biomass productivity and increased lipid content, resulting in higher lipid productivity than that under normal or high salinities (Figure 2). When the salinity shifted from 35 to 22 PSU, the lipid production was enhanced, without significant changing in fatty acids composition[36].

### 3.3. Metal components

Metallic components like iron, cadmium, silicon, magnesium, copper and zinc have also been found to affect lipid contents in some microalgae. It was reported that cadmium caused an increase in the total lipid content in *Euglena gracilis*[37]. Many diatoms tend to respond to the change in silicon concentration. *Cyclotella cryptica* produced an increased amount of TAG under silicon deficiency[2]. Lipid accumulation was achieved through silicon deficiency or iron supplementation in *Scenedesmus* and *Chlorella*[8,38,39]. In the investigation on the effect of iron on growth and lipid accumulation in *Chlorella vulgaris*[38], the culture in the late exponential growth phase, when supplemented with $Fe^{3+}$ at different concentrations, showed increased total lipid content of up to 56% biomass by dry weight. It was also reported for *Dunaliella tertiolecta* that the increased Fe (III) concentration was favorable for enhancing lipid content[40].

## 4. Conclusions

The different lipid induction techniques that can be used to stimulate lipid biosynthesis in microalgae have been discussed. Depending upon strains/species used, microalgae react to stresses by producing different amount of TAG and/or by altering their fatty acids composition. Therefore, the selection of the techniques to apply for the lipid induction should be determined through well-controlled laboratory experiments. Although the most widely applied method is the nitrogen

starvation, other potential factors, including nutrient surplus conditions and changes in salinity, pH, temperature and metal concentrations, should be considered for the commercial production of biodiesel.

If the selected stress method shows contrasting effects on cell growth and lipid production, separating lipid induction stage from cell growth is necessary to increase lipid productivity. In the case of nutrient limitation, biomass productivity should sacrifice to some extent although lipid accumulation is increasing, which results in the reduction of overall lipid productivity. The two-stage culture strategy can be an option to enhance lipid productivity in such cases, where the cultivation of normal nutrition is carried out with sufficient supply of required nutrients to obtain maximized high cell density as quickly as possible in the first stage and then, in the second stage, conditions are changed to stimulate lipid production.

In such two-stage processes, a cease of cultivation is inevitable for decanting the spent broth and supplying new medium or for transferring to separate reactor. Changing the conditions *in situ* (continuously in single reactor) from cell growth to lipid induction in the two-stage processes, especially towards creating a limitation of certain component (such as nitrogen, phosphate, salinity, metals, etc) in a short time, may be difficult to achieve on a large-scale cultivation system. However, adding something to create surplus stress condition is relative easy and achievable quickly. Therefore, screening the species/strains with lipid accumulating capability at high nitrogen or salinity condition would definitely be beneficial to enhance lipid productivity. Nitrogen surplus condition of proper level can favor both cell growth and lipid production.

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