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Microbial Biodiesel Production - Oil Feedstocks Produced from Microbial Cell Cultivations

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1. Introduction

Crude oil price has increased to over $100 per barrel, which is causing serious negative impact on the global and national economy. In 2005, the United States produced 8.3 million bbl/day, but consumed 20.8 million bbl/day, the balance of which was imported from other countries. For these fossil fuels, the U.S. used about 138 billion gallons of gasoline in 2006, accounting for about 44 percent of the world's gasoline consumption (EarthTrends, 2008). The annual U.S. usage of jet fuel was 21 billion gallons in 2006 (Energy Information Administration, 2008). The U.S. annual consumption of diesel fuel in 2006 was about 50 billion gallons (Energy Information Administration, 2008). Massive consumption of fossil fuels has already caused serious concern over global warming caused by greenhouse gases.

Biofuel offers an alternative to fossil fuels. It provides several benefits, such as alleviation from foreign oil dependence, carbon neutral process without greenhouse emission, and profits to local farmers. Bioethanol production from starch and lignocellulosic materials is a kind of an alternative to fossil fuels. It can be blended with gasoline in varying quantities up to pure ethanol (E100). The first generation of ethanol biofuel has been massively commercialized and dominated by the U.S. and Brazil. Fuel ethanol in the U.S. is primarily produced from corn, while Brazilian ethanol is produced mainly from sugarcane. These raw materials are in direct competition with human diet or the land to produce food, which triggers the controversy of food versus fuel. The second generation of ethanol is proposed to be produced from lignocellulosic biomass, which can be obtained from agricultural residue or other woody and herbal biomass from marginal land. Intense scientific research has been carried out over the past decade, focusing on this route in order to decrease the overall process cost and this process is gradually focusing on commercialization.

2. Biodiesel and current feedstocks

2.1 Biodiesel production

Another approach for alternative biofuels is biodiesel. The most common type of biodiesel is the methyl esters of fatty acid (FAME), obtained by transesterification of lipid with methanol or ethanol. It can be used in pure form (B100) or may be blended with fossil diesel at any rate. The commonly used biodiesel is B99 because 1% of fossil fuel is applied to

inhibit mold growth, which shortens its shelf life. Biodiesel is around 5-8% less efficient than conventional fossil diesel; yet, biodiesel has potential as a total or partial (in the cold regions) replacement to the fossil diesel, compatible to the current diesel engine. There are numerous environmental benefits to replace fossil diesel to biodiesel since combustion of biodiesel emits far less pollutants comparing to fossil diesel (except the NO_x emission) and the entire process is close to carbon neutral considering that the plant oil used to produce biodiesel is synthesized in the agriculture from CO_2 in the air. The production and utilization of biodiesel are significant in many aspects, for instance, increasing oilseed crop market, providing domestic job opportunities to the rural community, and decreasing the dependence on imported oil; therefore biodiesel has been commercialized around the globe. Another type of biodiesel is the hydrocarbon generated with direct decarboxylation of fatty acid or lipid. Although it shows superior features than current FAME biodiesel in many aspects, the chemical decarboxylation process to produce diesel still needs further development compared to the transesterification process for biodiesel production.

2.2 Agricultural feedstocks: Vegetable oil, animal fat and recycled grease

The biodiesel industry suffered due to limited raw materials such as soybean oil or vegetable oil. Although there are numerous potential renewable and carbon neutral feedstocks for the production of biodiesel, none seems capable of displacing fossil diesel. Table 1 shows a comparison of the oil yields of some sources of biodiesel, the land area for their cultivation, and the percentage of existing United States (U.S.) cropping area to meet half of the transport fuel needs. Using soybean as feedstock for the production of biodiesel requires 326% of the U.S. cropping area to meet the 50% of all U.S. transport fuel needs. Based on this rough estimation, none of the terrestrial crops is able to completely substitute crude oil.

Crop	Oil yield [Liters per hectare]	Land area needed [Mha][a]	Percent of existing US cropping area[a]
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae[a]	136,900	2	1.1
Microalgae[c]	58,700	4.5	2.5

[a] For meeting 50% of all transport fuel needs of the U.S.
[b] 70% oil (by wt) in biomass.
[c] 30% oil (by wt) in biomass.

Table 1. Comparison of some sources of biodiesel (Chisti 2007)

Various raw materials are proposed to produce biodiesel, including waste cooking oil and various oil-accumulating plants, which could help solve the shortage for raw materials but are not sufficiently available. New approaches need to be developed to use an alternative biomass as the substrate for biofuel production, for example, biomass leftover from agricultural harvest or the biomass produced in non-traditional agricultural land as the

source of sugars for biofuel and bioproducts. This potential has been gaining much attention recently as it is been defined as "second generation of bioenergy", compared to current technologies that primarily are limited to food materials. Oil accumulation with microalgae and other similar technologies started to show some potential to produce biofuel products with massive scales (Benemann, 1996; Chisti, 2007; Jarvis et al., 1994).

3. Microorganisms for microbial lipid production

3.1 Microalgae

Algae oil is being seriously considered because of the large oil yields it shows compared with other oilseeds. The Office of Fuels Development, a division of the Department of Energy, funded a program from 1978 through 1996 under the National Renewable Energy Laboratory known as the "Aquatic Species Program". The focus of this program was to investigate high-oil algaes that could be grown specifically for the purpose of widescale biodiesel production. NREL's research showed that one quad (7.5 billion gallons) of biodiesel could be produced from 200,000 hectares of desert land (200,000 hectares is equivalent to 780 square miles, roughly 500,000 acres). It would be preferable to spread the algae production around the country, to lessen the cost and energy used in transporting the feedstocks. Algae farms could also be constructed to use waste streams (either human waste or animal waste from animal farms) as a food source, which would spread algae production around the country. Nutrients can also be extracted from the algae for the production of a fertilizer high in nitrogen and phosphorous. By using waste streams (agricultural, farm animal waste, and human sewage) as the nutrient source, these farms essentially also provide a means of recycling nutrients from fertilizer to food to waste and back to fertilizer.

Microalgae are generally autotrophic eukaryotic cells, and contain one or more types of chlorophyll plus additional pigments known as carotenoids and biloproteins (also called phycobilins). Carotenoids are yellow, orange, or red water-insoluble linear hydrocarbons; biloproteins are blue or red water-soluble pigment-protein complexes. The color of the different groups of algae depends on the ratio of these pigments. Almost all the microalgae cells contain chlorophyll, but the green color can be masked by the carotenoids, giving them a brown or red color. Microalgae cells are mostly unicellular, but some species are colonial or filamentous. They can grow autotrophically and/or heterotrophically, with a wide range of tolerance to different temperature, salinity, pH and nutrient availabilities. Cyanobacteria, although sometimes also referred as algae, specifically as blue-green algae, are by definition prokaryotic bacteria, instead of microalgae. The eukaryotes microalgae we are referring to include green algae, diatoms, yellow–green algae, golden algae, red algae, brown algae, dinoflagellates and others; and only limited species have the capability to accumulate high content of lipids in their cell biomass. Microbial species that can accumulate over 20% of lipids in their cell biomass are considered oleaginous species.

One of the characteristics of algae that separate them from other oilseeds is the quantity of lipids and fatty acids algae have as membrane components; some algae have been found to contain over 80% of lipids, which is of great interest for a sustainable feedstock for biodiesel production. Different microalgae species are listed in Table 2 as examples of lipid accumulation research. For example, *Chlorella vulgaris* is a commercially important green microalgae because it has the potential to serve as a food and energy source due to its high photosynthetic efficiency, which can, in theory, reach 8%. It can grow with both autotrophic and heterotrophic modes, and its mixotrophic growth rate is the sum of its autotrophic

growth rate and heterotrophic growth rate, separately. *Chlorella protothecoides* is another single-cell green microalgae, which has high potential for the energy and food production. Heterotrophic growth of *C. protothecoides* supplied with acetate, glucose, or other organic compounds as carbon source, results in high biomass and high content of lipid in cells.

Microalgae	Cultures			Substrates	Growth Rate	Lipid Content
	AC	MC	HC			
Chlorella protothecoides	X		X	glucose, acetate / CO_2	3.74g/L - 144h	55.2%
Chlorella vulgaris	X	X	X	glucose, acetate, lactate / CO_2	0.098/h	
Crypthecodinium cohnii			X	glucose/ CO_2	40g/L - 60-90h	15-30%
Scenedesmus obliquus	X		X	glucose/ CO_2	double in 14h after adaptation	14-22%
Chlamydomonas reinhardtii	X	x	X	acetate/ CO_2	exponential during the first 20 Hr	21%
Micractinium pusillum		x	X	CO_2	0.94g/L - 24h	
Euglena Gracilis			X	CO2		14-20%
Schizochytrium sp				glycerol/ CO_2	130 -140h (28°C)	55%
Spirulina platensis	X		X	glucose/ CO_2	0.008 /h	
Botryococcus Braunii			X	CO_2	low growth rate	20 - 86%
Dunaliella salina	X	x		CO_2		~70%

AC: autotrophic cultures; MC: mixotrophic cultures; HC: heterotrophic cultures

Table 2. Some examples of microalgae cultivation for oil accumulation

Lipid accumulation occurs within the microalgae cells and it varies from strain and growth conditions. There are many nutritional and environmental factors controlling the cell growth and lipid contents, such as organic and inorganic carbon sources, nitrogen source, and other essential macro- and micro-nutrients like magnesium and copper, temperature, pH level, salinity, agitation speed (dissolved oxygen). Many microalgae species, for example, *C. protothecoides*, accumulated a higher content of lipids in cells and achieved higher growth rate when the culture was under heterotrophic mode. Like yeast and fungi, heterotrophic algae can accumulate biomass and lipids using organic carbon as its source instead of carbon dioxide and sunlight. Compared with autotrophic algae, the heterotrophic growth process has the advantages of no light limitation, a high degree of process control, higher productivity, and low costs for biomass harvesting (Barclay, Meager et al. 1994). Table 2 shows the oil production of autotrophically and heterotrophically cultured microalgae. Most heterotrophically cultured algae have greater than ten times the biomass concentration, while the lipid productivity is also significantly higher than the theoretical data for autotrophic cultivation. For certain algae strains, it was suggested the

heterotrophically cultured cells exhibited better capability for biomass and lipid production. Miao and Wu (2006) reported that the oil content of heterotrophically cultured *C. protothecoides* was approximately four times greater than that in the corresponding autotrophic culture. Liu et al. (2010) demonstrated that the heterotrophically cultured cells of *Chlorella zofingiensis* showed 411% and 900% increases in dry cell weight and lipid yield, respectively, compared to autotrophically cultured cells. Moreover, biodiesel produced from heterotrophically cultured algae oils had similar properties to diesel fuel in terms of density, viscosity, heating value, and H/C ratio (Xu, Miao et al. 2006). In addition to lipid production, high value byproducts can be obtained from heterotrophically cultured microalgae, including polyunsaturated fatty acids and carotenoids (Chen and Chen 2006).

3.2 Yeast and fungi

Besides microalgae, many yeast and fungi species (e.g., *Mucor circillenous* or *Mortierella isabellina*) also can accumulate a high content of lipids (Xia 2011; Heredia-Arroyo, Wei et al. 2011). Many oleaginous yeasts were studied for lipid accumulation on different substrates, such as industrial glycerol (Meesters, Huijberts et al. 1996; Papanikolaou and Aggelis 2002), sewage sludge (Angerbauer, Siebenhofer et al. 2008), whey permeate (Ykema, Verbree et al. 1988; Akhtar, Gray et al. 1998), sugar cane molasses (Alvarez, Rodriguez et al. 1992), and rice straw hydrolysate (Huang, Zong et al. 2009). The use of non-starch biomass is critical so that lignocelluloses can be used for organic carbon supply without concern of using food crops for fuel sources. Recent studies detailed conversion of hemicellulose hydrolysate into lipids by oleaginous yeast strains and their tolerance degrees to lignocellulose degradation compounds (Chen, Li et al. 2009; Hu, Zhao et al. 2009; Huang, Zong et al. 2009). However, these strains were unable to efficiently produce lipids in the presence of inhibitors in the hydrolysate, necessitating detoxification treatment prior to fermentation, which increases the cost of the process. Thus, using strains capable of growing in the non-detoxified hydrolysate is necessary for viable microbial lipid production in an industrial context. In addition, previous reports indicate that temperature is an key factor in regulating the fatty acid composition in fungi (Kendrick and Ratledge 1992; Weinstein, Montiel et al. 2000).

Similarly, some oleaginous filamentous fungi can also produce lipids by utilizing glycerol, acetic acid, soluble starch, wheat straw, and wheat bran. Dey et al screening two endophytic oleaginous fungi *Colletotrichum sp.* and *Alternaria sp*. with lipid content 30% and 58% respectively (Dey, Banerjee et al. 2011). Fifteen eukaryotic microorganism were tested for waste glycerol assimilation to produce lipid. Fungi accumulated lipid inside their mycelia (lipid content ranging between 18.1 and 42.6%) (Chatzifragkou, Makri et al. 2011). Such capabilities provide potential to utilize sugars in the pretreated lignocellulosic materials hydrolysate. Moreover, because the fatty acid profile of microbial oils is quite similar to that of conventional vegetable oils, oleaginous filamentous fungi are suggested as a favorable feedstock for a sustainable biodiesel industry (Peng and Chen 2008; Zhao, Hu et al. 2010).

4. Feedstocks for microbial lipid production

4.1 Light and carbon dioxide

Microalgae cells can generally utilize sunlight, carbon dioxide and nutrients from waste water for their cell growth (Brennan and Owende 2010). Lipid accumulation with microalgae cultivation is relatively efficient due to its high production efficiency and less

demand of agricultural land. Most microalgal ponds have a solar energy conversion efficiency of 1–4% under normal operating conditions and higher efficiencies can be achieved with closed photo-bioreactor systems. There is a considerable margin for improvement, which is being targeted through accelerated breeding programs and genetic modification. Autotrophic microalgae cells also absorb CO_2 as their carbon source to support their cell growth, which makes the microalgae an attractive option for the biological CO_2 fixation. Atmospheric CO_2 accumulation, derived mainly from fossil fuel combustion, is proved as the leading cause of global warming. Current mitigation methods such as physicochemical adsorption, injection into deep oceans and geological formations are not economically feasible due to the high cost of implementing these methods and possible CO_2 leakage. After CO_2 is fixed via microalgae assimilation, the cell biomass can be utilized to generate methane gas, biochar, or the oil can be extracted to generate biodiesel. Microalgae can fix not only atmospheric CO_2, but also the CO_2 from industrial exhaust gases and from the carbonate salt, which are chemically fixed. Atmospheric CO_2 levels (0.0387%) are not sufficient to support the high microalgae growth rates and productivities needed for commercial biofuel production, adding CO_2 into autotrophic microalgae culture is an effective method to accelerate the microalgae growth rate. Utilization of CO_2, for example, flue gas from electrical plant, by means of microalgae alleviates the impact of CO_2 on the environment (greenhouse effect) and renders algal biomass production less expensive. It was also reported that the CO_2 content was reduced from 44–48% to 2.5–11% when the microalgae cultivation was integrated with anaerobic digestion to remove impurities of biogas produced from anaerobic digestion. Microalgae also assimilated other impurities such as ammonia and hydrogen sulfide; and the gas leaving the algae pond had 88–97% by volume of methane [23]. Although there are conflicting results from different references about the toxicity of ammonia, hydrogen sulfide and other impurities on the growth of microalgae cells, integration of microalgae cultivation together with this industrial processes can be more sustainable and economically feasible than the individual microalgae cultivation process to generate oil for biodiesel production.

4.2 Wastewater

Culturing microalgae with nutrients from wastewater, such as nitrogen and phosphate, can decrease the cost of the raw materials and also provide some environmental benefits. Agricultural effluent and municipal wastewater, even after treated with anaerobic digestion (AD), cannot be disposed directly because of their high nutrient level (Levine, Costanza-Robinson et al. 2011). In contrast, these wastewater can be considered as a cost-effective candidate of raw materials for biodiesel production (Siddiquee and Rohani 2011). Cultivation of microalgae, yeast, or fungi can be integrated with AD system to reduce the remaining COD, phosphate, and ammonia. Microalgae were also studied to absorb metal ion, waste pharmaceutical chemicals and dye into their cell biomass in order to remove the pollutants from wastewater once their cell biomass were stabilized and harvested. A typical example is the recently developed high-rate algae pond (HRAP) in the tertiary wastewater treatment facility (El Hamouri 2009). HRAP functions behind a two-step upflow anaerobic reactor (pre-treatment) and was followed by one maturation pond (MP) for polishing. The HRAP was revealed to have no activity for removing the COD from the wastewater; however, it removed 85% of total N and 63% of total P. Nitrogen removal was discovered due to the assimilation of microalgae for their growth, and denitrification did not play any role in removing the nitrogen in this process. Phosphorus removal in this process was

attributed to chemical precipitation and biological assimilation (around 50% each). In removing ammonia, the HRAP is superior to the traditional bacterial nitrification–denitrification process, which requires the assimilation of extra-organic carbon as a carbon source. Phosphorus removal by microalgae is largely thought to be due to its uptake for normal growth, as an essential element required for making cellular constituents such as phospholipids, nucleotides, and nucleic acids. Under certain conditions microalgae can be triggered to uptake more phosphorus than is necessary for survival, in the form of polyphosphate (Powell, Shilton et al. 2011). Phosphorus removal by luxury uptake (amount of P uptake more than growth required) was confirmed to occur in the microalgae growing in the wastewater treatment facility; further research is needed in this prosperous field about the detailed mechanism and applications.

4.3 Lignocellulosic biomass

Several research studies revealed that organic carbon sources, if the algae have the capability to grow on heterotrophic mode, can significantly increase the cell growth rate and dramatically enhance the lipid content of the biomass. For example, *Chlorella protothecoides* can only accumulate 18-25% lipid in the autotrophic culture, while the lipid content can reach to 55.2% dry cell biomass if cultured with addition of organic carbons (Xiong, Li et al. 2008). However, the heterotrophic cultivation of microalgae mostly request pure mono-sugars, which are usually costly and limited. Alternative materials with relative abundance and zero or negative valued organic materials, such as lignocellulosic biomass from agriculture, are the only choice for this route. Agricultural feedstocks contribute a large part of renewable resource for biodiesel production, which are the target of biodiesel resource of cost reduction of biodiesel and non-human food resource discovery. Most of these substrates are locally available and thus are expected to support mainly small production facilities. Lignocellulosic materials are mainly composed of cellulose, hemicellulose, and lignin, which make up approximately 90% of the dry weight of most plant materials (Kumar, Barrett et al. 2009). Cellulose and hemicellulose can be converted to fermentable sugars for microbial lipid production. However, the direct enzymatic hydrolysis of cellulose and hemicellulose to sugars is impeded by the cell wall physico-chemical and structural composition. Thus, biomass pretreatment prior to enzymatic hydrolysis is essential to enhance enzymatic digestibility. Distinctly different from autotrophic microalgae cultures, this process is more similar to cellulosic ethanol production, where hydrolysis and fermentation are needed for conversion.

5. Conversion processes for microbial lipid production

5.1 Autotrophic microalgae cultivation and lipid accumulation

Three cultivation processes were designed to culture microalgae and other oleaginous cells, including open-pond system, photobioreactor, and fermentation. The open-pond system is typically a closed loop with a pump to create microalgae flow in the channel. The channel is 0.2-0.5 meter deep, and the pump keeps the microalgae cell well mixed for continuous growth. This type of open-pond system has been used for several years because it is easy to operate and inexpensive to maintain. The open-pond system also can be upgraded to large-scale microalgae production. On the other hand, a high cell density of microalgae cannot be reached because of limited capability to assimilate the sun light and low carbon dioxide concentration of air. Increasing the CO_2 concentration by using

flue gas instead of air can increase the microagale cell concentrations, but the final cell density is still limited to the mutual shading effects where light cannot penetrate through dense microalgae cell broth. Another problem is biological contamination during the long period of cultivation. The bacteria contamination or other non-oleaginous microalgae invasion can occur in stressed cultural conditions, where lipid accumulation usually is stimulated, such as nitrogen depletion or other nutrient imbalance. There is now extensive evidence that open-pond systems can operate for more than six months without significant contamination using a wide range of microalgae. Prolific strains of *Chlorella*, for example, are often dominant because they outgrow their competitors (and indeed can often be contaminants themselves in *Arthrospira* cultures or other microalgal strains). Extreme halophiles, such as *Dunaliella salina*, are also dominant in their optimal environments because they do not encounter much competition at high salinities. However, in the context of the wider microalgal industry, contamination issues are still of significant interest.

To enhance the productivity of microalgae, closed photobioreactor systems (tubular flat plate, Orcolumn are designed to increase the surface of microalgae broth exposed to sunlight. Closed photobioreactors are more costly than open-pond systems, but they have potential for higher productivity of cell biomass with less chance of contamination. The flat plate photobioreactors can receive greater sunlight for microalgae growth although there is potential for cell mixing. The microalgae cell density could reach up to 80g/L dry cell weight, significantly higher than the cell density of a pond system, which ranges within several g/L (Hu, 1998). Another design for the photoreactor is the tubular photoreactor, made with a diameter less than 0.1 m to maximize the sunlight harvest by microalgae. The tubular reactor can also expose the microalgae cells to sunlight from all the directions (Miron, Gomez et al. 1999; Ugwu, Ogbonna et al. 2002). There are a few reports about scale-up test of tubular photoreactor, such as the one in Hawaii with a size of 25M^3 (Olaizola 2000), and 700 M^3 in Germany (Pulz 2001). However, the tubular photobioreactors cannot scale-up indefinitely because of oxygen accumulation, carbon dioxide limitation, and pH changes (Eriksen 2008). The third type of photoreactor is the column photoreactor, the most controllable type among three because it most closely resembles the traditional bioreactor. The column containing microalgae is vertical, and the air is bubbled from the bottom. Sunlight is provided horizontally (Eriksen 2008).

In addition to individual open-pond system and closed photobioreactor, the hybrid system of microalgae cultivation is currently under intense investigation because it combines the open-pond system and closed photobioreactor to increase the cell productivity and reduce the cost. The first stage is autotrophy to avoid biological contamination, and the second stage is heterotrophy, which provides the stress condition for lipid accumulation.

5.2 Oleaginous microbial fermentation and lipid accumulation

Besides the pond system and photobioreactor, heterotrophic cell cultivation, including heterotrophic microalgae, oleaginous yeast and fungi, is usually limited to industrial fermentation tanks. These cells can be cultured in a dark fermentor with optional sunlight, and can usually reach a very high cell density (up to 200g/L). Due to its excellent controllability of all operation parameters, most of the industrial microalgae cultivations for nutraceutical production (e.g., polyunsaturated fatty acid) have been switched to the heterotrophic fermentations where sugar is provided to produce high-valued products, such as Docosahexaenoic acid (DHA).

Factor	Raceway	Photobioreactor	Fermenter
Cell density in culture	Low	Medium	High
Limiting factor for growth	Light	Light	Oxygen
Culture volume necessary to harvest a unit weight of cells	High	Medium	Low
Surface area-to-volume ratio	High	Very high	Not applicable
Control over parameters	Low	Medium	Very high
Commercial availability	Readily available	Usually custom built	Readily available
Construction costs per unit volume produced	Medium	High	Low
Operating costs	Medium	High	Low
Technology base	Readily available	Under development	Readily available
Risk of contamination	High	Medium	Low
Evaporative water losses	High	High[33]	Low
Weather dependence	High	Medium	Low
Maintenance	Easy to maintain	Difficult to maintain	Requires specialized maintenance
Susceptibility to overheating	Low	High	N/A
Susceptibility to excessive O_2 levels	Low	High[34]	N/A
Ease of cleaning	Very easy	Difficult	Difficult (must be sterilized)
Ease of Scale-up	High	Variable[35]	High
Land requirement	High	Variable	Low
Applicability to different species	Low	High	Low

Table 3. Comparison of different algae cultivation systems (Alabi. 2009)

Table 3 compares three cultivation methods and shows that the process cost of fermentation can be high due to its requirement of raw materials and oxygen, and sterilization of culture media during the cell growth. It is readily available both in the lab and in the industry, but is only suitable to produce high-valued products, of which biofuel products are not. The key barriers to apply this technology to biofuel production is the cost and availability of raw materials. Considering the competition with human diet, sugars cannot serve as the raw material for biofuel production; and alternative materials such as lignocellulosic materials should be used for the heterotrophic oil production. If the oleaginous cells are capable of generating the hydrolytic enzymes for lignocelluloses degradation, it will be the big plus for biodiesel production via oleaginous fermentation the overall system. Otherwise, external hydrolytic enzymes have to be used to release the monosugar, followed by lipid accumulation via olgeaginous microorganisms. Separated hydrolysis and fermentation (SHF) is a common working model to have these two steps separated. Two bioreactors will be necessary because the hydrolytic degradation of lignocellulose is preferred at 50°C, while the oleaginous microorganisms grow at much lower temperature (28^0C to 30^0C for most of the fungus). Simultaneous saccharification and fermentation is another working model currently under intense investigation, in which two steps are integrated into one.

Different fermentation processes are applied to obtain high productivity of lipids and high conversion ratio of substrate for the fermentation, such as batch cultivation, fed-batch cultivation, and continous cultivation. Fed-batch cultivatioin is a modified batch model that can reach high cell density and it has many applications in the fermentative lipid accumulation process. For example, *Rhorosporidum toruloides* reach much higher cell density with 48% lipid compared to its batch cultivation (Li, Zhao et al. 2007). The high productivity of fed-batch cultivation was conformed by *Phodotorula glutinis* (Xue, Miao et al. 2008), *C. curvatus* (Meesters, Huijberts et al. 1996), and *L. starkeyi* (Yamauchi, Mori et al. 1983). Continuous cultivation has advantages of easy maintenance and time-saving, although it is difficult to control the contamination. It has limited applicatioins in the fermentative lipid accumulation.

Besides the commonly used submerged cultivations, solid state fermentation, as a compact process for lipid production, showed many advantages, such as low requirements to the raw materails; low capital cost; low energy expenditure; less expensive downstream processing; less water usage and low water output; potential higher volumetric productivity; less fermentation space; easy operation and maintenance. The research for *Aspergillus oryzae* growing on rice bran and wheat bran through solid state fermentation resulted to the lipid content of cell biomass at about 10-11% (Da Silveira, Oliveira et al. 2010). The lipid yield reached 62.87 mg/gds in solid state fermentation on the 6th day after Plackett-Burman design (PBD) by *A. oryzae* A-4 (Lin, Cheng et al. 2010) . Currently, the solid state fermentation research is still in its infancy and many barriers are hindering this process from commercilization. The lipid yield is relatively low compared to submerge cultivation. Modern biotechnological approaches, such as heterogenous expression of hydrolytic enzymes and UV radiation, are available to enhance the hydrolytic enzymes production (Li, Yang et al. 2010; Awan, Tabbasam et al. 2011). Semi-solid state fermentation is used to avoid high sugar concentration on the surface of lignocellulose. An oleaginous fungus *M. isabellina* was cultured at semi-solid state fermentation with the results of 11g oil per 100g sweet sorghum (Economou, Makri et al. 2010) .

6. Cell harvest and lipid extraction

6.1 Cell harvest methods

The algae cell harvest from pond water and the subsequent water reuse have been one of the major obstacles for the algae-to-fuel approach. Microalgae cell harvest is technically challenging, especially considering the low cell densities (typically in the range of 0.3–5 g/L) of autotrophic microalgae due to limited light penetration, the small size of the oleaginous algal cells (typically in the range of 2–40 um), and their similar density to water (Li, Horsman et al. 2008). Oleaginous microalgae cells are usually suspended in the water and are hard to settle by natural gravity force due to their negative charges. The recovery of microalgae biomass generally requires one or more solid–liquid separation steps, and usually accounts for 20–30% of the total costs of production, according to one source (Uduman, Qi et al. 2010).

How to harvest microalgae cells is dependent on the characteristics of the microalgae, such as size and density(Olaizola 2003). All of the available harvest approaches, which include flocculation, flotation, centrifugal sedimentation, and filtration, have limitations for effective, cost-efficient production of biofuel (Shelef, Sukenik et al. 1984). For instance, flotation methods, based on the trapping of algae cells using dispersed micro-air bubbles, is

very limited in its technical and economic viability. Most conventional and economical separation methods such as filtration and gravitational sedimentation are widely applied in wastewater treatment facilities to harvest relatively large (>70 μm) microalgae such as *Coelastrum* and *Spirulina*. However, they cannot be used to harvest algae species approaching bacterial dimensions (<30 μm) like *Scenedesmus*, *Dunaliella*, and *Chlorella* (Brennan and Owende 2010), to which most oleaginous microalgae species belong. Centrifugation is a method widely used to recover microalgae biomass, especially small-sized algae cells; however, its application is restricted to algae cultures for high-value metabolites due to intensive energy needs and high equipment maintenance requirements. While flocculation is used to harvest small-sized microalgae cells, it is a preparatory step to aggregate the microalgae cells and increase the particle size so that other harvesting methods such as filtration, centrifugation, or gravity sedimentation can be applied (Molina Grima, Belarbi et al. 2003). Several flocculants have been developed to facilitate the aggregation of microalgae cells, including multivalent metal salts like ferric chloride ($FeCl_3$), aluminium sulphate ($Al_2(SO_4)_3$), and ferric sulphate ($Fe_2(SO_4)_3$), and organic polymers such as Chitosan (Li, Horsman et al. 2008). Chemical flocculation can be reliably used to remove small algae cells from pond water by forming large-sized (1–5 mm) flocs (Sharma, Dhuldhoya et al. 2006). However, the chemical reactions are highly sensitive to pH and the high doses of flocculants required produce large amounts of sludge and may leave a residue in the treated effluent. In summary, most technologies including chemical and mechanical methods greatly increase operational costs for algal production and are only economically feasible for production of high-value products (Park, Craggs et al. 2011).

Besides traditional methods mentioned above, there are several new technology developments in this field. DOE-ARPA-E recently funded a research project for Algae Venture Systems (AVS) to develop a Harvesting, Dewatering, and Drying (AVS-HDD) technology by using the principles of liquid adhesion and capillary action to extract water from dilute microalgae solutions. Attached algal culture systems have been developed for growing microalgae on the surface of polystyrene foam (Wilkie and Mulbry 2002) (Johnson and Wen 2010) to simplify the cell harvest. New bioflocculants, which are more environmentally friendly, are also proposed to address the cost and environmental concerns for current flocculation method (Uduman, Qi et al. 2010). All these methods are innovative and will decrease the harvest cost to some extent if developed successfully, but heavy investments on equipment and chemical supplies are still needed.

Dr. Bo Hu's research group at University of Minnesota developed an innovative approach to enhance natural algae aggregation and to encourage simple gravity settling or filtration by co-culturing filamentous fungal cells at the end of the microalgae cultures. Instead of suspended culture, this approach uses pelletized or granulized culture where cells form pellets in culture medium. In submerged cultures, many filamentous microorganisms tend to aggregate and grow as pellets/granules. They are spherical or ellipsoidal masses of hyphae with variable internal structure, ranging from loosely packed hyphae, forming "fluffy" pellets, to tightly packed, compact, dense granules (Hu and Chen 2007; Hu and Chen 2008; Hu, Zhou et al. 2009; Chunjie Xia 2011). Besides merits from the cell immobilization, there are several other advantages, especially for the micro-oil production: a). easy to harvest cells, and b). easy to re-use pond water (Johnson and Wen 2010; Xia 2011). As the first research group to introduce pelletized liquid fermentation (PLF) into biofuel production, this research group at University of Minnesota found key operational conditions that induce the fungal pelletization. They discovered that changing conditions

during cell cultivation can force fungal cells to aggregate and form pellets. This method avoids traditional approaches that use $CaCO_3$ powder to induce the fungal pelletization (Liao, Liu et al. 2007; Liu, Liao et al. 2008), which are costly and cause solid waste disposal issues. Self aggregated pelletization/granulation dramatically improves mass transfer and cell cultivation performance and facilitates cell harvest and separation. A simple filtration can be used to separate the cell biomass from the fermentation broth. This approach brings tremendous advantages to decrease the harvest cost of biofuel production, especially when the raw materials only contain very diluted sugar, (which are the cases for many agricultural waste). This would appear to be the most promising option to achieve both a high-quality treated effluent in terms of total suspended solids and economically recovering algal biomass for biofuel use (Uduman, Qi et al. 2010). It will also be more environmentally sound than current procedures which may need chemical addition.

6.2 Lipid extraction methods

Oil extraction also contributes a large part of the cost in the process to generate microbial biodiesel. Several oil extraction technologies are currently available to process the microbial biomass in order to meet the requirement of being low cost, easy and safe to operate, and environmentally friendly.

6.2.1 Mechanical methods

Mechanical methods include pressing, bead milling, and homogenization. Pressing is a technology to harvest lipids out of cells by high pressure. Bead milling works in a container to destruct the cell wall by high speed small beads. Homogenization provides a sudden pressure change when cells go through an orifice. The mechanical technologies are often used in combination with solvent methods to separate the lipid from the cell biomass. The mechanical methods are energy intensive and better operated at the high cell density condition; in addition, pretreatments are necessary to obtain high recovery ratio (Greenwell, Laurens et al. 2010).

6.2.2 Solvent extraction methods

Solvent extraction is a commonly used method for soybean processing, and it is also used to extract lipids from microbial cells. Organic solvents should be insoluble in water, be easy to obtain, have a low boiling point, and be reusable. Current industrial solvents for microlipids accumulation include hexane, chloroform, acetone, benzene, and cyclohexane, can dissolve lipid without residual cell. The extraction process is significantly affected by operation condition, such as temperature and pressure. Accelerated solvent extraction (ASE) is named when the operation temperature is higher than that of solvent boiling point, which can be used for oil extraction from dry biomass (Cooney, Young et al. 2009). Mixture chloroform and methanol (Bligh and Hyer method) is the most common organic solvent to extract oil from biomass. This organic mixture can extract oil not only from dry biomass but also from wet biomass. However, the efficiency is different at certain condition (Zhu, Zhou et al. 2002). The efficiency of oil extraction was not working well at wet *Mortierella alpina* biomass. The process generated large amounts of wastewater and solvent often contaminated the final products. Simultaneous extraction and tranesterification is more efficient (15-20%) than the separate process (Belarbi, Molina et al. 2000); however, the important point of the simultaneous process is to balance the reaction time for the best components of product (Lewis, Nichols et al. 2000).

6.2.3 Supercritical fluid extraction

Supercritical fluid extraction takes advantage that some chemicals behave as both a liquid and a gas, and have increasing solvating power when they are raised above their critical temperature and pressure points. Carbon dioxide is the most commonly used supercritical fluid, sometimes modified by co-solvents such as ethanol or methanol. Critical temperature and critical pressure of carbon dioxide is at 31°C and 74 bar, respectively (Cooney, Young et al. 2009). Supercritical fluids produce highly purified extracts without using toxic solvent; and the process is fast and safe for thermally sensitive products. Supercritical CO_2 extraction efficiency is affected by four main factors: pressure, temperature, CO_2 flow rate, and extraction time. Ethanol (10 -15%), co-solvent, lead to similar results of Bligh and Hyer method at extracting oil from *Arthrospira maxima* and *Spirulina platensis* (Mendes, Reis et al. 2006; Sajilata, Singhal et al. 2008). The limitation of supercritical fluid extraction is high capital cost and high cost for maintainence.

6.2.4 Other methods

Besides the methods mentioned above, numerous technologies are being tested at different labs to harvest lipids from cells. Genetic engineering has been applied to improve the porosity of the cell membranes in order to increase the release of lipids directly from the cells (Greenwell, Laurens et al. 2010). Enzymes treatment and pulsed electric field technology are other effective methods to break the cell wall and membrane and enhance the mass transfer across the cell membrane for oil extraction (Shah, Sharma et al. 2004; Guderjan, Elez-Martinez et al. 2007). Microwave technology is a portential pretreatment method, which heats the cell components in order to increase the release of oil. Oil yield increased from 4.8% to 17.7% from microalgae *Crypthecodinium chnii* when microwave was applied (Cravotto, Boffa et al. 2008). Microwave technology is featured for its time-savings, but its disadvantages include the oxidative damage to products and its intense energy need. Sonnication is a timely and efficient method, free of toxic materials. Cavitation occurs when high voltage is applied into cell lipids. Vapour bubbles form with negative pressure and cause a violent collapse when compressed under positive pressure while growing; then the cell contents are released (Wei, Gao et al. 2008). The sonnication is, however, difficult to scale-up.

7. Techno-economic analysis and life cycle assessment

A complete techno-econoic analysis for the microbial biodiesel production is difficult, especially considering that most of the technologies are still in the early research stage. Initial investment into microalgal biofuels has mostly failed and several early start-up companies have closed. Different versions of economic analysis for microalgae biofuel production have been published recently, and Table 4 lists an analysis conducted by Seed Science Ltd, sporsored by the British Columbia Innovation Council in Canada.

Table 4 shows that although photobioreactor has a higher cell concentration and utilizes CO_2, its cost to produce lipid is the highest of all methods. Heterotrophic fermentation, however, appears to be the most economically feasible route to produce microbial biodiesel. Techno-economic analyses may vary from different research group, but their conclusions are similar. The biomass and oil generated from heterotrophic fermentation are more close to current fossile fuel cost. Heterotrophic fermentation relies less on local climate conditions and can be carried out in close fermentors, which may facilitate their commercialization.

More effective, cost-efficient, and environmentally sound fermentation means to produce lipids are urgently needed, as well as adaptation of the fermentation cells to utilize lignocellulosic biomass. It is also widely indicated that currently microalgal biofuel systems are dependent on the production of coproducts (e.g., biochar, pigments, and nutriceuticals) for profitability. Considering the large scale of biofuel production, the market of the valuable byproducts will be the primary concern.

	Raceway		Photobioreactor		Fermentor	
Initial invertment ($/L)	52		111		2	
Production cost						
Labor cost	$4.03	26.69%	$2.96	11.90%	$0.29	10.88%
Other production cost	$3.71	24.59%	$6.37	25.59%	$2.07	78.45%
Capital cost	$7.35	48.71%	$15.56	62.50%	$0.28	10.66%
Total cost	$15.09		$24.89		$2.63	
Credit from sale of algae cake*	$0.65		0.29		$0.05	
Net total cost	$14.44		$24.60		$2.58	
Lipid content	15%		25%		50%	
Cost per kg of algae	$2.66		$7.32		$1.54	

*Assumes that the algae cake is sold to an ethanol producer for its carbohydrate content

Table 4. Cost comparison among different microalagae cultivation methods (Alabi. 2009)

8. Conclusions

Although microalgal biofuel systems theoretically have the potential to address both the food versus fuel challenges, to date no microbial biofuel system has achieved economic viability. Microbial lipid productivity must increase tremendously and the overall cost must significantly decrease before this approach can be commercially available.

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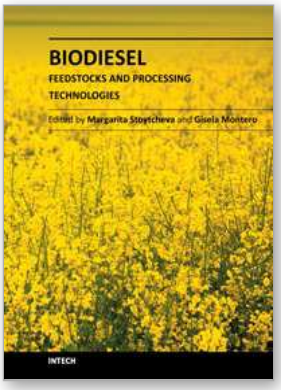

Biodiesel - Feedstocks and Processing Technologies

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The book "Biodiesel: Feedstocks and Processing Technologies" is intended to provide a professional look on the recent achievements and emerging trends in biodiesel production. It includes 22 chapters, organized in two sections. The first book section: "Feedstocks for Biodiesel Production" covers issues associated with the utilization of cost effective non-edible raw materials and wastes, and the development of biomass feedstock with physical and chemical properties that facilitate it processing to biodiesel. These include Brassicaceae spp., cooking oils, animal fat wastes, oleaginous fungi, and algae. The second book section: "Biodiesel Production Methods" is devoted to the advanced techniques for biodiesel synthesis: supercritical transesterification, microwaves, radio frequency and ultrasound techniques, reactive distillation, and optimized transesterification processes making use of solid catalysts and immobilized enzymes. The adequate and up-to-date information provided in this book should be of interest for research scientist, students, and technologists, involved in biodiesel production.

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