**LIFE CYCLE ANALYSIS OF A LARGE-SCALE LIMONENE PRODUCTION
FACILITY UTILIZING FILAMENTOUS N₂-FIXING CYANOBACTERIA**

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Abbreviations

AC: Activated carbon
AD: Anaerobic digestion
AISIM: Algae Income Simulation Model
CIP: Clean-in-place units
DAF: Dissolved air flotation
EC: Electrocoagulation
FARM: Farm-level Algae Risk Model
FU: Functional unit
GWP: Global warming potential
LCA: Life cycle analysis
mPt: Milipoint
NAABB: National Alliance for Biofuels and Bioproducts
NREL: National Renewable Energy Laboratory
PBR: Photobioreactor

Abstract

Due to the adverse effects of fossil fuel use, it is becoming increasingly important to produce next-generation biofuels from renewable, sustainable sources. Filamentous N₂-fixing strains of cyanobacteria have emerged as promising industrial microorganisms capable of producing a range biofuels and chemicals using CO₂, water, and sunlight. In this study, a life cycle analysis (LCA) was conducted on a hypothetical production facility that uses a genetically engineered strain of filamentous cyanobacteria to produce the cyclic hydrocarbon limonene. Two scenarios were evaluated in which the only difference between the scenarios was the limonene productivity by the engineered cyanobacteria strain. In Scenario 1, the cyanobacterium was assumed to produce limonene at a rate of 1.8 mg/L/h, resulting in an annual production of 32,727 L/yr of limonene. In Scenario 2, limonene productivity was 55.5 mg/L/h, resulting in annual production of 1,000,000 L/yr. Both scenarios were assumed to produce the same amount of cellular biomass, that was converted to biogas by anaerobic digestion and the biogas was

converted by gas turbines into electricity to power the facility. Excess electricity was assumed to be sold to the grid. The major environmental burdens of the facility, which were measured in eco-points and calculated based on the Eco-indicator 99 method, were the cyanobacteria nutrient supply (especially sodium nitrate) and the photobioreactor (PBR) electrical requirements. The lower output of limonene in Scenario 1 meant that less energy was required for product recovery, leaving more electricity for sale to the grid. Even though a higher limonene productivity will worsen the environmental profile of the process, both scenarios described in this study have less of a negative environmental impact than biodiesel production. This study strongly suggests that both scenarios of the theoretical limonene production facility described herein holds great potential as a future sustainable solution for producing next-generation biofuels directly from solar energy.

Keywords

Farm-level algae risk model; Filamentous cyanobacteria; Life-cycle analysis; Limonene; Next-generation biofuel

1. Introduction

Developing renewable, sustainable sources of biofuels is necessary to decrease the environmental burden created by the extensive use of fossil fuels. Fossil fuel reserves are finite and the adverse effects of fossil fuel-generated greenhouse gases are well established [1-3]. Biofuels can be categorized based on the type of feedstock used and/or the type of fuel produced. Each new generation of biofuel has been developed to overcome limitations or disadvantages of prior generations. This categorization has led to 4 generations of biofuels being defined [4].

While each biofuel type has its own advantages and disadvantages, together they have begun to decrease the burden of global fossil fuel consumption.

First generation biofuels were developed in the 1970s and 80s and consist of either: 1) ethanol produced via fermentation of sugar (primarily from sugar cane) or hydrolyzed starch (primarily from corn), or 2) biodiesel produced via trans-esterification of vegetable oil (primarily soybean oil or animal fats). The fuel ethanol process is well established and consists of feedstock pretreatment (milling, crushing, and solubilizing in water), saccharification (converting starch into sugars for the corn ethanol process), fermentation, distillation, and co-product recovery [5, 6]. First generation biofuels have three major disadvantages: production costs, market access, and competition for arable land with food crops. Because 1st generation biofuel feedstocks are also used for food, the feedstock usually accounts for more than 33% of total production costs, and this situation is unlikely to change as the world population and food demand continue to rise [7]. Second generation biofuels are typically defined as ethanol or other biofuels produced from lignocellulosic biomass, and includes a diverse range of by-products, wastes, and dedicated feedstocks [8]. The sustainability of 2nd generation biofuels is limited by land availability and competition for land use [9-12]

Due to the drawbacks associated with 1st and 2nd generation biofuels, 3rd and 4th generation biofuels have been developed. These are fuels derived from the fixation of CO₂ by photosynthetic algae and cyanobacteria [13], where the photosynthetic organism serves as both the photocatalyst and producer of biofuel [14]. At this time, algae and cyanobacteria appear to be the only sources of biofuel capable of meeting the global demand for transportation fuel [15-18]. While algal oil can potentially be used directly as a fuel, in most cases the oil is subsequently processed through traditional oil refinery or biodiesel technologies into biofuels [19-21].

Therefore, many researchers now suggest that the definition of 3rd generation biofuels be altered to photoautotrophic conversion of CO₂ into oil or algal biomass that is subsequently converted into biofuels [22]. This conversion step is a limitation to 3rd generation biofuels that does not exist with 4th generation biofuels. Fourth generation biofuel is the term used for the production of ‘drop-in’ biofuels directly from genetically engineered algae or cyanobacteria [19-21]. The benefit of using drop-in biofuels is that they can be mixed with crude derivatives without the need to develop new fuel infrastructures [23].

Heterocyst-forming filamentous cyanobacteria can fix atmospheric nitrogen, meaning that the cultivation medium does not need a combined nitrogen source, which is a considerable expense. This is one of the reasons that industrial microbiologists have focused on engineering filamentous N₂-fixing cyanobacteria to produce next-generation biofuels and high-value chemicals [24], including limonene [25], farnesene [26], myrcene [27], and linalool [28].

The biofuel producing strain of filamentous cyanobacteria evaluated in this study was previously described by Halfmann et al., [25]. In that study, a genetically engineered *Anabaena* sp. PCC 7120 (herein referred to as LimS-DXP *Anabaena*) produced limonene (Fig. 1) photosynthetically and it was postulated that this strain could be used for the large-scale production of limonene as a next-generation biofuel. However, production would need to be increased substantially before an economically feasible process could be achieved. Previously, our research group [29] performed an economic feasibility analysis on a theoretical limonene production facility which used the genetically engineered filamentous cyanobacteria as the limonene producer. This study showed that an economically feasible process is currently not possible due to the low limonene productivity of the cyanobacterial strain. However, if productivity was increased, an economically feasible process would be possible. Data from the

Halfmann et al., [25] study and our previous study [29] are used as the basis for substantial parts of this LCA study.

Figure 1: Molecular structure of limonene. (Structure drawn with ACD/ChemSketch Freeware).

The aim of this study was to evaluate the environmental profile of a hypothetical, next-generation biofuel production facility that uses genetically engineered cyanobacteria to produce limonene. To evaluate the environmental profile of the theoretical production facility, an LCA was conducted. LCA is a method commonly used to evaluate the environmental impacts of a process by quantifying resource demands, energy demands, and the resulting emissions [30]. LCAs have been commonly used to evaluate environmental profiles of algal and cyanobacterial chemical production facilities [30-32]. A cradle-to-gate strategy was applied to define the systems boundaries. Scenario 1 was defined based on a hypothetical production facility described by Johnson et al., in which 32,727 L/yr limonene was produced [29]. Scenario 2 was based on a hypothetical facility described by Halfmann, et al., that produced 1,000,000 L/yr of limonene [25]. The only difference in Scenario 1 and Scenario 2 was the total annual limonene production, due to different limonene productivities of the engineered cyanobacteria. The environmental profiles of both scenarios were then compared to the conventional production of

fossil-based diesel environmental profile. This study will provide evidence as to what the expected environmental effect of increased limonene production in the theoretical facility described below. Minimizing the negative environmental impact of this facility while maintaining economic feasibility will be essential.

2. Data and Methods

2.1. Production system overview

A process flow diagram of the limonene production process is shown in Figure 1, while Table 1 lists process inputs and parameters for both scenarios. The process includes photobioreactors (PBRs), clean-in-place units (CIPs), a limonene recovery unit, a biomass harvesting unit, an anaerobic digestion (AD) unit, and a wastewater treatment unit [33-36]. The system requires a CO₂ supply both as the primary carbon source for cyanobacteria (sodium bicarbonate from BG-11 also provides carbon) and to control culture pH [37]. In this system, a CO₂-enriched air flow will be applied for agitation and also to provide gas exchange by stripping out dissolved oxygen and limonene.

Figure 2: Process flow diagram for a cyanobacteria limonene production facility.

H: heat streams (red lines); W: clean water streams (blue lines); Nut: nutrients required for BG-11 medium;
Dashed lines (red): electricity, Eva: water evaporation; AC: activated carbon; Dashed lines (black): gas streams;
B₁: PBRs Unit, B₂: Limonene Recovery Unit, B₃: Solid Fraction Recovery Unit, B₄: Wastewater Treatment Unit, B₅:
Anaerobic Digestion Unit.

170 **Table 1: Limonene production inputs and parameters for Scenario 1 and Scenario 2.**

Process	Value (Scenario 1)	Value (Scenario 2)	Unit	Reference
<i>Adsorption unit</i>				
Limonene production capacity	32727	1000000	L/yr	
Required electricity for condensation	128	3947	kWh/yr	[54]
Electricity for adsorber unit (Adsorption)	7.2	222.0	MWh/yr	
Microwave power consumption	41.6	1282.6	MWh/yr	[74]
<i>Cultivation</i>				
Biomass production	14350	14350	kg biomass/batch ¹	
Required electricity in PBRs ^a	31.9	31.9	MWh/yr	
Log phase days per cultivation cycle	12	12	days	
Nutrients ^{b,c} required for cultivation				[62]
Sodium nitrate	87003	87003	kg/yr	
Ferric ammonium citrate	348	348	kg/yr	
Potassium phosphate dibasic anhydrous	2319	2319	kg/yr	
Magnesium sulfate	2119	2119	kg/yr	
Calcium chloride	2085	2085	kg/yr	
Citric acid	349	349	kg/yr	
Sodium carbonate	1160	1160	kg/yr	
Manganese chloride	105	105	kg/yr	
Boric acid	164	164	kg/yr	
EDTA	56	56	kg/yr	
Water consumption for cultivation	2391000	2391000	L/batch	[29]
Energy ^c required in PBR's- Cultivation broth	283	283	MWh/yr	[72]
Theoretical required CO ₂ (biomass retentate)	184441	184441	kg/yr	
Theoretical required H ₂ O(biomass retentate)	74560	74560		
Theoretical required CO ₂ (limonene)	87903	2710000	kg/yr	
Theoretical required H ₂ O(limonene)	28189	861352	kg/yr	
Energy required for supplying CO ₂	12.9	12.9	MWh/yr	[72]
<i>Harvesting</i>				
Cultivation broth ^d	159439	159439	L day ⁻¹	
Electricity required for mixing	20	20	kWh per 1000 m ³ of broth	[55]
Energy required in DAF unit	0.250	0.250	kWh kg ⁻¹ of biomass	[33]
Energy required in EC unit	0.039	0.039	kWh kg ⁻¹ of biomass	[76]
<i>Anaerobic digestion</i>				
Methane, 96 vol%	157850	157850	m ³ /yr	[87]
Biomass retentate	123000	123000	kg/yr	
Total electricity consumption	68.3	68.3	MWh/yr	[88]
Total electricity produced from biogas	943.338	943.338	MWh/yr	
Total heat produced from biogas	628.892	628.892	MWh/yr	

171 ^a: Based on 1 t/d biomass production; ^b: Considering a 50 kg/yr cut-off; ^c: Zinc sulfate, sodium molybdate, copper
172 sulfate, and cobalt nitrate were excluded from this analysis as their contribution to the environmental load was

assumed to be less than 0.05%; ^d: Energy required for pumping is included; ^e: Biomass concentration in the broth: 6 g/L.

In this study, both scenarios were defined assuming the PBRs can utilize solar light as their primary light source. Biogas produced in the AD unit is directed to the gas turbine and electricity produced from biogas is assumed to compensate for the electricity consumption of this process. In Scenario 1, electricity produced by the gas turbine is greater than the electricity consumption of the entire process and the excess electricity produced is assumed to be sold to the grid. In Scenario 2, electricity produced by the gas turbine is slightly less than the electricity consumed in the process. It is also assumed that hot gases from the gas turbine in both scenarios will be sent to a neighboring ethanol plant for energy recovery to be utilized in distillation processes. This is commonly calculated as negative heat consumption in chemical industries.

2.1.1. Cultivation

While it is possible to cultivate nitrogen-fixing filamentous strains of cyanobacteria in medium lacking combined nitrogen, the production data used in this study was derived from cyanobacteria cultivated in medium containing sodium nitrate. Thus, sodium nitrate was included in this model. A genetically engineered, limonene-producing strain of *Anabaena* sp. 7120 [25], was considered in this system. This strain contains the limonene synthase gene on a plasmid, requiring the addition of an antibiotic (100 µg/mL neomycin) to maintain plasmid retention [29]. This is not ideal due to the large cost associated with antibiotics. In an economic feasibility analysis study of this production facility, it was assumed that the gene can be integrated into the chromosome without any change in limonene production [29]. While the limonene production values may not translate because of the change in the copy number when integrating the limonene synthase gene into genome, for this LCA study productivity is assumed

to remain unchanged. The PBRs used in this system are tubular bubble columns with a pump used for gas circulation [37, 38], and will be cleaned via CIP units.

2.1.2. Limonene recovery

From an economic standpoint, limonene recovery from dilute process streams via gas-stripping may be preferred [39]. However, the gas stream exiting the PBRs would be very dilute and present a challenge for limonene recovery by simple condensation. To overcome this challenge, the gas stream is first passed through a column packed with activated carbon (AC) to absorb the limonene. After saturation, the adsorption column is heated to regenerate the AC and release the limonene into a much smaller volume of gas, which is then condensed via cold water [40]. Microwave regeneration of AC is considered in this study as it is believed to reduce downstream expenses for limonene recovery [41].

2.1.3. Biomass harvesting unit

A low-energy and low-cost method of harvesting cell biomass is essential to establish an economically feasible process. The harvesting unit should be integrated with cultivation facilities to reduce energy demands of the system. It should also have low environmental impact and low carbon or water footprint [42]. In this study, solid biomass will be recovered by the technology proposed by Richardson et al., [43]. Cultivation broth will be passed through the mixer and the settling tank that initially concentrates the cyanobacteria via autoflocculation [44]. The medium will then enter the dissolved air flotation (DAF) unit followed by the electrocoagulation (EC) unit that concentrates the slurry to a final concentration of 160 g/L [35, 45]. It has been suggested that this combination will minimize downstream costs [46].

2.1.4. Anaerobic digestion

In the proposed system described in this study, an AD unit is used to manage the large quantities of residual biomass produced and to improve the economic and energetic balance of the system. It is believed that conversion of cyanobacteria biomass into methane will allow for substantial energy recovery in the system [47]. Thus, in this process the majority of cyanobacterial biomass residue will undergo anaerobic digestion for energy recovery. This energy is considered the primary energy source in the system [48, 49], and the surplus of energy produced in the system can be sold to the grid.

2.2. LCA methodology

2.2.1. Goal and scopes

LCA modeling was carried out using SimaPro 8 software [50]. This software considers all four interrelated steps of an LCA including: “Goal and scope”, “Life cycle inventory”, “Life cycle impact assessment” and “Interpretation” according to ISO 14000 series (ISO 14041-43) [51]. Inventory data was collected from different sources, including: Greet 2014 [52], Ecoinvent 3.2 LCA database [53], technoeconomic reports [35, 54-59] and the literature [26, 29, 43-45, 60-63]. In this study, a cradle-to-gate strategy was considered to investigate the environmental profile and net energy balance of the limonene production system. A cradle-to-gate strategy means that the distribution of the produced limonene was not considered. This approach was used as the piping, pumping, and related distribution processes would be similar for both limonene and diesel. A well-to-pump strategy is often used for LCAs of transportation fuel production processes and it includes processes from the extraction of resources to delivery of

fuel at refueling stations. In this study, the distribution of limonene and diesel was considered to be similar between different scenarios, thus, a cradle-to-gate strategy was used.

Since different fuels have different heat values and efficiencies, the best approach for defining a functional unit (FU) in an LCA study consisting of different drop-in fuels is the fuel consumption of a standard car traveling a certain distance (e.g. 100 km). This approach requires the data of heat value and the efficiency of each fuel for the automobile that is going to be used. While the heat value of limonene is known, it is not currently used as fuel for automobiles, thus there is no data regarding its efficiency in diesel engines. Considering that limonene would be used in conventional diesel engines and its heat value is very similar to that of conventional diesel (10.508 kWh/L for limonene and 10.803 kWh/L for diesel), it is assumed that engine performance would be similar for the two fuels. The density of diesel is 830 kg/m³ with an energy content of 47 MJ/kg, and the density of limonene is 841 kg/m³ with an energy content of 44.98 MJ/kg. Due to these similarities, it is preferred to define the functional unit as 1 liter of limonene, which was used in this study.

2.2.2 Notable assumptions

Table 2 provides a list of notable assumptions that were considered in the different boundaries provided in Figure 1. The energy required in the process units for Scenario 1 was calculated based on 32,727 L/yr limonene production and a limonene productivity of 1.8 mg/L/h. This productivity was calculated based on a claim from Joule Unlimited, Inc. that cyanobacteria can be engineered to produce 10 mg/L/h ethanol [64]. Ethanol's molecular weight is 46 g/mole, while ethylene is 28 g/mole. Thus, the equivalent hydrocarbon productivity of 10 mg/L/h ethanol would be 6 mg/L/h. We chose a more conservative value of 1.8 mg/L/h [29] to represent a

realistic photoautotrophic productivity for limonene. In Scenario 2, energy required by the process units was calculated based on proposed limonene production by Halfmann et al. of 1,000,000 L/yr limonene and a limonene productivity of 55 mg/L/h [25]. While it is unlikely that 55 mg/L/h limonene production will be accomplished by cyanobacteria in the near-future, this will allow us to determine the effect of increased limonene production on the environmental profile of the hypothetical production facility described in this study. For cyanobacterial cultivation, a lag phase of three days was considered in which the microorganisms become accustomed to the growth medium followed by 12 days of production. Afterwards, the medium is sent for harvesting and energy recovery. Thus, a growth cycle of 15 days (22 batches per year) was considered for the production system [29]. CIP time is considered in the maintenance time required for the process, which is 35 days per year. The key area of sensitivity and uncertainty in the theoretical limonene production facility is the limonene productivity of the cyanobacteria, which is why output data is provided for two different production rates: 1.8 mg/L/h and 55.5 mg/L/h limonene as the expected margins.

The energy inputs for upstream CO₂ generation, purification, supply of turbine flue gas, and the energy required for CO₂ pumping into the system are summarized in “Energy required for supplying CO₂” in Table 1. In addition, “Cultivation broth” (Table 1) includes the energy inputs for cultivation of cyanobacteria, the energy required for recycling growth media, and the energy required for the CIP units. The energy required for anaerobic digestion and the energy required for water treatment unit are included in “electricity consumed at biogas production unit” (Table 1). “Water consumption” (Table 1) includes the required water (net) in the system, including: the evaporated water in the water treatment unit, algae pond, clarifier unit, the water dissipated in the anaerobic digestion units, and the water used to maintain volume in the PBRs.

The total biomass production is identical for Scenario 1 and 2, while the annual limonene production of the two scenarios differs. This occurs because in Scenario 2, the productivity of the cyanobacteria is assumed to be ~31 fold higher than Scenario 1, resulting in higher production of limonene from the same amount of cyanobacteria cultivated. In the study by Halfmann et al. [25] that described the development of the engineered strain of cyanobacteria evaluated in this study, biomass production, measured as chlorophyll mg/L, remained constant among strains that produced different concentrations of limonene. This was the basis of our assumption that biomass production will not decrease when limonene production increases.

In this study and our previous work, it was assumed that the PBRs receive adequate solar radiation to achieve the stated productivity values [29]. In these studies, the facility was assumed to be built in Southwestern USA and the bioreactors were assumed to require 50,000 m² of land. Per the National Renewable Energy Laboratory (NREL) USA solar map (<http://www.nrel.gov/gis/solar.html>), the average available solar energy in the Southwestern USA is 9 kWh/m²day (based on annual data). In the following calculations, we have considered a conservative estimate of solar irradiation (7.5 kWh/m²day) for that region. The solar energy provided to the whole system (50,000 m²) during the 12 days of production phase in each batch was: 7.5 kWh/m².day * (50,000) m² = 375,000 kWh/day. It was assumed that the days of productivity of the facility was 264 days out of 330 working days of the year:

$$375,000 * 264 = 99,000,000 \text{ kWh supplied to the facility.}$$

From this energy, 1,000,000 L limonene was produced from CO₂ and H₂O molecules. Considering the limonene heat value of 10.508 kWh/L, the facility captured 10,500,000 kWh, thus the solar energy conversion rate would be 10.6%.

Table 2: Notable assumptions used in this study.

Process	Assumptions	Reference
Adsorption unit	Annual limonene production: 32,433 L/yr (Scenario 1) and 1,000,000 L/yr (Scenario 2). Condenser efficiency: 90% Adsorption unit efficiency: 85%. Adsorption/Desorption period: 8:8 h/h. Activated carbon make up: 10% Activated carbon adsorption capacity: 30 % Activated carbon bed density: 480.554 kg m ³	[29] [54] [57] [57]
Cultivation	Nutrient demands for cyanobacterial cultivation: BG-11 medium chemical composition of Cyanobacteria: CH _{1.575} O _{0.459} N _{0.173} S _{0.006} P _{0.006} Productivity of strain: 1.25 kg. m ⁻³ day ⁻¹ limonene productivity: 1.8 mg.L ⁻¹ day ⁻¹ (Scenario 1) and 55.5 mg.L ⁻¹ day ⁻¹ (Scenario 2) Cyanobacteria growth cycle: 15 days. Log phase 12 days. Wastewater taken after secondary treatment Air enriched with 1% CO ₂	[62] [61] [44] [26, 60] [57]
Harvesting	Biomass concentration sent for harvesting: 6 g L ⁻¹ Settling tank: Autoflocculation: concentrates the cyanobacteria to 1% Dissolved air flotation (DAF): concentrates the cyanobacteria to 10%. Electrocoagulation unit concentrates the slurry to 16%. Dry biomass density: 1 kg L ⁻¹	[56, 89] [44] [35, 45] [84]
Energy recovery	Anaerobic digester: 0.5 m ³ methane per kg of residual biomass	[88]

2.2.3 System boundary

In this study, three different boundaries are defined including the boundary between the product system and the environment system, the boundary between the relevant and irrelevant processes (cut-off), and the boundary between the system under consideration and other product systems (allocation) [65, 66]. Here, the system boundary is considered as “cradle-to-gate” where only the energy and emissions necessary to produce the fuel are included. In some LCA studies, satisfactory results can still be obtained when excluding capital goods [67]. Figure 1 shows the boundaries defined in this study. However, capital goods may contribute up to 30% of the total

environmental impact and capital goods are included in some modern data bases (i.e. Ecoinvent and the USA Input-Output data bases) [65, 68].

2.3 *Data sources*

2.3.1 *Data collection and relating data to unit processes*

Data for the unit processes were acquired from the Farm-level Algae Risk Model (FARM) and other similar technologies. The FARM, formerly known as the Algae Income Simulation Model (AISIM), is a Monte Carlo farm level simulation model designed to simulate the annual cultivation, harvesting, extraction, and financial/economic activities of an algae farm [69]. The similarities between algae and cyanobacteria allow the model to be used for production facilities that use cyanobacteria [29]. The main difference between algal and cyanobacterial production facilities is algal systems typically produce oil that must subsequently be converted into biofuels, while cyanobacteria can be engineered to directly produce the biofuel that is excreted from the cell and volatilized into the culture headspace with minimal cell disruption. The data used to perform this LCA was composed of cultivation data, limonene recovery data, harvesting data, and data regarding energy recovery units presented in this system. Also, information was considered for five different areas: process information, materials information, equipment information, water treatment, and energy content recovery information [57].

In this study, data regarding cyanobacterial cultivation and growth were obtained from recent studies in which strains of filamentous cyanobacteria have been genetically engineered to produce limonene [25, 39]. For the cultivation phase, modified and updated data from the National Alliance for Advanced Biofuels and Bioproducts (NAABB) and the NREL, that were integrated into FARM, were the primary sources [59, 63, 70]. Other supplementary data on unit

operations was adapted from studies on algal biodiesel production and similar technologies, including pyrolysis and water treatment facilities [2, 71-73]. For limonene recovery, data was obtained from different sources for absorbers [41, 57, 58] (design factors, capacities and energy demands), a microwave regeneration unit (energy demands) [74], and the energy required for condensers [54]. For biomass harvesting, dewatering, and drying, supplementary data for energy consumption was obtained for mixing [55], the DAF unit [33, 75] and the EC units [45, 76].

2.3.2 *Software*

Previously, FARM has been used to evaluate the annual cultivation, harvesting, extraction, and financial/economic activities of an algae farm [77]. Recently, different scenarios were compared to determine the ideal strategies in algal biofuel production facilities [33, 63, 78, 79], and the most successful scenarios based on FARM were used in this LCA study. LCA software packages used in this study include Simapro 8.0 and GREET 1 2014 (for algal biodiesel and transportation) [80].

2.4 *Co-product allocation and displacement*

In energy recovery units, steam, electricity, and residual biomass from the AD are typical co-products. For systems with more than one product, ISO 14044 recommends utilization of system expansion instead of allocation when possible. As for the produced electricity and heat, the negated environmental burden can be easily quantified. In this study, the system expansion method is employed for dealing with the excess electricity and heat produced from biogas production. However, for the other co-product, the biomass retentate, it would be complicated to evaluate the avoided environmental burden via the system expansion method. In this case, the

allocation method is a better choice to deal with the biomass retentate. The produced steam, and electricity can provide the energy required for the operation and the excess electricity can be sold to the grid (considered to be sold as Electricity Low Voltage, National Average). Excess steam was considered to be sold to a neighboring ethanol plant.

In this study, 3.815 kg and 0.123 kg dry biomass retentate was considered to be produced per liter of limonene for Scenarios 1 and 2, respectively. It is assumed that the dry biomass retentate has the same economic value as dry wood chips. Conservatively, limonene is considered to have a similar economic value as diesel. Based on the economic value in the United States market [81, 82], 64% of the environmental load is allocated for limonene and 36% is allocated for biomass retentate for Scenario 1. In Scenario 2, 98% of the environmental load is allocated for limonene and 2% is allocated for solid residues from anaerobic digestion.

Allocation for the replacement of fresh water was considered to be 2% (0.4 % for Scenario 1) as the clean water from the water treatment facility was assumed to replace fresh water for cyanobacteria cultivation. Lastly, flue gas from the gas turbine was assumed to replace pure CO₂ for cultivation. CO₂ required for the cultivation of cyanobacteria is assumed to be supplied from a neighboring ethanol plant and turbine flue gas.

3. Results and Discussion

3.1 Overall energetic analysis

Table 3 summarizes the overall energy demand for unit processes, co-product allocations, and net energy demand per FU of limonene. Overall energetics of the process can be described as total energy input, net energy input, net energy balance, and net energy ratio as described below:

391 $Total\ energy = \sum sub-process\ energy\ inputs$

392 $Net\ energy\ input = Total\ energy\ input - by-product\ allocations$

393 $Net\ energy\ balance = Net\ energy\ input - energy\ in\ FU\ of\ main\ product$

394 $Net\ energy\ ratio = Net\ energy\ input / energy\ in\ FU\ of\ main\ product$

395

396 **Table 3: Energy demand for unit processes, co-product allocations, net energy demand per**

397 **FU of limonene, and CO₂ emissions allocated for limonene in Scenario 1 and Scenario 2.**

Scenario	Energy input (kWh/FU)		By-product allocation (kWh/FU)		Net energy input (kWh/FU)	
	1	2	1	2	1	2
Energy for PBRs	8.72	8.72	3.14	8.46	5.58	0.260
Electricity for PBRs	0.982	0.982	0.354	0.953	0.628	0.029
Energy for pumping CO ₂ in PBRs	0.397	0.397	0.143	0.386	0.254	0.012
Electricity for absorption unit	0.222	6.88	0.080	6.67	0.142	0.205
Electricity for microwave AC regeneration	1.28	39.7	0.462	38.5	0.821	1.18
Electricity for condensation unit	0.004	0.124	0.001	0.120	0.003	0.004
Electricity for mixing broth for harvesting	0.033	0.033	0.012	0.032	0.021	0.001
Electricity for DAF unit	2.44	2.44	0.881	2.37	1.56	0.073
Electricity for EC unit	0.382	0.382	0.138	0.371	0.244	0.011
Electricity for anaerobic digestion	2.11	2.11	0.762	2.05	1.35	0.063
Electricity from methane gas turbine	-29.2	-906	-10.5	-879	-18.7	-27.0
Steam from methane gas turbine	-19.4	-604	-7.01	-586	-12.4	-18.0
Total	-32.1	-1449	-11.5	-1405	-20.5	-43.1

398

399 Total energy and net energy input for the FU of limonene for Scenario 1 were calculated

400 as -32.147 and -20.574 kWh, respectively. Considering the limonene heat value of 10.508

401 kWh/L [83], the net energy balance was calculated as -31.082 kWh/FU. The net energy ratio of

402 Scenario 1 was -1.96. Total energy and net energy input for the FU of limonene for Scenario 2

403 were 0.423 and 0.414 kWh, respectively. The net energy balance was calculated as -10.094

404 kWh/FU. The net energy ratio of Scenario 2 was 0.04, which is substantially higher than

405 Scenario 1. This shows that Scenario 2 will consume electricity in the process, but its electricity

consumption is lower compared to algal biodiesel production technologies. These data provide evidence that by increasing limonene productivity of cyanobacteria, the overall environmental profile of the system is worsened. This is due to downstream processes of limonene being much more energy intensive compared to the downstream processes of the biomass produced. Also, by increasing the ratio of limonene to biomass, the overall energy consumption of the system increases.

The energy input for PBRs for both scenarios is identical even though volumetric productivities are different, because the cyanobacterial biomass production in both scenarios is identical. Also, most consumed energy for cultivation is solar energy, which is not considered in these values as it is supplied for free, and no corresponding environmental load is attributed. In both scenarios, identical PBRs with identical geometries and operational conditions are assumed, thus the energy required for the operation would be identical.

Figures 2 and 3 show the environmental load of each process stage based on Eco-indicator 99 for both scenarios [85]. The environmental load is measured in milipoint (mPt). mPt are a dimensionless Eco-indicator value established by the Eco-99 system, and reflect the ecological costs associated with a system or chemical. The highest mPt score correlates with the worst environmental performance [86, 87]. For Scenario 1, even though no artificial lighting was assumed, the electricity needed for the PBRs was the most significant individual contributor to the total environmental load. It was responsible for ~42% of the total energy consumption. In this technology, other downstream processes were considerably less energy intensive compared to processes that already exist in lipid based algal biodiesel production technologies. This superior energetic balance for downstream processes is the result of eliminating the process of oil extraction from the biomass, which is quite energy intensive. Thus, in this technology there is no

429 need for an energy intensive oil extraction processes as the limonene will be continuously
430 volatilized into the headspace during cultivation.

431

Figure 3: Environmental load of each process stage of Scenario 1 (A) and Scenario 2 (B). (Method: Eco-indicator 99 V2.10 / Single score / Excluding infrastructure processes).

Figure 3 shows that the negative eco-points (positive environmental load) assigned to the production of electricity and heat from biogas per FU of limonene has been drastically reduced in Scenario 2 compared to Scenario 1. Although the overall heat and electricity production of the facility remained almost identical to Scenario 1, it has been divided by a larger amount of limonene (~31-fold), and the environmental load allocated to limonene was increased from 64% to 98%. This translates into a ~20-fold reduction in the energy produced per FU allocated to limonene. This is similar to what occurs to the environmental loads caused by nutrients and electricity consumed by the PBR and biogas production units. “Allocation” of 64% for limonene in Scenario 1 means that 64% of environmental load per FU of limonene caused by each input-output category was attributed to 1 L of limonene. Using the same method in Scenario 2, ~98% of environmental load per FU was attributed to 1 L of limonene. Although the environmental load caused by the consumption of AC and electricity for the recovery unit per 1 L of limonene was identical for both scenarios, the amount allocated to 1 L of limonene was increased ~1.5-fold in Scenario 2.

Figure 4 shows the comparison of various categories of environmental loads on 3 scenarios: Scenario 1, Scenario 2, and diesel from fossil fuels. Each category was normalized to 100%. These results provide evidence that limonene production via both scenarios would be more environmentally favorable than fossil-based diesel. Scenario 1 was substantially more environmental friendly than Scenario 2 due to the environmental profile of the system becoming worse with increased limonene production.

Figure 5: Comparison of various categories of environmental loads for production of 1 L of limonene for Scenario 1 and 2 and 1 L 'Diesel, at refinery/L/US'. (Method: Eco-indicator 99 V2.10 / Characterization / Excluding infrastructure processes). (Each category is normalized to 100%).

Single score comparisons of environmental loads for the 3 scenarios is shown in Figure 5. This comparison provides more evidence that limonene production in Scenario 1 causes a substantially lower overall environmental load compared to Scenario 2. In Scenario 2, overall

electricity consumed by PBRs, the production of biomass, and electricity produced from biomass remains virtually identical to Scenario 1, yet, the production of biomass and electricity produced from biomass and electricity produced from biogas per FU has been reduced. Due to the very high energy balance of the AD and energy recovery unit, reducing the proportion of biomass production of the whole system dramatically affects the energy balance.

Figure 6: Damage assessment comparison of the environmental loads of production of 1 L of limonene for Scenario 1 and 2 and 1 L 'Diesel, at refinery/L/US'. (Method: Eco-indicator 99 V2.10 / Single score / Excluding infrastructure processes).

Figure 6 compares the global warming potential of different scenarios based on Greenhouse Gas Protocol v1.01 [53]. Results show CO₂ equivalent emissions allocated to 1 L of limonene in Scenarios 1 and 2 are equal to -16.81 and -0.435 kg/L limonene, respectively. For fossil-based diesel, it is 0.449 kg/L. The negative CO₂ equivalent emissions in Scenarios 1 and 2 indicates that in both scenarios, the amount of prevented greenhouse gas emissions (caused by electricity generation and fixing CO₂ in limonene and biomass) were higher than greenhouse gas emissions caused by the consumption of electricity and nutrients. This also provides evidence that the production of limonene from Scenarios 1 and 2 is more environmentally favorable than fossil-based diesel.

Figure 7: Comparison of global warming potential of production of 1 L of limonene for Scenario 1 and 2 and 1 L 'Diesel, at refinery/L/US'. (Method: Greenhouse Gas Protocol V1.01/CO₂ eq).

4. Conclusions

The life cycle analysis described in this study showed that production of limonene by genetically engineered filamentous cyanobacteria is less energy intensive than both fossil fuel based diesel and 3rd generation biodiesel production. Algal biodiesel production is more energy intensive due to the additional steps of drying biomass, separation of lipids, and conversion of lipids into biodiesel. Scenario 1 was found to be more environmentally friendly compared to

Scenario 2 because Scenario 1 has a higher ratio of biomass to limonene production (produced biomass per 1 L limonene in the system).

Although higher cyanobacterial productivities are more economically favorable for the production of limonene, the results of this study show that higher productivities do not necessarily improve the environmental profile of the process. This occurs because biomass production is substantially more environmental friendly than limonene production. Therefore, by increasing the ratio of limonene to biomass, the overall environmental profile of the system worsened. Thus, future research should target a more energy efficient limonene purification process. This study also strongly suggests that a limonene production facility using filamentous cyanobacteria that can use CO₂ emitted from coal-fired power plants and/or ethanol production plants as its carbon source could be a future sustainable solution for producing next-generation biofuels. Finally, this study also provided evidence that as cyanobacterial productivity of next-generation biofuels, such as limonene, are increased, there may be a decrease in the environmental profile for the entire process. It is not ideal that the environmental profile would be decreased with increased limonene production, however, increased production is necessary to achieve an economically feasible process.

A weakness of this work is that the cyanobacterial limonene productivities used are theoretical and have not been achieved yet. Targets for future research regarding the large-scale production of limonene from filamentous cyanobacteria include increasing limonene productivity from genetically engineered strains of cyanobacteria and scaling-up the cultivation vessels during limonene production. As improvements are made to either the cyanobacterial strains described in this study or the limonene production process, further modeling studies can be performed to determine what the effect of these improvements are on the environmental

profile of the process. Minimizing the environmental impact will be essential in developing next generation biofuel and high-value chemical production facilities.

Acknowledgements

This work was supported by the South Dakota Agricultural Experiment Station under grant [SD00H398-11], by NASA under award [NNX11AM03A], and by the US Department of Energy through contract [DE-EE0003046] to the National Alliance for Advanced Biofuels and Bioproducts (NAABB) and Texas A&M Agrilife Research.

Declaration of Contributions

All authors of this manuscript were involved in the conception and design of the study, acquisition and interpretation of data, and drafting the article and revising it critically.

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