

Biofuel production – tapping into microalgae despite challenges

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Abstract

Biofuels provided 2.7% of world's transportation fuel in 2015 which is expected to go up to 28% by 2050. However, most of the biofuel produced till date is from crops that can be used as food or feed. Microalgae or most famously the 3rd generation of biomass has the potential to overcome the problems associated with this food vs fuel debate. Microalgae are microscopic photosynthetic organisms which have the ability to fix CO₂. Thermochemical conversion via hydrothermal liquefaction is a favourable technology for recovering energy from algal biomass. Research is focused on discovering a viable and sustainable feedstock by cultivating and up-scaling the use of microalgae and then utilizing hydrothermal liquefaction to produce a workable biofuel. Synthetic biology and several genetic engineering techniques have also shown promising results in the production of biofuels. Plenty of research is being carried out in the field of using microalgae as biomass for biofuel generation; however, it still calls for a robust conversion technology to make the process commercially viable.

Keywords: Greenhouse gases, Biomass, Biofuels, Microalgae, Hydrothermal Liquefaction, Engineered algae, Transgenic algae, Synthetic biology

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40 1. Introduction

41 The world has witnessed the Paris Climate Conference (COP21) in December 2015. Over 195
42 countries adopted the first-ever universal, legally binding global climate deal. The agreement
43 focusses on to avoid the dangerous climate change by limiting global warming to below 2°C
44 by reducing carbon emissions and mitigating other climate changes. Since many decades, there
45 has been a global concern associated with higher fuel prices, climate variability and CO₂
46 emissions (Cuellar-Bermudez *et al.*, 2014). These are becoming more difficult to control due
47 to population explosion and consequent rise in energy needs. A further major complication is
48 the depletion of inexpensive non-renewable energy sources such as oil, diesel and ethanol
49 (Cherubini and Stromman, 2011; Franco *et al.*, 2015). Presently, transportation and energy
50 sectors are responsible for a huge proportion of greenhouse gas emissions. The global energy
51 crisis and political pressure to reduce greenhouse gas emissions has increased the volume of
52 research being carried out to discover a sustainable alternative method of producing energy
53 which is more economical and environment friendly (Cuellar-Bermudez *et al.*, 2014).

54 As a source of energy, biofuels are considered renewable through sustainable farming
55 techniques and are associated with low production costs (Franco *et al.*, 2015). The production
56 of biofuels from renewable feedstock is nothing new and has been going on from many
57 decades. The oil crisis in the 1970's led to an interest in producing and using biofuels as an
58 alternative to fossil fuels for use in transportation in many countries (Timilsina and Shrestha,
59 2011). However, the use of vegetable sugars and oil for producing biofuels (sugar beet, sugar
60 cane, corn and oily seeds) has been named as one of the main reasons for increasing food prices
61 and disputes over land use; thus the global sustainability of such a process comes under scrutiny
62 (Fung *et al.*, 2014; Franco *et al.*, 2015). The possible environmental benefits that could be
63 gained by replacing petroleum fuels with bioenergy and biofuels obtained from renewable
64 biomass sources remain the primary motivators for advancing the manufacture and use of
65 bioenergy and ls (Von Doderer and Kleynhans, 2014). **Table 1** shows the different types of
66 biomass used and the conversion technologies adopted so far. Biomass viz. plant waste and
67 microalgae is regarded as one of the most positive alternatives to conventional feedstock as it
68 is the only renewable source of fixed carbon that converts into solid, gaseous heat and liquid,
69 fuel and power (Jahirul *et al.*, 2012). The carbon sequestration during biomass growth and
70 ensuing release of carbon during the combustion process in the form of CO₂ can be regarded
71 as a carbon neutral part of the bioenergy system (Von Doderer and Kleynhans, 2014).

72 Biofuels can be classified as natural biofuels, first generation, second generation and third
73 generation biofuels (Noraini *et al.*, 2014).

74 Natural biofuels are usually obtained from organic sources such as animal waste, vegetables
75 and landfill gas. These are used for heating, cooking, brick kiln or electricity production. The
76 technologies used to produce biofuels are dependent on the type of feedstock used. The first
77 generation group comprises of the technologies that utilize the starch or sugar elements of
78 edible plants; sugar beet cereals, sugar cane and cascara being used as feedstock to produce
79 ethanol and those that use rapeseed, sunflower, oilseed crops, palm oil and soy bean to make
80 biodiesel (Timilsina and Shrestha, 2011; Noraini *et al.*, 2014). Second generation biofuels are
81 produced using technologies that convert fervent lignocellulose biomass such as *Sterculia*
82 *foetida*, *Ceiba pentandra*, *Miscanthus*, jatropha, switch grass, poplar forest and agricultural
83 residues into usable biofuels (Peters, *et al.*, 2011; Noraini *et al.*, 2014). Biofuels produced from
84 more advanced feedstock such as jatropha and microalgae are also included in the second
85 generation group (Timilsina and Shrestha, 2011). Thermochemical conversion by fast

86 pyrolysis is one of the most efficient methods of producing biofuels from the lignocellulosic
87 biomass. The acquired pyrolysis oil is a high density and moderate heat value liquid that can
88 be upgraded in a biorefinery into diesel and gasoline blend stocks (Peters et al., 2011). Third
89 generation biofuels use macro and micro algae as feedstock and have been widely accepted as
90 a potentially viable alternative energy source.

91 First generation biofuels are still commercially produced; however, despite the advantages of
92 biomass that can be used for second generation biofuel production, higher yields and lesser
93 requirement for land use, they are not presently being commercially produced due to lack of
94 efficient technologies (Noraini et al., 2014).

95 There is enormous research going on to examine the role of microalgae in biofuel production.
96 In recent years, microalgae have become a popular feedstock for triacylglycerol, neutral lipid
97 storage and biodiesel production (De Bhowmick, Koduru and Sen, 2015). By genetically
98 engineering the microalgae used in biofuel production, the target product yield could be
99 increased, improving the viability of the technology.

100

101 The present review gives an overview of this field of research throwing light on the global
102 concerns in bioenergy sector and if microalgae is the answer to the biomass required for biofuel
103 generation. Biotechnological approaches, their challenges and prospective opportunities have
104 been discussed in this review. Despite vast research in this field it seems an inexpensive and
105 commercially viable technology to convert microalgae into biofuel is yet to be discovered.

106 **2. Increasing energy demands – global concerns**

107 The global transportation sector is responsible for producing approximately 15% of all
108 greenhouse emissions, over 70% originating from road transportation (Soimakallio and
109 Koponen, 2011). The use of diesel and gasoline fossil fuels is set to double in the next 25 years
110 and, therefore, greenhouse gas emissions will certainly increase vastly unless preventative
111 measures are put in place (Soimakallio and Koponen, 2011). The world energy needs can be
112 predicted to increase by 44% from 2006 to 2030 (Cherubini and Stromman, 2011). This has
113 been made evident by the 4th Assessment Report of the Intergovernmental Panel on Climate
114 Change, IPCC, which reveals that the growing use of fossil fuels coupled with the current
115 population growth has resulted in a rapid increase in greenhouse gas emissions.

116 Environmental concerns are that every year the earth's atmosphere is subjected to more than
117 15 billion tonnes of CO₂. Fossil fuel combustion is a major contributor to the increase in the
118 levels of CO₂, which is a direct cause of global warming (Kolar and Civas, 2013). Worldwide,
119 oceans annually absorb approximately one-third of all CO₂ produced from human activity
120 (Cuellar-Bermudez et al., 2014). The continuous rise in the amount of CO₂ present in the
121 atmosphere increases the amount that is absorbed into oceans, which is an environmental
122 concern. This gradually changes the pH of the water making it more acidic and precipitates
123 immediate losses to the ecosystem diversity of both marine life and coral reefs (Li and Gao,
124 2012). If CO₂ production continues at the present rate there are huge implications for ocean life
125 and consequential effects to earth life (Cuellar-Bermudez et al., 2014).

126 Bioenergy has some advantages as it has an almost closed CO₂ cycle that produces only small
127 amounts of greenhouse gas emissions. However, there are some disadvantages to using
128 bioenergy as an alternative fuel source. The conversion processes are dependent on the addition
129 of external fossil fuels to produce and harvest feedstock, to handle and process biomass, to fuel

130 bioenergy plants and they are also required in the transportation of the feedstock and biofuels
131 (Von Doderer and Kleynhans, 2014).

132 Biofuels have also been identified as one of the principal reasons behind the increased food
133 prices. As the industrial use of biofuels has increased so has the need for agricultural land to
134 produce the feedstock (Kim *et al.*, 2013). Even if the land usage problem is solved there are
135 still key issues in relation to the use of crops to produce biofuels as a long-term alternative to
136 petroleum including inadequate scalability, minimal net energy reductions and insignificant
137 reduction in the production of greenhouse gases (Quinn *et al.*, 2014). These will need to be
138 investigated thoroughly and be overcome if crop biofuels are to be a future sustainable option.

139 There also have been some feasibility studies conducted globally to encourage the use of
140 biofuels. Brazil increased its national ethanol programme following a peak in oil prices in 1979.
141 The US launched a corn-based ethanol programme at the same time but on a much smaller
142 scale. China, Kenya and Zimbabwe were also motivated to try and produce biofuels but failed
143 (Timilsina and Shrestha, 2011). An example of where this type of feasibility study has been
144 implemented successfully is in Malaysia. Historically this is a country that produces huge
145 amounts of palm oil that was exported and sold to other countries in order to generate an income
146 from an otherwise surplus commodity. In 2011 a mandatory implementation of a programme
147 to use its palm oil yield to produce biodiesel for the transportation sector was ordered to meet
148 the countries contribution to carbon reduction and biofuel sustainability (Masjuki *et al.*, 2013).

149 Concerns over greenhouse gas emissions and the potential for rapid increases in petroleum
150 prices due to the limitations in supply-demand has activated a global search for an alternative
151 transport fuel and a high efficiency conversion technology that can achieve the maximum
152 motive power out of chemical fuels (Bergthorsen and Thomson, 2015). To reduce and limit the
153 levels of global greenhouse gases below the current 550 ppm CO₂ equivalent would require
154 huge emission reductions and would result in a total phase out of all fossil fuel emissions in
155 developed countries by 2050 (Ullah *et al.*, 2014).

156 All the data indicate that with the outburst in population, the demand for food and fuel is bound
157 to increase in the coming years. Therefore, an out-and-out sturdy technology where the biomass
158 such as microalgae can be inexpensively and conveniently converted into biofuel is the need
159 of the hour.

160 **3. Microalgae - the ultimate solution?**

161 Since the 1950's attempts have been made to extract fuels from algae and there has been
162 significant investment made worldwide, particularly by the military, the aviation industry and
163 energy companies. Large scale commercial production is only just starting to emerge, the
164 primary issue is whether the production of biofuels from algae is commercially viable (Benson
165 *et al.*, 2014). Several life cycle assessments (LCA) have been performed to evaluate the
166 microalgae biomass to biofuel and bio-product possibilities on a conceptual level, based on a
167 range of different approaches and methods. LCA are focused on determining the severity of an
168 environmental impact due to the production of microalgae-based biofuels (Benson *et al.*, 2014).
169 LCA are critical to validate usable technological innovation, with lower energy intensities and
170 improved environmental performance (Grierson *et al.*, 2013).

171 Microalgae are aquatic as well as terrestrial species and are photosynthetic microorganisms
172 that convert water, sunlight and carbon dioxide into biofuels, feed, food and high-value

173 bioactive compounds (Li and Savage, 2013; Chen *et al.*, 2014a). Autotrophic algae cultivation
174 can be performed in either enclosed photo-reactors or open pond raceways; however photo-
175 reactors are usually seen as too expensive for large-scale production of biofuels (Handler *et al.*,
176 2014). The use of microalgae in large-scale production of biofuels is inhibited by expense and
177 feasibility. Microalgae that store lipids are usually unicellular and found in suspensions with
178 low densities making separation problematic (Rawat *et al.*, 2013). One solution could be to
179 utilize all the constituent parts of the microalgae. The carbohydrate and lipid content is
180 approximately 70% and has several applications including bio-oil, bio-hydrogen, bio-ethanol,
181 bio-methane, plastics, fertilizers, nutrients, sorbents and animal feed (Rizwan *et al.*, 2015). The
182 use of pure strains or cultures causes problems in industrial applications due to contamination
183 therefore the utilization of mixed indigenous microalgae cultures could be a potential solution
184 with commercial capability (Cea-Barcia *et al.*, 2014).

185 Freshwater macroalgae, a largely overlooked class of phototrophic microorganisms, can show
186 high rates of areal productivity and usually form either substrate-attached turfs, or closely
187 packed floating mats that could mean huge reductions in the cost of harvesting and dewatering
188 compared to microalgae (Yun *et al.*, 2015). Typically macroalgal cultivation is synonymous
189 with seaweed growth and harvesting, over 16 million dry tonnes are produced yearly
190 worldwide (Yun *et al.*, 2015). Despite the economic and environmental advantages of using
191 macroalgae biomass to produce biofuels there remains several challenges that need to be
192 addressed. One such challenge is that macroalgae contains unique carbohydrates that means
193 the conventional biomass conversion process to produce biofuel cannot be utilized (Jung *et al.*,
194 2013).

195 Autotrophic microalgae are able to use carbon dioxide and solar energy to synthesize proteins
196 and lipids enabling them to grow. The production of biodiesel from autotrophic microalgae
197 mostly occurs in indoor photo-bioreactors. However autotrophic microalgae depends heavily
198 on light for photosynthesis resulting in higher energy outputs for illumination, a requirement
199 for shallow cultivation systems with large surface areas (Kim *et al.*, 2015; Mohan *et al.*, 2015).
200 In comparison there is a lot more flexibility in heterotrophic microalgae culturing as they can
201 grow without the addition of a light source and are capable of storing higher lipid contents in
202 their cells (Zhang *et al.*, 2013). In heterotrophic nutritional mode, microalgae use organic
203 molecules as their main carbon and energy source which assists in high biomass yields and
204 makes large-scale production much more feasible. The relative simplicity of operations, easy
205 maintenance and cost effectiveness are the primary benefits to heterotrophic microalgae
206 culturing (Mohan *et al.*, 2015).

207 A variety of biomass conversion technologies have been investigated in an effort to use
208 microalgae to produce biofuels commercially. Technologies for the extraction and conversion
209 of biomass include hydrothermal liquefaction (HTL), pyrolysis and lipid extraction. Two
210 thermochemical technologies, slow pyrolysis and HTL have been successful experimentally in
211 the conversion of microalgae into bio-oil. Both slow pyrolysis and HTL have the potential to
212 be used but there has been limited assessment of industrial-scale feasibility and the
213 environmental impact (Bennion *et al.*, 2015). HTL converts biomass into liquid fuels by
214 thermal conversion, operating in heated pressurized water conditions for a long period to break
215 down the hard polymeric structure into mostly liquid components. The process allows wet
216 materials to be treated without having to dry them first and to achieve ionic reaction conditions
217 by preserving a liquid water-processing medium (Elliot *et al.*, 2015).
218

219 Undoubtedly the algae-to-biofuel conversion is not an affordable and trivial process and has
220 several challenges. It certainly has limited market as of now but the majority of research in this
221 regard has shown promising consequences. There are several ongoing projects which when
222 completed seem to have a game-changing influence in this area. An overview of research
223 councils and companies which have invested in the micro and macro algae projects is shown
224 in [Table 2](#).

225

226 **4. Complications and opportunities in converting microalgae to biofuel to its scale-up**

227 Transportation fuels and energy industry are responsible for producing the majority of all
228 energy related emissions. Currently renewable energy only contributes about 11% to global
229 primary energy, although it is predicted that 60% of all energy will originate from renewable
230 sources by 2070 ([Ullah et al., 2014](#)). The first developments in discovering effective biofuels
231 for transportation purposes were based on the established process of converting plant sugars
232 into ethanol by fermentation and the upgrading of vegetable oils by trans-esterification
233 ([Bergthorson and Thomson, 2015](#)). Globally it is expected that there will be a rise in the
234 production and use of biofuels. But the overall contribution to the total energy demands,
235 particularly in the transport sector, will continue to be limited. This is mainly due to the
236 competition with fibre and food production for arable land use, lack of appropriately governed
237 agricultural practices in emerging markets, regionally constrained market structures and the
238 necessity for bio-diversity conservation ([Noraini et al., 2014](#)).

239 Current research is focused primarily on discovering a viable sustainable feedstock to produce
240 biofuels, upscaling the use of certain types of microalgae and then utilizing hydrothermal
241 liquefaction to produce a workable biofuel. There are negative and positive factors to be
242 considered for the upscaling of algal cultivation especially to the marine and coastal
243 environments ([Coelho et al., 2014](#)). Presently the technology required to make each stage of
244 the process economically feasible viz. microalgae cultivation, harvesting, transport,
245 pretreatment and successful conversion of biomass into high yield biofuels, has not yet been
246 discovered ([Coelho et al., 2014](#)).

247 For the successful mass production of biofuel from microalgae cultivation the problems that
248 would need to be resolved are locating the large amounts of fresh water needed, obtaining
249 enough nutrient sources of nitrogen, phosphorus and trace elements and over-coming the
250 shortage of cost effective and energy efficient procedures for the harvesting of algal biomass,
251 oil extraction and conversion. There is also a need for fully developed and tested technologies
252 to deal with CO₂ mitigation from microalgae and a system integration and evaluation ([Zhou et
253 al., 2014](#)). Algal cultivation could hypothetically provide a sustainable feedstock and has the
254 potential for CO₂ remediation when the microalgae biomass reaches a higher CO₂ fixation than
255 that of terrestrial biomass if these initial problems are addressed ([Coelho et al., 2014](#)).

256 Also, during the conversion process organic nitrogen transforms into ammonia in a reducing
257 environment and NO_x in an oxidising/combustible environment. During the production of
258 biogas the substantial levels of nitrogen biomass content causes ammonia toxicity throughout
259 the anaerobic process and may impede the bacterial decomposition of algal biomass. A prime
260 concern regarding this process is that nitrogen in biomass will also produce NO_x molecules
261 throughout the gasification process, which is performed with a limited oxygen supply, resulting
262 in NO_x release into the atmosphere. This is an environmental concern as it has greenhouse gas
263 properties and thus the requirement for the implementation of rigid emission regulations
264 ([Garcia-Moscosa et al., 2013](#)). Initial problems with developing the microalgae industry at

265 large scale are the massive installation and continuous operating costs, robustness of the strains,
266 quality of lipid for the production of biodiesels, the loss in lipid content during scale-up and
267 the difficulty managing the conditions of cultures, particularly outdoor cultivation (Ahmad *et*
268 *al.*, 2013; Yen *et al.*, 2014).

269 **Figure 1** shows an experimental new alternative to traditional conversion methods.
270 Thermochemical conversion via hydrothermal liquefaction (HTL) has a strong potential for
271 commercial production as it seamlessly merges with existing petroleum refining infrastructure
272 (Liu *et al.*, 2013). During the HTL process drenched algae biomass with a 85-90% water
273 content is transformed through temperature reactions and high pressure into four process
274 streams; non-aqueous bio-crude, made mainly of fatty acids, long chain alkanes and phenolic
275 compounds, an aqueous phase comprising of organic acids and nearly all of the phosphorous
276 and nitrogen in the biomass (30 – 50% wt.), a gas phase that contains CH₄ and CO₂ and the
277 other volatile compounds (1 – 8% wt.) and a solid phase comprising mainly of bio-char (-3%
278 wt.) (Liu *et al.*, 2013). The main advantage of this technique is that it is not a threat to food
279 crop production as it is a simple cultivation process in open sea (Anastasakis and Ross, 2015).

280 Most biomass can be processed by HTL due to its hydrothermal nature and the adequate ease
281 in producing water slurries from biomass particles at pump able concentrations usually about
282 5-35% dry-solids (Elliot *et al.*, 2015). High-moisture microalgae biomass often requires some
283 dewatering which helps in lowering costs of processing excess water. Using HTL to process
284 microalgae has various advantages over conventional methods as it can tolerate low cell
285 concentrations and allows conversion of low-lipid strains that usually have higher growth rates
286 than those optimized to acquire high lipid levels (Jazrawi *et al.*, 2015). The long-term
287 environmental and societal effects of biofuel and bioenergy production have certain concerns
288 associated with them that need to be overcome if a more sustainable global energy and fuel
289 source is to be discovered (Seay and Badurdeen, 2014).

290 There are a significant number of economic and technical challenges associated with the usage
291 of microalgae in the biofuels industry. Harvesting microalgae is a major problem. The
292 unicellular algae that stores lipids have low densities and are located in suspensions making
293 separations laborious. The extraction processes used for large-scale production are particularly
294 complex and are still in the early development stages (Rawat *et al.*, 2013). Microalgae
295 cultivated in open pond systems are prone to contamination. Bacterial contamination
296 aggressively competes for nutrients and oxidises the organic matter, which can lead to culture
297 putrefaction. They are also susceptible to protozoa and zooplankton grazers that consume
298 microalgae and may destroy the concentrations of algae in a short time (Rawat *et al.*, 2013). In
299 open pond systems there is also loss of water through evaporation and in order to maintain a
300 fixed volume and salinity in the culture it is necessary to add large quantities of freshwater
301 (Das *et al.*, 2015).

302 Other challenges that inhibit the commercialization of algal based biofuel production include;
303 difficulties in finding rapid growing algae strains with high oil content, photosynthetic
304 efficiency, simple algae culture harvesting systems, infrastructure, operation and maintenance
305 costs and the ability to develop economical photo-bioreactor designs (Adenle *et al.*, 2013).

306 **5. Genetics to Synthetic Biology – approaches and their challenges**

307 The use of microalgae in biotechnology has the potential to revolutionise the field, this potential
308 increases with the utilisation of transgenic or genetically modified algal strains (Rosenberg *et*

309 *al.*, 2008). Both genetic engineering and lately synthetic biology techniques have been
310 deployed to produce biofuels from microalgae.

311 How genetic engineering is applied in various processes can be polarising. Many genetic
312 engineering processes are considered the norm; such as therapeutic protein production,
313 however processes such as genetically modified crops or laboratory grown meat are much more
314 controversial. With the current demands for food and fuel for a growing population, the
315 economical production of algal biomass to be used in the fuel industry has placed focus on the
316 use of engineered algae (Henley *et al.*, 2013).

317 Transgenic or engineered algae can be produced through various methods. The well-
318 documented ones are transformation using electroporation and using *Agrobacterium*
319 *tumefaciens*. The second highly used approach is biolistics.

320 Previously the lipid production of *Phaeodactylum tricornutum* has been improved through
321 genetic modification; specifically the enhanced expression of *Phaeodactylum tricornutum*
322 Malic enzyme (PtME) by Xue *et al.*, (2015). Transformation in the Xue *et al.*, (2015) study
323 was accomplished through the use of electroporation. The process involved running a pulse of
324 electricity through the host cell to disrupt the cell membrane to allow for the introduction of
325 new genetic information. The resulting lipid yields increase 2.5-fold to a record 57.8% of dry
326 cell weight. Furthermore the growth rate of the cells is similar to that of the wild type. Neutral
327 lipid content increases by 31% in a nitrogen-deprived environment; a 66% improvement when
328 compared to the wild type. This could prolong the production of lipids in an environment where
329 a wild type algal species would reduce its lipid production due to nutrient restrictions. The
330 study commented on the ability to optimise electroporation by the management of plasmid
331 amount, concentrations of osmosis solution, the duration of the pulse and the voltage used to
332 create the pulse (Guo *et al.*, 2013), essential for the creation of effective genetically engineered
333 microalgae.

334 The utilization of *Agrobacterium tumefaciens* as a transformation method is a common
335 approach and is the most efficient method to transform plant cells (Sanitha *et al.*, 2014). This
336 transformation can occur in nuclear DNA or chloroplast DNA (Cheng *et al.*, 2012). The tumour
337 inducing (Ti) plasmid found in *Agrobacterium tumefaciens* is empirical to the introduction of
338 any gene of interest into the genome, specifically the segment of DNA known as the T-DNA
339 and its accompanying flanking regions (Lee *et al.*, 2012). T-DNA, can be replaced with the
340 gene of interest; such as a gene involved in lipid or carbohydrate synthesis for biofuel
341 production, and be used to manipulate the algal species used in biofuel production. A binary
342 plasmid approach; whereby the genetic information is split over two plasmids (Lee *et al.*,
343 2012), a T-DNA plasmid and a helper plasmid can be used to overcome issues such as limited
344 restriction sites and difficulty in recovery due to the size of the engineered plasmid (Cheng *et*
345 *al.*, 2012). The expression can be tailored through the use of promoters. An inducible promoter
346 will allow the lipid expression to be linked to a specific action such as a metabolic function.
347 The inclusion of a constitutive promoter allows for continuous expression of the gene of
348 interest. Figure 2 provides an example of how a binary plasmid, genetically engineered to
349 provide desirable traits, can be introduced via *A. tumefaciens*.

350 Biolistic particle delivery system or a gene gun provides an approach that circumvents the need
351 for marker genes. It is used for selection in other methods of genetic engineering (Bertalan *et*
352 *al.*, 2015). This process involves microscopic beads of an inert metal such as gold coated with
353 the genetic information that is to be incorporated into the genome. Biolistics nullifies the

354 obstacles of the cell wall and cell membrane as the gold is fired through these barriers at high
355 velocity into the cytoplasm of the cell, allowing for incorporation into the chloroplast or nuclear
356 DNA through the inclusion of homologous regions of DNA sequence (Martin-Ortigosa *et al.*,
357 2012b).

358 The use of genetically engineered microalgae to enhance the production of biofuel has been an
359 area of interest for scientists from a long time. The process of genetically enhancing algae
360 improves the yield of the final product however; it has its challenges which must be addressed
361 to assess the commercial viability of its use in biofuel production (Rawat *et al.*, 2013).

362 Studies have also been carried out to maximise photosynthetic ability of microalgae by
363 reducing the size of chlorophyll antenna; which has been shown to result in more efficient use
364 of light resulting in increased productivity (Sutherland *et al.*, 2015). However genetic
365 engineering can come with drawbacks, in this case the reduction in antenna size causes a
366 reduced ability for the cell to dissipate any excess photon energy which can cause susceptibility
367 to photo-damage (Simionato *et al.*, 2013). The processes involved in the reduction in
368 chlorophyll antenna involve gene knockout, however the addition of genes of interest through
369 methods such as *A. tumefaciens* also has difficulties; such as gene silencing or little to no
370 expression of target gene. This can be as a result of the compatibility with the host genome;
371 including usage of codons not reflecting the plants bias, premature poly-adenylation sites or
372 mRNA interference and the stresses that factors like these induce (Moshelion and Altman
373 2015).

374 The approaches known as the “omics” have made a significant contribution to the
375 understanding of the molecular processes of microalgae. Furthermore, the discoveries that
376 omics studies have made; such as the identification of genes involved in specific processes,
377 may be vital to engineering of enhanced microalgae (Winck, Melo and Barrios, 2013). To
378 assess the expression levels of the genes involved transcriptomics can be utilised, this involves
379 the sequence information gathered from reverse transcribed mRNA that is extracted from the
380 algal sample (Vanwonderghem *et al.*, 2014). The results will show gene expression *in situ* and
381 provide an understanding of expression rates and allow for optimisation of the target product.
382 Through the understanding of the levels of transcription and the gene activation data gathered
383 from transcriptomics, the effectiveness of the genetic alteration can be measured. Should the
384 new gene insert be operating at its optimum then the transcriptomics data should show an
385 increase in the mRNA of the target gene when compared to the wild type (if an increase in
386 output is the aim).

387
388 Apart from genetics, microalgae are commonly put under stress conditions such as temperature,
389 nutrient starvation or pH to enhance production of a target product such as lipids or
390 carbohydrates (Ho *et al.*, 2014). The result of the introduction of stress conditions is the
391 alteration of lipid synthesis pathways in many microalgae (Rawat *et al.*, 2013), a feature of
392 great interest to biofuel production. Omics techniques can again provide valuable insight into
393 this process. Metabolomics assesses the low molecular metabolite end products and are
394 indicative of response to stresses (Jamers, Blust and De Coen, 2009). A combination approach
395 would allow for optimisation of algal engineering, as the data gathered from transcriptomics
396 should show an increase in transcription in the gene of interest that coincides with a reduction
397 in metabolism caused by stress such as nutrient limitation; highlighted by metabolomics,
398 should the expression of the gene of interest be linked to a metabolism process. The application
399 of omics studies can not only ascertain the effectiveness of any genetic modification but can
400 also be used to optimise the scale up process. With the use of spatial and temporal omics studies

401 of systems such as raceways, used for algal growth, a deeper understanding of how algae will
402 perform in varying areas of the raceway can be gained allowing for process optimisation.

403 The technology for small-scale commercial cultivation of microalgae to produce nutraceutical
404 products and animal feed is already available, however the commercial production of biofuel
405 from algae still seems like a farfetched dream. There are many doubts and technical challenges
406 associated with large-scale algae biofuel manufacturing. As well as the long-term physical
407 impact on ecosystem health by the commercialization of open pond cultivation, the use of
408 genetically modified algae for biofuel production can affect the sustainability of the regional
409 ecosystem. This is particularly important in developing countries as the introduction of
410 invasive foreign species can endanger biodiversity. The appropriate experiments and clear
411 independent assessments should be used to evaluate genetically modified algae opportunities
412 and risks, especially in regard to regulatory issues and biosafety (Adenle *et al.*, 2013).

413
414 In the recent years, synthetic biology has made biology easier for genetic engineers. Using the
415 tools of synthetic biology the algal strains have been designed as per the environmental
416 conditions and yield requirements. Synthetic biologists are assembling genetic materials and
417 working on the manipulation of lipid content of the microalgae, along with the biomass
418 accumulation and increasing biofuel production. The promising results are surely going to
419 change the fate of biofuels industry from microalgae for better in the near future.

420 **6. Applications of microalgae**

421 Due to the increase in consumer concerns over the use of chemicals as ingredients in cosmetics
422 there has been a higher demand for more natural and environmentally sustainable products.
423 Microalgae biomass has a considerable market value as researchers have recently discovered
424 that compounds derived from algae, particularly those that express immune response, anti-
425 inflammatory and antibiotic potency, can be utilized in the production of cosmetics such as
426 anti-aging supplements and colouring pigments (Koller *et al.*, 2014; Wang *et al.*, 2015). The
427 phylogenetically archaic cyanobacteria produce material containing polyunsaturated fatty
428 acids (PUFA), anti-oxidative agents, heat induced proteins or immunologically effective and
429 viro-static compounds that could also be used in the production of cosmetics (Wang *et al.*,
430 2015). Marine algae have recently attracted attention in the search for natural tyrosinase
431 inhibitors that have skin whitening properties. Tyrosinase catalyses two separate reactions in
432 the synthesis of melanin; the hydroxylation of *L*-tyrosine to 3,4-dihydroxy-*L*-phenylalanine (*L*-
433 dopa) and the oxidation of *L*-dopa to dopaquinone, following further conversion into melanin.
434 Exposure to the sun increases the synthesis of both melanosomes, which mature into melanin
435 and tyrosinase. Melanin is transported to keratinocytes and degradation occurs to encourage
436 skin melanisation and tanning. Therefore the depletion of melanin by desquamation can
437 remove a tan (Wang *et al.*, 2015).

438 The rising cost of fodders has resulted in the use of microalgae in poultry aquaculture by adding
439 a specific amount into poultry rations for the commercial production of animal feed.
440 Microalgae biomass is suitable for food and feed as it is rich in proteins and minerals; it also
441 contains beneficial compounds such as enzymes, pigments, lipids that contain high value fatty
442 acids, sugars, vitamins (riboflavin, thiamine, niacin, pantothenic acid, *inter alia* β -carotene,
443 biotin, folic acid and pyridoxine) and sterols (Koller *et al.*, 2014).

444 *Scenedesmus almeriensis* is currently used to feed farmed sea bream and can be used to partly
445 replace fishmeal in practical diets (Zhu, 2015). The nutritional value of some microalgae

446 species is rich due to the high quality of their intrinsic proteins that are often of a better quality
447 than some common vegetable proteins (Das *et al.*, 2015). In addition to these proteins
448 microalgae also contain other cell components including simple sugar carbohydrates, peptides,
449 lipids, vitamins, pigments, minerals and trace elements (Das *et al.*, 2015).

450 Recent developments and findings from a life cycle assessment (LCA) have shown that
451 microalgae have a huge potential for producing and overcoming a lot of the problems
452 associated with long-term bioenergy production (Quinn *et al.*, 2014). The potential for
453 microalgae to be used to produce certain biofuels comes from the organisms' high efficiency,
454 productivity and the capacity for CO₂ fixation maximising production (Gerde *et al.*, 2013).
455 They also have ten times greater photosynthetic efficiency than land plants and produce larger
456 lipid and biomass content which equates to 5-50% of dry biomass (Ahmad *et al.*, 2013).
457 Microalgae's lipid content is very important as this is used to produce biodiesel through
458 transesterification (Garcia-Moscosa *et al.*, 2013). The nitrogen content of microalgae is
459 approximately 4-8 wt% depending on nutrient availability and the algae's physiological state.

460 **Concluding Remarks**

461 Biofuels remain the most environment friendly and practical solution to the global fuel crisis
462 however further research is needed to discover an effective, cheap, sustainable biomass and a
463 method of conversion that does not produce harmful emissions and is not reliant on the addition
464 of fossil fuels.

465 Microalgae have the potential to be used to produce certain biofuels without the controversial
466 issues associated with land use, the environment and sustainability. There is lot of focus on the
467 possibility of thermal conversion using hydrothermal liquefaction (HTL) to transform
468 microalgae biomass into usable biofuels. The feasibility of up-scaling a microalgae cultivation
469 system requires testing especially in terms of economic viability and product yield. The use of
470 microalgae in biotechnology certainly has the possibility to transform the field, this potential
471 increases with the utilisation of transgenic algal strains (Rosenberg *et al.*, 2008). The success
472 stories w.r.t. synthetic biology and genetically engineering microalgae indicate a bright future
473 for the biofuels industry. There already are several big players in the business of generation of
474 biofuels from microalgae in the USA i.e. Solazyme, Sapphire Energy, PetroSun, Joule
475 Unlimited, Green Fuel Technologies Corporation, Global Green Algae, Gevo, Algenol. In
476 Europe, there are: Powerfuel.de (Germany), Alpha Biotech (France), Algae-farms (Greece),
477 AlgaeLink (Spain), and Varicon Aqua Solutions Ltd and British Algoil Ltd (UK). All these
478 companies are already producing commercial scale biodiesel, bioethanol, algal oil, hydrogen,
479 and aviation fuel from algae. There is massive amount of research going on in this field, but
480 relaxing the legislation w.r.t growing genetically modified algae in open ponds and attracting
481 more innovative projects is the need of this field. Coming up with vigorous and cost-
482 competitive conversion technologies would be staggeringly beneficial to the biofuel industry
483 and to humankind in the long run.

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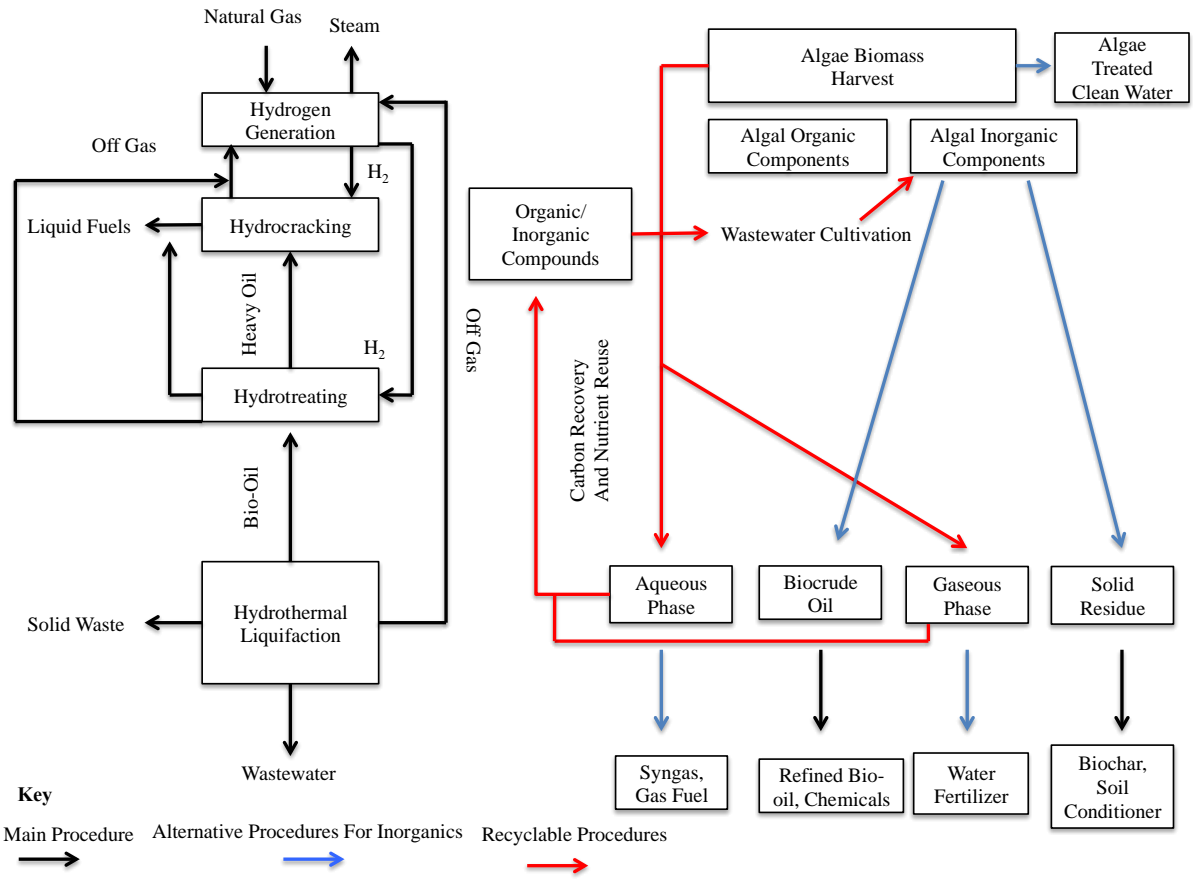
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710 **Figure 1.** The main procedures involved in hydrothermal liquefaction (HTL) conversion of
 711 algal biomass into usable biofuels (Li *et al.*, 2014a; Tian *et al.*, 2014; Zhu *et al.*, 2013).

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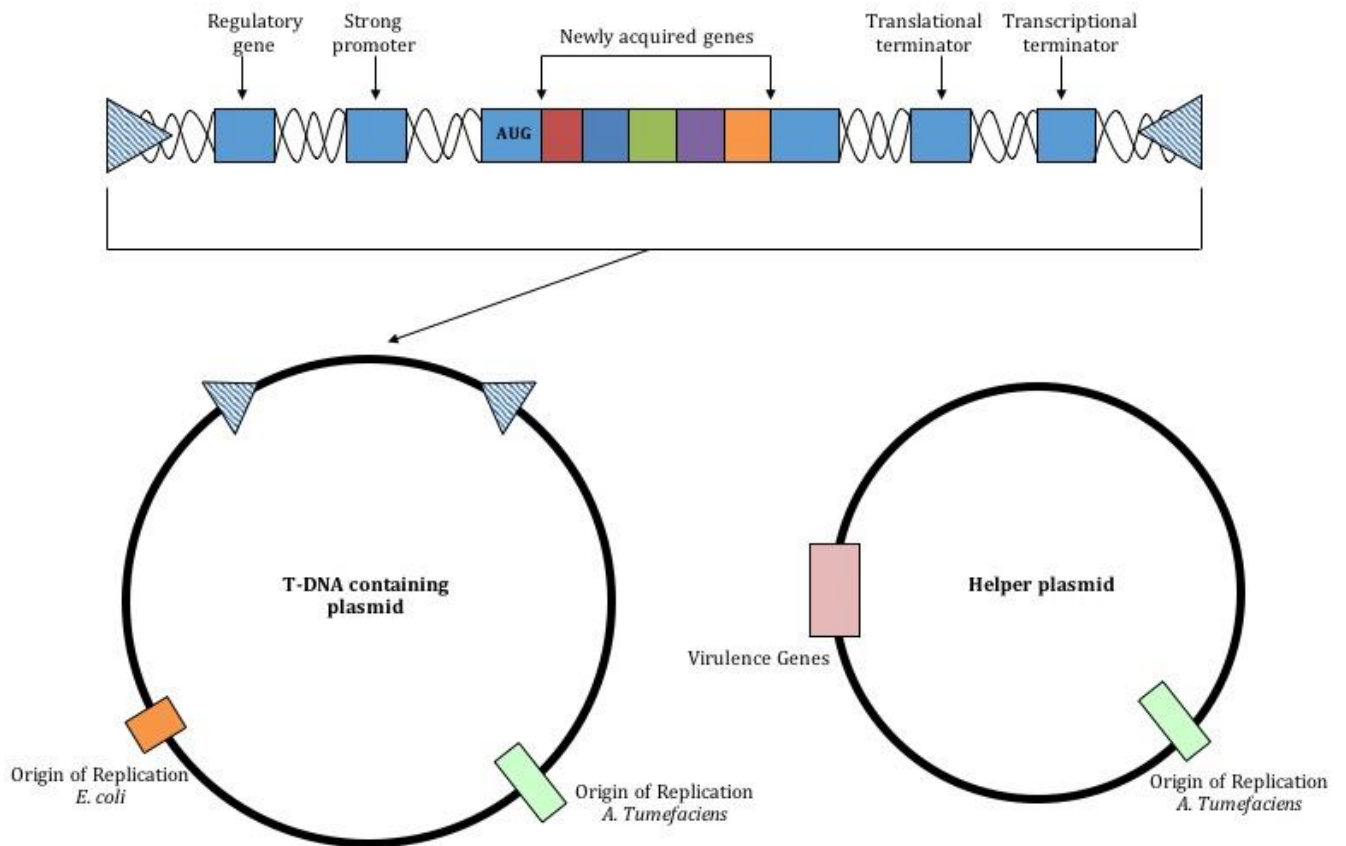
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Figure 2. A schematic diagram of how a plasmid containing T-DNA can be modified to accomplish genetic engineering of microalgae. The AUG start codon proceeds the region of newly acquired genes (genes of interest/target genes). Regulatory genes allow for the linking to a cellular function such as metabolism, and the inclusion of a strong promoter will increase transcription rates, termination sequences must also be included. The use of an origin of replication in *E. coli* allows for the use of this organism as a vector due to its ease of culturing. *vir* genes are found in the genome of *A. tumefaciens* and allow for the incorporation of T-DNA into the host genome.

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748 **Table 1.** Types of biomass and conversion technologies researched so far.

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Biomass	Conversion	Reference
Firewood	Combustion	<i>Guo et al., 2015</i>
Wood Chips	Combustion	<i>Esteban et al., 2015;</i> <i>Guo et al., 2015</i>
Charcoal	High Pressurised Palletisation	<i>Mwampamba et al., 2013;</i> <i>Guo et al., 2015</i>
Microalgae	Microalgae Fermentation	<i>Chen et al., 2015</i>
Municipal Solid Waste	Hydrothermal Conversion	<i>Zhao et al., 2014</i>
Microalgae	Transesterification	<i>Chen et al., 2015</i>
Non-edible Oilseed Jatropha	Heat Conversion and Palletisation	<i>Doshi et al., 2014</i>
Non-edible/ Edible Vegetable Oils, Waste Cooking Oils and Animal Fats	Direct Use and Blending Transesterification/Micro-emulsions and Pyrolysis	<i>Adewale et al., 2015</i>
Karanja Defatted Residue	Heat Conversion and Palletisation	<i>Doshi et al., 2014</i>
Microalgae	Hydrothermal Liquefaction	<i>Chen et al., 2015</i>
Lignocellulosic Materials	Acid Hydrolysis/Pre-Treatment and Enzymatic Hydrolysis	<i>Guo et al., 2012</i>
Sweet Sorghum	Advanced Solid State Fermentation	<i>Li et al., 2014b; Yu et al., 2014</i>
Sugar Cane, Sugar Beet, Sweet Sorghum, Corn Wheat, Barley, Potato Yam and Cassava	Fermentation, Distillation and Dehydration Process	<i>Guo et al., 2015</i>
Landfills and Wastewater Treatment Plants	Anaerobic Digestion of Organic Waste	<i>Surita and Tansel, 2015</i>
Coal Derived from Wood Pellets and Sawdust	Pyrolysis or Gasification and Torrefication	<i>Dudynski et al., 2015;</i> <i>Guo et al., 2015</i>
Microalgae	Anaerobic Digestion	<i>Allen et al., 2015</i>

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Table 2. An overview of the micro and macro algae projects underway at various institutes.

Research Councils/Companies	Research Institutes	Research Area	Reference
Netherlands- based AkzoNobel and US bioproduct company Solarzyme (2014)	Partnership research	A multi-year supply deal of up to 10,000 tonne/year of tailored algal oils. Oil will replace petroleum and palm-oil derived chemicals	Chemistry and Industry (London), 2014
BBSRC	Durham University and the Institute of Chemical Technology	Investigating the use of Green macro-algae found along UK coastlines to convert into usable biofuel. Harnessing the natural processes by which seaweeds are broken down in order to make use of enzymes and microbes that are capable of converting the seaweed biomass into advanced biofuels	BBSRC (2015)
BBSRC/DBT	The University of Sheffield and Bharathidasan University	Smaller water dwelling 'microalgae' to convert solar energy and carbon dioxide into the precursors of fuel	
BBSRC/DBT	Sustainable bioenergy and Biofuels (SuBB) initiative funding £4m	Renewable and sustainable fuel alternatives using microalgae/macroalgae	Algae Industry magazine (2013)
RCUK/BBSRC	University College London	Genetic engineering of the algal chloroplast to produce therapeutic proteins Development of genetic strategies to improve biofuel production from cyanobacteria and algae	UCL Algae Biotechnology (2015)
Natural Environment Council (NERC)	Algal Bioenergy Special Interest Group (AB-SIG)	Development of synthetic biology tools for metabolic engineering of algae Regulation of organelle gene expression by nuclear-encoded factors	
Innovate UK	Cardiff University (School Of Biosciences), University of Southampton (water Engineering Group)	To understand the opportunities and risks of the quality of freshwater and marine environments of using algal biomass as a source of renewable energy	NERC (2015)
PHYCONET (BBSRC NIBB)	Institute of structural & Molecular Biology, London	Development of a hybrid culture system for biomass production of "premium quality microalgae" for aquaculture and agriculture industry using wastewater in desert coastal areas	Innovate-UK-GOV.UK (2015)
Australian Energy Market Operator (AEMO)	Clean Energy Council (CEC)	From January 2014 continuing over the next five years their focus is on producing high value products from microalgae and cyanobacteria industrially cultured in a controlled and intensive system using photobioreactor and fermenter-based technologies	PHYCONET (2015)
		Investigated two possible futures in 2030 and 2050 by investigating the potential expense and feasibility of fuelling the electricity generation system using renewable fuels only.	<i>Azad et al., 2015</i>