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Research review paper

Dual purpose microalgae–bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a Biorefinery

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ABSTRACT

Excess greenhouse gas emissions and the concomitant effect on global warming have become significant environmental, social and economic threats. In this context, the development of renewable, carbon-neutral and economically feasible biofuels is a driving force for innovation worldwide. A lot of effort has been put into developing biodiesel from microalgae. However, there are still a number of technological, market and policy barriers that are serious obstacles to the economic feasibility and competitiveness of such biofuels. Conversely, there are also a number of business opportunities if the production of such alternative biofuel becomes part of a larger integrated system following the Biorefinery strategy. In this case, other biofuels and chemical products of high added value are produced, contributing to an overall enhancement of the economic viability of the whole integrated system. Additionally, dual purpose microalgae–bacteria-based systems for treating wastewater and production of biofuels and chemical products significantly contribute to a substantial saving in the overall cost of microalgae biomass production. These types of systems could help to improve the competitiveness of biodiesel production from microalgae, according to some recent Life Cycle Analysis studies. Furthermore, they do not compete for fresh water resources for agricultural purposes and add value to treating the wastewater itself. This work reviews the most recent and relevant information about these types of dual purpose systems. Several aspects related to the treatment of municipal and animal wastewater with simultaneous recovery of microalgae with potential for biodiesel production are discussed. The use of pre-treated waste or anaerobic effluents from digested waste as nutrient additives for weak wastewater is reviewed. Isolation and screening of microalgae/cyanobacteria or their consortia from various wastewater streams, and studies related to population dynamics in mixed cultures, are highlighted as very relevant fields of research. The species selection may depend on various factors, such as the biomass and lipid productivity of each strain, the characteristics of the wastewater, the original habitat of the strain and the climatic conditions in the treatment plant, among others. Some alternative technologies aimed at harvesting biomass at a low cost, such as cell immobilization, biofilm formation, flocculation and bio-flocculation, are also reviewed. Finally, a Biorefinery design is presented that integrates the treatment of municipal wastewater with the recovery of oleaginous microalgae, together with the use of seawater supplemented with anaerobically digested piggery waste for cultivating *Arthrospira* (*Spirulina*) and producing biogas, biodiesel, hydrogen and other high added value products. Such strategies offer new opportunities for the cost-effective and competitive production of biofuels along with valuable non-fuel products.

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1. Introduction

1.1. The strategy of producing biodiesel from microalgae

The International Panel on Climate Change has concluded that our planet's sustainability relies heavily on our capacity to generate enough renewable clean energy to satisfy future generations' demands (IPCC, 2007). Thus, many current sustainability issues, such as greenhouse gas emissions, climate change, fossil fuel depletion and energy security, can be mitigated (Subhadra, 2010). However, there are many practical challenges associated with the large-scale production of renewable energy. The primary constraint in future energy scenarios is the land and water resources required to harvest, grow or process the potential feedstock (Subhadra, 2011).

Using oleaginous microalgae to produce biodiesel has several advantages for producing renewable energy, making it the most promising biofuel option. Among the most important advantages are: a) oleaginous microalgae have an oil yield much higher than that of oleaginous plants; b) this biofuel has a small ecologic footprint because it requires less surface area compared to conventional crops; c) some oleaginous microalgae can be cultivated in seawater or brackish water (Table 1). Additionally, the fresh water species can be cultivated in municipal wastewater, avoiding competition for fresh water that is used to irrigate crops; d) microalgae are excellent at capturing CO$_2$, fixing 183 tons per every 100 tons of produced biomass; and e) biodiesel from microalgae is one of the very few biofuels with negative CO$_2$ emissions (-183 kg CO$_2$ MJ^{-1}) (Chisti, 2007, 2008).

Furthermore, there have been substantial efforts worldwide to produce renewable biofuels, resulting in an overwhelming amount of information in this field. Some recent reviews have offered an in-depth discussion of several issues within this topic (A. Singh et al., 2011; Greenwell et al., 2010; Lee, 2011; Loera-Quezada and Olguín, 2010; Mata et al., 2010; McGinn et al., 2011; Norsker et al., 2011; Park et al., 2011; Schenk et al., 2008; Singh and Olsen, 2011; Wijffels et al., 2010) and are therefore outside the scope of this review.

In addition to the interest expressed by academic and government entities in renewable energy technologies, private entities have also been created to explore these alternative strategies (Christenson and Sims, 2011). However, significant obstacles (Lam and Lee, 2012; Singh and Gu, 2010; Tredici, 2010) still need to be overcome before microalgae–based biofuel production becomes cost-effective and can impact the world's supply of transport fuel. Several recommendations have been made recently to overcome the economic constraints of microalgae production on a large scale. Among the most relevant

Table 1
Oil content and lipid productivity of some microalgae species (Loera-Quezada and Olguín, 2010).

Species	Oil content (% dry weight)	Lipid productivity (mg L^{-1} d^{-1})	Reference
Parietochloris incisa (f)	60[a]	N.R.	Solovchenko et al. (2008)
Nannochloropsis sp. (m)	60[a]	204	Rodolfi et al. (2009)
Neochloris oleoabundans (f)	56[a]	13.22	Gouveia et al. (2009)
Chlorella vulgaris (f)	~42[a]	12.77	Widjaja et al. (2009)
Crypthecodinium cohnii (m)	41.14[a]	82	Mendoza et al. (2008)
Scenedesmus obliquus (f)	43[b]	N.R.	Mandal and Mallick (2009)
Neochloris oleoabundans (f)	38[c]	133	Li et al. (2008)
Nannochloropsis sp. (m)	28.7[c]	90	Gouveia and Oliveira (2009)
Chlorella vulgaris (f)	27[c]	127.2	Francisco et al. (2010)
Nannochloropsis oculata (m)	30.7[c]	151	Chiu et al. (2009)
Dunaliella (m)	67[c]	33.5	Takagi et al. (2006)
Choricystis minor (f)	21.3[c]	82	Mazzuca-Sobczuka and Chisti (2010)
Chlorella protothecoides (f)	50.3[d]	N.R.	Xiong et al. (2008)
Chlorella vulgaris (f)	21[d]	54	Liang et al. (2009)
Scenedesmus rubescens (m)	73[e]	N.R.	Matsunaga et al. (2009)

(f) = freshwater; (m) = marine; N.R. = not reported.
[a] Cultured under nitrogen starvation.
[b] Cultured under nitrogen deficiency.
[c] Cultured with nutrient sufficiency.
[d] Heterotrophic culture.
[e] Nutrient starvation.

suggestions are: a) to recover the nutrients found in wastewater to cultivate the microalgae at a low cost with the additional benefit of eliminating pollutants from the environment (Park et al., 2011; Pittman et al., 2011); b) to combine the production of microalgae for biofuels with the production of bulk chemicals, food and feed ingredients (Wijffels and Barbosa, 2010); c) to use a biorefinery-based production strategies (Chisti, 2007; Milledge, 2010; Singh and Gu, 2010; Subhadra, 2010); d) to integrate microalgae cultivation with fish-farms, food processing facilities and wastewater treatment plants (A. Singh et al., 2011); e) to improve the capabilities of microalgae through genetic engineering and advances in engineering of photobioreactors (Chisti, 2007); f) to carry out more research to understand and potentially manipulate algal lipid metabolism (Greenwell et al., 2010); g) to apply a multidisciplinary approach in which systems biology, metabolic modeling, strain development, photobioreactor design and operation, scale-up, biorefining, integrated production chain, and the whole system design (including logistics) are considered (Wijffels et al., 2010); h) to consider anaerobic fermentation for biogas production as a final step in future microalgae-based biorefinery strategies (Mussgnug et al., 2010); i) to integrate the co-digestion of microalgae with wastewater sludge for biogas production (Kumar et al., 2010); j) to optimize the algal production and harvest from wastewater treatment High Rate Algal Ponds (HRAPs) because they are presently considered by many research groups as the most economically viable way to produce algal biomass for converting waste to biofuels with minimal environmental impact (Park et al., 2011); and k) to significantly improve the efficiency, cost structure and ability to scale up algal biomass production, lipid extraction, and biofuel production (Singh and Olsen, 2011).

In agreement with some of these recommended strategies, this review focuses on dual purpose systems aimed at treating wastewater with microalgae that are potential biofuel sources and that can also produce other non-fuel products within a Biorefinery strategy.

1.2. The strategy of using dual purpose microalgae–bacteria-based systems for treating wastewater and for producing biodiesel

Dual purpose systems that use microalgae for treating wastewater and producing biodiesel and chemical products are gaining popularity and are an attractive alternative to microalgae-based systems aimed solely at biodiesel production. Pittman et al. (2011) recently found that dual-use microalgae cultivation for wastewater treatment coupled with biofuel generation is an attractive option for reducing energy, fertilizer and freshwater costs, as well as reducing greenhouse gas emissions. Furthermore, Park et al. (2011) showed that the costs of algal production and harvesting using wastewater treatment in High Rate Aeration Ponds are essentially covered by the wastewater treatment plant capital and operation costs and thus have significantly less environmental impact, in terms of their water footprint, energy and fertilizer use, compared to cultivation systems that use freshwater and fertilizer. A multi-national group of researchers (Kumar et al., 2010) also noted that microalgae-mediated CO_2 fixation and biofuel production can become more sustainable by coupling microalgal biomass production with existing power generation and wastewater treatment infrastructures. Furthermore, cultivating microalgae consumes more commercial fertilizers compared to most common oleaginous plants. For instance, microalgae cultivation shows an N-fertilizer consumption in the range of 0.29 to 0.37 kg/kg oil, which is higher than that for *Jatropha* (0.24 kg/kg oil) and is nearly ten times higher than that for oil palm (0.048 kg/kg oil) (Lam and Lee, 2012).

Although there are some who argue that using wastewater as a source of nutrients poses contamination risks and that using fertilizer and freshwater should be preferred, some recently published Life Cycle Analysis (LCA) have confirmed that the use of wastewater for biofuel production with microalgae is a very useful approach to ensure the economic viability and the sustainability of the whole biofuel production process. LCA is a systems approach aimed at evaluating the environmental burden associated with the entire life cycle of the product of interest (e.g., microalgae) to avoid problem-shifting between life cycle stages and to identify technological innovation opportunities (Kumar et al., 2010). Another definition, ISO 10440, establishes that LCA is a "compilation and evaluation of the inputs and outputs and the potential environmental impacts of a product system throughout its life cycle" (Pfromm et al., 2011).

Lardon et al. (2009) carried out a LCA to assess the energetic balance and the potential environmental impacts of the whole process chain, from biomass production to biodiesel combustion. The key objective of this study was to identify the obstacles and limitations that should receive specific research efforts to make this process environmentally sustainable. The results indicated the necessity of decreasing the energy and fertilizer consumption of the process. Clarens et al. (2010) provided a stochastic life cycle model of algae cultivation processes compared to other biofuel feedstocks, namely, switchgrass, corn and canola, to understand which biofeedstocks produced the most biomass energy with the lowest environmental burden. Their results indicated that algae cultivation using freshwater and fertilizer addition had higher environmental impacts than the other feedstocks in terms of the energy use, greenhouse gas emissions and water consumption. These authors concluded that the large environmental footprint of algae cultivation is driven predominantly by upstream impacts, such as the demand for CO_2 and fertilizer. They recommended that flue gas and, more importantly, wastewater could be used to offset most of the environmental burdens associated with algae. Another Life Cycle Analysis focused mainly on the water and nutrient demands for microalgae-based biodiesel production (J. Yang et al., 2011) and found that there was a 90% reduction in the use of freshwater when wastewater was used to cultivate the microalgae. Furthermore, the required nitrogen was reduced by 94%, and the need for added potassium, magnesium, and sulfur from fertilizer was reduced 100% by replacing freshwater with wastewater. The same study indicated the usefulness of water recycling and established that 3726 kg water is required to generate 1 kg microalgae biodiesel if freshwater is used without recycling.

The results from other recent Life Cycle Analysis studies have also confirmed that substitutes for fertilizers currently used for cultivating the microalgae are needed and that this cultivation could easily include the production of co-products to improve the Energy Balance Ratio and the sustainability of the whole process. A LCA in which the energy and carbon intensity of the whole process of producing microalgae-based biodiesel was evaluated (Shirvani et al., 2011), indicated that current algae biodiesel production is 2.5 times as energy intensive as conventional diesel because energy input is both directly and indirectly needed for the production of fertilizers, ponds, and harvesting facilities, as well as transport. The authors concluded that the production costs can be partially lowered by displacing costly grid heat and electricity through the usage of oilcake residues via a combined heat-and-power unit and the use of glycerol as a livestock feed. The carbon footprint of the algae-to-biodiesel carbon cycle can only be minimized through the successful decarbonization of the heat and electricity grid and the sourcing of all indirect energy requirements for fertilizers, transport and building materials from low-carbon energy sources. Furthermore, the reliance on fossil-based CO_2 from power plants or fertilizer production renders algae diesel unsustainable in the long term based on a thorough mass balance approach to assess the sustainability of biodiesel production from microalgae (Pfromm et al., 2011).

1.3. The Biorefinery strategy

According to the International Energy Agency (IEA, 2008), a Biorefinery has been defined as "the sustainable processing of biomass into a spectrum of marketable products and energy". Additionally, a biorefinery may be defined as a facility that integrates biomass conversion processes and equipment to produce fuels, power, materials and/or chemicals from biomass (Cherubini, 2010; Singh and Gu, 2010). An extensive discussion of the biorefinery concept was recently published, emphasizing the integration of green chemistry (Cherubini, 2010) and of microalgae-based biorefinery and the wide spectrum of possible products (Singh and Gu, 2010; Subhadra, 2010).

This work reviews the most recent and relevant information related to the application of the biorefinery strategy using microalgae and cyanobacteria for treating wastewater to simultaneously produce biofuels and high added value products. Detailed information regarding several aspects of the treatment of municipal and animal wastewater with microalgae is provided. Additionally, some alternative technologies aimed at harvesting the biomass at a low cost are also reviewed because this step is one of the most common economic constraints in producing microalgae as a source of biodiesel. Finally, a dual purpose system is proposed that treats municipal wastewater with oleaginous microalgae and in separate raceways treats the effluents from anaerobically digested piggery wastewater using a well-known species, such as *Arthrospira*, to enhance the production of high added value products.

2. Treatment of municipal wastewater with microalgae–bacteria-based systems

The work of Oswald and his group (Oswald, 1963, 1988, 1995) in California more than six decades ago established the foundation for developing High Rate Oxidation Ponds (HROPs) to treat municipal wastewater while producing microalgae as a valuable sub-product. Modern developments use this type of open, shallow pond, also known as a raceway, to commercially produce certain microalgae and also to perform phycoremediation in general (Li et al., 2011a; Olguín, 2003; Park et al., 2011). In early work, the main objective of recovering microalgae was to use them as a source of feed and other chemical products (Sandbank, 1982). Currently, due to the urgent need for alternative and sustainable sources of biofuels, dual purpose wastewater treatment systems are considered one of the most promising strategies for producing microalgae to overcome the current economic viability limitations of large-scale processes for producing biodiesel from microalgae (Park et al., 2011; Pittman et al., 2011). This section reviews the more recent developments in this field and also highlights previous work, which may help to answer some of the questions driving most of the current research work.

2.1. Are there enough nutrients in municipal wastewater to support high microalgae productivity?

Several reports have focused on using certain fractions of municipal wastewater, trying to demonstrate that high biomass productivities can be achieved. Researchers have used settled raw sewage, primary settled sewage, activated sewage, primary clarifier effluent, secondarily treated sewage and a fraction derived from the activated sludge thickening process (Li et al., 2011a). The following section provides a summary of the most recent efforts to evaluate various types of wastewater for maximum biomass production.

Typical concentrations of ammonia nitrogen and phosphates in secondary-treated wastewater fall into the ranges of 20–40 mg L^{-1} and 1–10 mg L^{-1}, respectively, which are adequate to support high productivities from most fresh water microalgae strains (McGinn et al., 2011). However, the inorganic N/P ratio varies with the different fractions of wastewater within a municipal wastewater treatment plant (Wang et al., 2010). Those fractions collected before and after the primary settling showed N/P ratios of 5.9 and 4.7, respectively, while a range of approximately 6.8–10 is considered optimal. Conversely, the N/P ratio of the effluent from the activated sludge process was 53.2, much higher than the optimal ratio, indicating a high phosphorus limitation. On the other hand, the N/P ratio of the centrate (the liquid from the activated sludge thickening process) was 0.36, much lower than the optimal ratio, indicating a high nitrogen limitation. Although there were some nutrient limitations, the specific growth rate of the microbial population observed in each fraction was in the range of 0.343 to 0.948 day^{-1} and was the lowest for the secondary effluent and the highest for the centrate. Wang et al. (2010) concluded that the centrate is the best wastewater fraction within a treatment plant for achieving higher microalgae biomass productivity, while also removing a high percentage of nutrients.

Following the recommendation on using centrate, a very recent work (Li et al., 2011b) implements this innovative approach. According to these authors, centrate is rich in nutrients, including phosphorus, ammonium, and organic matter, reflected in a COD of approximately 1300 mg L^{-1}. They showed that a *Chlorella* strain isolated from wastewater was able to reach a productivity of 0.92 g $L^{-1}d^{-1}$ in continuous cultures on a bench scale. In batch cultures, the *Chlorella* strain showed a biomass yield of approximately 1.1 g L^{-1} and a biodiesel yield of 0.12 g L^{-1} of algae culture, with a fatty acid methyl ester (FAME) content of 11% of dry biomass. These productivities are comparable or higher than those previously reported for other fractions of municipal sewage.

Supplementation with CO_2 in the municipal wastewater is expected to increase the algal biomass productivity. In fact, Woertz et al. (2009) found that when they sparged CO_2 into semi-continuous cultures of a microalgae consortium cultivated in primary clarifier effluent, the algal biomass, estimated as the Volatile Suspended Solids (VSS), increased from 317 to 812 mg/L, and the lipid productivity increased from 9.7 to 24.4 mg L^{-1} d^{-1} in cultures operated at a Hydraulic Retention Time (HRT) (i.e., a measure of the average length of time that a soluble compound remains in a bioreactor) of 3 days. On a large scale, the external source of CO_2 should be inexpensive. There have been several proposals that suggest that microalgae cultivation plants or biorefineries should take advantage of residual CO_2 from thermo electrical plants (Van den Hende et al., 2011). When this strategy is not feasible, considering the costs of cleaning the exhaust gases, another alternative is to provide CO_2 from biogas (a mixture of CO_2 and methane) as proposed in Section 3.1.

The use of treated wastewater requires additional nutrients to support algae growth. Supplementing wastewater with 5 mM $NaNO_3$ has been recommended for maximal biomass productivity of *Chlorella* spp. cultivated in post-chlorinated wastewater, while supplementation with 25 mM of $NaNO_3$ was required to achieve a high increase in the lipid yield (Mutanda et al., 2011). Thus, using treated wastewater requires adding extra nutrients. Other sources of low-cost nutrients could be added as suggested in the next section to avoid using fertilizers.

2.2. Which feedstocks can serve as nutrient supplements for weak wastewater?

Section 1.2 mentioned that adding commercial fertilizers as a source of nutrients for microalgae cultivation increases the cost of the biomass to such a level that biodiesel production becomes non-competitive and unsustainable. Thus, other sources of nutrients that could be used as additives for weak wastewater should be selected according to the geographical location and the specific needs of the microalgae or cyanobacteria cultivation system. Apart from the need for CO_2 as a carbon source, the two more important nutrients required for microalgae growth are N and P. Their ratio should be close to the optimum nitrogen-to-phosphorus stoichiometry encountered in phytoplankton, which has been described to fall in the range 8–45 (Klausmeier et al., 2004). The chemical composition of various high organic strength and nutrient content wastewaters (Table 2) indicates that their N/P ratio is adequate for promoting microalgae growth,

Table 2
Composition of different wastewaters with high organic strength and nutrient content.

Source	COD	BOD	TN	TP	N/P	NH_4-N	Reference
Soybean processing wastewater	5000 –16,300	2250–8000	1700–2550	125–183	13.6–13.9	71–140	Zhu et al. (2012)
Sugar cane vinasses	100,000–150,000	– –	600–4200	100–3800	1.10–6	– –	Tang et al. (2006)
Sugar cane stillage	135,867	61,350	2975	238.50	12.5	432.50	Olguín et al. (2008)
Piggery wastewater	17,640	– –	– –	58.5[a]	– –	1931	Patil et al. (2010)
Meat processing wastewater	1544	646	– –	– –	– –	– –	Wahaab and El-Awady (1999)

All parameters units are mg L^{-1}.
[a] Soluble phosphorus.

although it is possible that dilution or pre-treatment could be necessary to avoid inhibiting growth by turbidity or high organic matter content. Furthermore, the preferred pretreatment of this type of wastewater is anaerobic digestion (Olguín, 2000), with the advantage of obtaining biogas, which serves as source of methane as well as source of CO_2. The latter gas can be fed into microalgae cultivation ponds/photobioreactors, with the additional advantage of increasing the productivity, as mentioned above. The chemical composition of anaerobic effluents (digestate) from various digested wastes also indicates an adequate N/P ratio (Table 3). One of the most important advantages of using anaerobic effluents as additives for weak wastewater is that they are also a source of dissolved CO_2 in the form of bicarbonate (Table 3). In fact, various kinetic parameters of the cultivated microalgae species indicate that the use of anaerobic effluents provided sufficient N and P, as well as additional nutrients, including bicarbonate. Furthermore, anaerobic effluents could also serve as a source of organic acids for the heterotrophic growth of microalgae. The anaerobic effluents from stillage or distillage wastewater from ethanol produced from cassava contain acetic acid (2.0 to 4.0 g/L), propionic acid (1.0 to 3.0 g L^{-1}) and butyric acid (0.4 to 2.5 g L^{-1}) (Zhang et al., 2010).

Finally, highly polluted urban rivers, which contain mainly organic matter and small concentrations of toxic or recalcitrant pollutants, could be used as another source of water for microalgae cultivation. One group is researching using anaerobic effluents of diluted raw wastes as the source of additional nutrients to enrich the water from an urban polluted river in Mexico (Olguín et al., 2010) for cultivating microalgae/bacteria consortia with the potential for biofuel production (Olguín et al., unpublished results). A similar approach using waste-enriched seawater could be harnessed to cultivate marine microalgae with the potential for biodiesel production.

2.3. How efficient are microalgae–bacteria-based systems at removing nutrients?

The economic viability of the dual purpose systems within a Biorefinery depends on several factors; one major factor is the efficiency of the microalgae–bacteria systems at removing nutrients and another important one is how much these factors contribute to the cost-benefit analysis.

The Hydraulic Retention Time (HRT) determines both the nitrogen removal efficiency and the distribution of nitrogen forms in the effluent of a High Rate Oxidation Pond treating municipal sewage (García et al., 2000). These authors reported that the annual average nitrogen removal was 73% for ponds operating at a higher HRT, compared to a removal of 57% observed at a lower HRT. They also concluded that the main removal mechanism for ammonia nitrogen was stripping, due to the high pH, followed by algae uptake.

Wang et al. (2010) have compiled data from early work performed with settled domestic sewage and secondary-treated domestic effluent supplemented with settled swine wastewater and reported that in all of these cases, the N removal efficiency was in the range of 92–95% although the phosphate removal efficiency was lower, approximately 62–80%. In this study, various fractions of the sewage in a MSTP were compared (mentioned in Section 2.1). The removal rates of N-NH_4 were 82.4%, 74.7%, and 78.3% for wastewaters before and after the primary settler (stream 1 and 2) and after the sludge centrifugation (stream 4), respectively, by a *Chlorella* sp. strain. However, these authors did not find N-NH_4 removal in the effluent from the activated sludge tank (stream 3), but they registered a 6.3-fold increase in the NO_2-N concentration, indicating an active denitrification process. The phosphate removal rates for the same study were 83.2%, 90.6%, and 85.6%, and the COD removal rates were 50.9%, 56.5%, and 83.0%, for streams 1, 2 and 4, respectively. In stream 3, only 4.7% of phosphorus was removed, and the COD increased slightly.

Thus, it seems that no generalizations can be made because the COD removal efficiency depends on various factors, such as the characteristics of the specific type of wastewater utilized and more important, the microalgae species or microbial consortia involved.

2.4. Which species perform better for higher biomass and lipid productivity?

Several different microalgae species have been tested under various experimental conditions (e.g., nutrient starvation, nitrogen starvation, nitrogen deficiency, nutrient sufficiency, heterotrophic conditions) and their lipid content and the lipid productivity of the culture have been reported (Table 1). However, only a few of them have been cultivated using municipal wastewater. As mentioned in Sections 2.1 and 2.2, the *Chlorella* species have been preferred over other microalgae by many researchers (Bhatnagar et al., 2010; Chu et al., 2009), especially because they are usually isolated from sewage treatment plants and can be considered as autochthonous species. In fact, *Chlorella kessleri* was found to produce a very high biomass density (2.01 g L^{-1}) when cultivated in the fraction of municipal wastewater known as centrate (Li et al., 2011b).

Natural consortia of various microalgae genera might be established during wastewater treatment operations, as reported by Woertz et al. (2009). The consortium was dominated by *Chlorella*, *Micractinium* and *Actinastrum* and had a maximum lipid productivity of 24 mg $L^{-1}d^{-1}$ at a Hydraulic Retention Time of 3 days using primary clarifier effluent.

Other microalgae have been used due to their special attributes. *Botryococcus braunii* has been selected by some researchers because it is a colonial chlorophyceae (green microalga) that produces extracellular polysaccharides (EPS), is widely distributed on all continents in freshwater, brackish and saline lakes and is able to accumulate unsaturated long-chain hydrocarbons at a concentration of 15% to 75% of its dry weight (Orpez et al., 2009). *B. braunii* UTEX 572 has been cultivated in secondarily treated piggery wastewater, yielding 0.95 g L^{-1} hydrocarbons (An et al., 2003).

In a recent study (Li et al., 2010a), an interesting strain of *Scenedesmus* sp. LX1 was isolated from stored tap water and was compared against other 11 strains. This strain showed the highest yield (0.11 g L^{-1}), highest lipid content (31–33%) and a maximum lipid productivity of 8 mg L^{-1} d^{-1} on day 10 in a batch culture using secondary effluent as the culture medium. In another study performed with the same strain (Li et al., 2010b), different nitrogen sources were tested. High removal percentages of total nitrogen (90.4% and 87.8%, respectively) and total phosphorus (nearly 100%) were observed when nitrate or urea was used as the nitrogen source. When

Table 3
Chemical compositions of anaerobic effluents from various digested wastes and microalgae kinetic parameters.

Source	COD [mg L^{-1}]	BOD [mg L^{-1}]	TN [mg L^{-1}]	TP [PO_4–P mg L^{-1}]	N/P	NH_4-N [mg L^{-1}]	Alkalinity [mg $CaCO_3$ L^{-1}]	Cultured species	Kinetic para-meters	Reference
Piggery waste	17,640[a]–2730 [b]	N.R.	N.R.	58.5[a]–71.75 [b]	– –	1931[a]–3185 [b]	N.R.	Microbial consortia	N.R.	Patil et al. (2010)
Piggery waste	3665–4157	N.R.	1458–1519	358–620	3.96–2.45	1320–1481	4416–5450	*Arthrospira* (*Spirulina*)	11.8[c]–14.4 [de]	Olguín et al. (2003a)
Swine slurry	3858	N.R.	1790	304	5.88	1664	N.R.	*Oocystis sp.* + *Scenedesmus*	332[f]	Molinuevo-Salces et al. (2010)
Cow manure[g]	N.R.	N.R.	60.14	6.0	10.02	41.94	N.R.	*Neochloris oleoabundans*	88.3[f]	Levine et al. (2011)
Poultry waste[h]	N.R.	884	2022	184	10.99	1465	N.R.	Microalgal consortia	76[f]	M. Singh et al. (2011)
Coffee processing	N.R.	122–252	84.3–99.2	38.5–81.6	2.19–1.22	59–64	N.R.	N.R.	N.R.	Olguín et al. (2003b)
Olive mill waste	1930	N.R.	14.0	7.0	2.0	N.R.	310	*Chlorella zofingiensis*	0.49[i]	Travieso et al. (2008)
Food and municipal waste	2500	N.R.	190	19	10	N.R.	N.R.	*Chlorella sorokiniana*	1.53[j]	Chi et al. (2011)
	3200	N.R.	140	14	10	N.R.			0.58[j]	
Municipal wastewater[k]	24	N.R.	7.0	0.46	15.22	0.50	N.R.	*Chlorella ellipsoidea*	0.425[j]	Y. Yang et al. (2011)

N.R. = Not reported.
[a] Thermophilic digester effluent.
[b] Mesophilic digester effluent.
[c] Productivity, average annual value, g m^{-2} d^{-1}.
[d] Productivity maximum value, g m^{-2} d^{-1}.
[e] Using seawater + 2% anaerobic effluents.
[f] Productivity maximum value, mg L^{-1} d^{-1}.
[g] Diluted 1:50.
[h] Diluted 6%.
[i] Specific growth rate, μ, d^{-1}.
[j] Algal biomass density, g L^{-1}.
[k] Secondary effluents.

ammonium was used as the nitrogen source, *Scenedesmus* sp. LX1 reached a very high specific growth rate of 0.82 d^{-1} (after 6 days of cultivation), although it was inhibited later on due to a decrease in the pH.

Chlamydomonas reinhardtii is another microalgae with the potential to produce oil and treat wastewater simultaneously (Kong et al., 2010). It was cultivated in wastewaters taken from three different stages of a municipal wastewater treatment plant (influent, effluent and centrate). High biomass productivity was found (2.0 g $L^{-1}d^{-1}$), with an oil content of 25.25% when cultured in a biocoil using 100% centrate. Under such conditions, a lipid productivity of 505 mg $L^{-1}d^{-1}$ was achieved, which may be the highest lipid productivity reported for microalgae in wastewater and is most likely because centrate is very rich in nutrients, as mentioned in Section 2.1.

Two major conclusions may be drawn from this subsection. One is that no specific species is the best for higher biomass and lipid productivity when used to treat wastewater. The species selection depends on various factors, such as the specific characteristics of the wastewater, the original habitat of the algal strain and the climatic conditions in the treatment plant, among others. In some cases, a natural selection of consortia of various microalgae will occur spontaneously. Thus, isolating and screening microalgae/cyanobacteria or their consortia from various wastewater streams are a very relevant field of research for selecting new strains or for carrying out research related to poorly known species with high potential for biodiesel production. During such screening processes, the use of dyes such as Nile red (a selective fluorescent stain for intracellular lipid droplets) or Sudan III (a lysochrome or fat-soluble dye used for staining of triglycerides and other cell lipids), is highly recommended. Furthermore, the use of Sudan III has been described as a fast and easy technique for staining microalgae lipids that does not require sophisticated equipment, which is in contrast to the use of Nile red (Loera-Quezada et al., 2011). A second conclusion is that the total lipid content of microalgae cultivated in wastewater may be lower than the one observed in synthetic medium. Thus, a trade-off balance should be considered where the advantages of using wastewater might be accompanied by lower lipid productivity than a more costly system fed with fertilizers. Furthermore, a cost-benefit analysis should be undertaken to justify such supplementation in cases in which the nutrients might not be sufficient for supporting algae growth and nutrient supplementation is necessary. The latter situation would apply to the use of treated wastewater at the end of the treatment sequence. The use of anaerobic effluents from animal or any other high strength organic waste as the nutrient source within integrated systems or Biorefineries also produces biogas during the anaerobic digestion of the waste. Additionally, mixotrophic cultures of microalgae/cyanobacteria can be established because organic acids are present in these anaerobic effluents.

2.5. What is the effect of potentially toxic compounds found in municipal wastewater?

Municipal wastewater contains several compounds that are potentially toxic to microalgae, such as heavy metals and other recalcitrant compounds, especially as they mix with industrial wastewater. Heavy metals are potent inhibitors of microalgal photosynthesis because they can replace or block the prosthetic metal atoms in the active site of important enzymes (Kumar et al., 2010). The maximum specific growth rate and biomass productivity of *B. braunii* cultivated in secondary effluents of a municipal sewage treatment plant were lower than those observed in a control synthetic medium, and the authors noted that the decrease in the value of such parameters could be due to the presence of phenolic compounds and heavy metals in the wastewater (Orpez et al., 2009). Although these types of studies are relevant, very few researchers have reported the effect of toxic compounds on biomass and lipid production, providing a niche opportunity for future research.

2.6. Do the energy balance and the economic viability of biodiesel production from microalgae improve when using wastewater?

The answer to this question is one of the most important challenges in current research. However, very few studies have answered it and provided concrete answers. Feng et al. (2011) performed analyses of the energy efficiency in semi-continuous cultures of *C. vulgaris* using synthetic wastewater, showing that the Net Energy Ratio for lipid production with a daily replacement of 50% of the culture volume was higher than the unit, reaching a value of 1.25. Positive values indicate a feasible process in terms of energy because this parameter is the ratio of the energy produced (energy content of the oil and residual biomass) over the energy requirements (Singh and Gu, 2010). Additionally, their cost analyses showed that the algal biomass could be competitive in the world market if the cost of a petroleum barrel was equal to or greater than US\$63.97, adding an additional credit for the wastewater treatment. In fact, Feng et al. (2011) calculated that 1443 m^3 of wastewater is treated during the production of 1 ton of algal biomass. Thus, if the credit for wastewater treatment at US\$0.4/m^3 is counted, the cost of producing 1 ton of biomass is reduced from US\$808.79 to US\$231.59. The system was able to remove 86% of COD, 97% of N-NH_4 and 96% of TP, respectively.

An energy balance of the microalgal production coupled with the nutrient removal from wastewater was performed in open ponds (Sturm and Lamer, 2011). The results showed that biofuel production was energetically favorable for open pond reactors that used wastewater as a nutrient source, even without an energy credit for nutrient removal. Direct combustion of the algal biomass may be a more viable energy source than biofuel production, especially when the lipid content of dry biomass (10% in this field experiment) is lower than the high values reported in lab-scale reactors (50–60%).

In conclusion, the use of dual purpose microalgae–bacteria-based systems for treating wastewater and producing biodiesel can improve energy efficiency, achieving positive values of the Net Energy Ratio and can improve the economic viability of the process. Furthermore, this type of comprehensive analysis needs to be applied to a variety of processes, especially at pilot and large scales; thus, there is a clear need for further research in this field, offering a niche opportunity.

3. Treatment of animal wastewater with microalgae–bacteria-based systems within Biorefineries

3.1. Animal waste as a cause of eutrophication and source of greenhouse gases (GHG) emissions

The use of animal wastewater for cultivating microalgae within a biorefinery serves a dual purpose of providing a source of water and nutrients for the microalgae and simultaneously treating this type of noxious and abundant wastewater to avoid eutrophication of surface and ground water bodies, as well as reducing greenhouse gases (GHG) emissions. Animal waste, also known as manure, is rich in organic matter, nitrogen (N) and phosphorus (P) and is excreted as a high percentage of the animal's weight. Pig and poultry manure are among the most polluting wastes due to their higher organic matter contents in terms of the Biological Oxygen Demand (BOD) and the N and P contents compared to other animal wastes (Laliberté et al., 1997).

The lack of proper procedures for manure storage and handling contributes the most toward marine eutrophication and terrestrial acidification and had the greatest impact on climate change in a Life Cycle Assessment (LCA) of the impacts of a large scale swine production facility in the Northern U.S. (Stone et al., 2010). Large-scale livestock operations are a major contributor (55.8%) of phosphorus to local surface waters in central China according to a phosphorus-flow analytical model developed using substance flow analysis (Yuan et al., 2011).

The problem of manure storage and handling is more acute in certain regions of the world in which large-scale production facilities lack the infrastructure to appropriately process and dispose of the waste. In countries in which a large number of animal heads are encountered (Fig. 1), the development of biorefineries for animal waste treatment with tandem microalgae biofuel production offers a large opportunity for developing new business, as well as treating the animal wastewater and enhancing environmental conservation. Although very little work has been reported using chicken manure for microalgae cultivation (Ungsethaphand et al., 2009), this type of waste seems to be very relevant in countries registered as the first five producer countries (FAOSTAT, 2011).

Anaerobic digestion has been proposed, and implemented in some cases, as the most appropriate technology for handling animal manure (Olguín, 2000). There are three clear benefits of this technology: a) the energy recovery from produced biogas, b) a net reduction in the emissions of greenhouse gases and c) a large increase in the overall productivity of the farming system (Michel et al., 2010). Additionally, anaerobic digestion produces maximum electricity from animal waste because biogas can be converted to electricity at high efficiency in a gas engine, while producing heat to run the digestion process (Prapaspongsa et al., 2010).

3.2. Use of microalgae–bacteria-based systems for treating raw or pre-treated animal manure

There have been some efforts to use raw or pre-treated animal manure as a source of nutrients for cultivating microalgae, which have been previously reviewed (Laliberté et al., 1997; Olguín, 2003; Pittman et al., 2011). In this section, a brief summary of the research performed only at pilot plant outdoor facilities is reviewed, together with some other recent work, to provide data for further work in scaling up efforts in the short and medium term.

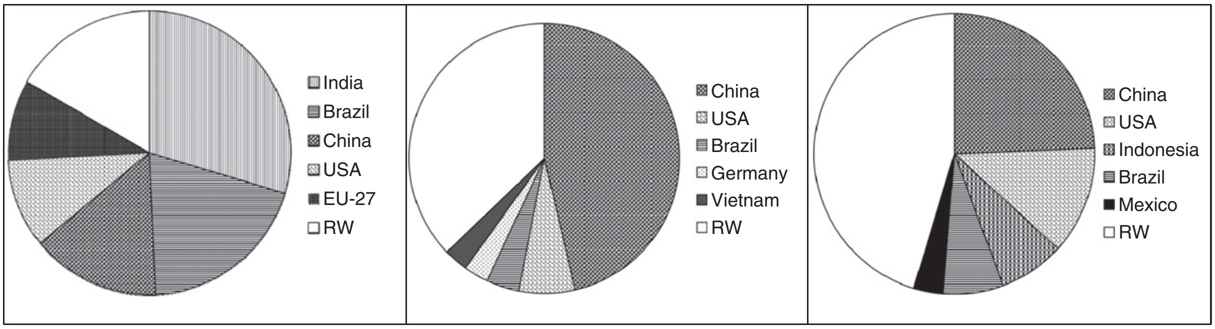

Fig. 1. The first five countries according to the number of animal heads encountered in each one: (1) cattle (2) pigs and (3) poultry. RW — rest of the world. (Data from FAOSTAT, 2011).

3.2.1. Treatment systems using green microalgae and cyanobacteria

The dominance of one particular species within a consortium of microalgae is determined by a higher tolerance towards the concentration of ammonia nitrogen in solution compared to other species in the context of animal, and specifically piggery, wastewater (de Godos et al., 2010). A comparative study was performed on two green microalgae (*Scenedesmus obliquus* and *Chlorella sorokiniana*), one cyanobacterium (*Arthrospira platensis*), one euglenophyte (*Euglena viridis*) and two microalgae consortia to evaluate their behaviors during the biodegradation of diluted piggery wastewater in batch tests. *C. sorokiniana* and *E. viridis* tolerated high ammonia concentrations, while the other microalgae were inhibited. *C. sorokiniana* showed the highest tolerance and was able to dominate in a continuous algal–bacterial photobioreactor initially inoculated with *C. sorokiniana*, *S. obliquus* and *S. platensis*. The authors found that nitrogen assimilation into the algal–bacterial biomass most likely only occurred via the ammonia nitrogen removal mechanisms in this system. Another study used a microalgae–bacteria-based system (dominated by *Oocystis* sp. and *Scenedesmus* sp.) to treat anaerobic effluents from piggery waste. By comparing two different configuration bioreactors, nitrogen recovery by biomass assimilation was higher in the open configuration reactors, ranging from 38 to 47%, than in the closed type reactors (31%) (Molinuevo-Salces et al., 2010).

In a recently published work (Levine et al., 2011), the oleaginous green algae *Neochloris oleoabundans* was cultivated using anaerobically digested dairy manure in batch cultures. The microalgae assimilated 90–95% of the initial nitrate and ammonium and contained 10–30% fatty acid methyl esters (by dry weight) after 6 days of cultivation. The authors mentioned that more work is needed to clarify the role of N deficiency and the influence of the pH on the lipid accumulation. A recent report used *Ettlia oleoabundans* (formerly known as *N. oleoabundans*) at a lab scale to process diluted (2% v/v) anaerobic digesters effluents from catfish processing waste, soybean field waste, and rice hulls (Y. Yang et al., 2011). All three effluents were deficient in phosphate and nitrate but were richer in ammonia and urea than the standard BBM medium. Although the best growth was observed with 2% (v/v) soy effluent, scant oil content was observed in the cells cultivated using all of the effluents. When the three effluents were mixed, the oil content in the biomass increased up to six fold, depending on the age of the effluent, although the growth rate did not increase significantly. Thus, this work indicates that more research work is needed at the biochemical and physiological levels to understand the inhibitory factors of lipid accumulation during cultivation of microalgae in waste streams.

Research on various carbon sources for the mixotrophic cultivation of microalgae has indicated that acetate can be used as a carbon source for *Chlorella vulgaris*, an organism with the potential for oil accumulation and biodiesel production (Heredia-Arroyo et al., 2011). Acetate is encountered in the effluents of anaerobic digesters under certain operational conditions; thus, anaerobic effluents could be used to cultivate green microalgae, similar to previous research on mixotrophic cultures of cyanobacteria of the genus *Arthrospira* (see Section 3.2.2).

3.2.2. Treatment systems using cyanobacteria of the genus Arthrospira with the recovery of high added value products

Cyanobacteria are usually a part of the microalgae group because they are a large and widespread group of photoautotrophic microorganisms that are able to perform oxygenic photosynthesis (similar to that of the chloroplasts), although they show typical prokaryotic features (Whitton and Potts, 2000). The cyanobacteria of the genus *Arthospira* (formerly known as *Spirulina*) is one of the very few microalgae/cyanobacteria cultivated on a large commercial scale because it is a source of nutraceuticals, poly-unsaturated fatty acids (PUFAs) and pigments and can be sold as a supplement for human and animal consumption (Vonshak, 1997). C-phycocyanin (C-PC) is a blue pigment with fluorescent and antioxidative properties that is found in cyanobacteria, rhodophytes and cryptophytes (Eriksen, 2008). It can be extracted and purified using a simple low-cost procedure (a modification of the non-chromatographic, rivanol-sulfate method) already reported to be very efficient when applied to *A. maxima* and *A. fusiformes* with recovery rates of 54% and 55% (w/w) from the crude extract, respectively (Minkova et al., 2007).

Phycocyanin and polyunsaturated fatty acids (PUFAs) of the genus *Arthrospira* are known to promote immune system health in human beings and animals (Belay et al., 1993, 1996). More recently, using *A. platensis* as a dietary supplement (5.0 g *Arthrospira* kg^{-1} diet) (Abdel-Tawwab and Ahmad, 2009) was found to promote growth and immunity when fed to Nile tilapia, *Oreochromis niloticus*, while the fish were challenged by pathogenic *Aeromonas hydrophila*. Furthermore, supplementation of the common carp (*Cyprinus carpio*) with *Arthrospira* induced a significantly higher level of survival and growth compared to supplementation with *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* (Ramakrishnan et al., 2008).

Thus, it is clear that using the genus *Arthrospira* within a biorefinery provides ample benefits, especially if it is produced at low cost, and can recover nutrients from wastewater. In countries in which legislation could prohibit the use of *Arthrospira* as a feed source when produced using wastewater, its use as a source of nutraceuticals, poly-unsaturated fatty acids (PUFAs) and pigments completely justifies its production. Thus, a review of the various reports that use *Arthrospira* (*Spirulina*) to treat animal wastewater is presented below.

There are several advantages in using this particular microorganism in phycoremediation (Olguín et al., 2003a): a) its capacity to flocculate makes harvesting easier and cheaper than for other microalgae, b) its biomass has the highest possible protein content (60–70% d. wt.) when grown under N excess conditions, c) it has been used successfully as a feed supplement for mammals and fish larvae, d) its content of polyunsaturated fatty acids (PUFAs) is high under certain culturing conditions (Olguín et al., 2001), e) it can be enriched in polysaccharides and used as a bioadsorbent for heavy metals (Hernández and Olguín, 2002), f) its ability to grow at high pH values reduces contamination by other species, g) some strains can grow at a very high $\mathrm{NH_4}$-N concentration (130 mg L^{-1}) (Olguín and Martínez, unpublished data) and h) some strains can grow under heterotrophic and mixotrophic conditions.

An integrated system developed for producing *Arthrospira maxima* while treating piggery wastewater has been evaluated at the laboratory level (Olguín et al., 1994) in outdoor raceways under subtropical (Olguín et al., 1997) and tropical conditions in México (Olguín et al., 2003a). The system has the advantage of producing biogas from the piggery waste, while also producing valuable *Arthrospira maxima* biomass for fish feed or as a source of pigments and nutraceuticals. After treating the piggery waste in anaerobic filters, the anaerobic effluents were treated using raceways containing diluted sea water (1:4) and anaerobic effluents (2% v/v) inoculated with *Arthrospira maxima*. Under tropical conditions, the maximum productivity of semi-continuous cultures in 23.6 m^2 ponds (containing 4720 L when operating at a column height of 0.2 m) during the summer was 15.1 g m^{-2} d^{-1}, equivalent to 55.12 ton ha^{-1} year^{-1}. The average annual productivity evaluated during 4 consecutive years, was 11.8 g m^{-2} d^{-1}, which is the highest value reported for an *Arthrospira* production system using seawater. The average protein content of the semi-continuous cultures was 48.9% ash-free dry weight. The $\mathrm{NH_4}$-N removal rate was in the range of 84–96% and the P removal was approximately 72–87%, depending on the depth of the culture and the season. More recently, a similar process was evaluated in Thailand using *A. platensis* and effluents (20%) from an up-flow anaerobic sludge blanket (UASB) digester processing piggery waste, supplemented with 4.5 g L^{-1} sodium bicarbonate and 0.2 g L^{-1} urea fertilizer. The average productivity of a semi-continuous culture grown under outdoor conditions in a

100 liter pond was 12 gm^{-2}d^{-1}. The biomass contained approximately 57.9% protein, 1.12% gamma-linolenic acid and 19.5% phycocyanin (Chaiklahan et al., 2010). Thus, both systems, one developed earlier in México and one developed recently in Thailand, promoted a similar average *Arthrospira* productivity of approximately 12 g m^{-2} d^{-1}, equivalent to 43.8 ton ha^{-1} year^{-1}, with a maximum of 55.12 ton ha^{-1} year^{-1} registered in México during the summer, demonstrating that anaerobic effluents from piggery waste can be treated while simultaneously recovering of high yields of this valuable cyanobacterium and generating of biogas. In fact, the system developed in México was called "Bioespirulinema" (Olguín, 2000) because it also produces lemnaceae, which can be used as poultry feed.

The chemical composition of *Arthrospira* has been found to vary with environmental conditions, as it is the case with all organisms. It has been demonstrated that *A. maxima* had a maximum protein content (70% dry weight of biomass) and low total lipids (8%) and polysaccharide content (7%) in Zarrouk medium when cultures were exposed to a low irradiance (66 μmol photon m^{-2} s^{-1}). In contrast, under nitrogen deficiency in a complex medium when cultures were exposed to an irradiance of 144 μmol photon m^{-2} s^{-1}, a minimum protein content (34%), a maximum polysaccharide content (29%) and a total lipid content of 18% were observed. The total lipid content increased to 28.6% when the cultures in the complex medium were exposed to the lower irradiance (66 μmol photon m^{-2} s^{-1}). Under these conditions, a very high percentage (28.13%) of linolenic acid (18:3), was observed (Olguín et al., 2001). Thus, a combination of nitrogen availability and irradiance level was the main factors determining the cell chemical composition in this particular case. The increase in PUFAs as a result of culturing *Arthrospira* at a low irradiance level was confirmed when 35 *Arthrospira* strains were grown at 30 °C and very low irradiance (10 μmol photon m^{-2} s^{-1}). Furthermore, it was observed that linoleic, gamma-linolenic acid, and palmitic acid formed 88–92% of the total fatty acid content (Muhling et al., 2005). The increase in the lipid content of *A. platensis* under nitrogen deficiency (up to 17.05% of its dry weight) has also been confirmed by a recent report (Uslu et al., 2011).

Other important factors impacting the growth of *Arthrospira* have also been investigated. One report showed a linear relationship between the rates of *A. platensis* growth and carbon dioxide removal from biogas, with 95% efficiency of carbon utilization for biomass production (Converti et al., 2009). Supplementing *Arthrospira* sp. cultures with 12% CO_2 and SO_2 and NO promoted a high PUFA content (29.37%) with palmitoleic acid as the predominant fatty acid (41.02%) out of the total PUFA content (Radmann and Viera-Costa, 2008).

Finally, the most important challenge is scaling up the production of *Arthrospira* from the laboratory level to the commercial production. That transition encounters many unforeseen problems, mostly related to design, scale, contamination, and external unknown variables, regardless of the type of microalgae culture. Furthermore, downstream processing also requires a lot of effort, taking special care to harvest only the biomass greater than the optimal areal density concentrations (Grobbelaar, 2009). Maximum productivities need to be optimized to establish an economically feasible process. In this respect, at least six important factors have been identified that determine productivity in mass algal cultures (Grobbelaar, 2007). These factors are (1) the culture depth or optical cross section, (2) turbulence, (3) nutrient content and supply, (4) cultivation procedure, (5) biomass concentration and areal density, and (6) photo-acclimation. The latter factor is very relevant because photoinhibition could reduce areal productivities by up to 30% and more. Furthermore, a significant loss in the productivity of *Spirulina* (*Arthrospira*) in open ponds has been observed in mid-summer due to high pH and high dissolved O_2 concentrations (Jiménez et al., 2003). The algal density in the pond and its productivity were found to reach a maximum value at pH values below 10.5 and a dissolved oxygen concentration below 25 mg L^{-1}.

4. A Biorefinery combining dual purpose oleaginous microalgae–bacteria-based systems for treating wastewater and cultivating *Arthrospira* using anaerobic effluents

A flow chart of the various stages needed to establish a wastewater treatment system utilizing microalgae is shown in Fig. 2, taking into consideration all the information mentioned in Sections 2 and 3. The first step is to characterize and condition the wastewater (e.g., adjustment of pH). Selecting the most appropriate species is another key step and takes into consideration the characteristics of the wastewater. Thus, fresh water species, such as those shown in Table 1, could be selected for cultivation in municipal wastewater. Additionally, isolating native microalgae species from the wastewater to be treated is highly recommended. An acclimation of the selected species in the specific wastewater to be used is a required third step. Using an alternative harvesting technology, such as immobilization or biofilm formation (see Section 5), may require pre-treatment

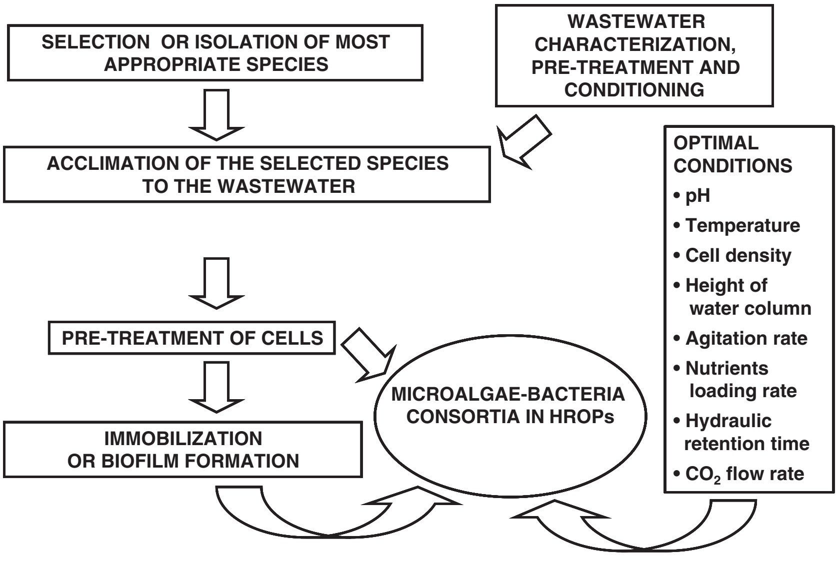

Fig. 2. A flow diagram of the stages and operational conditions needed in the establishment of microalgae–bacteria-consortia in dual purpose systems that treat wastewater and produce biodiesel and chemical products.

of the cells. A mixed population of one or more microalgae species together with a bacterial population will be established as a consortium and will follow a particular population dynamics when establishing a culture, depending on the setting of optimal culturing conditions for the species of the most interest. There is a large niche for research in relation to population dynamics and structure of useful consortia. Controlling grazing and algal species is also a major problem that requires more research (Park et al., 2011). Furthermore, the important factors that determine productivity in mass algal cultures (Grobbelaar, 2007) mentioned in Section 3.2.2. need to be considered. In this respect, the experience accumulated over a long period of time dealing with culturing microalgae in outdoor ponds (reviewed in Borowitzka, 2005) should be taken into account.

Fig. 3 shows the flow chart for a Biorefinery with the double purpose of producing oleaginous microalgae grown in wastewater and *Arthrospira* grown in seawater added of anaerobic effluents from animal waste for the production of biofuels and high added value products, which is an ambitious and original scheme aimed at producing three different types of biofuels from oleaginous microalgae: biodiesel, biogas and hydrogen. Additionally, biogas is generated from animal waste, and valuable products can be extracted from *Arthrospira* biomass, such as phycocyanin and PUFAs, and from the residues of the biomass (useful as fish feed), which can be commercialized. Section 3.2.2 mentioned that an integrated system processing piggery waste through anaerobic digestion was reported that produced biogas and *Arthrospira* biomass (grown in seawater supplemented with anaerobic effluents) at a pilot scale (Olguín et al., 2003a). Using seawater is a key issue because it provides nutrients and the culture does not compete for fresh water for agricultural purposes, as previously highlighted (J. Yang et al., 2011). The produced biogas can be used as a source of CO_2 for the cyanobacteria. As demonstrated previously (Olguín et al., 1994, 1997, 2003a), high productivities of *Arthrospira* can be attained using High Rate Oxidation Ponds (HROPs), depending on the environmental and culturing conditions. Furthermore, HROPs or raceways are significantly more environmentally sustainable than closed air-lift tubular bioreactors (Stephenson et al., 2010). The advantages of using HROPs for microalgae cultivation have been extensively reviewed recently (Park et al., 2011) and are not dealt with in this review.

On the other hand, cultures of oleaginous microalgae using municipal wastewater have been already investigated, as mentioned in Section 2, and are expected to become a consortium of microalgae and bacteria in which a particular population dynamic will be established according to the environmental and culturing conditions. The use of wastewater is highly recommended because the footprint of water used is one of the most important factors related to the sustainability of the Biorefineries (J. Yang et al., 2011). Furthermore, an original attempt to use the nutrients from municipal wastewater, which has not received proper treatment and has been discharged into rivers and small streams, is under progress (Olguín et al., unpublished results). A recent study whose goal was to assess the quality of the water of an urban river polluted by the City of Xalapa in México (Olguín et al., 2010) indicated that the Sordo River is a potential source of nutrients for cultivating oleaginous microalgae. This study could be taken as a model for improving the quality of many urban and rural rivers in developing countries, especially in regions where there is not enough infrastructure to treat municipal wastewater. Thus, treating polluted rivers through phycoremediation and phytoremediation (Olguín and Sánchez-Galván, 2010) could be linked to producing oleaginous microalgae (fresh water species).

Flate Plate Photobioreactors (FPPs), built according to Molina-Grima et al. (1999) and Sierra et al. (2008), have been incorporated in the Biorefinery (Fig. 4) to achieve high biomass productivities of oleaginous microalgae and to provide an inoculum for establishing massive cultures in High Rate Oxidation Ponds (HROPs). A combination of reactors has been already successfully applied for commercial-scale cultivation of *Haematoccocus pluvialis* (Huntley and Redalje, 2007) and for the cultivation of *Nannochloropsis* sp. for lipid production (Rodolfi et al., 2009).

Cultures of oleaginous microalgae can be subjected to various types of physiological stress to enhance the lipid productivity (see Section 3.2.1). Several reports of cultures in synthetic medium that provide detailed information about the various factors and strategies that can be followed may be consulted elsewhere (Hu et al., 2008;

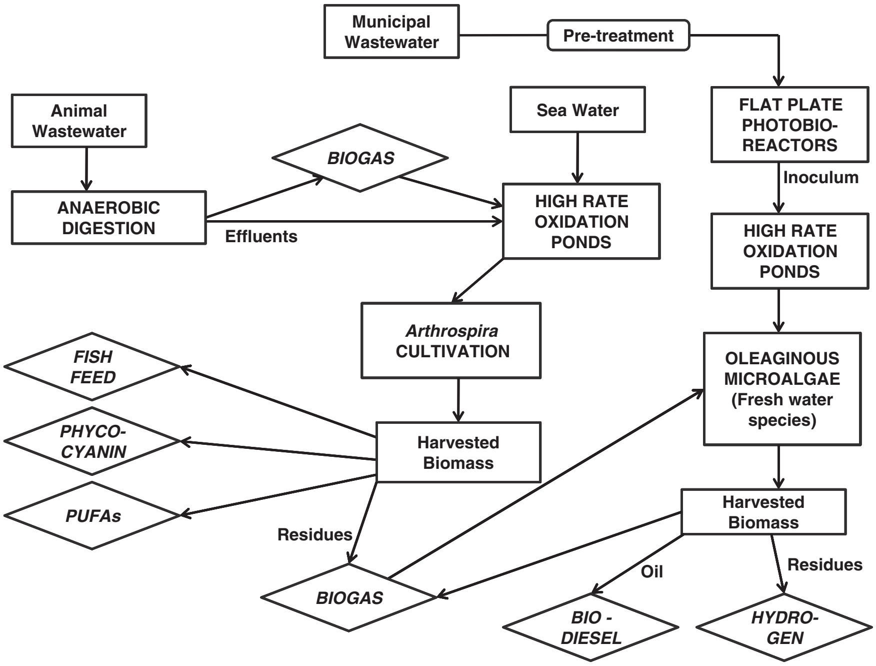

Fig. 3. A Biorefinery combining dual purpose oleaginous microalgae–bacteria-based systems for the treatment of wastewater and *Arthrospira* cultivation using anaerobic effluents for the production of biofuels and high added value products.

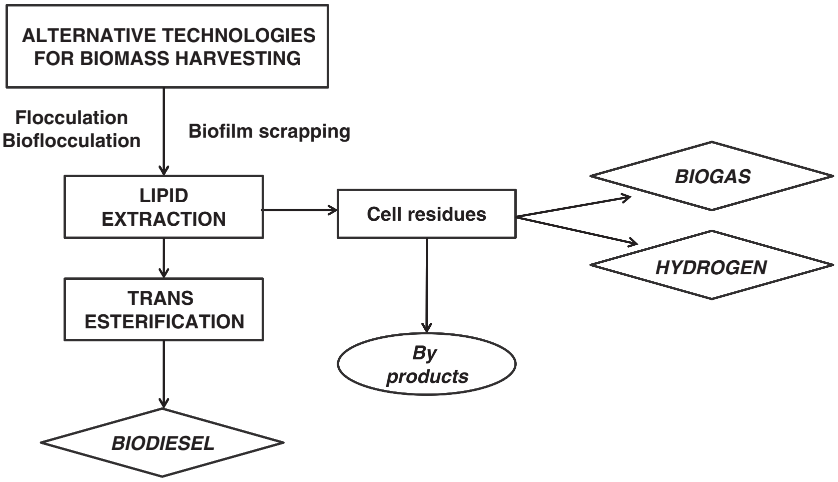

Fig. 4. Downstream processing of microalgae biomass for production of biofuels and by-products.

Subramaniam et al., 2010). Several alternative technologies may be used for harvesting the biomass of oleaginous microalgae at a low cost (see Section 5). Finally, the harvested biomass has to be subjected to downstream processing (Fig. 4) to extract the oil or the non-fuel valuable products and to produce biogas, biodiesel and hydrogen from the cell residues, depending on the cultivated microalgae species. Detailed information of these processes is outside the scope of this review and may be consulted elsewhere (Kumar et al., 2010; Rawat et al., 2011). However, it is important to mention that the energy density in the microalgal biomass from a variety of species growing under optimal conditions is remarkably consistent, ranging between 19 and 25 GJ ton^{-1}, which is similar to the energy density of bituminous coal (McGinn et al., 2011). Furthermore, the conversion of algal biomass after lipid extraction into methane is a process that can recover more energy than the energy from the cell lipids (Sialve et al., 2009). However, three main bottlenecks have been identified for efficient digestion of the microalgae biomass: a) the biodegradability of microalgae can be low; b) ammonia toxicity, if there is a high protein content, is present in the microalgae biomass and c) the toxicity of sodium derived from marine species can also affect the digester performance (Sialve et al., 2009). Despite these limitations, algal productivities in the range of 24–30 ton VS ha^{-1} year^{-1} can be produced in a closed-loop system in which the microalgae biomass is converted into biogas, producing 0.5 m^3 biogas kg^{-1}algal VS (De Schamphelaire and Verstraete, 2009). Recent reports about the production of hydrogen from microalgae note the particular advantages and production technologies of this type of biofuel that can be derived from the microalgae cell residues after the lipids or valuable pigments are extracted (Demirbas, 2011; Rashid et al., 2011).

5. Alternative technologies aimed at reducing the cost of harvesting

The harvesting cost is constantly mentioned as one of the major limitations to the economic viability of wastewater treatment with microalgae and/or in the production of biodiesel from microalgae (Christenson and Sims, 2011; Loera-Quezada and Olguín, 2010; Oswald, 1988; Uduman et al., 2010). There are at least three basic problems: a) the cell size is very small, ranging between 3 and 30 μm; b) the relatively low cell density, especially in the raceways (<0.5 kg m^{-3} of dry biomass) and the large volumes of water being harvested; and c) the contribution of the harvesting cost to the total cost is in the range of 20–40%. In the case of some high added value products, such as metabolites or nutraceuticals such as Eicosapentanoic acid (EPA), the recovery cost could even represent 60% of the total cost (Molina-Grima et al., 2003). The use and limitations of several conventional biomass harvesting technologies, such as centrifugation, chemical flocculation, filtration and screening, gravity sedimentation, flotation, and electrophoresis techniques, have been extensively reviewed (Chen et al., 2011; Uduman et al., 2010) and are not the subject of discussion in this section. However, not enough research has been performed in the field of alternative technologies, such as those that could allow harvesting of the algae biomass without centrifugation or that could be applied prior to centrifugation to dewater the biomass, thereby decreasing the energy input and offering large niche technology development opportunities. This section reviews the various alternative technologies or approaches that are promising for the recovery of biomass because they are aimed at improving the energy balance and the economic viability of microalgae mass production within a Biorefinery. Cell immobilization has been previously highlighted as one of the best alternatives for cost-effective wastewater treatment with microalgae (Olguín, 2003). Biofilm formation, flocculation and bioflocculation are also very promising alternatives that deserve further attention in research and development.

5.1. Cell immobilization

Immobilized algal systems should contain certain properties, such as retaining cell viability and the capacity for adequate photosynthetic rates, to maintain high cell densities and a continuous productivity (Mallick, 2002). Additionally, the matrix should be selected on the basis of physical and chemical resistance properties while retaining the biomass for long-term use. Additionally, they should be non-toxic and should be produced without the need for complex immobilization techniques (Mallick, 2002; Olguín, 2003). On the other hand, for wastewater treatment, the selection of the microalgae should take into account their capacity for high nutrient removal, high growth rates over or in the matrix with low cell leakage and the facility for being handled (Pérez-Martinez et al., 2010).

Among the several immobilization techniques that have been described and tested, gel entrapment in natural polysaccharide matrices, such as carrageenan, agar and alginate, has been the preference of several researchers. These types of matrices are advantageous because they are a renewable resource extracted from various types of algae. Carrageenan and agar are extracted from red algae, while alginates come from brown algae. Alginate gels may be destabilized by the presence of phosphate ions because the Ca^{2+} ions, which are used to form the gel, may be sequestered as a phosphate salt (Moreno-Garrido, 2008). Thus, alginate gels are not chosen for wastewater treatment very often because they lack long-term stability in the presence of phosphate ions. However, several synthetic matrices (acrylamide, polyurethane, polyvinyl, resins) have also been tested and are more stable and less vulnerable to microbial degradation compared to the naturally produced matrices (de-Bashan and Bashan, 2010).

Chitosan and polyvinyl foams are low-cost polymers with a long-term performance that consider various selective criteria (Olguín, 2003). Chitosan-immobilized cells of the marine cyanobacterium *Synechococcus elongates* have been found to be very efficient at removing nitrogen and phosphorus from synthetic wastewater simulating aquaculture wastewater (Aguilar-May and Sánchez-Saavedra, 2009). These authors reported that ammonia removal was higher in free cells (54%) compared to immobilized cells (29%), although nitrate removal was similar in both groups (38% immobilized vs. 44% free). The phosphorus-removing capacity of the free cells was higher (88%) than the immobilized cells (77%). de-Bashan and Bashan (2010) reviewed the literature over the last two decades and concluded that the removal of nitrogen is favorable in immobilized systems in most cases, although phosphorus removal still represents a challenge that needs to be optimized to enhance its removal.

Another important factor for immobilized systems for treating wastewater is selecting those species with a higher potential for nutrient removal, although this issue has been poorly addressed (Pérez-Martinez et al., 2010). Eight benthonic microalgae species isolated from different sources of pig manure were immobilized in calcium alginate beads and were tested based on the hypothesis that autochthonous species remove nutrients more efficiently compared to other species. The results show that phosphate removal rates for the unicellular self-aggregating benthic species (*Palmellopsis gelatinosa*, *Chlorosarcinopsis* sp., and *Macrochloris* sp.) were much higher than those for other species. Similarly, nitrogen removal rates were highest for *Macrochloris* sp., *Chlorosarcinopsis* sp., and *Euglena* sp. and were comparable to the maximum rates obtained by other authors (Pérez-Martinez et al., 2010).

Thus, more research is required to solve the following key issues before this technology can be applied at a large scale: 1) matrix selection that takes into consideration strong physical and chemical hardiness, with the proper retention of biomass for long-term use in wastewater treatment systems. Additionally, they should be non-toxic and should be produced without the need for complex and costly immobilization techniques; 2) selection of microalgae considering their capacity for high nutrient removal and high growth rates in mixed cultures where other contaminating microorganisms will compete for nutrients; 3) development of easy-to-use harvesting devices to collect the immobilized beads at a large scale and a further treatment to extract the cells from the beads.

5.2. Biofilm formation

Another alternative technology for reducing the harvesting costs in massive microalgae cultivation is to induce biofilm formation on the surface of various substrates, which may be removed easily from the cultivation medium. In this approach, the information derived from other types of fields, such as biofilm formation that results in fouling and corrosion, may be very useful when applied to cultivating microalgae in wastewater for biofuel production or other purposes. Studying the adhesion of *C. vulgaris* (chlorophyceae), *Nitzschia amphibia* (bacillariophiceae) and *Chroococcus minutes* (cyanobacteria) to hydrophobic (perspex, titanium and stainless steel 316-L), hydrophilic (glass) and toxic (copper, aluminum brass and admiralty brass) substrata provided important data (Sekar et al., 2004). The attachment varied significantly with respect to the surface properties (wettability and roughness) and composition of the material. Adhesion was higher on rough surfaces compared to smooth surfaces, and all three organisms attached more on titanium and stainless steel and lower on copper and its alloys. The attachment was also influenced by pH, organic film, culture age, culture density, cell viability and the presence of bacterial films.

Another approach for designing substrate materials is to use a relatively low-cost surface material similar to the brushes for cleaning glassware or for washing cars. A continuous system operated at a Hydraulic Retention Time (HRT) of 4 days showed removal efficiencies of TP, TN, NH_3-N, and COD that reached 95.38%, 83.93%, 82.38%, and 92.31%, respectively, and were very stable for 24 days (Wei et al., 2008).

A recent report showed successful biofilm formation of *Chlorella* sp. on polystyrene foam using dairy manure wastewater as the growth medium. This study resulted in a high biomass yield (25.65 g/m^2, dry basis) and a high fatty acid yield (2.31 g/m^2) (Johnson and Wen, 2010). Furthermore, the total nitrogen removal was in the range of 61–79% and the total phosphorus removal ranged from 62 to 93%. The biomass was harvested from the attached culture system by scraping and was a paste-like pulpy slurry.

Biofilm formation has large potential as an alternative harvesting technology, although there is little information about its application in dual purpose systems for treating wastewater and recovering microalgae for biodiesel production. There is definitely a need to improve biofilm designs to optimize the algae biomass production (Christenson and Sims, 2011) and the harvesting of it.

5.3. Flocculation

Microalgae and cyanobacteria are covered by extracellular polysaccharides (EPS), which give them a negatively charged surface. The EPS of cyanobacteria are complex anionic polymers, mainly composed of uronic or pyruvic acids, peptides, acetyl radicals or sulfated molecules (De Philippis et al., 2001). The negatively charged surface allows flocculation or aggregation of the cells using cationic metals or other flocculating agents. Thus, flocculation was proposed decades ago as a means of recovering microalgae at a low cost prior to centrifugation and has therefore been specifically proposed for harvesting microalgae in wastewater treatment (Lee et al., 2009; Oswald, 1988). Four different types of flocculating agents have been tested: 1) cationic metals, such as Al^{3+} or Fe^{3+}; 2) organic polymers, such as chitosan and other commercially produced polymers; 3) cationic starch and 4) hydroxide salts, such as NaOH and KOH. A brief account of the most recently reported experiences with each type alone or in combination is presented below.

One study compared the efficiency of two ferric salts against five commercial polymeric flocculants (Drewfloc 447, Flocudex CS/5000, Flocusol CM/78, Chemifloc CV/300 and Chitosan) for their ability to flocculate three different green microalgae (*C. sorokiniana*, *S. obliquus*, *Chlorococcum* sp.) and a wild type *Chlorella* in symbiosis with a bacterial consortium cultivated in the supernatant of pig wastewater (de Godos et al., 2011). The polymeric flocculants had similar removal efficiencies using a much lower dosage (25–50 mg L^{-1}) than that required for the ferric salts (150–250 mg L^{-1}). This comparative study is useful because prior studies showed that microalgae biomass for biofuel purposes should be free of metallic ions (Lee et al., 2009) and thus provides evidence that polymeric flocculants might be an alternative, although the authors discuss their limitations in the presence of organic colloidal matter. Chitosan was used in a different study to flocculate phytoplankton from shrimp cultivation tanks. The optimal concentration of this polymer was 0.75 and 0.5 g L^{-1} for sulfate and chloride salts (Lertsutthiwong et al., 2009), indicating that lower concentrations can be used under certain water quality conditions. In another comparative study, twelve metallic flocculants salts were tested for their ability to flocculate *Chlorella minutissima* (Papazi et al., 2010). Aluminum salts were the most efficient, although they caused some cell lysis, which prevents their use in any Biorefinery design. Ferric and zinc salts had the second and third best flocculation efficiencies, respectively.

Alternatively to the use of cationic polymers (either metallic or natural), the neutralization of the negatively charged surface of cells with NaOH or KOH has been tested for flocculation purposes. Several microalgae that are useful as live feed in aquaculture have been flocculated with an efficiency equal to or above 80% using NaOH to adjust the pH to 10–10.6 with the addition of Magnafloc LT-25, a non-ionic polymer, to yield a final concentration of 0.5 mg L^{-1} (Knuckey et al., 2006). Moreover, a harvesting efficiency greater than 90% has been achieved with *Chaetoceros calcitrans* using NaOH or KOH to adjust the pH to 10.2 (Harith et al., 2009). To neutralize the negative surface charge, cationic starch has been successfully used for the flocculation of freshwater microalgae (*Parachlorella* sp. *and Scenedesmus* sp.) but not for marine microalgae (*Phaeodactylum* sp. and *Nannochloropsis* sp.). Of the two commercial cationic starch flocculants tested, Greenfloc 120, which is used in wastewater treatment, was more efficient than Cargill C*Bond HR (Vandamme et al., 2010).

A different approach was investigated by Lee et al. (2009), who demonstrated a recovery percentage of 90% and a concentration factor of 226. In this study, a marine microalgae, *Pleurochrysis carterae*, was cultivated in the presence of organic substrates, such as acetate, glycerol and glucose, to induce formation of exo-polysaccharides and flocculation. Mixing was found to be one of the major factors involved in this flocculation process. The authors performed a modeling study to estimate the order of magnitude of the mixing energy required for a hypothetical 1 km^2 High Rate Oxidation Pond (HROP). They found that the mixing energy required to harvest the microalgae produced in the system was equivalent to 0.1 m of hydraulic head or 0.9 $kWh*t^{-1}$ of flocculated dry mass. The overall cost of microalgae harvesting was estimated to be A$0.13 (Australian dollars) per m^3 of culture medium (Lee et al., 2010).

One limitation of the reported alternative harvesting technologies is that very few economic assessments have been performed. Danquah et al. (2009) presented a comparative economic study on the use of chemical flocculation and tangential flow filtration (TFF) for dewatering a *Tetraselmis suecica* culture. The authors showed that by using TFF, the microalgae were concentrated up to 148 times, and the process consumed 2.06 $kWh*m^{-3}$ of energy. In contrast, when flocculation was used, the microalgae were concentrated up to 357 times, and the process consumed 14.81 $kWh*m^{-3}$. An economic evaluation showed that TFF required a higher initial capital investment over polymer flocculation. However, the payback period for TFF at the upper extreme of microalgae revenue was approximately 1.5 years while that for flocculation was approximately 3 years. This example demonstrates the usefulness of economic assessments and indicates that TFF is more convenient than flocculation in this particular case. More studies of this type are needed to draw a clear conclusion about the limitations or advantages of flocculation on a larger scale.

5.4. Bio-flocculation

Bio-flocculation using microalgae species with self-aggregation properties was described a few decades ago as a potential harvesting procedure (Olguín, 2003). In a previous study (Borowitzka, 1988), a cyanobacterium of the genus *Phormidium* was isolated because of its capacity to release a bio-flocculant. This cyanobacterium secreted a large molecular weight polymer that contained polysaccharide, fatty acid and protein moieties. In another work, a filamentous bio-flocculating microalga of the genus *Chlorhormidium* was used to successfully treat secondary municipal effluents (Sérodes et al., 1991). There is still limited knowledge of the factors that influence bio-flocculation, and more research is needed to apply this approach on a larger scale. The literature in this field is limited, indicating that this area offers a very promising niche for research and development. A recent report on this subject used a combination of chemical flocculants together with a bio-flocculant polymer that was naturally produced by a bacterium (*Paenibacillus polymyxa*). Kim et al. (2011) reported the sequential addition of 8.5 mM $CaCl_2$, 0.2 mM $FeCl_3$ and 1% of a bio-flocculant extracted from the culture broth of *P. polymyxa* AM49. They observed a high flocculating activity (up to 95%) of dense cultures of *Scenedesmus* sp. The possibility of reusing the culture medium after harvesting the biomass to further cultivate microalgae was also investigated.

Another promising approach involved mixing flocculating (*A. falcatus*, *S. obliquus* and *T. suecica*) and non-flocculating (*C. vulgaris* and *N. oleoabundans*) microalgae in order to enhance the recovery efficiency of the mixture (Salim et al., 2010). The lipid content of the species used was, on average, more than 25% of their dry weight. This factor helped maintain a recovered product with the expected oil content for biodiesel production; no addition of chemical flocculants was required. Taking into account the cost of production of the flocculating microalgae in a separate cultivation system, the authors recognized that more work is required in order to determine the economic viability of bio-flocculating microalgae on a larger scale.

6. Concluding remarks and future perspective

Currently, there are a number of technological, market and policy barriers that affect the economic feasibility of producing biodiesel from microalgae. Nevertheless, a number of potential business opportunities exist if the production of these alternative biofuels becomes a part of an integrated Biorefinery system. In this scenario, high-value biofuels and chemical products are produced and contribute to the economic viability of other products. To this end, dual purpose microalgae–bacteria based systems that treat wastewater and produce biofuels and chemical products offer substantial savings over microalgae biomass production. These systems have the advantage of not competing for the fresh water resources needed for agriculture and add to the value of treated wastewater.

A number of recommendations can be made to overcome the technological constraints of a Biorefinery system that uses microalgae to simultaneously treat wastewater and produce biofuels and high added value products: a) scale up the laboratory results and establish pilot plants (data from these large scale facilities should be the subject of economic feasibility analysis and Life Cycle Analysis); b) avoid extrapolations of biomass, lipid, high added value products and biofuel productivity using data generated at the laboratory level; c) intensify research on integrating systems that treat wastewater with microalgae, especially in the areas of nutrient availability, toxic compound effects and population dynamics in mixed cultures and consortia; d) use polluted rivers as source of nutrients for microalgae cultivation, simultaneously treating the water through phycoremediation and phytoremediation; e) demonstrate the efficiency and cost effectiveness of various alternative biomass harvesting technologies on a large scale; f) provide clear answers to measure the Energy Balance and economic viability of biodiesel production from microalgae when using wastewater; and g) assess the economic benefit of an integrated system for cultivation of oleaginous microalgae and *Arthrospira* (using different ponds) as sources of high value added products and perform an Energy Balance analysis and Life Cycle Analysis of each biofuel production.

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