Prospects for microbial biodiesel production

Shuobo Shi; Juan Octavio Valle -Rodríguez; Verena Siewers; Jens Nielsen*

Department of Chemical and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden

Keywords: Biofuel, Fatty acids, Metabolic engineering, Microbial biodiesel

*Corresponding author:

Professor Jens Nielsen,

Mailing address: Department of Chemical and Biological Engineering, Chalmers University of Technology, Kemivägen 10, SE-41296, Göteborg, Sweden.

Telephone: + 46 31 772 3804.

Fax: + 46 31 772 3801.

E-mail: nielsenj@chalmers.se.

Abstract

As the demand for biofuels for transportation is increasing, it is necessary to develop technologies that will allow for low-cost production of biodiesel. Conventional biodiesel is mainly produced from vegetable oil by chemically transesterification, but this production has relatively low land-yield and is competing for agricultural land that can be used for food production. There is therefore an increasing interest in developing microbial fermentation processes for production of biodiesel as this will allow for use of a wide range of raw-materials, including sugar cane and biomass. Production of biodiesel by microbial fermentation can be divided into two different approaches, (1) indirect biodiesel production from oleaginous microbes by transesterification *in vitro*, and (2) direct biodiesel production from the redesigned cell factories. This work reviews both microbial approaches for renewable biodiesel production and evaluates the existing challenges in these two strategies.

1. 1. Introduction

Due to population growth and industrialization, the demand for energy has increased rapidly in recent years, and the world energy consumption is projected to increase by 49% from 2007 to 2035 (http://www.eia.doe.gov/oiaf/ieo/highlights.html). However, the primary source of energy, fossil fuels, is now widely recognized to be unsustainable and will be exhausted in the foreseeable future. Moreover, fossil fuel emissions are believed to be a major contributor to global warming [1]. Consequently, worldwide concerns have been raised to search for sustainable, alternative, and renewable fuels that have a lower environmental impact and that can satisfy the energy needs in the future [2, 3].

The development of different biofuels as alternative, sustainable fuels is expected to relieve the current energy crisis [4]. Currently, the most widely used biofuels are biodiesel and bioethanol. However, bioethanol is presently not viewed as the ideal biofuel in the future because of its low energy density and incompatibility with the existing fuel infrastructure [5, 6]. There is therefore much interest to introduce other biofuels, e.g. butanol [6, 7], that can be easier blended into gasoline and is non-corrosive and can hence be implemented in the current fuel infrastructure. There is also much interest in biodiesel (fatty acid esters) which are easily fitting into the existing infrastructure and has been thoroughly tested as an alternative fuel on the market. As a fuel, biodiesel is similar to petro-diesel in combustion properties, allowing it to work well in conventional diesel engines and making it compatible with the existing fuel infrastructure [8]. Besides, biodiesel is better than petro-diesel in several characteristics, such as environmental friendliness, renewability, reduced emission, higher combustion efficiency, improved lubricity, better safety, etc. [9].

In light of these demands, the total world biodiesel production has been constantly increasing, with a 16 fold increase over the last 10 years, and was estimated to amount to about 4 billion gallons in 2009, mainly produced in the European Union and the USA [10]. Currently, biodiesel is mainly produced from plant oils by transesterification with alcohol (methanol or ethanol) in the presence of a base, an acid or an enzyme catalyst (Fig. 1A). For cost reasons methanol is the reagent most frequently used for transesterification in a molar ratio of 1:1. The plant oils account for a large percent of the overall production cost [11]. Currently, the high cost and limited availability of plant oils has become a rising problem for large-scale commercial viability of biodiesel production, and different ways have been explored to address this problem. For example, microbial oils, genetically modified crops, soapstocks, used cooking oil and animal fat could be explored as the alternative feedstock to lower the cost of biodiesel [12-17]; additionally, engineered microbes could be used to produce fatty esters (biodiesel) directly from simple sugars, which avoid using the costly feedstock [16-18].

Production of biodiesel using microorganisms has been considered as a promising alternative solution for biodiesel production. First, it is well known that many microbes, such as microalgae, bacteria, fungi or yeast, can accumulate intracellular lipids (mainly triacylglycerol) to a large percent of their biomass (Table 1). These oleaginous microbes oil could represent a promising raw material for biodiesel production through transesterification in line with the plant based process (Fig. 1B) [13, 16, 19]. In particular, through the use of fast growing microbes it is possible to use a wider variety of feedstocks such as sugar cane that has a substantially larger yield per hectare compared with rapeseed and biomass, and hence allows for biodiesel production with less use of arable land.

On the other hand, with the help of metabolic engineering and synthetic biology, interest has grown to engineer well-studied microbes such as *Escherichia coli* and *Saccharomyces cerevisiae* into biodiesel cell factories by introducing an ester synthesizing pathway, which could lead to direct production of fatty acid ethyl esters (FAEEs) by direct esterifying ethanol with the acyl moieties of the CoA thioesters of fatty acids (Fig. 1C) [18, 20, 21]. These engineered cell factories could produce biodiesel directly from cheap and widely available sugars such as glucose or abundant lignocellulosic biomass circumventing the need for a transesterification process, which requires complex pretreatment involving isolation and purification. Clearly including the entire transformation process in one step will be the most convenient and cost-effective way for large-scale production of biodiesel.

In the following we will review the two different approaches to production of biodiesel by microbial fermentation.

1. 2. Indirect biodiesel production from oleaginous microbes

Oleaginous microorganisms could be new lipids feedstock for biodiesel production. The oils can be extracted from the fast grown microorganisms and transesterified with short-chain alcohols, yielding high quality biodiesel esters that comply with the currently existing standards [35, 36]. Besides, the wide array of microbial lipids makes it feasible to vary the biodiesel property such that it exhibits a combination of improved fuel properties [37]. Added to these advantages, using oleaginous microbes for the production of biodiesel will not compromise the production of food or products derived from crops.

Microalgae for biodiesel production

Microalgae are photoautotrophic-microorganisms that can convert carbon dioxide directly to biodiesel or other biofuels [38-40]. These microorganisms can produce different types of biofuels: biodiesel [22, 40], methane [41] and biohydrogen [42, 43].

Microalgae seem to be one of the most promising feedstock for providing large amounts of lipids that can be furtherly directed to synthesize renewable biodiesel to substitute fossil diesel, due to a very high oil yield (3.4 times more than corn oil yield) and a very low land area needed for its cultivation (3.4 times smaller lands required in comparison with corn cultivation). Furthermore, they have a higher content of oil than macroalgae [44], they grow very quick and some species are very rich in oil. They can double their biomass in 3.5 h during the exponential growth phase in batch cultures, and their common doubling time is around 24 h.

The microalgae oil content usually ranges between 20 to 60% by weight of dry biomass (Table 1); and in some genera such as *Botryococcus*, *Nannochloropsis* and *Schizochytrium* it can be close to 80%. Microalgae can produce many different kinds of lipids, hydrocarbons and complex oils, depending on the species [45]. Not all of them are satisfactory for the biodiesel production, but the suitable ones occur commonly. These oils produced in microalgae are mainly unsaturated fatty acids: palmitoleic (16:1), oleic (18:1), linoleic (18:2) and linolenic (18:3) acids. Saturated fatty acids such as palmitic (16:0) and stearic (18:0) acids are also present in low concentration [13]. In certain species, polyunsaturated fatty acids can be synthesized [46], but biodiesel produced from these compounds oxidizes faster than petroleum diesel, forming sediments that affect the combustion engine. The faster the microalga grows and the higher its oil content, the higher the biodiesel productivity will be.

Microalgae can contain high amounts of lipids, but compared to other oleaginous microorganisms they require large areas of land due their photosynthetic activity and other microbes, such as bacteria and yeasts can grow faster and easier than them [13]. Besides this, due their sunlight requirement, the daily and seasonal variations affect their growth and they also need low density cultures which implies big amounts of water required and its further treatment, increasing the production costs [26].

Bacterial biodiesel production

Bacteria can also be used as source for lipids production to finally obtain the esters that can constitute biodiesel.

Most bacteria produce mainly complex lipids, only few species can produce lipids that can be used as precursors of biodiesel [47]. The main source of lipids in these specific bacteria are triacylglycerols (TAGs), which only few genera of the actinomycetes class can accumulate TAGs to high levels, as in the case of *Acinetobacter* [48], *Mycobacterium* [49] and *Streptomyces* [50]. These TAGs are accumulated inside the cell specially when bacteria are grown on simple carbon sources under stress conditions [28]. It has been found that strains from *Rhodococcus opacus* can accumulate up to 87% (by dry weight) [51]; the TAG bodies of these bacteria are mainly composed of TAGs (87%), diacylglycerols (~5%), free fatty acids (~5%), phospholipids (1.2%) and proteins (0.8%) [52]. The TAGs were mainly formed by hexadecanoic acid (16:0) and octadecenoic acid (18:1) [53]. Other bacteria genera, such as *Gordonia* sp. can accumulate up to 72% TAG with a predominant composition of docosanoic acid (22:0) and hexanoic acid (6:0) [29]. Genera such as *Streptomyces* synthesize TAGs, but only in the absence of a nitrogen source [50].

With the advance of systems biology and metabolic engineering, there is the possibility to engineer common production hosts such as *E. coli* to greatly increase its fatty acids production ability [54]. Metabolically modified *E. coli* can produce fatty acids at 2.5 g/L by knockingout the *fadD* gene(encoding the fatty acyl-CoA synthetase) and by overexpressing ACC and *E. coli* or plant thioesterase. This effort opens the door to harnessing multiple metabolic tools in constructing an efficient fatty acids producing cell from a non oleaginous microbe. Although bacteria accumulate low concentrations of lipids (compared to microalgae, for example); they have other advantages related to biodiesel production: they possess a higher specific growth rate (usually reaching high biomass levels in 12 – 24 h) and they are easy to cultivate.

Fungi for biodiesel production

Fungi can also be utilized as a lipid source for biodiesel production. Some species can produce high concentrations of lipids such as *Humicola lanuginosa* (75%) [13]. In other fungi different levels of lipids can be obtained: it has been reported that *Aspergillus oryzae* can accumulate 57% lipid [13], and in *Mucor rouxii* a lipid content of 30% was found, among these lipids the one that was present in the highest concentration was linolenic acid (3-17%) [55].

Biodiesel esters can be produced from the filamentous fungus *Mucor circinelloides*, from which a lipid content of 19.9 % (by wt) was reported using as extraction solvent mixture of chloroform andmethanol (2:1 ratio). There were two procedures followed for the formation of esters: transformation of extracted lipids and direct transformation of dry fungus biomass. The transesterification reaction was realized during 8 h at 65 ºC in the presence of an acid catalyst (in this case: BF_3, H_2SO_4 or HCl). Surprisingly, the direct method produced fatty acid methyl esters at a higher yield and purity (>99% for all catalysts) than those from the two steps process (91.4 – 98.0 %). These esters produced can be used directly as biodiesel [56].

Among fungi, oleaginous yeasts are distinguished by their capacity to accumulate high concentrations of lipids (over 20% of their biomass). Species such as *Rhodosporidium toruloides* and *Lipomyces starkeyi* have been found to accumulate lipids around 60% and 70% of dry cell weight (Table 1), these lipids are mainly constituted by TAGs [57]. Besides conventional batch culture, other fermentative systems applied to *Rhodosporidium toruloides* can achieve a higher productivity of lipids synthesis (0.54 g/L/h in fed-batch culture), these lipids were mainly oleic, palmitic, stearic and linoleic acids [58].

Lipids produced from yeasts can be converted into esters to constitute biodiesel. In the case of *T. fermentans*, a high methyl esters yield (92%) was found by transesterification of fatty acids extracted from cells [59]. A direct transesterification process from yeast biomass could be achieved but low yields (less than 20%) have been found. In a particular study, methanolysis of *L. starkeyi* biomass was performed under mediation of alkali metal hydroxides under heating at 70 ºC for 24 h [31]. Another option that can be applied is utilizing different mineral acids (H_2SO_4, HCl and H_3PO_4) as catalysts. The reaction was started mixing powdered cells with a methanolic solution of a mineral acid and heated at 70 ºC. The methyl esters yields were 60 and 53%, for H_2SO_4 at 0.1 M and HCl at 0.2 M, respectively, in a reaction with a biomass:methanol ratio of 1:20 (w/v) for 16 hours [31]. The factors influencing this esterification yield are: acid catalyst selected and its concentration, time course of the reaction, temperature and biomass:methanol ratio.

As mentioned above, in order to process the transesterification reaction with the oils from oleaginous microbial cells, several unit operations should be performed. Conventionally, the transesterification is perfomed with the alcohol (methanol) and triacylglycerides extracted from dried microbial biomass, but a novel single-step method has been developed that transesterifies lipids by direct alcoholysis of dried microbial biomass, without previous lipid extraction (Fig. 2) [24, 31]. However, even the single-step method requires an additional expense for the pretreatment of biomass. It would further reduce the cost of the inexpensive oleaginous microbe feedstock, if methods without drying of the biomass could be developed. The current catalysts used for transesterification are chemical catalysts, due to their high conversion efficiency at low costs, but they involve complex operations such as treatment of contaminated water and recovery of biodiesel esters. Recently, biocatalytic transesterification techniques using lipases have been presented as a less energy intensive and environmentally friendly method, and with yields exceeding 90% [60]. Biocatalytic transesterification has therefore received much attention, especially in the area of immobilization [61] and whole-cell biocatalysis [62, 63].

2. 3. Direct biodiesel production from engineered cell factories

Much research efforts have focused on direct production of biodiesel through microbial conversion from abundant and cost-effective renewable resources without any additional modifications, and this became feasible with the finding of a novel bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT), which could synthesize wax esters from alcohols and fatty acid coenzyme A thioesters (acyl-CoA) [64]. Biodiesel produced in this process is primarily ethanol-yielding fatty acid ethyl esters (FAEEs), which have better performances than methanol-yielded fatty acid methyl esters (FAMEs). Furthermore, used methanol for transesterification is largely derived from non-renewable natural gas and is toxic and hazardous (Fig. 1A and B), and there are therefore many benefits for making biodiesel (FAEEs) directly using a redesigned microbial cell factory (Fig. 1C). Currently, the two model organisms, *E. coli* and *S. cerevisiae*, are being used to develop direct biodiesel production.

The idea of wax-ester production was first accomplished by Kalscheuer et al [20]. They successfully expressed the WS/DGAT gene (*atfA*) from the *Acinetobacter baylyi* strain ADP1 in combination with the ethanol production genes (*pdc* and *adhB*) from *Zymomonas mobilis* in *E. coli* and applied the recombinant *E. coli* for biodiesel production (Fig. 3). A final fatty acid ethyl ester (FAEE) content of 1.28 g/L was achieved after 72 h of fermentation supplemented with exogenous fatty acids. The research is an excellent demonstration of feasibility for microbial direct production of fatty acid esters although the metabolism needs to be further optimized.

Furthermore, Steen et al harnessed the extensively investigated fatty acid metabolism in bacteria to engineer *Escherichia coli* to produce biodiesel directly from simple sugars [18]. The flux through the.fatty acid pathway was increased to improve production of free fatty acids and acyl-CoAs by eliminating β-oxidation, by over-expressing thioesterases and acyl-CoA ligases. Biodiesel was produced by expressing a wax-ester synthase and ethanol producing genes (Fig. 3). In the presence of glucose, the yield of produced biodiesel could reach 674 mg/L. By further introducing xylanases, the engineered *E. coli* could produce biodiesel to 11.6 mg/L directly from hemicellulose, a major component of plant-derived biomass.

Production of biodiesel directly from microorganisms has also been reported in recent patent applications [65-67], all owned by LS9 Inc. (Fig. 3). Briefly, the metabolically engineered *E. coli* strain was manipulated to be able to produce biodiesel and fatty acid derivatives thereof (short and long chain alcohols, hydrocarbons, fatty alcohols, waxes, etc). The *fadE* gene was first disrupted in *E. coli*, which was not capable of degrading fatty acids and fatty acyl-CoAs. Then the enforced fatty acids biosynthesizing ability and fatty acid derivatives production ability were accomplished through the overexpression of several genes encoding for enzymes like thioesterase (*tesA*), acyl-CoA synthase (*fadD*), acetyl-CoA carboxylase (*accABCD*), fatty acid synthase (*fabH*, *fabD*, *fabG*, *fabF*), acyl carrier protein (*acpP*), wax synthase (*atfA*), alcohol acyltransferase, alcohol dehydrogenase, and different kinds of fatty alcohol forming acyl-CoA reductases. To further enhance fatty acids production, genes *aceEF* had been suggested to express in a production host, accompanied by attenuating glycerol-3-phosphate dehydrogenase (*gpsA*), lactate dehydrogenase (*ldhA*), pyruvate formate lyase I (*pflB*), phosphate acetyltransferase (*pta*), pyruvate oxidase (*poxB*), acetate kinase (*ackA*), and glycerol-3-phosphate O-acyltransferase (*plsB*). Later, in US patent publication 2010/0071259 inventors from the same company showed that by adding a mixture of at least two different alcohols to a medium containing the engineered fatty esters producing *E. coli* strain, at least two different fatty esters could be produced. In particular, by selecting various types and/or amounts of alcohols, it was possible to produce a desired fatty ester composition, i.e. designed biodiesel, which would possess improved fuel properties, such as desired cloud point, cetane number, viscosity and lubricity [68].

On the one hand, it should be noticed that ethanol, one of the two substrates for biodiesel, is not naturally produced by *E. coli*. Establishment of heterologous ethanol biosynthesis is a prerequisite for *E. coli* biodiesel producer. In this regard, a far better choice of microbial cell factory for industrial production of biodiesel would be the yeast *S. cerevisiae*, which is a well-known organism used in the production of ethanol through the fermentation of glucose [69].

Using the same principle as for *E. coli*, it has been reported that novel lipids, including FAEEs and fatty acid isoamyl esters (FAIEs), could be produced in *S. cerevisiae* H1246 with oleic acid addition by expressing the *A. baylyi* bifunctional WS/DGAT enzyme [70]. This study indicated that the un-specificity of WS/DGAT from *A. calcoaceticus* ADP1 could lead to the biosynthesis of a large variety of lipids *in vivo* in a eukaryotic expression host.

A recent patent application, namely US patent 2009/0117629 by Schmidt-Dannert and Holtzapple [71] also describes a method for the production of biodiesel and wax esters by heterologous expression of wax synthase (WS2) from *Marinobacter hydrocarbonoclasticus* in *S. cerevisiae* by exogenous supply of fatty acids. The WS2 from *M. hydrocarbonoclasticus* performs a higher wax synthase activity for ethanol compared the *A. baylyi* bifunctional WS/DGAT enzyme. Moreover, unlike the *A. baylyi* bifunctional WS/DGAT enzyme, the WS2 does not have DGAT activity, which catalyzes the formation of TAG from fatty acids. TAG synthesis would function as competitive pathway for biodiesel production. Hence, the WS2 from *M. hydrocarbonoclasticus* is very suitable in the particular purpose of producing biodiesel, and gives a titer of ethyl oleate to approximately 62 mg/L in the oleate added (0.11%, w/v).

3. 4. Conclusions and Perspectives

The use of biodiesel has grown dramatically during the last few years, and is believed to increase even further in the future. The conventional production of biodiesel through transesterification of triacylglycerols derived from plant oils requires the involvement of limited plant resources and petro-chemically derived methanol (Fig. 1A). Furthermore, the high costs involved in the use of plant resources associated as well as the issue of using food/feed grade products for fuel production is prohibitive for large-scale biodiesel production using the conventional technology.

Biodiesel production through microbial systems is therefore receiving increasing attention as a cost-effective, sustainable alternative. Press releases from several different companies indicate that there are several microbial biodiesel projects ongoing, e.g. ExxonMobil Corp., Dow Chemical Co., LS9 Inc., Amyris Biotechnologies Inc., Codexis Inc, BP and Martek Biosciences Corp.

The application of microorganisms for efficient production of biodiesel will require a significant de-regulation of lipid metabolism, which represents a big challenge due to its complexity and limited knowledge [18, 72, 73]. Recent progresses in synthetic biology and systems biology have accelerated the ability to analyze and implement metabolic pathways with unprecedented precision [74-76]. More importantly, *in silico* metabolic models enabled the systematic elucidation and design of biology systems with desired properties, e.g. enhanced lipid accumulation, or engineered pathways for *de novo* biodiesel production *in vivo*, making microorganism an ideal platform for future biodiesel production.

Acknowledgements

The authors would like to thank …………..

The authors have declared no conflict of interest.

Reference:

[1] International Energy Agency, World Energy Outlook, OECD Publication Service, Paris, 2008.

[2] Hirsch, R. L., Bezdek, R., Wendling, R., Peaking of World Oil Production and Its Mitigation. *AIChE J.* 2006, *52*, 2-8.

[3] Kerr, R. A., CLIMATE CHANGE: Global Warming Is Changing the World. *Science* 2007, *316*, 188-190.

[4] Schubert, C., Can biofuels finally take center stage? *Nat. Biotech.* 2006, *24*, 777-784.

[5] Stephanopoulos, G., Challenges in Engineering Microbes for Biofuels Production. *Science* 2007, *315*, 801-804.

[6] Atsumi, S., Hanai, T., Liao, J., Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* 2008, *451*, 86-89.

[7] Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K*., et al.*, Fermentative butanol production by clostridia. *Biotechnol. Bioeng.* 2008, *101*, 209-228.

[8] Demirbas, A., Diesel fuel from vegetable oil via transesterification and soap pyrolysis. *Energy Sources* 2002, *24*, 835-841.

[9] Demirbas, A., Importance of biodiesel as transportation fuel. *Energy Policy* 2007, *35*, 4661-4670.

[10] Brown, L., *Plan B 4.0: mobilizing to save civilization*, New York: W.W. Norton & Company 2009.

[11] Miao, X., Wu, Q., Biodiesel production from heterotrophic microalgal oil. *Bioresour. Technol.* 2006, *97*, 841-846.

[12] Canakci, M., Sanli, H., Biodiesel production from various feedstocks and their effects on the fuel properties. *J. Ind. Microbiol. Biotechnol.* 2008, *35*, 431-441.

[13] Meng, X., Yang, J., Xu, X., Zhang, L., *et al.*, Biodiesel production from oleaginous microorganisms. *Renew. Energ.* 2009, *34*, 1-5.

[14] Vega-Sánchez, M. E., Ronald, P. C., Genetic and biotechnological approaches for biofuel crop improvement. *Curr. Opin. Biotechnol.* 2010, *21*, 218-224.

[15] Lin, L., Cunshan, Z., Vittayapadung, S., Xiangqian, S., Mingdong, D., Opportunities and challenges for biodiesel fuel. *Appl. Energ.* 2010, *DOI: 10.1016/j.apenergy.2010.09.029*.

[16] Li, Q., Du, W., Liu, D., Perspectives of microbial oils for biodiesel production. *Appl. Microbiol. Biotechnol.* 2008, *80*, 749-756.

[17] Peralta-Yahya, P. P., Keasling, J. D., Advanced biofuel production in microbes. *Biotechnol. J.* 2010, *5*, 147-162.

[18] Steen, E., Kang, Y., Bokinsky, G., Hu, Z., *et al.*, Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* 2010, *463*, 559-562.

[19] Ahmad, A. L., Yasin, N. H. M., Derek, C. J. C., Lim, J. K., Microalgae as a sustainable energy source for biodiesel production: A review. *Renew. Sustain. Energ. Rev.* 2010, *DOI: 10.1016/j.rser.2010.09.018*.

[20] Kalscheuer, R., Stolting, T., Steinbuchel, A., Microdiesel: *Escherichia coli* engineered for fuel production. *Microbiology* 2006, *152*, 2529-2536.

[21] Schmidt-dannert, C., Holtzapple, E. K., Isoprenoid wax ester synthases, isoprenoid acyl coa-synthetases, and uses thereof, US patent 2009/0177629. 2009.

[22] Banerjee, A., Sharma, R., Chisti, Y., Banerjee, U., *Botryococcus braunii*: A Renewable Source of Hydrocarbons and Other Chemicals. *Crit. Rev. Biotechnol.* 2002, *22*, 245-279.

[23] Illman, A. M., Scragg, A. H., Shales, S. W., Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. *Enzyme Microb. Technol.* 2000, *27*, 631-635.

[24] Mata, T. M., Martins, A. A., Caetano, N. S., Microalgae for biodiesel production and other applications: A review. *Renew. Sustain. Energ. Rev.* 2010, *14*, 217-232.

[25] Khozin-Goldberg, I., Cohen, Z., The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte *Monodus subterraneus*. *Phytochemistry* 2006, *67*, 696-701.

[26] Chisti, Y., Biodiesel from microalgae. *Biotechnol. Adv.* 2007, *25*, 294-306.

[27] Khozin-Goldberg, I., Bigogno, C., Shrestha, P., Cohen, Z., Nitrogen starvation induces the accumulation of arachidonic acid in the freshwater green alga *Parietochloris incisa* (*Trebouxiophyceae*). *J. Phycol.* 2002, *38*, 991-994.

[28] Alvarez, Steinbüchel, Triacylglycerols in prokaryotic microorganisms. *Appl. Microbiol. Biotechnol.* 2002, *60*, 367-376.

[29] Gouda, M., Omar, S., Aouad, L., Single cell oil production by *Gordonia* sp. DG using agro-industrial wastes. *World J. Microbiol. Biotechnol.* 2008, *24*, 1703-1711.

[30] Chen, H.-C., Chang, C.-C., Production of γ-Linolenic Acid by the Fungus *Cunninghamella echinulata* CCRC 31840. *Biotechnol. Prog.* 1996, *12*, 338-341.

[31] Liu, B., Zhao, Z., Biodiesel production by direct methanolysis of oleaginous microbial biomass. *J. Chem. Technol. Biotechnol.* 2007, *82*, 775-780.

[32] Čertík, M., Baltészov, L., Šajbidor, J., Lipid formation and γ-linolenic acid production by *Mucorales* fungi grown on sunflower oil. *Lett. Appl. Microbiol.* 1997, *25*, 101-105.

[33] Ratledge, C., Wynn, J., The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv. Appl. Microbiol.* 2002, *51*, 1-44.

[34] Angerbauer, C., Siebenhofer, M., Mittelbach, M., Guebitz, G. M., Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production. *Bioresour. Technol.* 2008, *99*, 3051-3056.

[35] Vicente, G., Bautista, L. F., Gutiérrez, F. J., Rodríguez, R. a*., et al.*, Direct Transformation of Fungal Biomass from Submerged Cultures into Biodiesel. *Energy Fuels* 2010, *24*, 3173-3178.

[36] Xu, H., Miao, X., Wu, Q., High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. *J. Biotechnol.* 2006, *126*, 499-507.

[37] Knothe, G., Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Process. Technol.* 2005, *86*, 1059-1070.

[38] Belarbi, E.-H., Molina, E., Chisti, Y., RETRACTED: A process for high yield and scaleable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil. *Proc. Biochem.* 2000, *35*, 951-969.

[39] Metting, B., Pyne, J. W., Biologically active compounds from microalgae. *Enzyme Microb. Technol.* 1986, *8*, 386-394.

[40] Roessler, P. G., Brown, L. M., Dunahay, T. G., Heacox, D. A., *et al.*, Genetic Engineering Approaches for Enhanced Production of Biodiesel Fuel from Microalgae, *Enzymatic Conversion of Biomass for Fuels Production*, American Chemical Society 1994, pp. 255-270.

[41] Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., Commercial applications of microalgae. *J. Biosci. Bioeng.* 2006, *101*, 87-96.

[42] Fedorov, A. S., Kosourov, S., Ghirardi, M. L., Seibert, M., Continuous Hydrogen Photoproduction by *Chlamydomonas reinhardtii*, in: Davison, B. H., Evans, B. R., Finkelstein, M., McMillan, J. D. (Eds.), *Twenty-Sixth Symposium on Biotechnology for Fuels and Chemicals*, Humana Press 2005, pp. 403-412.

[43] Kapdan, I. K., Kargi, F., Bio-hydrogen production from waste materials. *Enzyme Microb. Technol.* 2006, *38*, 569-582.

[44] Shay, E. G., Diesel fuel from vegetable oils: Status and opportunities. *Biomass Bioener.* 1993, *4*, 227-242.

[45] Guschina, I. A., Harwood, J. L., Lipids and lipid metabolism in eukaryotic algae. *Prog. Lipid Res.* 2006, *45*, 160-186.

[46] Thomas, W., Tornabene, T., Weissman, J., Screening for Lipid Yielding Microalgae: Activities for 1983. *SERI/STR-231-2207. p. 31* 1984.

[47] Shaojin, Y., Yiping, Z., Research and Application of Oleaginous Microorganism. *China Foreign Energy* 2006, *11*, 90-94.

[48] Makula, R. A., Lockwood, P. J., Finnerty, W. R., Comparative analysis of the lipids of *Acinetobacter* species grown on hexadecane. *J. Bacteriol.* 1975, *121*, 250-258.

[49] Barksdale, L., Kim, K., Mycobacterium. 1977, *41*, 217-372.

[50] Olukoshi, E. R., Packter, N. M., Importance of stored triacylglycerols in *Streptomyces*: possible carbon source for antibiotics. *Microbiology* 1994, *140*, 931-943.

[51] Alvarez, H. M., Kalscheuer, R., Steinbüchel, A., Accumulation and mobilization of storage lipids by *Rhodococcus opacus* PD630 and *Rhodococcus ruber* NCIMB 40126 *Appl. Microbiol. Biotechnol.* 2000, *54*, 218-223.

[52] Alvarez, H. M., Mayer, F., Fabritius, D., Steinbüchel, A., Formation of intracytoplasmic lipid inclusions by *Rhodococcus opacus* strain PD630. *Arch. Microbiol.* 1996, *165*, 377-386.

[53] Waltermann, M., Luftmann, H., Baumeister, D., Kalscheuer, R., Steinbuchel, A., *Rhodococcus opacus* strain PD630 as a new source of high-value single-cell oil? Isolation and characterization of triacylglycerols and other storage lipids. *Microbiology* 2000, *146*, 1143-1149.

[54] Lu, X., Vora, H., Khosla, C., Overproduction of free fatty acids in *E. coli*: Implications for biodiesel production. *Metab. Eng.* 2008, *10*, 333-339.

[55] Somashekar, D., Venkateshwaran, G., Sambaiah, K., Lokesh, B. R., Effect of culture conditions on lipid and gamma-linolenic acid production by mucoraceous fungi. *Proc. Biochem.* 2003, *38*, 1719-1724.

[56] Vicente, G., Bautista, L. F., Rodríguez, R., Gutiérrez, F. J., *et al.*, Biodiesel production from biomass of an oleaginous fungus. *Biochem. Eng. J.* 2009, *48*, 22-27.

[57] Ma, Y.-l., Microbial oils and its research advance. *Chin. J. Bioprocess. Eng.* 2006, *4*, 7–11.

[58] Li, Y., Zhao, Z., Bai, F., High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. *Enzyme Microb. Technol.* 2007, *41*, 312-317.

[59] Zhu, L. Y., Zong, M. H., Wu, H., Efficient lipid production with *Trichosporon fermentans* and its use for biodiesel preparation. *Bioresour. Technol.* 2008, *99*, 7881-7885.

[60] Parawira, W., Biotechnological production of biodiesel fuel using biocatalysed transesterification: A review. *Crit. Rev. Biotechnol.* 2009, *29*, 82-93.

[61] Tan, T., Lu, J., Nie, K., Deng, L., Wang, F., Biodiesel production with immobilized lipase: A review. *Biotechnology Advances* 2010, *In Press, Uncorrected Proof.*

[62] Gao, B., Su, E., Lin, J., Jiang, Z.*, et al.*, Development of recombinant *Escherichia coli* whole-cell biocatalyst expressing a novel alkaline lipase-coding gene from *Proteus* sp. for biodiesel production. *J. Biotechnol.* 2009, *139*, 169-175.

[63] Fukuda, H., Hama, S., Tamalampudi, S., Noda, H., Whole-cell biocatalysts for biodiesel fuel production. *Trends Biotechnol.* 2008, *26*, 668-673.

[64] Kalscheuer, R., Steinbuchel, A., A Novel Bifunctional Wax Ester Synthase/Acyl-CoA: Diacylglycerol Acyltransferase Mediates Wax Ester and Triacylglycerol Biosynthesis in *Acinetobacter calcoaceticus* ADP1. *J. Biol. Chem.* 2003, *278*, 8075.

[65] Del Cardayre, S., Cockrem, M., Charles, M., Systems and methods for the production of fatty esters, *WO2009009391* 2009.

[66] Keasling, J., Hu, Z., Somerville, C., Church, G.*, et al.*, Production of fatty acids and derivatives thereof, *WO2007136762* 2007.

[67] Hu, Z., Valle, F., Enhanced production of fatty acid derivatives, *WO2008119082* 2008.

[68] Knothe, G., ‘Designer’ Biodiesel: Optimizing Fatty Ester Composition to Improve Fuel Properties. *Energy Fuels* 2008, *22*, 1358-1364.

[69] Bro, C., Regenberg, B., Forster, J., Nielsen, J., In silico aided metabolic engineering of *Saccharomyces cerevisiae* for improved bioethanol production. *Metab. Eng.* 2006, *8*, 102-111.

[70] Kalscheuer, R., Luftmann, H., Steinbuchel, A., Synthesis of Novel Lipids in *Saccharomyces cerevisiae* by Heterologous Expression of an Unspecific Bacterial Acyltransferase. *Appl. Environ. Microbiol.* 2004, *70*, 7119-7125.

[71] Schmidt-dannert, C., Holtzapple, E. K., Isoprenoid wax ester synthases, isoprenoid acyl coa-synthetases, and uses thereof, *US patent 2009/0177629 A1* 2009.

[72] Prather, K. L. J., Martin, C. H., *De novo* biosynthetic pathways: rational design of microbial chemical factories. *Curr. Opin. Biotechnol.* 2008, *19*, 468-474.

[73] Mukhopadhyay, A., Redding, A. M., Rutherford, B. J., Keasling, J. D., Importance of systems biology in engineering microbes for biofuel production. *Curr. Opin. Biotechnol.* 2008, *19*, 228-234.

[74] Gibson, D., Glass, J., Lartigue, C., Noskov, V., *et al.*, Creation of a bacterial cell controlled by a chemically synthesized genome. *Science* 2010, *329*, 52.

[75] Tyo, K., Alper, H., Stephanopoulos, G., Expanding the metabolic engineering toolbox: more options to engineer cells. *Trends Biotechnol.* 2007, *25*, 132-137.

[76] Stephanopoulos, G., Alper, H., Moxley, J., Exploiting biological complexity for strain improvement through systems biology. *Nat. Biotechnol.* 2004, *22*, 1261-1267.

Figure Legends

Figure 1. Biodiesel synthesis by (A) chemical or enzymatic transesterification reaction using oils from plants; (B) chemical or enzymatic transesterification reaction using oils from oleaginous microorganisms; and (C) direct synthesis using redesigned cell factories.

Figure 2. Process followed to synthesize biodiesel esters from oleaginous microorganisms.

Figure 3. Overview of engineered pathways for production of Biodiesel (fatty acid ethyl ester) from hemicelluloses or glucose in recombinant *E. coli* discussed in this review. Overexpressed genes or operons are indicated with red arrows; deleted or attenuated genes are indicated with red crosses; endogenous genes and endogenous pathways are highlighted in blue; introduced heterologous genes and heterologous pathways are highlighted in green (*C. stercorarium*), dark blue (*B. ovatus*), plum (*Z. mobilis*), olive green (*A. baylyi*), and yellow (*S. cerevisiae*).

Table 1. Lipid content of some oleaginous microorganisms

Microorganism	Lipid content (% dry wt)	Reference
Microalgae		
Botryococcus braunii	25 - 75	[22]
Chlorella emersonii	25 - 63	[23]
Dunaliella tertiolecta	16 - 71	[24]
Monodus subterraneus	39.3	[25]
Nannochloropsis sp.	31 - 68	[26]
Neochloris oleoabundans	29 - 65	[24]
Nitzschia sp.	45 - 47	[26]
Phaeodactylum tricornutum	18 - 57	[24]
Parietochloris incisa	>35	[27]
Schizochytrium sp.	50 - 77	[26]
Bacteria		
Arthrobacter sp.	>40	[28]
Acinetobacter calcoaceticus	27 - 38	[28]
Bacillus alcalophilus	18 - 24	[13]
Gordonia sp.	72	[29]
Rhodococcus opacus	24 - 25	[13]
Fungi		
Aspergillus oryzae	57	[13]
Cunninghamella echinulata	35	[30]

Humicola lanuginosa	75	[13]
Mortierella isabellina	53.2	[31]
Mucor mucedo	62	[32]
Yeasts		
Candida curvata	58	[13]
Cryptococcus albidus	65	[13]
Cryptococcus curvatus	58	[33]
Lipomyces starkeyi	68	[34]
Rhodosporidium toruloides	58	[31]

Figure 1A.

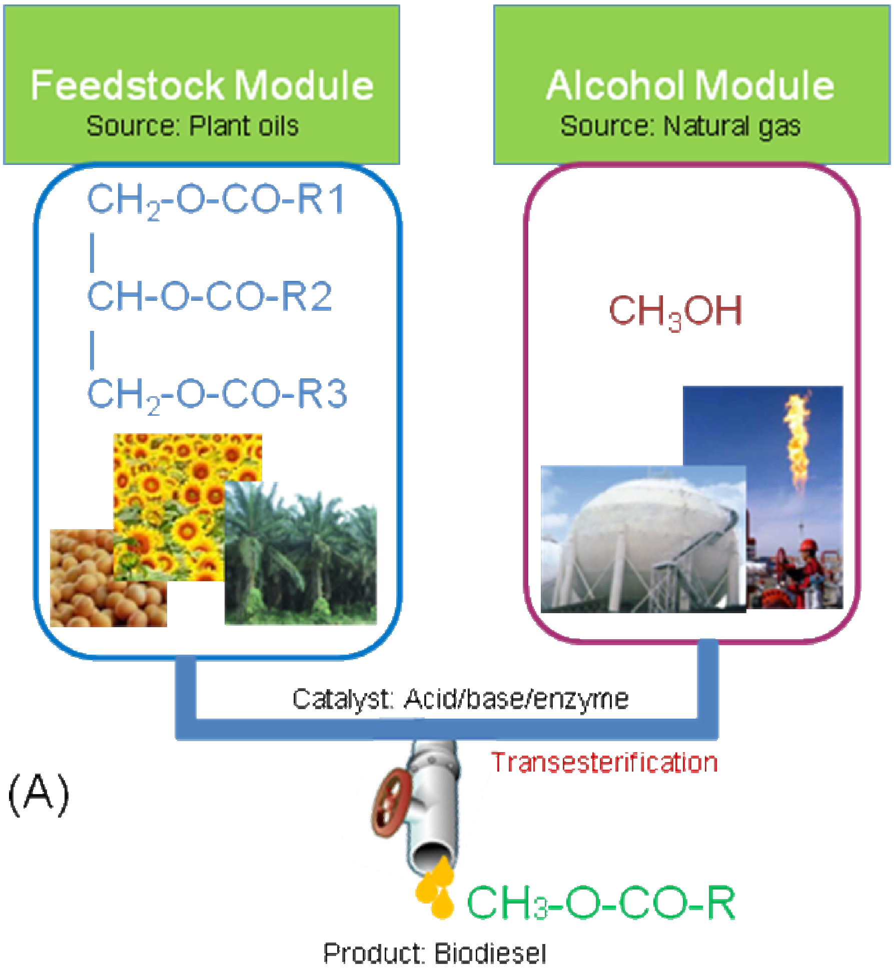

Feedstock Module
Source: Plant oils
Alcohol Module
Source: Natural gas
CH2-O-CO-R1
CH-O-CO-R2
CH2-O-CO-R3
CH3OH
Catalyst: Acid/base/enzyme
(A)
Transesterification
CH3-O-CO-R
Product: Biodiesel

Figure 1B.

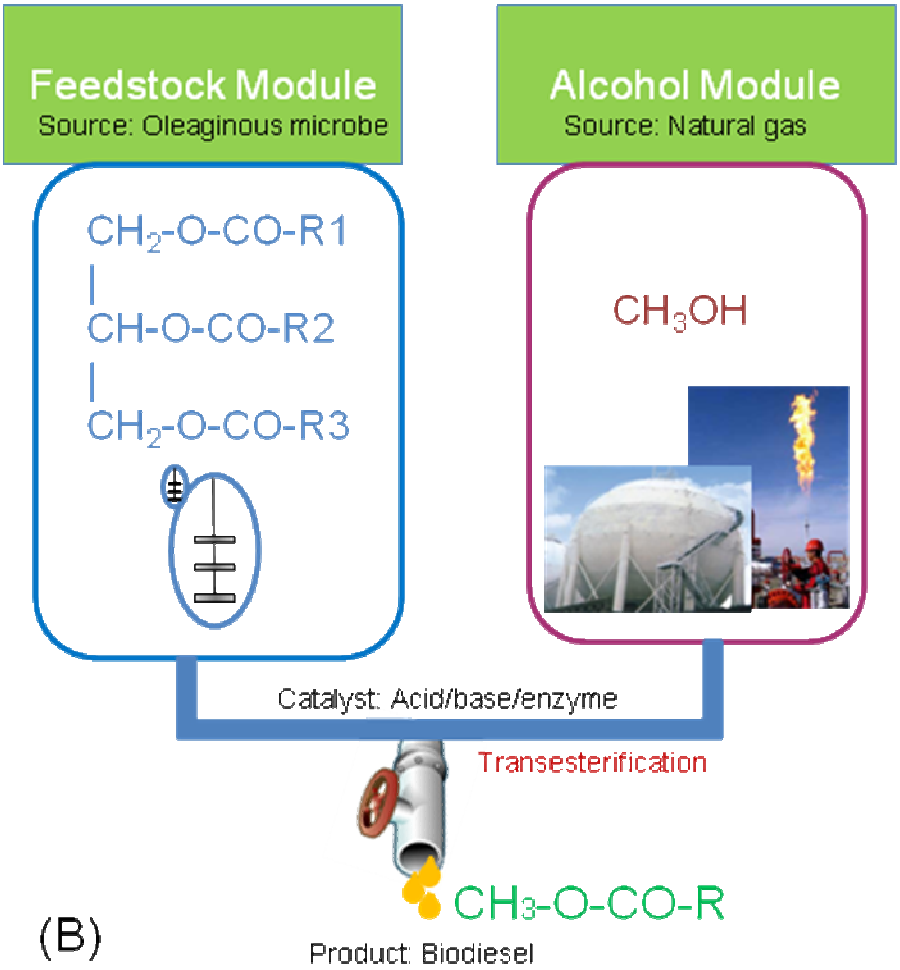

Feedstock Module
Source: Oleaginous microbe
CH2-O-CO-R1
CH-O-CO-R2
CH2-O-CO-R3
Alcohol Module
Source: Natural gas
CH3OH
Catalyst: Acid/base/enzyme
Transesterification
CH3-O-CO-R
Product: Biodiesel
(B)

Figure 1C.

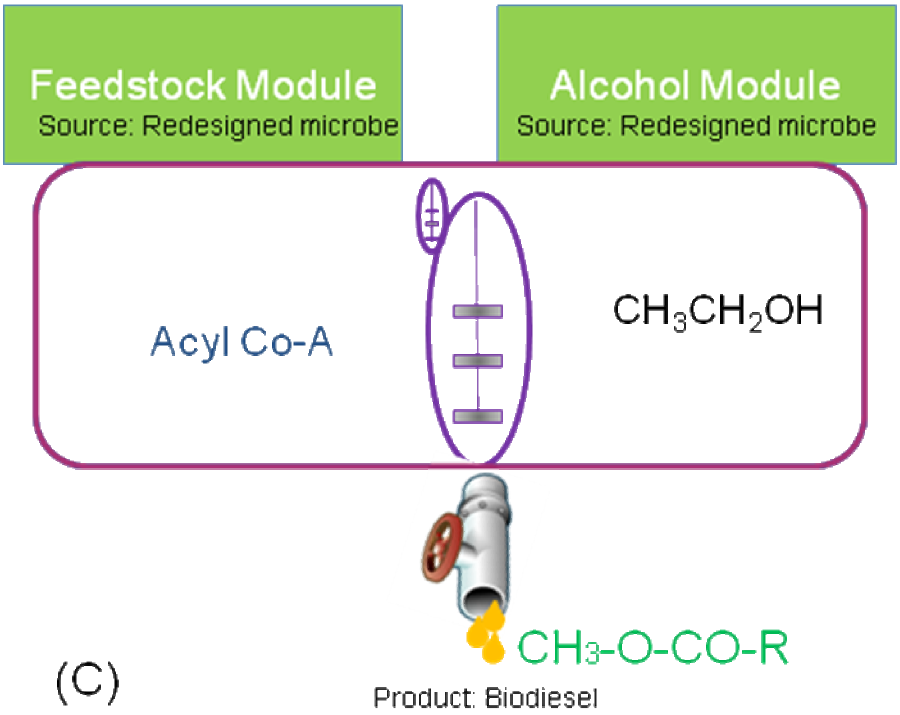

Feedstock Module
Source: Redesigned microbe
Alcohol Module
Source: Redesigned microbe
Acyl Co-A
CH_3CH_2OH
CH_3-O-CO-R
(C)
Product: Biodiesel

Figure 2.

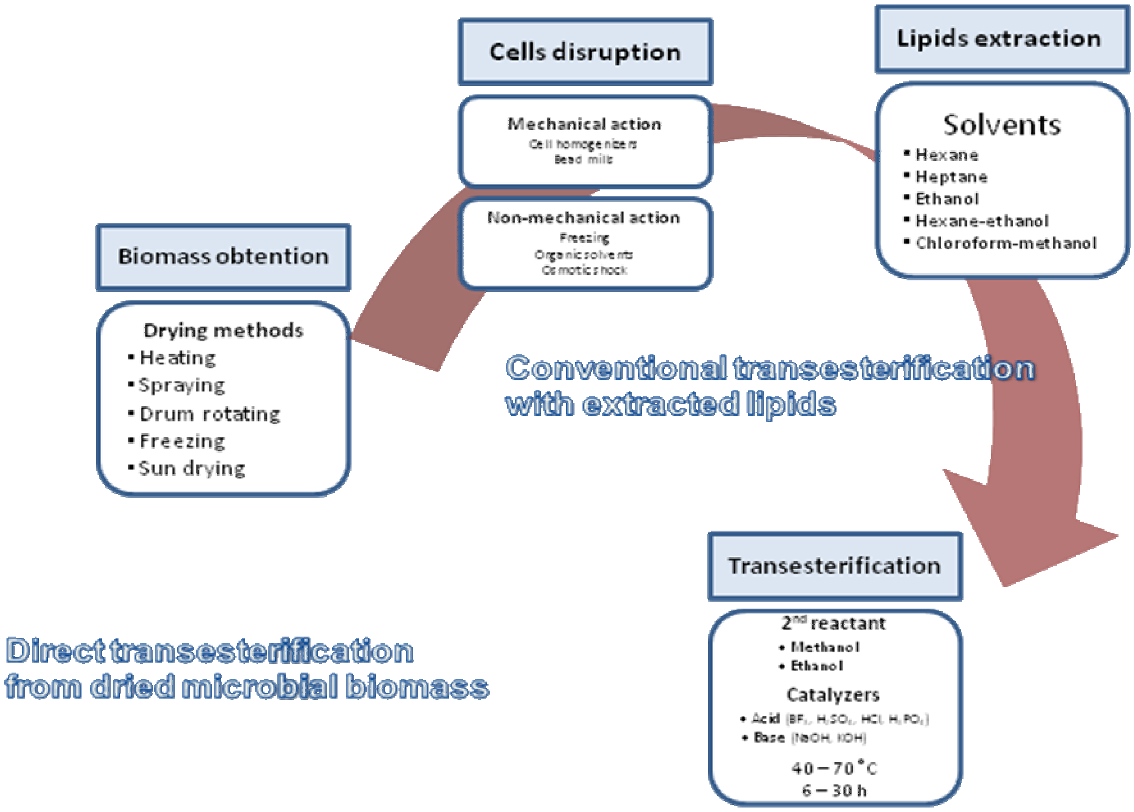
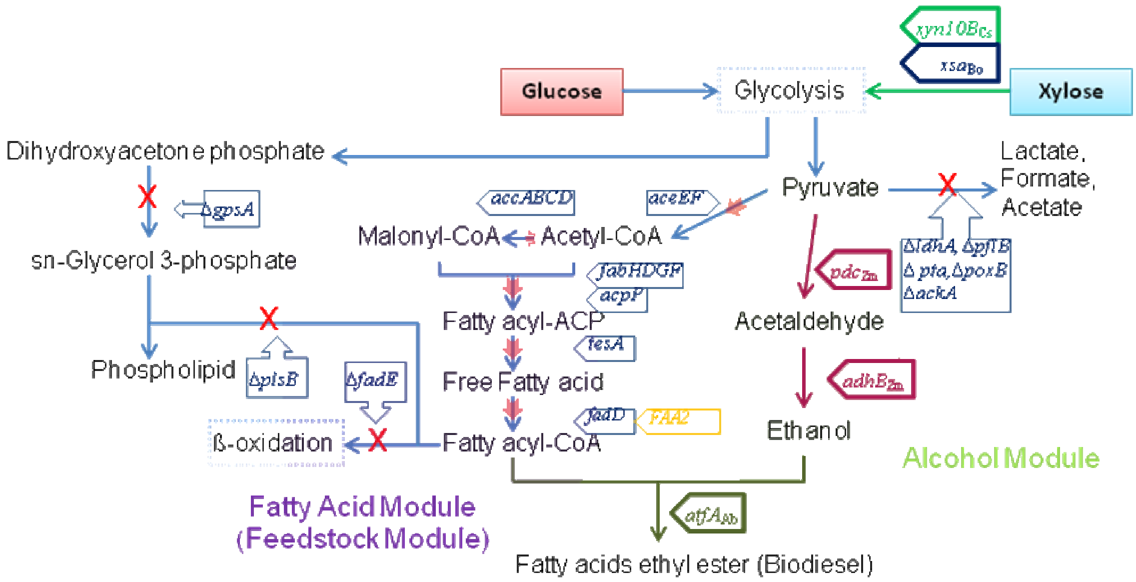

Cells disruption
Lipids extraction
Mechanical action
Cell homogenizers
Bead mills
Non-mechanical action
Freezing
Organic solvents
Osmotic shock
Solvents
▪ Hexane
▪ Heptane
▪ Ethanol
▪ Hexane-ethanol
▪ Chloroform-methanol
Biomass obtention
Drying methods
▪ Heating
▪ Spraying
▪ Drum rotating
▪ Freezing
▪ Sun drying
Conventional transesterification with extracted lipids
Transesterification
2nd reactant
• Methanol
• Ethanol
Catalyzers
• Acid (BF_3, H_2SO_4, HCl, H_3PO_4)
• Base (NaOH, KOH)
40 – 70 °C
6 – 30 h
Direct transesterification from dried microbial biomass

Figure 3.


xyn10BCs
xsaBo
Glucose
Glycolysis
Xylose
Dihydroxyacetone phosphate
ΔgpsA
sn-Glycerol 3-phosphate
accABCD
aceEF
Malonyl-CoA
Acetyl-CoA
Pyruvate
Lactate,
Formate,
Acetate
ΔldhA, ΔpflB
Δ pta,ΔpoxB
ΔackA
fabHDGF
acpP
pdcZm
Fatty acyl-ACP
Acetaldehyde
tesA
Phospholipid
ΔplsB
ΔfadE
Free Fatty acid
adhBZm
fadD
FAA2
Ethanol
ß-oxidation
Fatty acyl-CoA
Alcohol Module
Fatty Acid Module
(Feedstock Module)
atfAAb
Fatty acids ethyl ester (Biodiesel)