Review

Recent developments of downstream processing for microbial lipids and conversion to biodiesel

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PII: S0960-8524(18)30151-2
DOI: https://doi.org/10.1016/j.biortech.2018.01.129
Reference: BITE 19486

To appear in: *Bioresource Technology*

Received Date: 21 November 2017
Revised Date: 26 January 2018
Accepted Date: 27 January 2018

Please cite this article as: Yellapu, S.K., Bharti, Kaur, R., Kumar, L.R., Tiwari, B., Zhang, X., Tyagi, R.D., Recent developments of downstream processing for microbial lipids and conversion to biodiesel, *Bioresource Technology* (2018), doi: https://doi.org/10.1016/j.biortech.2018.01.129

Recent developments of downstream processing for microbial lipids and conversion to biodiesel

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Abstract

With increasing global population and depleting resources, there is an apparent demand for radical unprecedented innovation to satisfy the basal needs of lives. Hence, non-conventional renewable energy resources like biodiesel have been worked out in past few decades. Biofuel (e.g. Biodiesel) serves to be the most sustainable answer to solve "food vs. fuel crisis". In biorefinery process, lipid extraction from oleaginous microbial lipids is an integral part as it facilitates the release of fatty acids. Direct lipid extraction from wet cell-biomass is favorable in comparison to dry-cell biomass because it eliminates the application of expensive dehydration. However, this process is not commercialized yet, instead, it requires intensive research and development in order to establish robust approaches for lipid extraction that can be practically applied on an industrial scale. This review aims for the critical presentation on cell disruption, lipid recovery and purification to support extraction from wet cell-biomass for an efficient transesterification.

Keywords: Biomass harvesting, wet biomass, cell wall disruption, lipid recovery, transesterification, biodiesel purification

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1. Introduction

Worldwide fossil fuel (non-renewable fuel) demand is increasing day by day and its depletion concerns over greenhouse gas emission (GHG). With this rapid consumption, the oil resources will be exhausted within 40 years (Shafiee & Topal, 2009). Hence, the development of renewable fuels is attracting researchers. In order to mitigate the heavy reliablity on the raw material (vegetable oil or animal fat) availability, it is necessary to find some alternative feedstock to produce biodiesel. It has been widely reported that oleaginous microorganisms could be used as raw materials for producing biodiesel (Munch et al., 2015; Patel et al., 2015; Sitepu et al., 2014). Various studies have been done using Oleaginous microorganisms (lipid-producing microorganisms) like yeast, fungi, microalgae, and bacteria they can accumulate lipids in the form of TG (triglycerides), FFA (free fatty acids), sterols, polar lipids, hydrocarbon, and pigments. During downstream processing (bio-refinery process), harvested biomass is fractionated into biofuel, value-added co-products, and energy in order to create cost-effective biomass-based industry (Grima et al., 2013).

The biggest obstacle to biodiesel production from an oleaginous microorganism is the high cost (Amanor-Boadu et al., 2014; Benemann et al., 2011; Santander et al., 2014). It requires around minimum 6-8 $ to produce per gallon of biodiesel from an oleaginous microorganism which was only 2 to 3 $ for per gallon biodiesel produced from vegetable oil and animal fat (Davis et al., 2011; Delrue et al., 2012; Ramos Tercero et al., 2014). Autotrophic microorganisms (microalgae) use sunlight as driven power to convert carbon dioxide to lipid which requires zero cost in carbon utilization. However, the cultivation requires large land occupation and the lipid accumulation is slow (Bellou et al., 2014; Meng et al., 2009). Heterotrophic microorganisms are promising to produce lipid due to their ability to accumulate

high lipid content and rapid growth rate. The downstream processing such as, biomass harvesting, drying, cell wall disruption and transesterification are costly, which is the main cause of high biodiesel production cost (Koutinas et al., 2014). In order to lower the cost, multiple steps of downstream processing need to be reduced.

In literature, vigorous reviews have been published on downstream processing using microalgae as a feedstock. But there was a lack of knowledge using oleaginous yeast, fungi, and bacteria as a feedstock for biomass harvesting, wet cell wall disruption, and in-situ transesterification to obtain final product biodiesel.

This review mainly highlights important considerations involved since last five years (2013 – 2017) of literature on downstream processing of biodiesel obtained from oleaginous yeast, fungal and bacterial lipids. Advancement in biomass harvesting and mechanism of wet biomass cell wall disruption with various recent technologies has been introduced. This review presents a critical discussion about lipid recovery and its mechanism using organic and environmentally friendly solvents and its effects after lipid separation. This review contains in-depth analysis and discussion about lipid extraction and transesterification from wet biomass slurry and recent trends of biodiesel purification and challenges for researchers to make biodiesel an industrially feasible economical process.

2. Oleaginous microorganisms for lipids production

All microbes including prokaryotes and eukaryotes like fungi and yeast are known to produce lipids for regular cellular metabolism and structural purposes but recent research has identified many microbes mostly yeast and algae which are found to be accumulating a significant amount of intracellular lipids in the form of lipid vesicles which account for over 20% of dry biomass

weight. These organisms are classified as oleaginous microorganisms (Liang & Jiang, 2013). From last one decade, several studies have been done on microbial lipid production using yeast, fungi, and bacteria under lab scale and it consists of 30 to 80% wt lipid content. Origin of single cell oil dates back to 1985 when first single cell oil was produced from *Mucor circinelloides.* Since then many new microbes have been discovered, such as, *Cryptococcus sp, Lipomyces sp, Rhodosporidium sp, Rhodotorula sp, Trichosporon sp, Yarrowia sp, Aspergillus sp, Mortierrela sp, Thamnidium sp, Candida sp, Zygosacchromyces sp, Zygorhynchus sp, Mucor sp, Torulopsis sp and Pichia sp* (Levering et al., 2015). Table 1 gives a non-exhaustive list of the oleaginous microorganism employed for the production of single cell oil so far.

Table 1. Different types of lipids found in oleaginous microorganisms.

Name of Organism	**Neutral lipids (N.L) % w/w**	**Polar lipids (P.L) % w/w**	**References**
Fungi and yeast			
Cryptococcus curvatus ATCC20509	66.0	15.5	(Liang et al., 2012)
	35.9	7.6	(Gong et al., 2014)
Cryptococcus sp.	63.5 61.3 (fed batch)	9.4, 10.8 (fed batch)	(Chang et al., 2013)
Lipomyces starkeyi DSM 70295	56.39	13.3	(Angerbauer et al., 2008)
Lipomyces starkeyi	47	17.2	(Huang et al., 2014)
Microsphaeropsis sp	32.5	8	(Xiaowei & Hongzhang, 2012)
Rhodosporidium toruloides 21167	63.63	22	(Wang et al., 2012)
Rhodosporidium toruloides AS2 1389	69.66	26.7	(Xu et al., 2012)
Yarrowia lipolytica SKY 7	58.77	11.4	(Yellapu et al., 2016)

3. Biomass Harvesting

The initial step in the downstream processing for biodiesel production is the biomass harvesting from the fermented broth. Due to a tiny cell size of yeast, fungi, and bacteria (less than 5 µm in diameter), separation of biomass from the medium is the key bottleneck for the biodiesel production. After lipid accumulation, harvesting is the preliminary step for processing of biomass to biofuel, where water removal from yeast, fungi, and bacteria by centrifugation accounts 20-30% of total production cost (Dickinson et al., 2017).

Table 2. Different harvesting technologies used for microbial biomass separation

Harvesting method	Feasibility	Advantages or Effectiveness	Disadvantages	References
Centrifugation	• Microalgae, yeast, fungi, and bacteria	• Fast method. • 95-100% efficiency.	• Expensive method. • High energy requirements.	(Dassey & Theegala, 2013)
Chemical coagulation/Flocculation	• Microalgae.	• Low energy requirement • 95% efficiency	• Chemical flocculants may be expensive • Recycling of culture medium is limited	(Barros et al., 2015)
Flotation	• Microalgae	• Feasible for large-scale application. • Low-cost method. • 90-95% efficiency.	• Oversized bubbles break up the floc • Unfeasible for marine microalgae harvesting.	(Kurniawati et al., 2014)
Filtration	• Microalgae and fungi	• Allows the separation of shear sensitive species. • 70-89% efficiency	• High operational and maintenance cost. • Membrane replacement and pumping represent the major associated costs.	(Zhao et al., 2017)
Magnetic based separation	• Microalgae	• High recovery efficiencies. • Recovery and utilization of nanoparticles	• Nanoparticles are cost effective. • It is under lab-scale study.	(Yang et al., 2018)

3.1 *Recent trends in biomass harvesting*

Harvesting method has a great importance for economics and industrial biodiesel production using microbial lipid. The harvesting method depends upon characteristics of the microorganism. A vigorous research was done and critical reviews were published on biomass harvesting using microalgae, a brief overview was presented in Table 2 such as centrifugation, coagulation, filtration, and flotation. A huge knowledge gap has been observed in literature on biomass (yeast, fungi, and bacteria) separation without any intensive and cost-effective process.

According to literature, till date biomass (Yeast, fungi, and bacteria) harvesting was done by using batch centrifugation (Dassey & Theegala, 2013). Recently in our lab, oleaginous yeast biomass has been harvested by settling using extra polymeric substances (EPS) as a bioflocculant with a combination of calcium chloride and biomass settling was observed in less than 10 min (unpublished data).

4. Existing technologies for cell wall disruption

Another critical challenge in biodiesel production from microbial lipid is cell wall disruption followed by lipid recovery. Recently researchers have a major focus on cell wall disruption using wet biomass. Depending upon the nature of the material, mechanism of cell wall disruption can change. Cell wall disruption using dry biomass is based upon physical mechanism that directly acts upon cell wall in presence of co-solvent as a medium.

Cell disruption is the most important step in lipid extraction from the microbial biomass because the efficiency of this step has a direct influence on subsequent downstream processing efficiency (Senanayake & Fichtali, 2006). Microbial cells synthesize both extracellular and intracellular products, where extracellular products can be easily separated by filtration or

centrifugation; while recovery of intracellular products (lipid in the form of bilayer cell membrane and lipid droplets in the cytoplasm) requires cell disruption. Traditional lipid extraction methods developed by Folch (Folch et al., 1957) and Bligh & Dyer (Bligh & Dyer, 1959) requires a co-solvent system, a mixture of a non-polar solvent (chloroform) and a polar solvent (methanol), to extract the lipids from the dry biological material. The total dry lipid obtained from the microbial biomass was considered as 100% (w/w) and it was compared using different alternative and economic technologies to know the lipid extraction efficiency. In literature, cell wall disruption was much reviewed. Therefore in this section, **Table-3**, an overview of mechanical cell wall disruption techniques and critical discussion of recent biomass disruption technologies will be explained.

4.1 Mechanical Cell Disruption Methods

Mechanical cell disruption results in non-specific cell wall breakdown due to high shear stress, abrasion. Mechanical cell disruption methods show great industrial potential due to their less dependency on species and applicability on an industrial scale (Klimek-Ochab et al., 2011). Bead milling, homogenization, and ultrasonication are commonly used mechanical methods. Therefore, these methods were briefly explained in following sections.

4.1.1 Bead Milling

Bead milling is an effective and suitable method for a wide range of microbes. Compaction and shearing action of glass, ceramic, or steel beads result in cell disruption. Disruption efficiency depends on the size and type of beads, agitation velocity, cell concentration, flow rate, bead loading, and microorganisms (Doucha & Lívanský, 2008). Bead milling has been proved to be an effective disruptive method for algal species, e.g., *Botryococcus* sp., *Chlorella* P12, *Chlorella*

Table 3 Comparison of Various Cell Disruption Methodsbacterial species

Microorganism	Type	Cell disruption technology	Moisture content %	Lipid recovery %(w/w)	Limitations	References
Scenedesmus sp.	Microalgae	Enzymatic	93.2	75	Need specific enzyme cocktails for every microorganism. Very expensive	(Taher et al., 2014)
Scenedesmus sp.	Microalgae	Surfactant-MTAB*, 3_DAPS*	-	98	Requires subsequent process to remove the detergent	(Lai et al., 2016)
Chlamydomonas reinhardtii	Microalgae	Osmotic shock	99	84	High cost of additives	(Lee et al., 2010)
Nannochloropsis occulata	Microalgae	SDS*	30	98	Efficiency of the method depends on surfactant concentration	(Salam et al., 2016)
Nannochloropsis sp	Microalgae	Oligomeric surfactant	30	78.8		(Wu et al., 2017)
Yarrowia lipolytica	Yeast	Detergent	83.2	98.2		(Yellapu et al., 2016)
Trichosporon oleaginous	Yeast	Ultrasonication	-	100	Non-specific cell disruption. High heat generation. Generation of harmful free radicals	(Zhang et al., 2014)
Rhodotorula glutinis	Yeast	Pressurized CO2	-	99	High energy consumption	(Duarte et al., 2017)
Rhodosporidium diobovatum	Yeast	Ionic liquid	80	97.1	Not suitable for large scale	(Ward et al., 2017)
Cryptococcus curvatus	Yeast	Acid digestion	95.2	98.9	Not applicable for industrial process. Acid will corrode reactor	(Yu et al., 2015)
Mortierella isabelina	Fungi	Soxhlet	97.5	100	High energy and solvent consumption	(Yu et al., 2015)
Mucor fragilis AFT7-4	Fungi	Soxhlet	-	95.4		(Huang et al., 2015)

vulgaris, Scenedesmus sp. (Doucha & Lívanský, 2008; Lee et al., 2010) , yeast species, e.g., *Rhodotorula gracilis, Candida boidinii, S. cerevisiae, S. carlsbergensis* (Channi et al., 2016) , e.g., *Bacillus cereus, Rhodococcus* sp., *E. coli* as well as for fungal species, e.g., *Penicillium citrinum,* (Klimek-Ochab et al., 2011).

4.1.2 Ultrasonication

Ultrasonication attributes to formation, growth and collapse of gas bubbles. Microscopic bubbles at various nucleation sites in fluid were formed during ultrasonication, which has two phases, namely, rarefaction and compression phase. The bubbles grow during the rarefaction and are compressed during compression phase, which cause the collapse of the bubbles. Ultrasonication has been widely applied in industry for protein extraction, chemical synthesis, disinfection, and cell disruption with reduced chemical addition. The study has been performed by utilization of ultrasonication on lipid extraction from *Nannochloropsis oculata* (Adam et al., 2012). The highest lipid yield was 0.21% w/w which is lower than solvent (chloroform and methanol) extraction yield (5.47% w/w). In another study by Zhang et al. (2014), ultrasonication (50 Hz, 2800 W) was applied for lipid extraction from *Trichosporon oleaginous* and SKF-5 (an oleaginous fungal strain). They compared the efficiency of water, methanol, hexane and 1:1 v/v chloroform/methanol under ultrasonication. In case of *Trichosporon oleaginous,* highest lipid recovery was 43.2% with hexane, 10.2% with water, 75.7% with methanol and 100% w/w with chloroform/methanol. Similarly for SKF-5, 100% w/w lipid recovery was obtained with chloroform/methanol at ultrasonication frequency of 50 Hz with 2800 W power input for 15 min as compared to water (9.3% w/w), methanol (65.1% w/w) and hexane (33.2% w/w). So, more efforts are required to increase lipid recovery with ultrasonication.

4.2 Non-Mechanical Cell Disruption Methods

4.2.1 Physical Methods

4.2.1.1 Microwaves assisted lipid extraction

Microwave-assisted lipid extraction is an efficient extraction process that results in an increased yield and quality within short time. The mechanism of microwave technology works on non-ionizing electromagnetic oscillating waves. In the range of 300 MHz to 300 GHz, they generate

heat in the polar material by the electric field- induced polarization and the reorientation of the molecules that results in friction (Jeevan Kumar et al., 2017). These microwaves interact with free water molecules present inside the cell and give a hasty non-uniform rise in temperature resulting in increased intracellular pressure, thereby, causing spontaneous cell rupture. Lipid extraction can be potentially quick and inexpensive with microwave-assisted solvent extraction with the following benefits- a) requirement of the reduced amount of organic solvent (eg: chloroform and methanol) b) elimination of pre-drying of biomass, c) increased yield in comparison to simple thermal treatment (Rakesh et al., 2015).

The limitations of microwave use at industrial scale, involves damage of PUFA (Polyunsaturated fatty acids) due to development of heat and free radicals, hence affecting the product quality (Günerken et al., 2015).

4.2.1.2 Electroporation

Electroporation is used for lipid extraction from yeast, cyanobacteria, diatoms, and other microalgae (Coustets et al., 2013; Coustets et al., 2015; Sheng et al., 2011). In this system, two electrodes (anode and cathode) are connected with an electrical power supply in a PEF (pulsed electric field) treatment chamber and then the aqueous medium (culture medium) is passed between the electrodes by applying a voltage of 0.5 V to 50 kV. The electrical power is pulsed at a frequency range of 1 Hz to 50 kHz. Pulsation results in the fracture of the cell wall, thereby, releasing oil content. PEF does not lead to cell flocculation, and hence no cellular component come out of the cell. The fractured cells then undergo healing, thereby, remain viable. Thus, this same microbial batch can be reused for further high valued materials (HVM) extraction (Reep & Green, 2012)

Several recent studies have been conducted on PEF process for lipid extraction. Flisar et al. (2014) have investigated the effect of PEF on lipid extraction from *Chlorella vulgaris* in a continuous flow system. *Chlorella vulgaris* consists of 50-58% of lipid content based on dry biomass weight. In this study, PEF treatment chamber was fabricated with stainless steel as electrodes with a gap of 15mm between them. They obtained 50% lipid yield (wt%) when an electric field strength of 2.7 kV/cm was applied for 21 pulses in 100 µs. Eing et al. (2013) also used stainless steel as electrodes in PEF treatment chamber with a 4mm gap between them. Here, an electric field strength of 35 kV/cm was applied to the target sample (*Auxenochlorella protothecoides*) for 1 µs duration and obtained 22% lipid yield. In another study done by (Zbinden et al., 2013), lipid has been extracted from *Ankistrodesmus falcatus* using this approach. At an electric field strength of 45 kV/cm for 100 ms, PEF resulted in the electroporation of 90% of algal cells and thus led to 6.1 mg/L lipid yield. Liu et al. (2011) have reported electroporation of 87% of the cells of *Synechocystis* PCC 6803 by applying electric field strength of 35 kWh/m^3 that resulted in 25-75% lipid recovery. Thus PEF has high potential to be used at high scale due to its low energy consumption which makes it economical.

4.3 Chemical Cell Disruption Methods

4.3.1 Organic Solvent Extraction

Biocompatible organic solvents have been used for lipid milking (removal of accumulated lipid without killing the oleaginous microbes so that these microbes can undergo repetitive milking) from microalgae. Here, the microbial biomass is exposed to a biocompatible hydrophobic solvent that is absorbed by the cells. The solvent creates pores and openings in the cell membrane and results in the secretion of lipids (inside cytosol) outside the cells. The partition coefficient of the biocompatible solvent should be high (log P >5) in order to obtain

highly efficient extraction and also to maintain a separation between the extracellular chemicals and the aqueous cytosolic content so that cell culture can be prevented from being contaminated (Dong et al., 2016). The high partition coefficient prevents irreversible membranous damages (such as uncontrolled cracks and holes), thereby, extends the cell life for milking. The concept of algal milking using this process has been demonstrated to extract β-carotene from *Dunaliella salina* culture using a biphasic reactor (consisting of two phases- an aqueous phase and a biocompatible organic solvent phase) (Jackson et al., 2017).

4.3.2 Surfactant-assisted lipid extraction

Surfactant-assisted lipid extraction is non-toxic and uses biodegradable chemicals and has a potential for the cell wall disruption without requiring any specific equipment (Zeng et al., 2007). Surfactants are differentiated by hydrophobic and hydrophilic moieties. Cell membranes possess negative charges because of functional groups. As a result, they can be disrupted easily using a hydrophobic domain (Jeevan Kumar et al., 2017). Surfactant application in enzyme isolation is well studied, where cationic, anionic and zwitter ions are employed. The state-of-art of using surfactant is for wet biomass disruption. Recently few papers have been published on the subject. (Lai et al., 2016) investigated Myristyltrimethylammonium bromide (MTAB)- and 3-(decyldimethylammonio)-propanesulfonate inner salt (3-DAPS)-surfactants for lipid recovery from wet biomass slurry of *Scenedesmus* sp and lipid extraction efficiency was almost 100% as compared with standard chloroform and methanol (2:1) method. A similar study was conducted by Yellapu et al. (2016) using N-lauryl sarcosine (N-LS) as a biodegradable anionic surfactant for lipid extraction from oleaginous yeast wet biomass with 82.3% (w/w) moisture content. The maximum lipid extraction efficiency obtained was 98.2 % w/w in less than 10 min reaction time. There are similar studies using different surfactants, such as oligomeric surfactant and sodium dodecyl sulphate (SDS), lipid recovery

in both cases was greater than 90% w/w (Salam et al., 2016; Wu et al., 2017) also showed that the technique has an important potential in the development of an industrial-viable approach for lipid extraction. However, studies on variables (concentration of surfactant, reaction temperature and pH) that limit the efficiency of surfactant action are scanty and systematic research should be carried out.

4.3.3 Supercritical Fluid Lipid Extraction

In recent years, supercritical fluid extraction (SFE) has grabbed considerable attention (Duarte et al., 2017). SFE achieves the lipid extraction by manipulating the chemicals, which behave as both a liquid and a gas in their critical temperature and pressure. In critical stage, solvating power of the compound used in SFE is increased and then it plays as a solvent to extract the product from cells. Mostly, carbon dioxide is used due to its low viscosity (<100 μPa.s), high diffusivity (<0.1 mm^2/s), and suitable critical temperature (31.1 °C) and pressure (72.8 atm). In an extraction vessel, oil-bearing substances contact with supercritical carbon dioxide for certain time (several hours). During the process, oil will be solubilized in CO_2 and extracted. CO_2 which contains oil is then collected and depressurized to allow the escape of CO_2, and finally, oil is obtained. The application of supercritical CO_2 lipid extraction from microorganisms has been extensively reported.

5. Separation of microbial lipids for transesterification

The foremost requirement for industrialization of biodiesel production using microbial lipid is the efficient extraction of lipid from biomass. The downstream recovery of microbial lipid in conventional method requires a large amount of chemical solvent which contributes around 70-80% of the total biodiesel production cost (Dong et al., 2016). Moreover, the chemical solvents employed for lipid extraction (chloroform, methanol) have high toxicity and flammability which

consequently raise a concern regarding its impact on the environment. The lipid in microbial cells is enclosed by the solid matrix. Therefore, the ideal solvent should be able to penetrate solid matrix and solvate the lipid. In last decades, a large number of studies were performed for developing the efficient and green process for lipid extraction; this section discussed the recent research efforts taken towards the optimization of extraction procedure, their challenges, and impact of physical properties of solvent on extraction and green environmental friendly solvent extraction techniques for biodiesel production.

5.1 Lipid separation mechanism and their challenges

The extraction of lipid from microbial biomass is a two-step process. In the first step, physical, chemical and enzymatic disruptions of the cell wall are performed by various means (Refer to section 3.2). The second step involves the use of a chemical solvent for oil recovery, and it is associated with specific conditions such as temperature and processing time. In the conventional method, the definite proportion of chloroform and methanol were used for extraction of microbial oil (Bligh & Dyer, 1959). With the advancement in the field of science, various extraction techniques have been developed for lipid extraction, such as ultrasonication assisted, microwave assisted, supercritical fluid extraction, pressurized fluid extraction, Soxhlet extraction and many more. In the microbial cell, lipid exists in three forms which are neutral lipids, free fatty acids, and polar lipids. Principally, the neutral lipid exists as globules in the cytoplasm of the cell and they form complex with the non-polar organic solvent through van der waal forces and diffuse out from the cell via concentration gradient separation. While polar lipids attached to protein in cell membrane via hydrogen bonding requires a polar solvent to disrupt the strong binding between the polar lipid and membrane proteins. However, some neutral lipid complexes with the polar lipid and hence not extracted via non-polar organic solvent, therefore in order to

ensure efficient and complete recovery, co-solvent mechanism or mixture of polar and non-polar organic solvent were utilized. In the co-solvent system, polar solvent breaks the hydrogen bond between the neutral lipid and polar lipid followed by its van der waal interaction of non-polar solvent which surrounds the neutral lipid and comes out via diffusion. Once the lipid comes out from the cell membrane with solvent, the lipid portion is recovered by addition of non-polar organic solvent and water which perform biphasic separation of lipid molecules from the other contaminants such as carbohydrates and proteins. The choice of solvent for lipid extraction from microbial cells depends on factors like initial lipid content, solvent-cellular interaction, type of microorganism and reaction time (Ranjan et al., 2010).

The mixture of chloroform and methanol is the most commonly used solvent for extraction due to its characteristic feature of being fast and quantitative, and also it does not require the complete dewatering of biomass, rather the water present in the cell works as ternary substance and helps in complete extraction of polar and neutral lipid (Jose & Archanaa, 2017). However, high toxicity of the chloroform and methanol limits its application on an industrial scale. A study reported that hexane could be more suitable for oil recovery because it was found to be more selective for neutral lipids which in turn reduces the downstream purification step, however, hexane was unable to extract polar lipids which cause the loss of lipid that ultimately affects the economy of the process. This study clearly demonstrates that the efficiency of extraction depends on the polarity of the solvent. However, complete extraction of lipid via solvent was not reported until date.

The physical disruption technique is a prerequisite for the complete and efficient recovery of lipid. In a study of mechanistic assessment of lipid extraction, it was reported that neither soxhlet method (with hexane) nor Bligh and Dyer method was able to disrupt the cell completely

(Ranjan et al., 2010). Boyd et al. (2012) investigated the use of Switchable Hydrophilicity Solvent (SHS) N, N-dimethylcyclohexylamine without any prior treatment of cell disruption such as sonication or microwave heating. The study reported 22 wt% recovery of oil from microalgal biomass whereas the lipid extraction efficiency by the conventional method was 52%. Zhang et al. (2014) evaluates the efficiency of lipid extraction of four solvent after ultra-sonication treatment (Water, hexane, methanol, chloroform and conventional chloroform-methanol mix) and observed 100% lipid extraction efficiency of chloroform at low temperature and shorter time duration. These studies strongly support the necessity of physical disruption technique before solvent extraction.

The recovered lipid from wet microalgae was subjected to treatment using persulfate-based oxidation with ferric chloride as a coagulant in order to eliminate the dewatering step. In this study, microalgal cells were first harvested by adding 200 mg L^{-1} of $FeCl_3$ (as a coagulant) and extraction was performed using persulfate based oxidation by addition of potassium persulfate, which eventually lead to the recovery of 95% of lipid (Seo et al., 2016).The persulfate based extraction does not require organic solvent for extraction process and can be directly applied to wet biomass.

5.2 Effect of physical properties of solvent upon lipid extraction

The physical properties of a solvent such as partition coefficient, density, and solubility of water are the critical parameters which determine the efficient extraction of lipid from the solvent.

The partition coefficient is the quantitative measurement of an organic compound distributed between the organic and aqueous phase. The partition coefficient of solvent defines the polarity of solvent which in turn determines the degree of interaction of solvent with the lipid

molecule. However, a hydration shell enclosed the polar lipids because of electrostatic attraction of water. Therefore, it requires additional energy input for the extraction (Dong et al., 2016). The solubility of solvent in the water affects the recovery of the solvent after extraction. A polar solvent such as methanol, ethanol, isopropanol is miscible in water, which means these solvents require the additional step of distillation for the recovery. A large difference in density of solvent and water help in the formation of the biphasic system.

5.3 Green Recovery of lipid

Traditional extraction procedures require harsh organic solvent which has reported to have a high environment, health and safety risk score (Zbinden et al., 2013). These limitations of the organic solvents lead to the investigation of green recovery system with an eco-friendly and natural solvent for lipid extraction. Pulsed electric field, lipolytic enzyme degradation, simultaneous distillation and extraction process, solvent-free extraction via non-woven fabric are the research effort taken towards the development of clean and green extraction system (Liu et al., 2011; Shang et al., 2015; Tanzi et al., 2013; Zbinden et al., 2013).

A recent study on utilization of non-woven fabric demonstrates the solvent-free extraction of lipid from the yeast *Rhodotorula glutinis.* The fermented broth of *Rhodotorula glutinis* was concentrated and homogenized in order to rupture the cell wall. The non-woven fabric (using polypropylene) was then immersed in the fermented broth, which adsorbed lipid with other impurities. Then the lipid was recovered from the fabric by mechanical extrusion, and the recovery of 10.4g of oil per gram of fabric was reported (Shang et al., 2015). This technique seems to be beneficial in terms of oil separation, recyclability, and environment-friendly features.

Ionic liquids are non-aqueous organic salts which consist of asymmetric organic cation and an inorganic or organic anion. Their non-volatile nature and thermal stability make them a suitable option for green recovery of lipid. Until date only one study was conducted using ionic liquids for recovery of microalgae lipid, the study reported a meager lipid content of 19% wt; however, conventional Bligh and Dyer's method process resulted in only 11% wt lipid recovery in the same study (Cooney & Benjamin, 2016). Although the extraction efficiency was quite low compared with other methods, the technical and economic viability is important. Therefore, research efforts should be directed to explore the potential of the ionic liquid as a solvent. In our lab, we identified petroleum diesel can act as a co-solvent to recover microbial lipid after cell wall disruption and transesterification (unpublished data). And this process will also help to avoid further blending of petroleum diesel and fatty acid methyl esters (FAMES).

All the extraction procedure investigated so far, either employing a green extraction process or organic solvent demonstrate the dependency of extraction process on lipid composition, type of lipid fraction (neutral or polar lipid), and their interaction with a membrane protein. An ideal extraction process should not only efficiently recover oil but also reduce the contamination, increase mass transfer and simplify downstream processing. Therefore further research has to be carried out regarding the scalability, extraction efficiency, and energy consumption and downstream process.

6. Microbial lipid to biodiesel conversion (Transesterification)

Biodiesel is produced by transesterification of triglycerides present in the microbial lipids, plant oils and animal fats in the presence of catalyst and alcohol to produce the fatty acid alkyl esters (FAAE) and glycerol as a byproduct. Transesterification of microbial lipids to biodiesel is being carried out by both homogeneous and heterogeneous catalysts. The homogeneous alkali catalysts

such as sodium hydroxide (NaOH) or potassium hydroxide (KOH) have been mostly used for transesterification due to certain advantages such as faster reaction under mild reaction conditions of low temperature and atmospheric pressure. However, due to the presence of high content of free fatty acids in the microbial lipids, homogeneous alkali catalysts are not suitable for transesterification process as they lead to formation of soap in the presence of free fatty acids, which causes the difficulty in biodiesel separation and further purification process (Hidalgo et al., 2013). To overcome this limitation, the acid catalysts such as sulfuric acid (H_2SO_4) or hydrochloric acid (HCl) have been considered as they can be used in the presence of free fatty acid content higher than 1%. However, they require higher temperature as well as higher reaction time as compared to alkali catalysts (Vonortas & Papayannakos, 2014). In various studies, both acid and alkali catalysts have been used. Primarily, the acid catalyst is being used to reduce the free fatty acid content to less than 1%, thereafter, alkali catalyst is being considered to conduct transesterification of triglycerides to FAAE. Enzyme catalytic process has gained the attention of researchers since last decade due to certain advantages such as accessibility for every feedstock, insensitivity to free fatty acid content as well as high purity of products (Channi et al., 2016). However, high production cost, the unstable behavior of enzymes as well as lower conversion yield as compared to homogeneous alkali and acid catalysts makes this process less considerable at the industrial scale biodiesel production process.

The use of heterogeneous catalysts such as alkali exchanged zeolite, potassium exchanged alumni, etc. for transesterification process has been considered as one of the emerging technology due to their advantage for removal of undesirable free fatty acid impurities, easy recovery, and production of cleaner biodiesel. Moreover, removal of washing and purification steps from the process steps due to the usage of heterogeneous catalysts makes them much

preferable for transesterification process (Degirmenbasi et al., 2015). In spite of certain advantages, these catalysts require high temperature and pressure as well as longer reaction time due to the formation of three different phases of reactants. However, researchers are continuously working to overcome these limitations (Lee & Wilson, 2015).The catalysts play an efficient role in transesterification process. However, the transesterification process of microbial lipid to biodiesel is a challenging process due to the presence of high water content in the biomass. There is two type of transesterification methods for microbial lipids.

6.1 Conventional method

The conventional method of biodiesel production using microbial lipids include multiple steps such as biomass drying, microbial cell disruption by mechanical, chemical or biological methods, oil extraction, separation, and transesterification. These multiple steps involved in conventional methods are considered as highly energy intensive as they require high temperature, a large number of solvents and longer reaction time, which adds up to high biodiesel production cost (Cheirsilp & Louhasakul, 2013). Moreover, use of toxic organic solvents in conventional transesterification method is deeming them unfeasible for industrial-scale biodiesel production. However, it has been reported that the drying step of biomass consumes a huge amount of energy and the researchers are trying to develop the method for production of biodiesel using wet biomass to avoid the drying step. Very few studies have been reported using wet biomass and further transesterification using conventional method. The first study was reported by Nagle and Lemke (1990), where wet concentrated microalgal biomass was used after harvesting and successful extraction of lipids by using 1-butanol, ethanol, hexane, and 2-propanol. The high recovery yield of 90% (w/w) was reported for lipids using 1-butanol as a solvent and high conversion yield of 93% was reported using conventional method. Most

recently, Yellapu et al. (2016) reported the detergent assisted lipid extraction approach for wet biomass of yeast *Yarrowia lipolytica* and used "response surface methodology" for optimization of principal parameters to obtain maximum lipid extraction efficiency of 95.3% (w/w). Further, transesterification was performed using conventional chloroform and methanol method and lipid to FAME conversion efficiency of 94.3% (w/w) was achieved.

Even though researchers are shifting towards lipid extraction using wet biomass and further transesterification process, but intensive research and development are required to establish the robust and economic process to be used at industrial scale biodiesel production. Moreover, the necessity to reduce the multiple steps involved in the conventional method as well as to reduce the use of solvents has shifted the researchers towards direct transesterification, which is also referred as in-situ transesterification.

6.2 In-situ or direct transesterification

In this process, biomass is treated with the methanol and catalyst (acid or base catalyst) in the single reactor, which results in the reactive extraction of lipids as FAAE (Fatty acid acyl esters). The methanol serves two functions, one as extraction agent and another as esterification agent. In some of the studies, an additional solvent such as chloroform or hexane is being used for easy extraction of oil from the microbial cells and also to enhance the contact of microbial oil with the esterification agent (Cao et al., 2013). Direct transesterification process has several advantages such as the elimination of multiple steps, reduction in the use and the potential loss of solvents during the extraction process and consequently reducing the processing units and costs. Several studies have been conducted for direct transesterification of dry microbial biomass. Thliveros et al. (2014) reported 97.7% FAME yield from yeast *Rhodosporidium toruloides* by using 4g/L of NaOH at 50^{o}C in 10 h reaction time in the presence of methanol. In another study

by Carvalho et al. (2017), conventional and in-situ transesterification reaction were performed using dried and wet microbial biomass obtained from fungal strain *Mucor circinelloides* in the presence of a heterogeneous catalyst supported on alumina as well as ethanol. Both reactions achieved high FAME yield of 97% (w/w). However, the conventional method has not been recommended due to higher energy intensive process as well as the use of huge amounts of toxic organic solvents.

The direct transesterification of lipids present in wet microbial biomass has been investigated using homogeneous acid catalysts. Liu and Zhao (2007) reported the acid catalyzed in-situ transesterification using wet microbial biomass obtained from two yeasts *Lipomyces starkeyi*, *Mortierella isabellina*, and one fungus *Rhodosporidium toruloides* and high FAME yield of up to 90% (w/w) was obtained using 0.2mol/L of H_2SO_4 at 70^0C in 20h reaction time in the presence of the methanol. In another study conducted by (Vicente et al., 2009), direct and conventional transesterification reactions were compared for wet biomass obtained from fungal strain *Mucor circinelloides* in the presence of three different solvent systems, chloroform: methanol, chloroform: methanol: water. The direct transesterification reaction gave high purity FAME of >99% as compared to conventional transesterification (91.4- 98%) using an acid catalyst for 8h at 65^0C in the presence of methanol to oil molar ratio of 60:1. Im et al. (2015) also reported in-situ transesterification of the wet microbial biomass of microalgae *N. oceanica* and obtained high FAME conversion yield of 91.1% using 0.3g of H_2SO_4 catalyst for 90 min at 95^oC in the presence of chloroform and methanol.

For direct transesterification reaction, high amount of methanol as well as sulfuric acid is required, which is not feasible at industrial scale biodiesel production as the use of methanol could be costly and presence of sulfuric acid can corrode the reactor. Therefore, researchers are

developing advanced strategies for in-situ transesterification in order to decrease the use of a solvent as well as sulfuric acid.

6.3 Factors affecting in-situ transesterification and advanced strategies used

Though direct transesterification offers shorter processing time, less use of solvents and lower production cost of biodiesel from microbial biomass as compared to conventional transesterification, there are many factors, which affect the conversion efficiency of in-situ transesterification. Water content, cell wall disruption, selection of catalyst as well as solvent extraction are the important factors that need to be discussed (Yousuf et al., 2017). Therefore, further investigation is required to improve these factors and researchers are continuously working to make this process feasible from lab scale to industrial scale.

6.3.1 Moisture content

The moisture content present in the microbial biomass significantly affects the efficiency of direct transesterification process and hence biodiesel production costs. Three types of effects have been discussed by Sathish et al. (2014): a) reversible reaction, i.e., hydrolysis of biodiesel into methanol and free fatty acids, b) shield the oil, thereby interference in reaction, c) deactivation of the acid catalyst due to competition of ions present in the water with protons present in the reaction. Hence, with increased moisture content, the conversion efficiency of lipids to FAME decreases (Hidalgo et al., 2013). Ehimen et al. (2010) also reported the similar results with an increase in moisture content from 0 to 72 % (w/w). In another study reported by Im et al. (2015), effect of moisture content on the product yield was studied by fixing the microalgal cell weight and increasing the moisture content from 0 to 90 wt.% and drastic decrease in the product yield was observed with the increase in moisture content more than 50 wt. %.

In spite of the limitations of transesterification reaction due to the presence of moisture or water level, the energy associated with the drying process is very high and hence the production cost. Therefore, it is necessary to use the wet microbial biomass for in-situ transesterification process. Kim et al. (2015) used the wet microalgae biomass for in-situ transesterification in the presence of HCl catalyst and methanol. Here, a mixture of wet algal cells, HCl and methanol were heated at 95 ^{o}C, resulting in <90% FAME yield. The high affinity of HCl with water resulted in low impact of moisture content on FAME yield and 15 wt.% higher FAME yield was obtained as compared to the H_2SO_4 catalyst. In order to improve the FAME yield using wet biomass, a number of other techniques have been implemented such as increasing methanol dosage, integrating mechanical processes and using supercritical methanol. The efficiency of in-situ transesterification process can also be improved by integrating microwave or ultra-sonication technologies in order to improve the mass transfer rate between immiscible phases and subsequently reducing the reaction time even by using wet biomass (Hidalgo et al., 2013).

6.3.2 Cell wall disruption

The disruption of microbial cell wall during direct transesterification is very important in order to release the lipids outside the microbial cells and further partitioned into solvents such as hexane and pentane (Halim et al., 2012). The knowledge of the structure of microbial cell wall is important for the choice of suitable cell disruption method. The oil-rich microalgae cell wall is comparatively thick and tough as compared to prokaryotic cells. The yeast cell wall is also rigid due to the presence of polysaccharides and proteinaceous network, which provide integrity and shape to the cells and provide stability in the osmotic environment (Backhaus et al., 2013). Therefore, cell disruption method has to be integrated with direct transesterification process in order to obtain high FAME yield. Several methods of cell disruption such as ultrasonication,

microwave, and supercritical processes have been developed to disrupt the cell wall and to bring out lipids from inner compartments of microbial cells to the solvents. (Zhang et al., 2016) reported the ultrasonication assisted biodiesel production using dried biomass-derived lipids and in-situ transesterification process was performed. For lipid recovery, ultrasonication process along with chloroform and methanol (1:1 v/v) mixture exhibited the best performance among all the solvents (hexane, methanol) and 95.3% (w/w) recovery was reported. Ultrasonication assisted in-situ transesterification gave maximum biodiesel yield of 95% (w/w) within 20 min reaction time as compared to 24h, without ultrasonication. Sara et al. (2016) also compared the microwave and ultrasonication assisted in-situ transesterification for dried biomass of *Trichosporon oleaginous* and maximum FAME conversion of 99% (w/w) was achieved with microwave assisted in-situ transesterification in the presence of 183 : 1 molar ratio of methanol to lipid and 2% (w/w) NaOH within 20 min at 100°C. In case of ultrasonication assisted in-situ transesterification, 95.1% (w/w) FAME yield was obtained by using 183 : 1 molar ratio of methanol to lipid and 3% (w/w) NaOH in 20 min at 25°C. Jazzar et al. (2015)used supercritical methanol for in-situ transesterification without catalyst and achieved 45.62 wt.% biodiesel yield.

The other techniques of cell destabilization for the wet biomass includes the use of surfactants, ionic liquids and use of nanoparticles, which are known to cause weakening of the cell wall (Park et al., 2015). The surfactants have been reported to enhance FAME yield along with catalyst for wet microbial biomass as they have high water tolerance ability and hence can cause the disruption of cell as well as phospholipid membrane layer. Yellapu et al. (2016) reported the N- Lauroyl sarcosine (N-LS) assisted ultrasonication aided in situ transesterification for biodiesel production using oleaginous yeast wet biomass. The maximum FAMEs yield of 96.1 ± 1.9 and 71 ± 1.4% (w/w) was obtained with or without N-LS treatment respectively in

24 h reaction time. The maximum FAMEs yield after N-LS treatment of biomass followed by with or without ultrasonication revealed 94.3 ± 1.9% and 82.9 ± 1.8% w/w respectively using methanol to lipid molar ratio 360:1 and catalyst concentration 360 mM (64 μL H_2SO_4/g lipid) within 5 and 25 min reaction time, respectively. (Yoo et al., 2014) studied cell disruption using wet biomass of microalgae using functional membrane coated with a cationic polymer [tertiary-amine cations deposited on poly-dimethylaminomethylstyrene (pDMAMS) film] and gained a cell disruption yield of 26% in 6h reaction time. However, the cell disruption yield was comparatively low but this process can also be combined with in-situ transesterification process as it was proposed to be a simple and efficient process.

6.3.3 Catalyst selection

In the transesterification reaction, catalyst selection plays an important role. During in-situ transesterification, acid catalysts are recommended due to the presence of high moisture content in the biomass. However, heterogeneous catalysts (acid and base, a mix of solid acid and solid base) have gained more attention due to easy separation, regeneration, reusability as well as easy product purification (Dong et al., 2016). Solid acids (silica-based, carbon-based, zeolite based, polymer-based, zirconia-based and hydroxyapatite based) are also preferred due to problems of corrosion and the environmental problem associated with the disposal of liquid acid catalysts. Solid super acids have the ability to perform simultaneous esterification and transesterification of fatty acids and hence can be used easily for a high content of free fatty acids. However, the limitations of heterogeneous catalysts such as longer reaction time and lower reaction rate have been considered as challenging aspects of ongoing research. Ma et al. (2015) reported the in-situ heterogeneous transesterification of microalgae using combined microwave and ultrasound irradiation. By using KF/CaO catalyst prepared by wet impregnation method

along with microwave and ultrasound technology gave 93.07 ± 2.39% FAME yield in the presence of 12 wt. % of catalyst and a methanol to biomass ratio of 8:1 at 60 °C for 45 min. It was reported that the combination of US and MW (US–MW) irradiation could overcome the limitations associated with the use of heterogeneous catalyst and has been successfully designed and well documented for product synthesis, decrease in reaction time and energy consumption, improved and enhanced yield, and selection of products (Zbancioc et al., 2014).

In other studies, researchers also performed the direct enzymatic (lipase) transesterification of wet microbial biomass. Tran et al. (2013)reported the direct enzymatic transesterification of *Chlorella vulgaris* lipids using immobilized *Burkholderia* lipase as a catalyst and obtained 95.7% of FAME conversion. The wet microalgae biomass with 86–91% water content was pre-treated by sonication to disrupt the cell wall and then directly mixed with methanol and solvent in the presence of immobilized *Burkholderia* lipase with 1.65 molar ratio of hexane/methanol at 45^{o}C and 500-600 rpm. Navarro López et al. (2016) also performed the optimization for the production of FAME using wet *Nannochloropsis gaditana* microalgal biomass by direct enzymatic transesterification. The wet microalgal biomass was homogenized at 140 MPa to enhance cell disruption and high FAME conversion of 99.5% was achieved using oil: mass ratio of 0.32 with methanol/oil and t-butanol/oil ratios of 4.6 and 7.1 $cm^3\ g^{-1}$, respectively, at 40 °C for 56 h. However, FAME conversion decreased to 57% after catalyzing three reactions with the same lipase. In addition, the presence of moisture, methanol, and biodiesel also contributed to the degradation of lipase immobilization support in N435. The key point of direct enzymatic transesterification technology is that the microbial biomass should have a high lipid content to obtain an efficiency of 90% FAME conversion, using lower biocatalyst loading and better lipase recycle efficiency. However, this technology is not economical and

feasible at industrial scale for microbial biomass with the low lipid content. In the latest study by Kim et al. (2017), in-situ transesterification was performed without any catalyst by combining hydrothermal liquefaction (iTHL) technology with in-situ transesterification. It was found that the chlorinated hydrocarbon solvents such as dichloromethane, chloroform, and dichloroethane (DCE), improve FAME production by providing hydrogen chloride in an ionized form that can act as an acid catalyst. The most effective solvent is DCE with the FAME selectivity of 91.85% at 185.08°C with 4.69 mL ethanol and 1.98 mL DCE/g of dry algal cells.

6.3.4 Solvent extraction

In most of the direct transesterification studies, extraction of lipids from wet microbial biomass was done by using solvents such as chloroform, hexane, 2-propanol, and ethanol (Park et al., 2015). The use of a solvent for direct transesterification reaction facilitates extraction and increases the contact between oil and esterification agents, thus ensuring superior ester formation. In the study reported by Li et al. (2011), the reaction mixture of wet microalgae biomass along with methanol and the sulfuric acid catalyst was stored at 120°C and was supplemented with 2, 4, 6, 8 and 10 ml of hexane over a reaction time of 120 min. It was observed that with the increase in hexane content in the reaction mixture (from 2 to 10 ml), FAME yield increased significantly from 16.6% to 94.5% as hexane enhanced the solubility of the oil.

However, the heating requirement for the solvent extraction step also requires high energy consumption and also suffers from challenges in the solvent extraction and scale-up process. The idea of direct transesterification of fatty acids in lipid without the use of solvent extraction step could substantially reduce both the time and solvent and biodiesel production cost (Halim et al., 2012). Cheirsilp and Louhasakul (2013) developed a method for direct

transesterification without using nonpolar solvent. The FAME yield of 58% was obtained by using 125:1 molar ratio of methanol/biomass for 6h, while 65-69% FAME yield was obtained with an increase in methanol/biomass ratio to 209:1 in 1h. Liu and Zhao (2007) also achieved 60% FAME yield using direct transesterification of oleaginous yeast in the presence of methanol and sulfuric acid within the longer reaction time of 20h. Nevertheless, the use of excess methanol is a cost-effective process as compared to traditional solvent extraction method because it is recoverable and reusable for the next batch.

7. Purification of biodiesel

After the trans-esterification reaction, the biodiesel-glycerol mixture contains many impurities like metal ions, water, acid, soap which needs to be separated in order to have better fuel performance and emission characteristics (Shirazi et al., 2013). Many downstream purification processes of biodiesel have been reported in the literature like dry-washing, wet-washing, and membrane separation technology. However, for actual purification process, it is inevitable to separate glycerol from the biodiesel as the pre-treatment step. It is usually done by gravitational settling, which involves lengthy separation of polar denser phase including glycerol from lighter non-polar phase (mono-alkyl esters of long-chain fatty acids) with both phases containing impurities.

If in-situ transesterification is performed using biomass, then the first step is to separate biomass from the trans-esterified mixture using filtration or centrifugation. The reported studies for biodiesel purification using in-situ trans-esterified mixture were mostly done by wet-washing technique. A study has been reported where ultrasonication assisted in-situ transesterification was performed using algal biomass and then the biomass was filtered followed by settling/ phase separation of the filtrate, wet-washing of filtrate using water and drying using anhydrous sodium

sulphate (Suganya et al., 2014). Another process has been reported where biodiesel was produced using microwave mediated in-situ transesterification of algal biomass. Once crude biodiesel was obtained, alcohol was devolatilized using vacuum distillation. N-hexane was mixed with the remaining product of vacuum distillation and passed through centrifugation. Three layers were obtained after centrifugation- upper organic phase containing biodiesel, lower aqueous phase containing glycerol, alcohol and other impurities and algal biomass layer. The uppermost layer was treated with anhydrous sodium sulfate followed by filtration and the purified biodiesel was analyzed for purity and impurities (Patil et al., 2013). The schematic diagram for biodiesel purification is shown in figure 1.

7.1 Biodiesel glycerol separation

7.1.1 Salt assisted gravitational settling vs Centrifugation

Although salt assisted gravitational settling has applications in batch process, centrifugation can be employed for the continuous process where the oil is continuously fed into the trans-esterification reaction and continuous purification takes place. In such scenario, gravitational settling, which requires longer incubation time is not feasible.

The continuous centrifuge can be employed for glycerol-biodiesel separation as the exit streams contain two liquid phases with two different densities. Due to high centrifugal force, settling is faster than the gravitational method. Centrifugation is apt where biodiesel is produced using in-situ transesterification. In this situation, separation of biomass and aqueous impurities including metal ions take place simultaneously (Patil et al., 2013). Although, continuous centrifuge has many advantages over salt-assisted gravitational settling method in terms of process time and productivity salt assisted gravitational method can be effective in a batch

process and smaller scale biodiesel production industries due to lower capital investments, lower operational and maintenance cost. Moreover, it is 4 times faster than conventional gravitational. settling method.

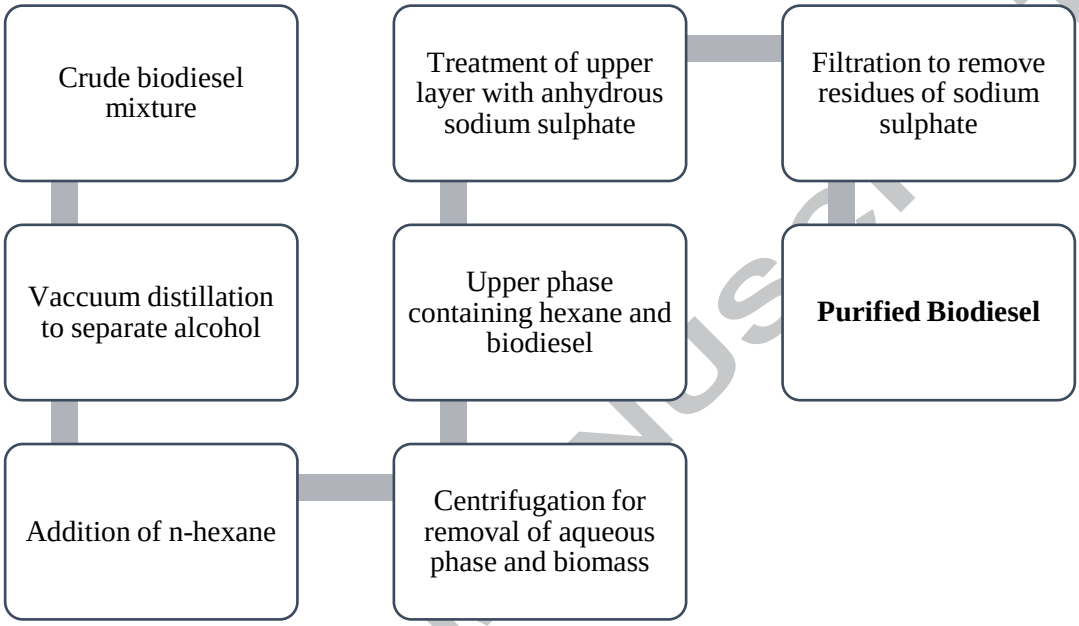

Figure 1. Scheme of biodiesel purification of in-situ trans-esterified biomass

7.2 Biodiesel Purification techniques

Once biodiesel is separated from the glycerol, it still contains soap, metal ions, water, acid ions, catalyst, residual alcohol and bound glycerol in form of mono-, di- and triglycerides. There are various techniques for biodiesel purification including wet washing, dry washing and membrane technology. The biodiesel purified after several techniques should meet quality standards as specified by ASTM (American society of testing and materials) and EN (European Union) (Banga et al., 2014).

7.2.1 Wet-washing

The common methodology for wet-washing is highlighted in figure 2. The glycerol free biodiesel is washed with water/ acid/ organic solvents followed by phase separation for 10-60

min. Two phases will be obtained after phase separation – (1) washed water/ solvent/ acid with impurities and (2) treated biodiesel. The process is repeated 2-3 times to obtain pure biodiesel.

(Mendow et al., 2012) reported biodiesel purification with two-different wet-washing techniques: two consecutive washing; (1) washing with aqueous solution of 5 wt.% HCl (aqueous phase: 30% v/v with respect to the biodiesel phase) followed by water saturated with CO_2 (30% v/v of water relative to the biodiesel phase); (2) washing with neutral water (10% v/v relative to biodiesel phase) followed by water saturated with CO_2 (30% v/v of water relative to the biodiesel phase). Later, the treated biodiesel obtained after phase separation was allowed for stripping with nitrogen at 80-100^{o}C for removing residual water. The results of the study indicated that second method of washing with neutral water followed by water saturated with CO_2 was more effective in removing acidity (0.32 mg KOH/g) as the first method with HCl imparted some H^+ ions in the mixture increases the acidity value (1.29 mg KOH/g). According to the international standards, the maximum value of acidity in purified biodiesel should be less than 0.5 mgKOH/g. However, both the methods were successful in reducing the soap and glycerine content to 0 from initial values of 11.28 g soap/kg biodiesel and 0.39% glycerine content.

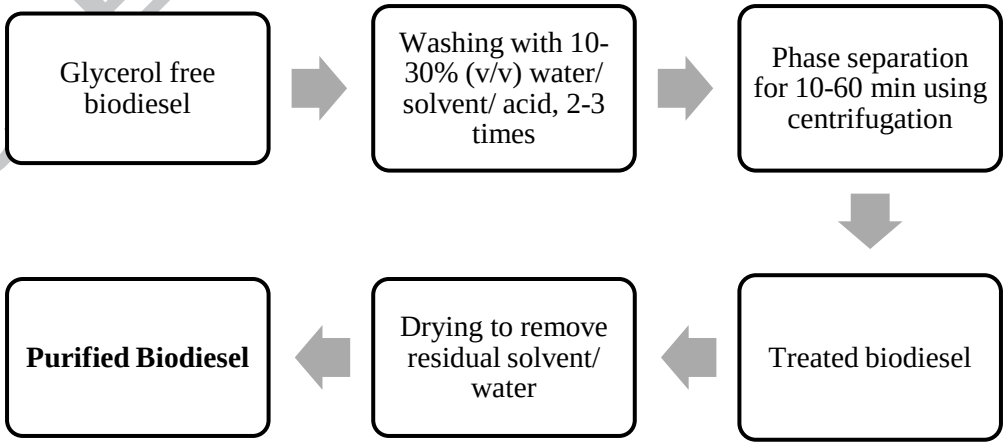

Figure 2. wet-washing technique for biodiesel purification

Besides, water and acid, organic solvents like glycerol have also been used for biodiesel purification (Berrios et al., 2011). Biodiesel purification was performed in single or multiple steps with different concentrations (5, 10 and 15 wt%). The mixture was vigorously shaken followed by settling for 10 min and centrifugation step for 10 min to obtain the final product. It was found that 15% wt glycerol was more effective than distilled and tap water in reducing the acid value and water content. Not only with regard to acid value and water content, glycerol was effective in purifying biodiesel as per international quality standards (European Union 14214). It can be concluded that by using water as a solvent in wet-washing technique imparts water content in the biodiesel while glycerol being hydrophilic in nature, it is soluble in water and hence it is able to remove water content as per quality standards.

However, wet-washing techniques have many disadvantages like huge amount of wastewater produced during the process, use of centrifugation for the phase separation making the process more expensive and more energy intensive, 2-3 times washing increasing the operation time, requirement of additional drying step and use of more holding tanks leading to the decreased productivity of the operation. Besides it, using water and acid in wet-washing methods imparts high water content and acidity values in the biodiesel respectively, not meeting the international quality standards of biodiesel.

7.2.2 Dry washing

7.2.2.1 Ion-exchange resins

Dry-washing method does not use solvents in biodiesel purification. Dry-washing can be attained using ion-exchange resins like AMBERLITE®, PUROLITE®, and LEWATIT®. These resins having negatively charged sulphonate groups (SO_3^{2-}) are able to bind positively charged

impurities like water, glycerol, metal ions, acid ions and soap leading to purified biodiesel. (Banga et al., 2014) has reported a comparative study of purification of Jatropha Curcas based biodiesel using ion-exchange resins like Amberlite® BD10 DRY, Purolite® PD206, and Tulison® T-45BD and the results were compared with wet washing methods. Here, crude biodiesel treatment with Amberlite® with 3% concentration at 65^{o}C for 25 min was most effective in removing soap, potassium, and methanol but the treated biodiesel didn't meet ASTM standards for water and acid value. Similar values were obtained after treatment with Purolite® at 3% concentration. Also, the temperature of 65^{o}C was more effective than room temperature even at low concentration of resin. This is because the temperature had an impact on the adsorption capacity of the resin, since, at room temperature, the resin is surrounded by water layers leaving no site available for the binding of other impurities while at high temperature, the water is removed from the surface sites of the resins, which can bind with other impurities. Hence, Amberlite® was effective in removing free glycerol and bonded glycerol, potassium ions and residual methanol up to quality standards.

7.2.2.2 Adsorbents

Dry-washing is also accomplished by using adsorbents like silica, Magnesol® which are inorganic in nature and have the high surface area, which can adsorb all sorts of impurities irrespective of their nature. They have excellent mechanical properties, very good solvent stability, and good chemical resistance. Due to these reasons, their application in biodiesel purification is apt. In one of the studies, a single step biodiesel purification has been attained with silica as an adsorbent (Manuale et al., 2014). In the study, 100 cm^3 of biodiesel were treated with silica Trisyl 3000 (1g and 3 g) at different temperatures varying between 50^{o}C-90^{o}C for different contact times, 15-100 min. Vacuum condition of 0.2 bar was maintained for the

treatment. After the treatment, treated biodiesel was filtered and liquid obtained was analyzed for impurities. It was found that reaction time of 90 min, a reaction temperature of 90^{o}C, adsorbent concentration of 1.1% and a vacuum pressure of 0.2 bar gave maximum adsorption capacity towards methanol, soap, water and other impurities. At high temperatures residual methanol and water were evaporated, reducing their content in purified biodiesel. This technology is advantageous as it is a single step purification method with no pre-treatment to remove excess methanol and glycerol.

7.3 Recent advancements in biodiesel purification techniques

7.3.1 Simultaneous production of FAMEs and purification using ion-exchange resins

In one of the studies, simultaneous production of high-quality biodiesel and glycerine from Jatropha oil using ion-exchange resins as catalysts and adsorbents has been reported (Shibasaki-Kitakawa et al., 2013). The cation-exchange resin, Diaion PK208LH acted as the catalyst for transesterification reaction and the anion-exchange resin, Diaion PA306S were used to adsorb impurities. The temperature of each resin was maintained at 50^{o}C by hot-water circulation through the jacket. The solution mixture of crude oil and methanol was fed to the bottom of the first column and elute coming from the final column was analyzed for reactants, FFA, triglyceride, products and FAME. The reaction was stopped when FAME concentration in the elute from the final column started decreasing due to loss of anion exchange resins catalytic activity. The operating conditions were first optimized with different flow-rates and reaction time. It was found that feed flow-rate of 0.233 dm^3/h with time between 4-16 h gave maximum FAME yield as after 16 h unreacted triglycerides started increasing.

The biodiesel purified using anion exchange resin Diaion PA306S met the EN 14214 quality standard values for the impurities and FAME content. The process has many advantages

like no requirement of upstream processing for refining the crude biodiesel, simultaneous trans-esterification and purification reducing the process time and increasing the productivity while glycerine adsorbed by anion-exchange resin can be easily recovered by supplying methanol. No decrease in catalytic activity of cation exchange resin while decrease in catalytic activity of anion exchange resin was observed which can be regenerated by sequential pass of i) methanol to recover glycerine, ii) acetic acid in methanol to displace fatty acid ion from resin, iii) NaOH aqueous solution to displace acetic acid ion, iv) deionized water to remove NaOH solution and v) methanol to restore the resin. The process could be helpful at continuous large scale operation as scale-up of column operation can be easily performed based on FAME productivity per hour per anion-exchange resin's weight (dm^3/h/kg-resin weight). However, a large number of solvents required to regenerate the anion-exchange resin is a slight disadvantage of the process.

In one of the studies, solid waste from ceramic industry (chamotte clay) was used as glycerol adsorbent for biodiesel purification by dry washing method (Santos et al., 2017). In the study, a face-centered composite design was used to analyze the combined effect of chamotte concentration (varied between 2-8% w/v) and temperature (varied between 30-50^{o}C) on glycerol removal. Based on graphical optimization models, optimum glycerol concentration (2.4 wt%) and temperature (45^{o}C) were determined. Glycerol removal reached 1282 mg/g of resin within 30 min adsorption time. Biodiesel obtained using biological (immobilized lipase) and chemical catalysts (Niobium oxide impregnated with sodium) was purified using chamotte clay and free glycerol was removed as per ASTM standard (< 0.02% wt). High adsorption capacity can be related to high silica (56 % w/w) and alumina content (36 % w/w) with porous structure and large surface area. But, chamotte clay was unable to be regenerated with organic solvents at 50^{o}C. However, it has benefits like chamotte-glycerol composite can be reused in a brick

formulation. Chamotte clay is a low-cost material with good adsorption capacity, which can be a promising adsorbent in biodiesel purification. But non-regeneration of the adsorbent is the main concern for its use in industrial processes. However, studies need to be conducted for its regeneration at high temperatures.

In another study, raw sugarcane bagasse was used for biodiesel purification using dry washing technique (Alves et al., 2016). Raw sugarcane bagasse was first cleaned with distilled water and dried at 80^{o}C for 24 h. Crude biodiesel of 100 mL was treated with adsorbent loading concentration ranged from 0.1-3% (w/v) at 120 rpm and 30^{o}C for 120 min. It was observed that 0.5% w/v sugarcane ash resulted in 40% removal of crude glycerol to bring down the glycerol content in biodiesel to less than 0.02% wt. These results for biodiesel purification were comparable to that of Magnesol®. However, sugarcane bagasse ash (obtained by heating raw sugarcane bagasse at 700^{o}C for 4 h) performed poorly as compared to raw sugarcane bagasse due to its low content of the cellulosic material. From the adsorption kinetics, it was concluded that with the addition of 3 wt% sugarcane bagasse, the necessary glycerol removal was achieved after only 10 min of the adsorption process. The process has many advantages like low-cost adsorbent with lower process time as compared to wet-washing technique. However, there are disadvantages of the process, such as, it was unable to remove water as per ASTM standard and regeneration studies have not been performed on the adsorbent.

7.3.2 Use of membrane technology

In one of the studies, solvent-resistant polymeric membranes were synthesized and used for biodiesel purification (Torres et al., 2017). The synthesized nanofiltration membranes were composed of poly (vinylidene fluoride) (PVDF) as a support and poly(dimethylsiloxane) as a coating material. The membranes were prepared by phase inversion process. MWCO (Molecular

weight cut-off) of the membrane was evaluated by passing organic solutes (300-1000 g/gmol) with ethanol that was based on rejection coefficients. The membrane showed high stability during adverse conditions like pH 12 and 60^{o}C temperature. Rejection coefficient of impurities was calculated based on following formulae, % R = (1 – Cp/Cr)*100 where Cp = concentration of impurity in permeate and Cr = concentration of impurity in the retentate. The experiments conducted at 60°C, pH 12 & 15 bar pressure revealed that rejection coefficients of 70% glycerol, 69% glycerides were obtained with a permeate flux of 7.4 M/m^2.h. High membrane stability was displayed with flux recovery ratio of 0.94-0.95 even after 20 cycles of use. Moreover, the presence of alcohol in biodiesel had little effect on rejection coefficients. Membrane separation in biodiesel can be economical as they have lower capital and operating costs. However, high membrane purchase cost is a disadvantage of membrane technology at industrial scale and the presence of high amount of soap in the crude biodiesel can lead to concentration polarization and membrane fouling.

7.3.3 Fiber-based bio sorbents

Yang et al. (2017) reported biodiesel purification using fiber-based dry-washing technique; BD-Zorb, sawdust, and wood shavings. Biodiesel purification was conducted in 3 cylindrical separator funnels (125 mL) filled with 18 g of adsorbent while biodiesel was allowed to pass with a flow-rate of 100 mL/h. The results revealed that BD-Zorb exhibited the best performance for soap removal capacity from the crude camelina biodiesel. The soap removal capacity of BD-Zorb, sawdust and wood shavings were 51.1 mg/L, 24.4 mg/L, and 9.5 mg/L. However, acid content (for BD-Zorb) and water content of biodiesel purified from three bio sorbents did not meet biodiesel quality standards indicating that the additional steps are required to decrease the acid and water content of biodiesel purified by fiber-based dry washing. A lower

purification capacity of sawdust and wood shavings implied more frequent replacements of adsorbents, leading to increased labor costs. Table 4 represents various advantages and disadvantages of different biodiesel purification techniques.

8. Current Challenges and Future Prospects

There are many technical challenges that must be resolved for profitable biodiesel production. A major challenge is to reduce the high feedstock cost by using low-cost feedstock, including waste cooking oil (WCO), algal oil, and animal fats, etc. However, these feedstocks also contained high amounts of free fatty acids (FFAs) and water that may lead to saponification, thereby, require further pretreatment and purification steps. These problems must be addressed in order to produce sustainable biodiesel.

8.1 Vegetable oil and Non-food crops as feedstock for biodiesel: Microalgae have been proved to solve most of the energy-crop associated problems. But main drawback using microalgae is that their growth rate is very slow. Therefore use of heterotrophic microorganisms (Yeast and fungi) is alternative approach due to their fast growth and less rigid cell wall than microalgae. However, the extraction technologies require major advancements for the sustainable commercial production.

8.2 Biomass harvesting: Several harvesting technologies (Flocculation, Auto-flocculation, magnetic separation etc.,) are developed to separate microalgae. But there are not many studies in the literature on techniques for harvesting of fast growing heterotrophic microorganisms such as yeast, fungi, and bacteria.

8.3 Effects of moisture and FFA: Presence of FFA and moisture content (contaminants) in the feedstock can badly affect transesterification process. Due to these type of contaminants in the

feedstock, acid catalysts are used for transesterification reaction. However, there are several problems using an acid catalyst such as a) it will increase water content in the biodiesel b) reaction temperature higher than 80^{o}C is required c) Lipids to biodiesel conversion efficiency will be low and d) it will corrode reactor tank. Therefore further research is need to be conducted to remove water and free fatty acids from feedstock using an economical process.

8.4 Supercritical alcohol process: Supercritical alcohol process takes only 4 to 10 min of residence time to generate biodiesel due to efficient mixing (Deshpande et al., 2017). However, there are some limitations with this process owing to the requirement of high temperature and pressure. The major limitation is the scale-up of the process to the commercial level adding extra cost for high energy requirement and high alcohol: oil (42:1) molar ratio. Researchers are trying to employ co-solvents, including CO_2, CaO, and hexane, in order to control the operating conditions, thereby, increasing product yield (Duarte et al., 2017). This became possible due to increase in homogeneity of reactants with the help of co-solvent. Supercritical CO_2 is another eco-friendly co-solvent that can be obtained at affordable cost. It can be safely recovered from the reaction via depressurization. This supercritical process combined with co-solvents helps in an increase in product yield, reduction in process time and a significant decrease in overall production cost. However, detailed systematic research is required in this field.

7.5 Use of co-solvents: Co-solvents, e.g. MTBE (methyl tertbutyl ether), THF (tetrahydrofuran), increase the rate of reaction and also overcome the mass transfer limitations. They help in the production of high-quality FAMEs under moderate conditions, i.e., 30 °C for 10 min. But there is a need for large “leak proof” reaction vessels. Also, the co-solvents must be completely removed from the product.

Table 4. Advantages and disadvantages of various biodiesel purification techniques

Purification method	Advantages	Disadvantages
Wet-washing	• Excellent in removing soap, methanol and free glycerol	• Not effective in reducing water content - Centrifugation step required for phase separation • Large amount of wastewater discharge - Additional drying step to remove water present in the crude biodiesel. • Not applicable in continuous operation
Dry-washing using resins	• Excellent in removing soap, methanol and free glycerol. • Lower capital investments. • Less energy intensive. • No wastewater production. • Applicable in continuous operation. • No drying step required. • Methanol used during regeneration can further be re-used during trans-esterification reaction	• Most of the resins are shipped with H^+ ions, impart acidity and water in the treated biodiesel - Chemical composition of the resin, sometimes is difficult to predict • No reported studies on performance of resin after regeneration
Dry-washing using commercial adsorbents	• High FAME yield, adsorbs residual water. • Less energy intensive. • No wastewater production. • No drying step required. • Faster than wet-washing technique	• Adsorbents are usually non-recyclable, frequent replacements of adsorbent leads to increased labour costs
Dry-washing using industrial/ agricultural wastes	• No wastewater discharge. • Environmental benefits - Used waste can act as soil corrective/ brick formulation	• Regeneration studies not reported • Wastes are unable to remove all the impurities (water and acidity) from the crude biodiesel; needs further treatment increasing the costs
Membrane technology	• High fuel quality and excellent performance. • Lower capital and operating cost. • Performance comparable to wet-washing technique	• High soap content can foul the membranes, leading to frequent replacements of membranes which are expensive

8.5 Biodiesel/glycerol separation and FAME quality: Separation of FAMEs and glycerol is a necessary step due to their soluble nature. This is usually done by phase separation. However, an excess of unreacted methanol in the reaction increases the solubility of ester in glycerol and vice versa, thereby, increasing the post-production cost. Also, all the trace elements must be removed from triglycerides because the emulsion layer formed by these trace elements interfere with the separation of glycerol and makes the product expensive. In case of continuous biodiesel production process after transesterification, phase separation between biodiesel and glycerol will be time-consuming process. Therefore further research needs to be conducted to separate crude glycerol from biodiesel with less time and low cost.

8.6 Biodiesel purification: Regeneration studies for the use of adsorbents and resins in dry washing biodiesel purification technique were missing in the literature. Regeneration studies should be conducted with different solvents and temperature to avoid frequent replacement of resins and adsorbents at large scale. Most of the studies reported for use of industrial/ agricultural wastes as adsorbents in biodiesel purification were conducted at lab-scale. Pilot scale studies should be conducted for their industrial feasibility along with techno-economic evaluation. Studies reporting membrane technology for biodiesel purification were conducted at lab-scale. Membranes which are resistant to soap and organic solvents should be looked upon to prevent fouling of membrane.

9. Summary

Biomass harvesting is one of the major task for biodiesel production using oleaginous biomass. Micro-algal biomass harvesting using flocculation has been rigorously studied during last few years but still, there is no harvesting method available except centrifugation that can be applied to oleaginous yeast, fungi, and bacteria. Various physical and chemical technologies

have been developed for lipid extraction. Each of the methods has its advantage and disadvantage. Physical method is clean but high energy consuming, while chemical extraction has the high possibility of contamination of the lipid due to the presence of the residual solvents when toxic organic compounds are used as solvents. However, lipid extraction from wet microbial biomass faces several challenges such as very limited lipid accessibility, reduced mass transfer, and formation of stable emulsions. To eliminate these problems, surfactant assisted cell wall disruption is a novel approach to extract total lipid from the wet biomass under lab scale. The process needs to be scale up followed by techno-economic analysis to ascertain the overall cost of the extraction process and to guide on the improvements required at a large scale.

Numerous green ecofriendly extraction methods were investigated, which are highly efficient in lipid recovery. However, all the extraction procedure investigated so far either employing a green extraction process or utilized organic solvents; demonstrated the dependency of extraction process on lipid composition, types of lipid fraction (neutral or polar lipid), and their interaction with membrane protein. Further, an ideal extraction process should not only efficiently recover oil but also reduce the contamination, increase mass transfer and simplify the downstream processing. Therefore, further research has to be carried out regarding the scalability, extraction efficiency, and energy consumption and downstream processing. The techno-economic analysis of the whole extraction process is also required, which provides guidance for improvement and modification of the process.

Biodiesel purification has been achieved by wet-washing methods using distilled water, glycerol and acid in the reported studies. Both wet and dry washing are applicable where biodiesel is produced from oil while wet washing is more convenient when biodiesel is produced through in-situ transesterification using wet-biomass. Wet-washing using glycerol is more

effective than using water and acid as they impart high water content and acid values, respectively in the treated biodiesel. Although they are excellent in removing soap, methanol and free glycerol, wet-washing method has several disadvantages: large amount of polluting wastewater produced during the operation, additional drying step for removal of water, requirement of centrifugation for phase separation after the process, requirement of holding tanks making their application in large scale continuous operation unfeasible. Membrane technology can be a good option for biodiesel purification as it produces zero-water discharge, is less energy intensive and more-ecofriendly. But there are concerns about membrane fouling due to high soap content in crude biodiesel, moreover, high cost of membrane is also a concern for their commercialization at large scale.

10. Conclusion

Currently, biodiesel industries are facing many challenges and competition among energy sources, advancement and acceptability of technologies. Lipid production using heterotrophic microorganisms is a substantial approach for microbial lipid production and conversion to biodiesel. But the process is under lab scale study due to high energy was required to harvest biomass, cell wall disruption and lipid recovery from wet biomass under biodiesel downstream process. Moreover, use of organic solvents for lipid separation can affect the cost and are industrially unsafe. Therefore, low cost industrial viable environment friendly solvents are necessary to be investigated for microbial lipid separation and conversion to biodiesel. Hence, a considerable effort of research is needed to obtain industrial acceptable biodiesel production process.

Acknowledgements

Sincere thanks are given to the Natural Sciences and Engineering Research Council of Canada (Grant A 4984 and Canada Research Chair) for their financial support.

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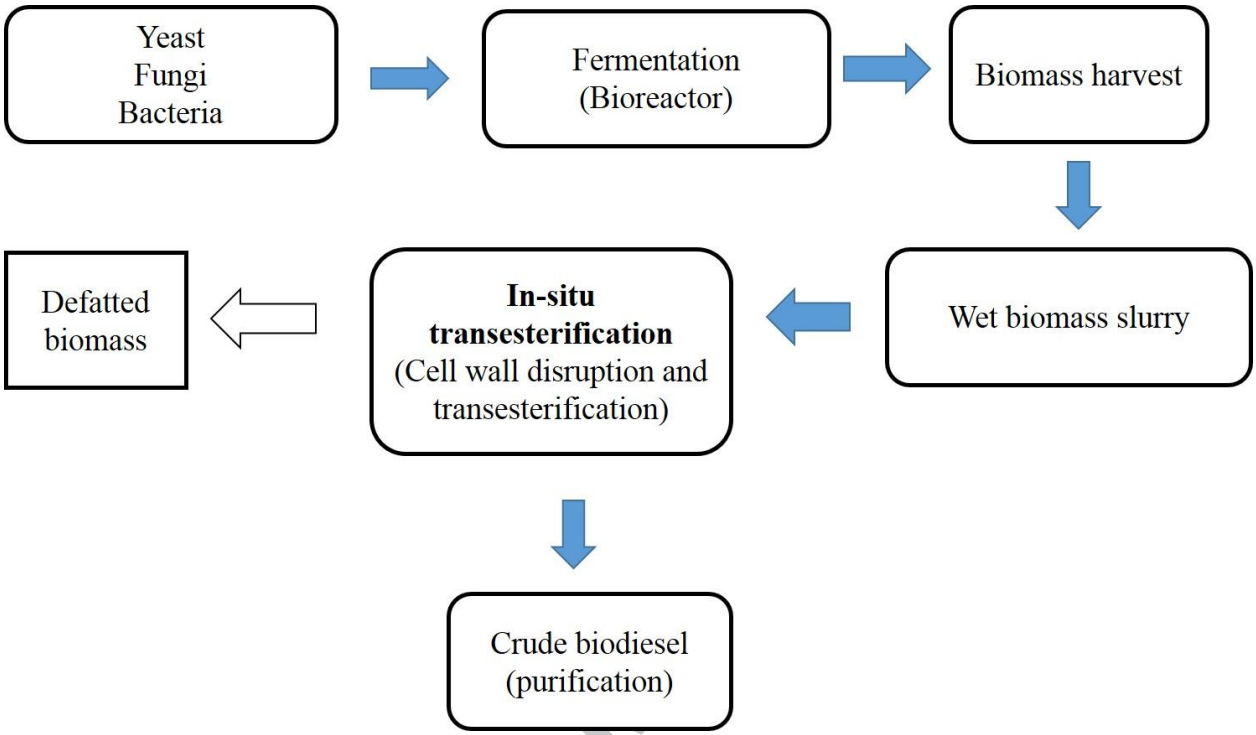
Graphical abstract



Highlights

- Biomass harvesting using centrifugation and flocculation.
- Applications of efficient cell-disruption methods.
- Lipid recovery and mechanism using organic solvents and their effects.
- *In-situ* Transesterification from wet biomass slurry.
- Purification techniques to meet quality standards of ASTM and EN.