**Production of third generation biofuel from oleaginous bacteria- an approach for utilization of lignocellulosic substrates biodiesel production**

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**Abstract**

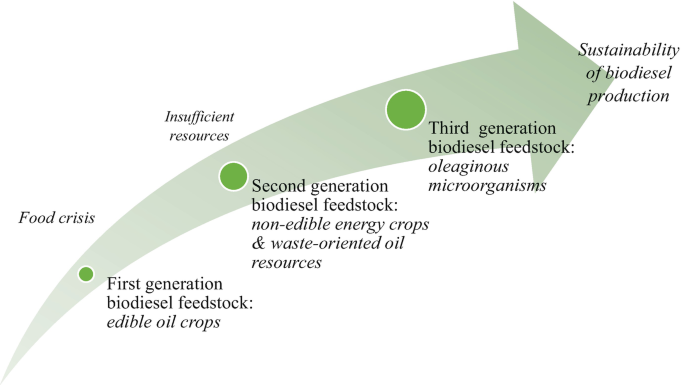
Population growth is a serious issue nowadays since it is increasing at an alarming rate while the earth's resources are still being degraded. Resources for conventional fossil fuels are exhausted and unsustainable.  Alternatives to fossil fuels include biofuels. They possess qualities such as sustainability, low production costs, great productivity, short incubation times, etc. Microorganisms with an oily nature are harnessed for the production of third-generation biofuels, aiming to address the limitations of both first and second-generation methods. Single cell oil is the name given to the lipid that the microorganisms create. The biofuel production from oleaginous bacteria is the recent interest area in the research field which uses lignocellulosic biomass as their substrate. This review discussed the lipid contents in bacteria and its extraction by bioprocessing technologies and the utilization of lignocellulosic biomass.

**Keywords;** Oleaginousmicrobes, lignocelluloses, transesterification, metabolic engineering

**Introduction**

Rapid population increase, grossly unbalanced provision of food, declining petroleum reserves, and depletion of natural resources have all triggered the emergence of the world's energy threats [1]. Around eight times the amount of fossil fuels that were consumed in 1950 have been utilized since that year. This pattern of consumption has remained relatively consistent since 1980 [2]. Along with the rising petroleum price, the reserves of fossil fuels are exhausted, non-renewable, and exploiting the natural environment. To cope with these issues, we need a novel approach to sustainable utilization of energy. Biodiesel stands out as a type of renewable energy source. It stems from renewable biomass, which undergoes a transformative process known as transesterification. This process yields altered forms of lengthy fatty acids, coupled with brief alcohol chains, notably forming fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs). When compared to conventional petroleum diesel, biodiesel showcases certain advantages: it contains more oxygen, boasts improved combustion efficiency, and possesses lower levels of sulfur and aromatic components. Furthermore, biodiesel demonstrates environmental friendliness, holds a superior cetane number, and boasts a higher flash point. Importantly, it emits fewer greenhouse gases than regular diesel, and it doesn't contribute to elevated atmospheric carbon dioxide or sulfur levels [3-6].

Four  generations  of  biodiesel  were  identified based on  the  feedstock  used  in  manufacturing[7-9]. First-generation biofuel is created using a variety of dietary sources, including animal fat and edible plant oils. The non-edible feedstock used to make second-generation biofuel includes things like non-edible oil, food waste, animal-based waste, and crop residue [9][10]. Microbiologically generated biodiesel is a type of third-generation biofuel. [12-14]. Fourth-generation biofuels come into being through hydro-refining techniques akin to those employed in petroleum production. They also make use of innovative methods and cutting-edge biochemical procedures, such as Joule's special "solar-fuel" system, that fail to neatly fall into any recognized type of biofuel [15].



**Fig 1:Generations of biofuels**

**Table 1: Different generations of biodiesel: their sources, advantages, and difficulties (Leong et al., 2018; Sigh et al., 2020)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **S.No** | **Biofuels** | **1st generation** | **2nd generation** | **3rd generation** | **4th generation** |
| 1 | Source | Feedstocks for edible oils, such as soybean, rapeseed, sunflower, and corn, among others | Unusable feedstocks include jatropha seeds, food scraps, animal fat scraps, and other scraps of a variety of industries. | Bacteria, fungi, yeast, microalgae, and other oleaginous microorganisms | Electro-fuels, photobiological solar fuels, and synthetic cells |
| 2 | Benefits | Renewable energy, environmental friendliness, and straightforward biodiesel conversion method | Biodegradable, renewable, and not in competition with food crops | These biofuels possess qualities such as renewability, biodegradability, lack of competition with food crops, absence of land requirements, freedom from climate dependence, and a faster growth rate. | High energy content, ample supply, and renewable |
| 3 | Challenge | land, labor-intensive farming, and growing food costs | Costly to produce since it needs additional land and labor for cultivation. | Low production for commercialization, challenging to maintain, etc. | Research focuses on early childhood. |

Microorganisms rich in oils, commonly referred to as oleaginous microorganisms due to their lipid content exceeding 20%, present promising potential for generating fatty acids as a viable and sustainable biofuel option [16][17]. The process of biologically crafting lipids through such oleaginous microorganisms—ranging from microalgae and yeast to fungi and bacteria—has been extensively explored through various studies [21-26]. These microorganisms are harnessed as substitute raw materials for manufacturing oil and fat [18].International interest in single-cell oils, microbial lipids used in the production of biodiesel, has grown significantly [19]. Most of the lipids generated by oleaginous microorganisms consist of straight carbon chains spanning from 4 to 28 carbons in length. These lipids exhibit the potential to be either saturated or unsaturated fatty acids, a characteristic determined by the configuration of the carbon chain and the count of double bonds it accommodates [20]. As metabolic byproducts of metabolizing fatty acids and triacylglycerol (TAG), various microbes form hydrocarbons. Eukaryotic organisms including yeast, fungus, plants, and animals utilise TAG as an energy reserve. Triacylglycerols (TAG) can be effectively produced by bacteria groups from a wide range of carbon sources, including carbohydrates, aromatic acids, ethanol, n-alkanes, extended alkanes, phenylalkanes, lipids, and even coal-derived lipids, though this process is still poorly understood. According to Bharti et al. (2014a) and Kumar et al. (2020), the fatty acids and TAG produced by microbial bacteria could serve as initial materials for cultivating microbial lipids, a valuable source for biodiesel generation. When compared to vegetable oil, bio-lipids originating from oleaginous microorganisms possess an advantageous fatty acid composition. Importantly, this composition can be tailored as needed by adjusting nutrient or substrate availability, alongside employing metabolic engineering strategies. Additionally, noteworthy findings indicate that oleaginous bacteria adeptly convert high-carbon waste into lipids through an efficient process [36][37]. Oleaginous bacteria, such as Arthrobacter sp. [18], Rhodococcus opacus [27], and Acinetobacter calcoaceticus [28], demonstrate rapid growth rates and have the capacity to accumulate oil content that can make up to 87% of their dry biomass. Additionally, they generate substantial amounts of biomass in a short span [28-31]. Recent research has concentrated on Rhodococcus sp.'s capacity to degrade fiber and then integrate each of its constituent parts into the pathway for lipid formation [32] [33]. In one study [34], when grown on aromatics derived from the organosolv treatment of loblolly pine and mixed with lignocellulosic pretreatment byproducts comprising a variety of carbohydrates, Rhodococcus opacus showed a lipid content of 26.8% w/w. The same species was also used to convert lignin from Kraft exposed to oxygen into useful lipids [35]. This review article will focus over the extraction of biofuel from oleaginous bacteria using lignocellulosic substrate (dry matter of plants) and it gives the significance of third generation biofuel and the role of oleaginous bacteria in utilizing the lignocellulosic subtrates.

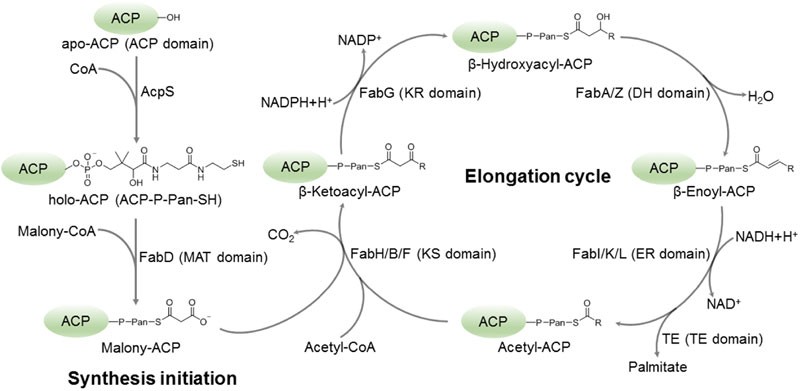
### Lipid content in various oleaginous bacteria

Although bacteria demonstrate rapid cell growth rates, they accumulate fewer lipids in comparison to fungi and microalgae. Employing simple cultivation methods, bacterial lipids are produced within cell cytoplasm as small droplets, while maintaining high cell growth rates. Additionally, certain strains have the ability to amass oil under specific environmental conditions [40]. In conditions of ample carbon supply coupled with limited availability of essential nutrients, primarily nitrogen, bacteria initiate the production of lipids. To promote favorable lipid accumulation, the carbon-to-nitrogen ratio of the culture medium must be high. Extra carbon in the cell is transformed into the lipid triacylglycerol [38][39]. Polyhydroxyalkanoic acids are a highly prevalent class of neutral lipids present in a wide range of microbial species. These acids act as both energy and carbon storage molecules within the body [41]. The lipid synthesis in bacteria is influenced by the various factors such as pH ,temperature, nutrients etc., The highest amounts of triacylglycerols are produced by various bacterial genera, including *Rhodococcus, Mycobacterium, Arthrobacter, Streptomyces, Nocardia, Acinetobacter, Clostridium etc,.* R. opacus was the oleaginous bacterial strain that attracted the most study interest in terms of fermentation and optimization. According to reports, species like Arthrobacter and Rhodococcus can store fatty substances up to 87% of their air-out cellular weight, demonstrating significant biomass [42]. It has yet to be determined that gram negative species have a significant lipid content, in contrast to these gram positive bacterial species [42][43].

**Lipid biosynthesis in bacteria**

In cells, fatty acid biosynthesis (FAS) is an essential activity. For the construction and metabolism of cells, fatty acids are crucial. The increasing fatty acid chain is stabilized and transported by an acyl carrier protein (ACP) throughout the enzymatic modules of the FAS system for stepwise catalysis [44]. The monoenoic C18 acids contain various double-bond locations and often lack polyunsaturation. Some bacteria produce 3-hydroxy acyl acids, whereas others produce branched-chain fatty acids. Type I and type II are the two primary chemical pathways for the synthesis of fatty acids (FAS). The method of fatty acid synthesis in the type I system, which is typically found in mammals, is centered on one huge polypeptide unit with many domains. In contrast, the type II system, observed in bacteria, plants, and protozoa, involves the involvement of the acyl carrier protein (ACP) alongside other components [45][46]. Since the majority of bacteria have the ability to integrate external fatty acids into their membrane phospholipids, it is crucial to determine whether this ability will enable them to get around FASII inhibitors by obtaining the fatty acids they require from the host [47]. The synthesis start and elongation phases of fatty acid elongation are carried out in succession by the FAS system using various enzyme modules.

### Initiation

**A covalent bond is created between the terminal sulfhydryl of the 4-phosphopantetheine arm, also known as the 4′-Pan-arm, and the carrier protein for the substrate at the early stage of fatty acid synthesis start. A complete acyl carrier protein (holo-ACP) is the name of this transporter protein. There is a short acetyl (or malonyl) group involved in this interaction. To facilitate the elongation of fatty acids, the ACP sequentially interfaces with about four distinct enzymatic modules within the elongation cycle. During each cycle, an extra two carbon units are integrated into the substrate chain, with this process repeating until the final product is liberated. The connections between ACP and enzyme modules within FASN or specific enzymes within FAS-II have only been briefly studied, with the exception of a few rare cases like the incomplete enoyl reduction (FabI)-ACP complex and the covalently linked -hydroxyacyl-ACP dehydratase (FabA)-ACP structure. This is a result of ACP's naturally high degree of flexibility and diffuse nature [48][49]. Consequently, the fundamental mechanisms governing the recognition and manipulation of ACP by enzyme modules for substrate catalysis, particularly during the elongation cycle, remain largely uncharted.

**Elongation**

An acyl-enzyme or acyl thioester (such as acyl-ACP or acetyl-CoA, notably in the case of FabH) participates in a Claisen fusion process involving malonyl-ACP during the cycle of fatty acid synthesis. The result of this interaction is the formation of a 3-ketoacyl-ACP molecule, as well as the release of the enzyme in its loose state, ACP (or CoA), and the emission of carbon dioxide (CO2). Three E. coli-specific enzymes that were originally known as synthases I, II, and III have since come to be known by their gene names, FabB, FabF, and FabH, respectively. The catalysis of the 3-ketoacyl-ACP synthesis processes is carried out by those enzymes. The enzymes FabB and FabF, structured as dimers, are capable of driving both saturated and unsaturated fatty acid synthesis processes. Due to this step being an irreversible element of the growth process involved in the fatty acid synthesis process, 3-ketoacyl-ACP synthases control the spread of products created by this pathway [50].

**Reduction**

The 3-keto-thioester, also known as 3-ketoacyl-ACP, proceeds through reduction that is aided by NADPH-dependent 3-ketoacyl-ACP reductase (referred to as Fab G), producing 3-hydroxy acyl-ACP as a byproduct. There is just one 3-ketoacyl-ACP reductase in E. coli, and it is active across the entire range of acyl chain lengths [51].

**Dehydration**

The enzyme 3-hydroxy acyl-ACP dehydratase, which is also identified as FabZ, assists in eliminating a water molecule from the substrate. This process leads to the creation of enoyl-ACP. The dehydratase enzyme has demonstrated its capability to successfully dehydrate both short-chain and long-chain 3-hydroxy acyl-ACPs, including those with saturated and unsaturated properties [52].

##### **Reduction**

The action of enoyl-ACP reductase (FabI) leads to the generation of an acyl-ACP through a reduction pathway. This enzyme, the final one in the fatty acid cycle, plays a pivotal role in governing the precision of fatty acid production. FabI exerts control over the reversible phases of the cycle by influencing the activity of other enzymes, namely FabG and FabZ. The resulting acyl-ACP can act as a starting point for further expansion or, as necessary, for the development of sufficiently prolonged chains, which may eventually result in the production of complex lipids. In the context of E. coli, the prominent saturated fatty acid is crafted through the elongation of trans-3-decanoyl-ACP by FabI. Subsequently, either FabB or FabF follows this step [52].

### Lipid extraction from oleaginous bacteria:

Effective solvent extraction of intracellular lipids and the breakdown of protective cell walls in microorganisms, a biomass pretreatment approach such as cell disruption is commonly employed. This process not only improves lipid accessibility by reducing cell wall barriers but also enhances mass transfer and streamlines subsequent processing stages [53]. The advancement of lipid extraction methods has been driven by the need for high-quality products. These procedures include, among others, solvent extraction, the Soxhlet method, the Folch strategy, the Bligh and Dyer plans, the use of supercritical fluids, and ultrasonic treatment [52]. An innovative approach, ultrasonication, has gained widespread use in boosting the yield of bioproducts from various organic waste sources. It has been integrated into a number of processes, including the production of biofuels and oil from crude recovery, and has been proven to be scale-up-applicable. [54][55]. In-situ trans-esterification, a revolutionary technique for making biodiesel, enables bacteria to directly transform oil into fuel without changing its chemical makeup. [55]. In addition to lipid extraction, a number of pretreatment techniques improve lipid recovery. The series employs enzymatic, chemical, and physical approaches to pretreat isolated lipids [56].

**Transesterification**

### Transesterification is a chemical process that entails converting one carboxylic acid ester into another form. The most common transesterification method normally involves an acid's catalyzed contact between an ester and an alcohol. Triacylglycerols (TAGs) and free fatty acids (FFAs) are important lipids from microbial oil while producing biodiesel. By using alcohols like methanol or ethanol, these lipid components can go through transesterification. The development of fatty acid (m)ethyl esters is the result of this reaction being aided by the presence of an acid, an alkali, or an enzyme catalyst [56].

### Scheme-1-Transesterification-process-for-biodiesel-production-Adapted-with-permission.png

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### Fig 3: Transesterification process:(Linganiso, Ella & Tlhaole et al.,2022)

### Enzymatic transesterification, the dynamics of homogeneous acid-base reactions, and the characteristics of heterogeneous acid-base transesterification represent merely a subset of the various transesterification methods available.

### Biodiesel production by oleaginous bacteria from lignocellulosic substrate

### The widely available and renewable resource known as lignocellulosic biomass (LB) is mostly made up of the polysaccharides cellulose and hemicellulose as well as the aromatic polymer lignin. Three polymers make up : lignin (10–25%), hemicellulose (20–40%), and cellulose (35–55%). Sugar-rich lignocellulosic biomass can promote the growth of heterotrophic organisms. The production of biofuels, biosourced chemicals, and minerals using LB has a significant potential as a substitute for fossil resources without endangering the world's food supply[57][58][59]. For instance, lipid made up to 70% of the DCW under nitrogen-deficient conditions. The process of fermenting lignocellulosic biomass to produce biogas or ethanol has been studied extensively. Triacylglycerols (TAGs), a precursor for the synthesis of biodiesel, have also been investigated as a potential outcome of these experiments [60][61]. Oleaginous microorganisms (OMs) can use cheap feedstocks, such as waste substrates and lignocellulosic substrates (LCSs), to accumulate more lipid [62]. Delignification, saccharification, the use of microorganisms in fermentation to increase lipid synthesis, and the final transformation through transesterification are the four basic steps in the production of biodiesel from lignocellulosic biomass . [63][64].

### Fig 4: Diagrammatic representation of the oleaginous bacteria used in the production of lipids from lignocellulosic biomass.fmicb-12-658284-g001.jpg

### Application of metabolic engineering in microbial cells' synthesis of lipids:

Significant attention is being paid to microbial sources of lipids that can be employed as nutraceuticals or as sources of energy [65]. More than others, the gene regulatory mechanisms for fatty acid production in bacteria are well understood. The most recent method of altering the metabolism of microorganisms through genetic engineering is known as metabolic engineering. Metabolic engineering primarily focuses on the enhancement of existing biochemical pathways or the integration of necessary components [66][67]. When it comes to boosting lipid production in bacteria, specific techniques within the realm of metabolic engineering are employed [52].

**Conclusion**

The utilization of fossil fuels, urban expansion, and population growth have exerted a notable influence on the economies and resource reserves of numerous countries. Among the prominent solutions to promote sustainability in the environment, biofuels stand out as a highly significant renewable energy source. An developing method for the productive synthesis of third generation biofuels uses oleaginous bacteria and lignocellulosic biomass as a substrate. The negative effects of using both edible and non-edible feedstocks are considerably reduced by the use of oleaginous microorganisms and lignocellulosic biomass.The modification of these cells at genetic and metabolical level is quite easy. There are many ongoing research projects which implement the efficient utilization of microbial cells in the production of biofuels by metabolic engineering technologies.

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