Biocatalysis and Agricultural Biotechnology 22 (2019) 101321

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: http://www.elsevier.com/locate/bab

Strategies to improve microbial lipid production: Optimization techniques

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ARTICLE INFO

Keywords:
Oleaginous microbes
Metabolic engineering
Statistical optimization
Systems biology
SIGEX

ABSTRACT

The exponentially depleting fossil fuel reserves has led to the growth of lipids being used as an alternate potential feedstock, derived from varied sources, including plants, animals and microbes. In comparison to plant and animal sources, microbial sources can generate higher fractions of polyunsaturated fatty acids but their extraction is not industrially feasible or economical. Although statistical optimization techniques have enhanced lipid accumulation, these aren't sufficient to compete on a commercial scale. Advances in genetic engineering and systems biology tools have helped us get a better insight of the complex pathways involved, hence has a more directed and definite approach. This review discusses about the recent advances in microbial lipid accumulation, focusing on the metabolic engineering strategies.

1. Introduction

Lipids are a diverse group of organic compounds which serve important functions such as storage of metabolic energy, constructing the membrane structure, protecting the organism against dehydration and pathogens, local hormone regulation and intracellular signaling to name a few (Muro et al., 2014). The ability of lipids to store metabolic energy has been exploited in the recent years and considerable progress has been made in terms of their utilization. Lipids derived from plant and animal sources are being extensively used as an alternative feedstock to replace conventional fossil fuels in many industrial processes and also as transport fuel (Mujeeb et al., 2016). However, the oil yield of common oil crops is very poor, often roughly less than 5% of their biomass (Chhetri et al., 2008). These crops are usually cultivated separately from other food crops; hence the need for fertile land leads to a competition (Gallagher, 2008). Recovered animal lipids which are derived from waste oils are also limited and cannot meet the high demands as an alternative source of fuel (Martins et al., 2010). Another important disadvantage seen in case of fuel production from animal and plant source is the extraction of the fuel via suitable solvents. If glycerol is to be extracted using methanol and then distilled, there might be a chance of glycerol being removed earlier from the system. In that case, the recovery rate of glycerol is limited and for purification purpose another step of distillation must be included which leads to increase in production cost (Ma et al., 1998).

Microorganisms on the other hand can accumulate neutral lipids having fatty acid composition similar to vegetable oil, under specific cultivation conditions, which may constitute up to 70% of their biomass (Guerzoni et al., 1985). They require a limited space for their cultivation, can be cultivated under controlled conditions (not dependent on external factors like weather or soil condition), can be cultivated on a variety of inexpensive substrates, short generation time, can be easily grown in bioreactors, rapid growth rates and easier to scale up (Li et al., 2008). Thus microbial lipids can be a potential alternative to replace or reduce dependency on petro-based hydrocarbon sources and may be able to generate sufficient lipids without compromising yield of food crops. However, the fermentation and extraction cost of lipids from microbial sources is not economically feasible at an industrial scale (Yousuf et al., 2010). Furthermore, the quantity of lipid obtained from microbial sources is less, compared to animal and plant sources.

These technical constraints have led to the development of various approaches to enhance the yield and to relatively cut down the cost of production. These approaches include identifying candidate strain with high lipid content, statistically optimizing culture conditions for the selected strains and genetically modifying their metabolic pathways. However, the initial approaches mentioned, alone cannot give a turnover over a specific rate and applying metabolic engineering approaches, further enhances the productivity of the oleaginous microorganism. The primary aim of the metabolic engineering approach is to restrict the production of by-products, which interfere with the process of extraction or inhibit the accumulation of lipids and to increase the accumulation of lipids by overexpression of enzymes or multiple

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https://doi.org/10.1016/j.bcab.2019.101321
Received 28 April 2019; Accepted 27 August 2019
Available online 28 August 2019
1878-8181/

genes (Marella et al., 2018). This review gives an overview of the various strategies used over the years to enhance lipid productivity and analyses the need of metabolic engineering approaches (Fig. 1).

1.1. Strain selection and culture conditions

The first step of optimization is to identify and select the candidate strains. The desirable characteristics of selection are summarized in Table 1.

Mostly marine microalgae and yeasts have been extensively studied for lipid production, largely being utilized for biodiesel production. Oleaginous yeasts have exceptional lipid accumulation capabilities and have wide-ranging adaptabilities to various substrates (Xu et al., 2016; Chang et al., 2015). On the other hand, marine microalgae, can be easily cultivated with water which is otherwise unsuitable for human use like salt water and waste water (Wahlen et al., 2013). The extreme conditions also reduce the chances of contamination (Amaro et al., 2011).

2. Statistical methods

The selection of a candidate strain is aided by a wide range of optimization techniques ranging from one variable at a time to statistical techniques like Plackett-Burman Design. These techniques help in maximizing the yield further. An overview of the most widely used mathematical and statistical optimization methods are discussed below.

Table 1

Characteristics	Advantages	Reference
Low nutritional needs	Can be grown on residual organic matters	Bull et al. (1979)
Optimum temperature of organism above 40 °C	Reduces cooling costs of industrial fermenters	Bull et al. (1979)
Genetic Stability	Ensures consistent production	Bull et al. (1979)
Rapid growth rate	Gives the strain competitive edge; reduces required culture area; ensures high biomass productivity	(Borowitzka, 1992)(Griffiths and Harrison, 2009)
Poor response to variation in growth environment	Reduces contamination; minimal control required over culture conditions	Borowitzka (1992)
Shear force tolerant	Cheaper pumping and agitation methods can be employed	Borowitzka (1992)
Ease of biomass harvesting	Reduces cost of extraction and further processing	Amaro et al. (2011)
High lipid quantity and quality	High Lipid quantity is desirable; quality can be compromised depending on industrial needs and cost of production	Amaro et al. (2011)

2.1. One variable at a time (OVAT)

This is a classical optimization technique, wherein only one parameter is varied over a period of time, while keeping the other factors constant. The ease of this technique helps scientists to design the medium composition during the initial stages, if the metabolite to be

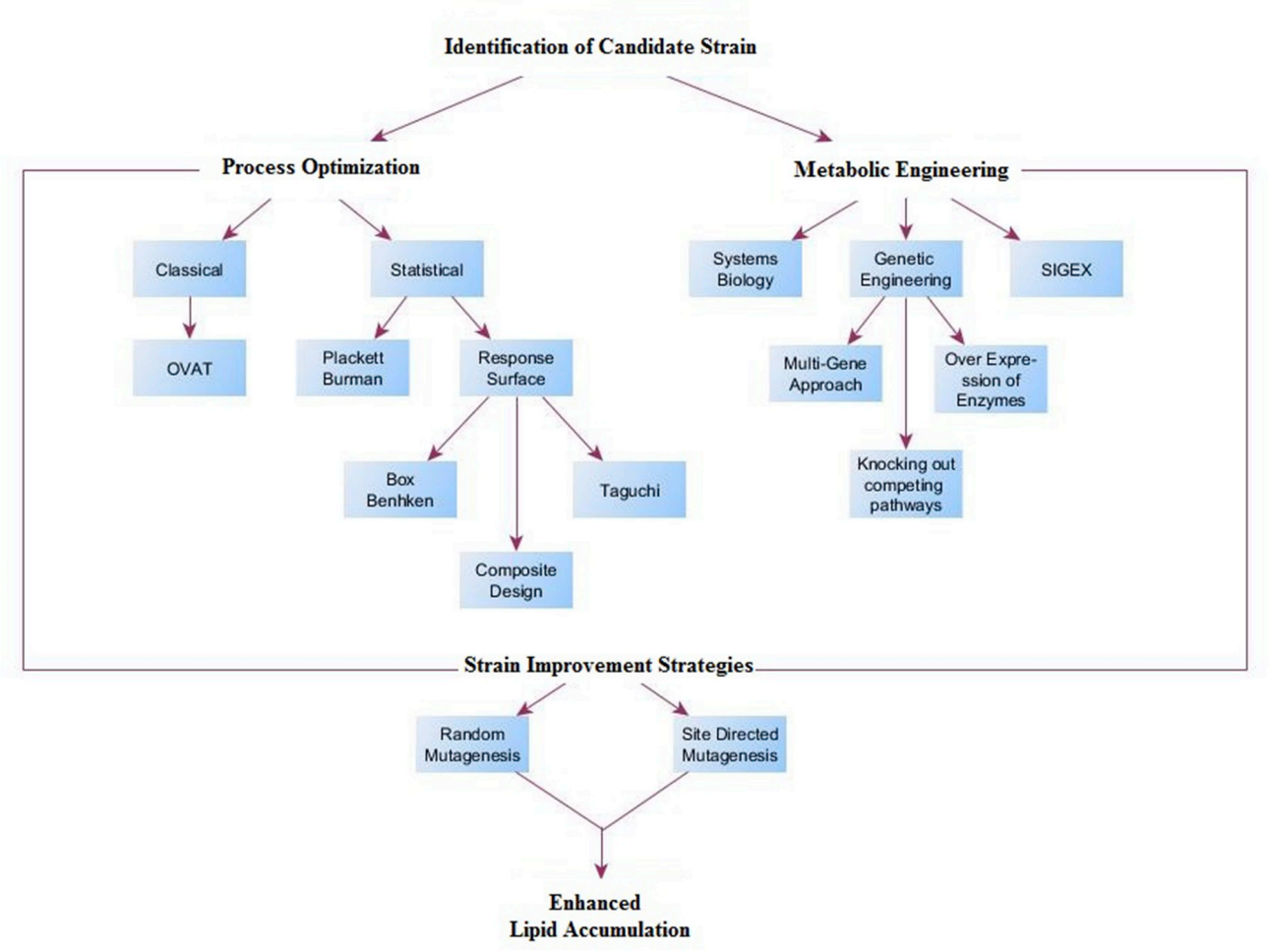

Fig. 1. Strategies used to enhance lipid production.

produced or its source is new (Singh et al., 2017). Although being a relatively simple method, OVAT fails to study the interactions between different variables and is a very costly and time-consuming approach when the number of parameters is large enough. Thus, a statistical approach is needed to reduce the number of trials.

2.2. Plackett-Burman Design

This design helps us determine the significance of the quantitative or qualitative alterations of the variables in the system. It is usually used when there are more than five independent variables to be examined. Thus helps us to identify the important variables or *factors* in the system that affects the output (lipid productivity in this case) and helps us to rank them according to the order of importance. In this technique X-1 variables can be evaluated against X variables where X is a multiple of 4.

Zhao et al. tried optimizing the medium conditions based on the design by selecting oleaginous yeast *Lipomyces starkeyi*. This design helped him shortlist 4 significant variables out of the 9 variables studied, the variables being mixed sugar, yeast extract, $FeSO_4$ and $MgSO_4$. Thus the next step was to determine the optimum level of each important variable.

2.3. Response surface technique

The second step is to study the effects of these *factors* on dependent variables or *response*. The experiments are designed to run at specific factor values called *levels*. Thus the objective is to optimize the output variable (*response*) being influenced by the independent input variables (levels). The input variables are changed accordingly in order to identify the reason for changes in the outer response and to optimize the same (Bezerra et al., 2008).

2.3.1. Box- Behnken design (BBD)

This design requires atleast 3 factors and is highly efficient in estimating second order responses. The primary advantage of using BBD is that it doesn't contain combinations wherein all the input variables are at their highest or lowest levels simultaneously. Its main drawback is that it lacks an embedded factorial or factorial design making it unsuitable for sequential experiments (Ferreira et al., 2007).

Zhao et al. carried out a design with 3 factors and 3 levels to optimize the media composition for lipid production by oleaginous yeast *Lipomyces starkeyi*. The result indicated that the optimum medium composition through co-fermentation of xylose and glucose is 73.9 g/L of mixed sugar, 7.9 g/L of yeast extract and 4 mg/L of $FeSO_4$. Thus according to the design these conditions would most likely give the best response yielding maximum lipid productivity of 61%, which represents a 2.03 and 1.59 fold increase in lipid and cellular lipid contents (Zhao et al., 2008).

2.3.2. Central composite design (CCD)

This is the most commonly used response surface design. This design helps in optimizing the overall desirability of multiple variables. Unlike Box-Behnken design this design includes embedded factorial design thus useful for sequential experiments (Gilmour, 2006).

Areesirisuk et al. carried out a CCD to find a relationship between production efficiency and cultivation process using oleaginous yeast *Pseudozyma* sp. Glucose proved to be a key substrate affecting the cell growth and hence lipid production. Nitrogen was also an important factor as its deficiency affected the protein metabolism and induced lipid production. It was also reported that the increasing concentration of KH_2PO_4 had a negative impact on lipid productivity (Areesirisuk et al., 2015).

2.3.3. Taguchi design

This design utilizes mixed-level fractional factorial design and is generally applied for large screenings. This design is often referred to as "offline quality control" as it ensures good productivity in the design stage of the process. The main advantage of this design is that the outcome of lot of experiments can be evaluated without actually conducting them (Kumar et al., 2000). This design is based on two ideas:

2.3.3.1. Primary design. The aim of this design is to make the process less variable with respect to the variations over which we have little or no control. Thus the primary step is to divide the factors into categories; inner factors- factors which can be controlled or modified and outer factors- which can't be modified as such.

2.3.3.2. Tolerance design. This design decides how or when to specify tightened tolerances for a process to enhance the productivity. In other words it helps us in identifying the critical components to be targeted when tolerances are tightened. Thus helps us in focusing the resources to reduce and control the variation (Zhang et al., 2007).

Enshaeieh et al. carried out a Taguchi design using oleaginous yeast *C.albidus* based on these parameters- Glucose and nitrogen concentrations, pH, agitation rate, time and temperature of incubation. Glucose, pH and time each have 4 levels, Nitrogen having 3, and time and temperature each having 2 levels. The analysis showed that temperature, time of incubation, glucose and nitrogen concentration had significant effect on lipid production when compared to pH and rpm. The optimum conditions yielded lipid content of 60.1% as predicted by the Taguchi design (Enshaeieh et al., 2014).

3. Metabolic engineering

The metabolic network present inside a cell is complex-regulated via various enzymes which catalyze the individual reactions. However, these natural networks need to be optimized for industrial applications. Metabolic engineering is a tool used to rewire or reconfigure the internal metabolic network of a cell so that it can enhance cellular performance either by enzymatic manipulation or changing the transport and regulatory pathways inside a cell (Nielsen, 2001, 1998; Nielsen and Keasling, 2016). From time immemorial, the genetic makeup of a microbial cell has been changed for enhanced performance via random mutagenesis. However employing mutagenesis does not give us control over the manipulation and further fails to inform us about the point of mutation which is giving rise to the enhanced performance. Due to this reason metabolic engineering has been employed and has been exploited considerably to make known the changes inside the cell in a calculated way so as to have control on these microbial bioreactors (Bailey, 1991; Stephanopoulos et al., 1998).

The process of metabolic engineering follows a generalized route for any product enhancement process (Voll and Börnke, 2010). The first step is to create the stoichiometric matrix. This is done to theoretically calculate the amount of production of metabolic intermediates or products that cannot be practically determined. This approach helps to complement the empirical approach of metabolic quantification where the latter fails. The stoichiometry of the system can be represented as in Eq. (1).

$$\sum_{i=1}^{N} Aji.Si + \sum_{i=1}^{M} Bji.Pi + \sum_{i=1}^{P} Cji.Ii + \sum_{i=1}^{Q} Dji.Xi = 0 \quad (1)$$

Here 'i' refers to the i^{th} substrate (S), product (P), intracellular intermediate (I) or biomass constituent (X) in the jth reaction. A, B, C and D are the stoichiometric matrices respectively.

The second step involves calculating and analyzing the metabolic flux; also referred to as metabolic flux analysis (MFA). This step helps elucidate how fast the various metabolites are forming or getting transformed to another form inside the metabolic network. However before proceeding for manipulation it is important to understand whether the system is determined, underdetermined or overdetermined. Studies have shown that for a metabolic pathway of considerable

complexity, the system is generally underdetermined. In that case the unknown fluxes are expressed in terms of fluxes of metabolites which can be assayed or determined. Flux analysis makes use of the matrix model where the transpose of stoichiometric matrix (D) is multiplied by the known fluxes (v) as shown in equation (2).

$$D^{T}v = 0 \quad (2)$$

Just knowing the flux of the individual metabolites is futile when it comes to cellular manipulation. The control points of these networks need to be analyzed in order to understand the exact points of control. This is referred to as metabolic control analysis (MCA). MCA additionally enables us to understand individual enzymes exact a control over the entire metabolic process (Stephanopoulos, 1999; Woolston et al., 2013).

Metabolism inside any cell follows the bow-tie model, where catabolic and anabolic processes happen one after the other (Backman et al., 2018). The catabolic processes, lead to the formation of a linking agent (also referred to as the 12 precursors) between the two subdivisions of metabolism, hence the model narrows down at this point. Anabolism leads to a diverging process which opens up from this narrowed down area to give rise to metabolites, intercellular intermediates and biomass constituents. The 12 precursors are, glucose-6-phophate, fructose-6-phosphate, ribose-5-phosphate, erythrose-4-phosphate, glyceraldehyde-3-phosphate, 3-phosphoaglycerate, phosphoenol pyruvate, pyruvate, acetyl coA, 2-oxoglutarate, succinyl coA and oxaloacetate.

3.1. Metabolic engineering and lipid biosynthesis

Metabolic engineering in order to enhance lipid production has been an evolving field for quite some time now. Before we understand the metabolic engineering strategies implemented, it is important to understand some of the pathways which lead to inherent lipid synthesis inside a cell.

One of the anabolic pathways used for lipid production is *de novo* fatty acid biosynthesis (Fig. 2). The pathways precursor molecule is acetyl coA which after carboxylation and a cyclic series of elongation process leads to the synthesis of different fatty acyl coAs having different carbon lengths (C14, C16, C18 *etc.*). The fatty acetyl coAs then get transformed to various lipids such as steryl ethers and triglycerides.

Almost all metabolic engineering strategies that have been employed till date usually start from overexpressing the desired pathway genes which are usually followed by knocking out competing pathways that drain precursors, products or cofactors (Liao et al., 2016). Other

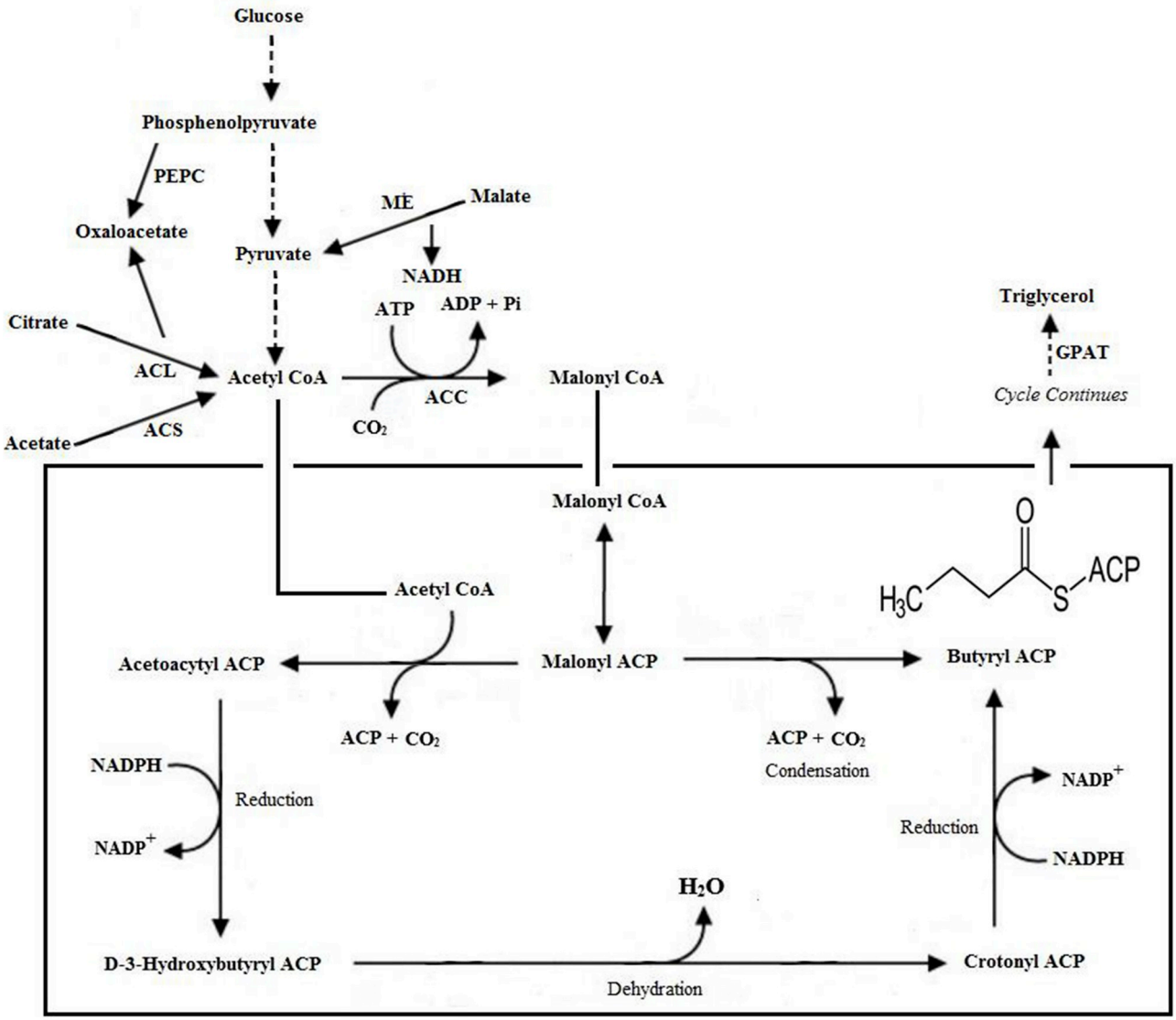

Fig. 2. de novo Fatty Acid Synthesis.

favorable strategies include constructing a new pathway for precursor supply (Martin et al., 2003), circumventing or deleting the native regulatory loops (Nielsen et al., 2009; Choi and Lee, 2013), constructing an artificial regulatory circuit (Farmer and Liao, 2000; Zhang et al., 2012), global mutation and selection strategies (Caspeta et al., 2014; Alper et al., 2006). Singling out the driving force for the pathway flux helps in indentifying the useful strategies for increasing production (Angermayr et al., 2015; Shen et al., 2011). In order to be an efficient metabolic pathway, they need to be driven by both kinetic and thermodynamic driving forces, which can further be manipulated through the size of the metabolite or cofactor pool. The following metabolic engineering approaches have been thoroughly used for enhanced lipid synthesis.

3.1.1. Genetic engineering

This is a commonly used method for the implementation of metabolic engineering. Understanding the genes required for the production of lipids inside a system and overexpressing them via recombinant DNA technology is an efficient method of increasing lipid production by the microbial cell. Kang et al. increased the lipid production in *Nannochloropsis salina* by expressing AtWRI1 transcription factor (obtained from *Arabidopsis thaliana*) (Kang et al., 2017). This transcription factor controls Wrinkled 1 (WRI1) which is an important regulator of lipid accumulation in the seeds of *Arabidopsis*. WRI1 in turn regulates fatty acid biosynthetic genes. On inserting the gene into the microalgae the lipid production was found to be increased by 36.5% in normal conditions and 44.7% under osmotic stress. Thaps3_264297 is a multifunctional lipase/phospholipase and acetyltransferase. Trentacoste et al. showed that by introduction of an antisense strand increased lipid production without affecting growth in diatom *Thalassiosira pseudonana* (Trentacoste et al., 2013). In this case the lipid catabolism was disrupted which led to increased lipid accumulation which could be extracted for specific purposes. Knockdown of the above gene resulted in disruption of the *de novo* pathway of lipid synthesis; this did result in decreased lipid remodeling which may be considered as a drawback of this transformative process. Another method of increased production of lipid was carried out by Subrahmanyam and Cronan where they the overexpressed the KAS subunit of fatty acid synthetase in *E.Coli* (Subrahmanyam and Cronan, 1998). It resulted in C2 concatenation however it led to increased toxicity which led to death of the transformants. Acyl-CoA:diacylglycerol acyl-transferase (DGAT) is an enzyme that catalyzes the last step of TAG production. cDNA isolated from *Arabidopsis* was inserted into yeast and a 200–600 fold increase in DGAT was seen. Furthermore, 3–9 fold increase in TAG accumulation was observed. The list of DNA based manipulation is endless.

When we discuss about genetic engineering, it is to be mentioned here that any method employed by man to bring about some manipulation in the genetic level can be talked about. Two such techniques that have been used for increased lipid production is site directed mutagenesis (SDM) and directed evolution. Ouyang et al. had observed that Glycerol-3-phosphate acyltransferase (GPAT) was responsible for the acylation of glycerol-3-phosphate (Ouyang et al., 2016). The cDNA of GPAt from *Lobosphaera incisa* was transformed into yeast, however implementation of SDM led to the development of a mutant (Arg195His). Upon carrying out lipid profile analysis, it was found out that the mutant containing yeasts had increased levels of phospholipids inside their systems. The process is not commonly used for increased lipid production due to the tediousness of the methodology. Directed evolution makes use of random mutagenesis and a usage of a proper selection pressure. Based on the selection pressure the mutants are forced to behave according to stipulated conditions and the 'fittest' strain survives. The application of random mutagenesis in strain improvement may not be implicit always. Sometimes just exposing the strain to harsh or changing environmental conditions can cause inherent alterations inside the genome of the organism being modified (Sen et al., 2007). In 2016, Smythers et al. reported attempts of exposing *Chlorella vulgaris* to varying concentrations of sethoxydim, which is an herbicide. *C.vulgaris* naturally is an extremely high lipid producing microalgae (Smythers et al., 2016). Sethoxydim leads to over expression of Acetyl coA carboxylase and has already shown promising results in *Nannochloropsis salina*. On exposure to the chemical the lipid content of *N. salina* was seen to increase 4 fold than that of the wild strain. The work pertaining to *C. vulgaris* is still in progress and has shown moderate success rates.

As of now, the research approaches mainly focus on marketing microbial lipid as biodiesel. TAG being the main material required for biodiesel, its production pathways are being engineered to get suitable microbial lipids.

Three broad approaches applied can be divided into (i) Multi-gene transgenic approach (ii) Knocking out competing pathways (iii) Overexpression of enzymes (M. M. Liang and Jiang, 2013; M. H. Liang and Jiang, 2013).

3.1.1.1. Multi-gene approach. This strategy deals with overexpressing more than one important gene involved in the TAG synthesis pathway.

Tai et al. co-expressed two important genes (ACC1+DGAT1) of *Yarrowia lipolytica* involved in the lipid production pathway (Tai and Stephanopoulos, 2013). This engineered strain accumulated 62% lipid of its dry weight resulting in almost a five-fold increase in its lipid productivity. The increased productivity due to co-overexpression is probably due to an improved balance between the TAG and fatty acid synthesis pathways.

3.1.1.2. Knocking out competing pathways. A simple strategy to increase lipid yield would be to decrease lipid catabolism. This can be achieved by knocking out genes involved in β-oxidation of fatty acids.

Scharnewski et al. knocked out genes involved in β-oxidation in yeast *Saccharomyces cerevisiae* which led to an increased intracellular fatty acid secretion and in some instances extracellular secretion also (Scharnewski et al., 2008). The mutant strain involved a combined deletion of genes which restricted the synthesis of Fatty acid transport protein 1 and 2. This dysfunction of Acyl coA-oxidase led to the repression of β-oxidation pathway.

3.1.1.3. Overexpression of enzymes. Though not directly involved in lipid metabolism, a few enzymes have been involved in influencing the rate of lipid accumulation. These enymes are usually responsible for increasing the pool of essential metabolites involved in lipid biosynthesis.

Davis et al. cloned the genes of E.coli responsible for the production of Acetyl-CoA carboxylase (ACC) (Davis et al., 2000). The induction of gene expression led to overproduction of the ACC subunits subsequently leading to increase in ACC activity hence overexpression. The study revealed that there is strong positive correlation between ACC overexpression and fatty acid synthesis pathway leading to a six-fold increase in the rate of fatty acid synthesis over the controlled.

3.1.2. Systems biology

Systems biology (SB) has been considered to be a multidisciplinary field which utilizes mathematical and computational modeling in order to simulate the biological system for better understanding. The process of biological manipulation is extremely costly and labor intensive. The field of SB helps curb this by simulating and attaining a comprehensive understanding of the same. SB is an interesting way of understanding the interaction of the metabolic networks inside the cell along with its manipulation. The engineered microbial strains are effectively designed, built and tested through SB approaches, to help develop a better understanding of the microbial metabolism. The employed approaches are either used to obtain an insight into the molecular mechanisms through mathematical modelling or vice versa (Nielsen, 2009). SB has helped implement strategies like codon-optimization, directed evolution, screening enzyme libraries, incorporating non-natural amino acids to

name a few in enhancing enzymatic activities (d'Espaux et al., 2015). However, we need to make a distinction between SB and bioinformatics. Bioinformatics employs an overall *in-silico* approach in order to understand the target system. Generally dealing with creation of software and tools to interpret or model. SB cannot be carried out until and unless we have a data set collected from *in-vitro* work. Hence, SB builds upon *in-vitro* experimentation (He et al., 2016; García-Granados et al., 2019).

Strain improvement using systems level analysis of metabolic network data, fluxomics, regulatory pathways *etc.* are the most looked into subjects when it comes to SB (Lee et al., 2018). With the passing of years and advancement of sequencing strategies huge amount of data has been generated for genetic model building when it comes to cellular systems. Algorithms help create genome scale metabolic network along with regulation patterns embedded which help in rewiring the intrinsic circuits inside a cell (Stephanopoulos, 2012). The E-cell project has helped devise models of parts of a cell with the basic aim of simulating the entire cell (Tomita et al., 2000, 1999). The software uses a structured substance reactor model which assumes all simulations to be carried out in dynamic equilibrium. Hence, the state of the cell can be expressed in terms of ordinary differential equations. The simulations have been successfully used to test phenomena of starvation where the substrate was limited and the cells started to die. When the cell again received the substrate they would get revived but it depended on the period of starvation. The models mimicked membrane lipid synthesis and fatty acid synthesis pathways. Not only were the major pathways simulated but gene regulatory pathways were also explored. There have been many instances of usage of SB for enhancing lipid production in microbes. Brem et al. had conducted large scale genotype to phenotype mapping to understand genes required for lipid synthesis in yeast *Rhodosporidium toruloides* (Brem et al., 2017). This enabled them to establish a suitable model for increased lipid production for usage as biofuels. Thompson et al. did a systems biology examination of *Bacillus megaterium* strain SR7 to develop it into a biofuel production platform by creating a continuous flow bioreactor which are resistant to contamination and utilizes supercritical CO_2 for extraction of the biofuel compounds (Freedman et al., 2018). Advent of SB has also made it easier to look into lipidomics and see its crosstalk with the glycome, proteome and genome. This enables us to look at the bigger picture of the cell, in other words, helps us understand the macrocosm and its constant regulations via microsystems and interconnections.

3.1.3. Substrate induced gene expression (SIGEX)

This method has generally been utilized as a screening process for various strains. SIGEX was initially established to isolate novel catabolic genes from environmental metagenomes. The process was usually directed towards those genes which were difficult to obtain using normal genetic engineering methods. The metagenome library is restriction digested to give fragments which can be ligated into vectors and transformed into cloning hosts. The entire library is then subjected to substrate level gene induction assays and the relevant genes are selected (Uchiyama et al., 2005; Uchiyama and Miyazaki, 2010). Yu et al. carried out a SIGEX experiment to ascertain the relationship between lipase gene expression and aflatoxin production *Aspergillus flavus* (Yu et al., 2003). They did however show that there exists no correlation between the two pathways. SIGEX can be used as an interesting tool when it comes to metabolic engineering. SIGEX can not only screen for relevant genes but display interconnection and cross talks between pathways. Hence pathway interconnections can be determined. These can help give valuable insight in the controlling parameters in a metabolic network. By this way the metabolic map and the flux map can be changed and the dynamic nature based on substrate change can be incorporated too.

4. Conclusion and future prospects

There are a wide range of microbes that can accumulate oil, however the use of these microbes depends on their lipid productivity, oil yield, lipid coefficient and volumetric productivity. Among the heterotrophs, yeast has a fast growth rate and also high lipid content. On the other hand autotrophic algae use inexpensive raw materials like sunlight and carbon dioxide for lipid production. The main challenge here is the effect of the intensity of light on production and thus the need for developing low cost photobioreactors to cut down the cost of production. Also wastewater such as industrial effluents can be being utilized as cheap and renewable carbon and nitrogen sources. Thus the strains studied for production depends on access to resources and the required profile of lipid accumulation. The main advantages of microbial lipid are its renewability, high growth rate and the non-requirement of arable lands.

The advancement in molecular biology and genetic engineering tools have provided a new platform for overproduction of lipids in oleaginous microorganisms. However, only a few oleaginous microbes like *Y.lipolytica*, have been studied extensively. There are numerous other attractive hosts like *R. toruloides and B. braunii* which have shown a higher lipid accumulation and adaptability to a wide range of substrates. A broader perspective of the intricate metabolic networks of these potential hosts are required along with a deeper understanding of the wide array of subcellular functions and sections that influence the lipid anabolism and catabolism. Identification of these genes and networks are essential in order to address any metabolic engineering strategy, to optimize the lipid metabolism. A variety of genetic tools have been developed which focus on promoters, terminators, standardized integration sites, pathway assembly, vectors, GEMs and CRISPER-based systems (Shi and Zhao, 2017). However these tools need to be extended from model organisms to oleaginous microbes to facilitate convenient strain engineering. Further, an integrated approach, combining genomics, transcriptomics, metabolomics and lipidomics techniques will provide a deeper insight into lipid production hence enabling a more rapid and convenient strain engineering strategy to optimize the fatty acid profiles and their production and accumulation. From an economic viewpoint, the cost of extraction should be limited and the quantity and quality of the accumulated lipids should be enhanced through genetic manipulation and subsequent optimization strategies. The extraction methods used for extracting lipids from plants or animals is not efficient when it comes to microbes, owing to its small size and risk of emulsification. The profitability lies in escalating cell densities achieved per unit time, enhancing product purity i.e. production of a single desired fatty acid and development of efficient downstream operations, which essentially depends on cell disruption efficiency as it influences subsequent downstream operations and overall extraction efficiency.

References

Alper, H., Moxley, J., Nevoigt, E., Fink, G.R., Stephanopoulos, G., 2006. Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314, 1565–1568. https://doi.org/10.1126/science.1131969.

Amaro, H.M., Guedes, A.C., Malcata, F.X., 2011. Advances and perspectives in using microalgae to produce biodiesel. Appl. Energy 88, 3402–3410. https://doi.org/10.1016/j.apenergy.2010.12.014.

Angermayr, S.A., Gorchs Rovira, A., Hellingwerf, K.J., 2015. Metabolic engineering of cyanobacteria for the synthesis of commodity products. Trends Biotechnol. 33, 352–361. https://doi.org/10.1016/j.tibtech.2015.03.009.

Areesirisuk, A., Yen, T., Chiu, C., Liu, C., Guo, J., 2015. Optimization on Yeast Lipid Production of Psuedozyma Sp . With Response Surface Methodology for Biodiesel Manufacturing 2, pp. 13–18. https://doi.org/10.12720/joaat.2.1.13-18.

Backman, T.W.H., Ando, D., Singh, J., Keasling, J.D., Martín, H.G., 2018. Constraining genome-scale models to represent the bow tie structure of metabolism for 13C metabolic flux analysis. Metabolites. https://doi.org/10.3390/metabo8010003.

Bailey, J.E., 1991. Towards a science of metabolic engineering. Science 252, 1668–1674.

Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., Escaleira, L.A., 2008. Response Surface Methodology (RSM) as a Tool for Optimization in Analytical Chemistry. Talanta. https://doi.org/10.1016/j.talanta.2008.05.019.

Borowitzka, M.A., 1992. Algal biotechnology products and processes - matching science and economics. J. Appl. Phycol. 4, 267–279. https://doi.org/10.1007/BF02161212.

Brem, R., Coradetti, S., Skerker, J., Geiselman, G., Arkin, A., Pinel, D., 2017. Development of the Oleaginous Yeast Rhodosporidium Toruloides as a New Model Organism for a Systems-Level Analysis of Lipid Productivity. Buck Institute for Research on Aging.

Bull, A.T., Ellwood, D.C., Ratledge, C., 1979. The changing scene in microbial technology. Soc. Gen. Micro. Symp. 1–28.
Caspeta, L., Chen, Y., Ghiaci, P., Feizi, A., Buskov, S., Hallström, B.M., Petranovic, D., Nielsen, J., 2014. Altered sterol composition renders yeast thermotolerant. Science 346, 75–78. https://doi.org/10.1126/science.1258137.
Chang, Y.H., Chang, K.S., Lee, C.F., Hsu, C.L., Huang, C.W., Jang, H. Der, 2015. Microbial lipid production by oleaginous yeast Cryptococcus sp. in the batch cultures using corncob hydrolysate as carbon source. Biomass Bioenergy. https://doi.org/10.1016/j.biombioe.2014.11.012.
Chhetri, A.B., Tango, M.S., Budge, S.M., Watts, K.C., Islam, M.R., 2008. Non-edible plant oils as new sources for biodiesel production. Int. J. Mol. Sci. 9, 169–180. https://doi.org/10.3390/ijms9020169.
Choi, Y.J., Lee, S.Y., 2013. Microbial production of short-chain alkanes. Nature. 502, 571–574. https://doi.org/10.1038/nature12536.
d'Espaux, L., Mendez-Perez, D., Li, R., Keasling, J.D., 2015. Synthetic biology for microbial production of lipid-based biofuels. Curr Opin Chem Biol. 29, 58–65. https://doi.org/10.1016/j.cbpa.2015.09.009.
Davis, M.S., Solbiati, J., Cronan, J.E., 2000. Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in Escherichia coli. J. Biol. Chem. https://doi.org/10.1074/jbc.M004756200.
Enshaeieh, M., Nahvi, I., Madani, M., 2014. Improving microbial oil production with standard and native oleaginous yeasts by using Taguchi design. Int. J. Environ. Sci. Technol. https://doi.org/10.1007/s13762-013-0373-2.
Farmer, W.R., Liao, J.C., 2000. Improving lycopene production in Escherichia coli by engineering metabolic control. Nat. Biotechnol. 18, 533–537. https://doi.org/10.1038/75398.
Ferreira, S.L.C., Bruns, R.E., Ferreira, H.S., Matos, G.D., David, J.M., Brandão, G.C., da Silva, E.G.P., Portugal, L.A., dos Reis, P.S., Souza, A.S., dos Santos, W.N.L., 2007. Box-Behnken design: an alternative for the optimization of analytical methods. Anal. Chim. Acta. https://doi.org/10.1016/j.aca.2007.07.011.
Freedman, A.J.E., Peet, K.C., Boock, J.T., Penn, K., Prather, K.L.J., Thompson, J.R., 2018. Isolation, development, and genomic analysis of Bacillus megaterium SR7 for growth and metabolite production under supercritical carbon dioxide. Front. Microbiol. https://doi.org/10.3389/fmicb.2018.02152.
Gallagher, E., 2008. The Gallagher review of the indirect effects of biofuels production. Rev. Lit. Arts Am. 1–92. https://doi.org/10.1111/j.2008.0908-8857.04218.x.
García-Granados, R., Lerma-Escalera, J.A., Morones-Ramírez, J.R., 2019. Metabolic engineering and synthetic biology: synergies, future, and challenges. Front. Bioeng. Biotechnol. https://doi.org/10.3389/fbioe.2019.00036.
Gilmour, S.G., 2006. Response surface designs for experiments in bioprocessing. Biometrics. https://doi.org/10.1111/j.1541-0420.2005.00444.x.
Griffiths, M.J., Harrison, S.T.L., 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. https://doi.org/10.1007/s10811-008-9392-7, 493–507.
Guerzoni, M.E., Lambertini, P., Lercker, G., Marchetti, R., 1985. Technological potential of some starch degrading yeasts. Starch - Stärke 37, 52–57. https://doi.org/10.1002/star.19850370205.
He, F., Murabito, E., Westerhoff, H.V., 2016. Synthetic biology and regulatory networks: where metabolic systems biology meets control engineering. J. R. Soc. Interface. https://doi.org/10.1098/rsif.2015.1046.
Kang, N.K., Kim, E.K., Kim, Y.U., Lee, B., Jeong, W.J., Jeong, B.R., Chang, Y.K., 2017. Increased lipid production by heterologous expression of AtWRI1 transcription factor in Nannochloropsis salina. Biotechnol. Biofuels. https://doi.org/10.1186/s13068-017-0919-5.
Kumar, P., Barua, P.B., Gaindhar, J.L., 2000. Quality optimization (multi-characteristics) through Taguchi's technique and utility concept. Qual. Reliab. Eng. Int. https://doi.org/10.1002/1099-1638(200011/12)16:6<475::AID-QRE342>3.0.CO;2-0.
Lee, H.-M., Vo, P., Na, D., 2018. Advancement of metabolic engineering assisted by synthetic biology. Catalysts. https://doi.org/10.3390/catal8120619.
Li, Q., Du, W., Liu, D., 2008. Perspectives of microbial oils for biodiesel production. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-008-1625-9.
Liang, M., Jiang, J., 2013. Progress in Lipid Research Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. Prog. Lipid Res. 52, 395–408. https://doi.org/10.1016/j.plipres.2013.05.002.
Liang, M.H., Jiang, J.G., 2013. Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. Prog. Lipid Res. https://doi.org/10.1016/j.plipres.2013.05.002.
Liao, J.C., Mi, L., Pontrelli, S., Luo, S., 2016. Fuelling the future: microbial engineering for the production of sustainable biofuels. Nat. Rev. Microbiol. 14, 288–304. https://doi.org/10.1038/nrmicro.2016.32.
Ma, F., Clements, L.D., Hanna, M.A., 1998. Biodiesel fuel from animal fat. Ancillary studies on transesterification of beef tallow. Ind. Eng. Chem. Res. 37, 3768–3771. https://doi.org/10.1021/ie980162s.
Marella, E.R., Holkenbrink, C., Siewers, V., Borodina, I., 2018. Engineering microbial fatty acid metabolism for biofuels and biochemicals. Curr. Opin. Biotechnol. https://doi.org/10.1016/j.copbio.2017.10.002.
Martin, V.J., Pitera, D.J., Withers, S.T., Newman, J.D., Keasling, J.D., 2003. Engineering a mevalonate pathway in Escherichia coli for production of terpenoids. Nat. Biotechnol. 21, 796–802. https://doi.org/10.1038/nbt833.
Martins, A., Caetano, N.S., Mata, T.M., 2010. Microalgae for biodiesel production and other applications . A review 14, 217–232. https://doi.org/10.1016/j.rser.2009.07.020.
Mujeeb, M.A., Vedamurthy, A.B., T, S.C., 2016. Current strategies and prospects of biodiesel production: a review. Pelagia Res. Libr. Adv. Appl. Sci. Res. 7, 120–133.
Muro, E., Atilla-Gokcumen, G.E., Eggert, U.S., 2014. Lipids in cell biology: how can we understand them better? Mol. Biol. Cell 25, 1819–1823. https://doi.org/10.1091/mbc.E13-09-0516.
Nielsen, J., 2001. Metabolic engineering. Appl. Microbiol. Biotechnol. 55, 263–283.
Nielsen, J., 1998. Metabolic engineering: techniques for analysis of targets for genetic manipulations. Biotechnol. Bioeng. 58, 125–132.
Nielsen, J., 2009. Systems biology of lipid metabolism: from yeast to human. FEBS Lett. 17, 3905–3913. https://doi.org/10.1016/j.febslet.2009.10.054.
Nielsen, J., Keasling, J.D., 2016. Engineering cellular metabolism. Cell 164, 1185–1197. https://doi.org/10.1016/j.cell.2016.02.004.
Nielsen, D.R., Leonard, E., Yoon, S.H., Tseng, H.C., Yuan, C., Prather, K.L., 2009. Engineering alternative butanol production platforms in heterologous bacteria. Metab. Eng. 11, 262–273. https://doi.org/10.1016/j.ymben.2009.05.003.
Ouyang, L.-L., Li, H., Yan, X.-J., Xu, J.-L., Zhou, Z.-G., 2016. Site-directed mutagenesis from Arg 195 to his of a microalgal putatively chloroplastidial glycerol-3-phosphate acyltransferase causes an increase in phospholipid levels in yeast. Front. Plant Sci. https://doi.org/10.3389/fpls.2016.00286.
Scharnewski, M., Pongdontri, P., Mora, G., Hoppert, M., Fulda, M., 2008. Mutants of Saccharomyces cerevisiae deficient in acyl-CoA synthetases secrete fatty acids due to interrupted fatty acid recycling. FEBS J. https://doi.org/10.1111/j.1742-4658.2008.06417.x.
Sen, S., Venkata Dasu, V., Mandal, B., 2007. Developments in directed evolution for improving enzyme functions. Appl. Biochem. Biotechnol. https://doi.org/10.1007/s12010-007-8003-4.
Shen, C.R., Lan, E.I., Dekishima, Y., Baez, A., Cho, K.M., Liao, J.C., 2011. Driving forces enable high-titer anaerobic 1-butanol synthesis in Escherichia coli. Appl. Environ. Microbiol. 77, 2905–2915. https://doi.org/10.1128/AEM.03034-10.
Shi, S., Zhao, H., 2017. Metabolic Engineering of Oleaginous Yeasts for Production of Fuels and Chemicals. Front. Microbiol. 8, 2185. https://doi.org/10.3389/fmicb.2017.02185.
Singh, V., Haque, S., Niwas, R., Srivastava, A., Pasupuleti, M., Tripathi, C.K.M., 2017. Strategies for fermentation medium optimization: an in-depth review. Front. Microbiol. https://doi.org/10.3389/fmicb.2016.02087.
Smythers, A.L., Adkins, P.E., Kolling, D., 2016. Using directed evolution to increase lipid formation in Chlorella vulgaris for use in biofuels. In: Proceedings of the West Virginia Academy of Science. Hungtington.
Stephanopoulos, G., 2012. Synthetic biology and metabolic engineering. ACS Synth. Biol. https://doi.org/10.1021/sb300094q.
Stephanopoulos, G., 1999. Metabolic fluxes and metabolic engineering. Metab. Eng. 1, 1–11. https://doi.org/10.1006/mben.1998.0101.
Stephanopoulos, G.N., Aristidou, A.A., Nielsen, J., 1998. Metabolic Engineering: Principles and Methodologies, Metabolic Engineering. https://doi.org/10.1016/B978-0-12-666260-3.50019-4.
Subrahmanyam, S., Cronan, J.E., 1998. Overproduction of a functional fatty acid biosynthetic enzyme blocks fatty acid synthesis in Escherichia coli. J. Bacteriol.
Tai, M., Stephanopoulos, G., 2013. Engineering the push and pull of lipid biosynthesis in oleaginous yeast Yarrowia lipolytica for biofuel production. Metab. Eng. https://doi.org/10.1016/j.ymben.2012.08.007.
Tomita, M., Hashimoto, K., Takahashi, K., Matsuzaki, Y., Matsushima, R., Saito, K., Yugi, K., Miyoshi, F., Nakano, H., Tanida, S., Saito, Y., Kawase, A., Watanabe, N., Shimizu, T.S., Nakayama, Y., 2000. E-CELL project: towards integrative simulation of cellular processes. New Gener. Comput. https://doi.org/10.1007/BF03037563.
Tomita, M., Hashimoto, K., Takahashi, K., Shimizu, T.S., Matsuzaki, Y., Miyoshi, F., Saito, K., Tanida, S., Yugi, K., Venter, J.C., Hutchison, C.A., 1999. E-CELL: software environment for whole-cell simulation. Bioinformatics. https://doi.org/10.1093/bioinformatics/15.1.72.
Trentacoste, E.M., Shrestha, R.P., Smith, S.R., Gle, C., Hartmann, A.C., Hildebrand, M., Gerwick, W.H., 2013. Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth. Proc. Natl. Acad. Sci. https://doi.org/10.1073/pnas.1309299110.
Uchiyama, T., Abe, T., Ikemura, T., Watanabe, K., 2005. Substrate-induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. Nat. Biotechnol. https://doi.org/10.1038/nbt1048.
Uchiyama, T., Miyazaki, K., 2010. Product-induced gene expression, a product-responsive reporter assay used to screen metagenomic libraries for enzyme-encoding genes. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.00464-10.
Voll, L.M., Börnke, F., 2010. Metabolic engineering. In: Biotechnology in Agriculture and Forestry. https://doi.org/10.1007/978-3-642-02391-0_11.
Wahlen, B.D., Morgan, M.R., McCurdy, A.T., Willis, R.M., Morgan, M.D., Dye, D.J., Bugbee, B., Wood, B.D., Seefeldt, L.C., 2013. Biodiesel from microalgae, yeast, and bacteria: engine performance and exhaust emissions. Energy Fuels. https://doi.org/10.1021/ef3012382.
Woolston, B.M., Edgar, S., Stephanopoulos, G., 2013. Metabolic engineering: past and future. Annu. Rev. Chem. Biomol. Eng. https://doi.org/10.1146/annurev-chembioeng-061312-103312.
Xu, J., Du, W., Zhao, X., Liu, D., 2016. Renewable microbial lipid production from Oleaginous Yeast: some surfactants greatly improved lipid production of Rhodosporidium toruloides. World J. Microbiol. Biotechnol. https://doi.org/10.1007/s11274-016-2076-6.
Yousuf, A., Sannino, F., Addorisio, V., Pirozzi, D., 2010. Microbial conversion of olive oil mill wastewaters into lipids suitable for biodiesel production. J. Agric. Food Chem. 58, 8630–8635. https://doi.org/10.1021/jf101282t.
Yu, J., Mohawed, S.M., Bhatnagar, D., Cleveland, I.E., 2003. Substrate-induced lipase gene expression and aflatoxin production in Aspergillus parasiticus and Aspergillus flavus. J. Appl. Microbiol. https://doi.org/10.1046/j.1365-2672.2003.02096.x.

Zhang, F., Carothers, J.M., Keasling, J.D., 2012. Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. Nat. Biotechnol. 30, 354–359. https://doi.org/10.1038/nbt.2149.

Zhang, J.Z., Chen, J.C., Kirby, E.D., 2007. Surface roughness optimization in an end-milling operation using the Taguchi design method. J. Mater. Process. Technol. https://doi.org/10.1016/j.jmatprotec.2006.11.029.

Zhao, X., Kong, X., Hua, Y., Feng, B., Zhao, Z., 2008. Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast Lipomyces starkeyi. Eur. J. Lipid Sci. Technol. https://doi.org/10.1002/ejlt.200700224.