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Enhancement of Omega-3 Fatty Acid Production by Metabolic Engineering or Genetic Modification of Microorganisms

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Abstract

The use of Omega-3 fatty acids is garnering increasing interest due to its substantial effects on human health. Fish are a major dietary source of ω3FA; however, they are not the primary producers. The primary producers are microalgae and other microorganisms, which serve as the original source of omega-3s in the food chain. The marine fish however, has not been able to meet the global demands. Recently, the generation of fatty acids has been investigated from another source, namely microorganisms. Omega-3 fatty acids are naturally produced by the microorganisms. However, the use of metabolic engineering has provided evidence for improvements in production. The current article reviews research on microorganisms using engineering ways to accumulate ω3FA such as docosahexaenoic acid or DHA and eicosapentaenoic acid or EPA. These studies have demonstrated that modulation of existing pathways, as well as reconstitution of biosynthetic pathways, has a high potential for increasing the fatty acid yield. However, certain bottlenecks limit the yield of fatty acids in various host organisms. These may include the fatty acid flux of diates that exists between various lipid pools. Even though the heterologous and native microbes show fatty acid flux under acyltransferases control, there is evidence that modulation of even a single acyltransferase by genetic approach can provide significant alteration for producing fatty acids. The microbes with oleaginous properties are being identified rigorously and are expected to further advance the process of engineering which leads to enhanced production of fatty acids in microbes.

Keywords: Omega-3 Fatty Acids, Eicosapentaenoic Acid (EPA), Docosahexaenoic Acid (DHA), Metabolic Engineering, Desaturase and Elongase Pathway, Polyketide Synthase (PKS) Pathway

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INTRODUCTION

Omega-3 fatty acids, their source, and use

Omega-3 fatty acids (ω3FA) contribute significantly to human cell membranes and are extensively dispersed in nature.[1] α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are the main long chain polyunsaturated omega-3 fatty acids.[2] These fatty acids are essential for lipid metabolism. The fatty acids impart energy and form vital functional components in body functions like immunity, endocrine system, lungs, heart and blood vessels.[3,4] ALA is not produced in the human body, making it a vital component that must be obtained from meals and beverages. The transformation of ALA gives rise to EPA and DHA that are mandatory for retina and brain function.[2] Thus, the supplementation of such essential fatty acids is vital for humans.

The researchers have proposed alternative sources such as fish oil, transgenic plants, microalgae, etc.[3] The fatty acids production by microbes is presently considered for applications in commercial industries in the world.[5] The oil from microbes has various advantages including benefits to health due to the presence of squalene or phytosterols and are not having odor or cholesterol.[6] Another advantage of using these microbes is that they can be cultivated easily, they are independent of season and available full-year.[6-8] It is because of these features that fatty acid production using microbes is an effective and attractive approach for application in the industry.

Studies on various kinds of microbes have reported anabolic pathways for fatty acids, and there are several studies performed on the cultivation strategies. The technique of single-cell oil production is also reported previously for the manufacture of DHA and ARA.[9] Various oleaginous fungus and microalgae have been used to commercialize DHA and ARA production.[10] To fulfill the worldwide demand for these fatty acids, it is critical to design an efficient method for their manufacturing.[3] The application in the industry can be boosted by the metabolic engineering process that can improve production of fatty acid by using oleaginous microorganisms.

Microbial source of omega-3-fatty acid

The various types of fatty acids can be synthesized from a wide range of microbes that include yeast, diatom, fungi, and algae. The microbe species that can synthesize high levels of ARA, DHA, EPA include *Thraustochytrium, Mortierella alpina, Phaeodactylum tricornutum,* and *Chlorella minutissima.*[11,12] DHA producers are *Crypthecodinium cohnii, Thraustochytrium* sp.*, Aurantiochytrium* sp.*,* and *Ulkenia* sp., while ARA producer includes *M. alpine.*[13,14]

Genetic Engineering to Increase Omega-3 Fatty Acid Yield

The ALA production was observed to be increased when *Yarrowia lipolytica, Mortierella alpina, Schizochytrium,* and *Crypthecodinium cohnii* strains were grown using agro-industrial wastes.[15] The genetic engineering of *Yarrowia lipolytica* yeast yielded a high amount of DGLA, ARA, and EPA.[16] The genetic engineering of *Rhodosporidium toruloides* was found to boost ALA production to nearly 49% by introduction of Δ12 desaturase (FAD2) and ω3 desaturase (FAD3) gene.

The pollutants made by humans such as polychlorinated biphenols, dioxins, and methyl mercury are present in some fish oils and due to the decline of stocks of fish worldwide, there is an issue of their utility for DHA and EPA production.[2] The stability and sustainability of microbial oils with high amount of DHA and EPA in comparison to oil from fish is more. In addition, the oils from microbes are enrich in naturally produced antioxidants that aids in the prevention of oxidative damage.[2,9] Thus, the utilization of various microbes is possible for the special oil production. For example, omega-3 oils from the microbial source like *Schizochytrium* and *Crypthecodinium* species.[17] The DHA and ARA are breast milk components that are involved in the development of neonates.

The use of high throughput technology like whole-genome mutagenesis have improved fatty acid synthesis successfully in traditional strain.[2] The whole-genome mutagenesis was performed for *M. alpine* for production of fatty acids and Di-homo-γ-linolenic acid (DGLA) rich oil.[18] The process of mutagenesis has been applied to *Thraustochytrid* strains for an alteration of fatty acids through mutations.[1]

IMPORTANCE OF OMEGA-3-FATTY ACID

There are varieties of health benefits that ω3FA have. These include anxiety and depression, eye, development of brain during the time of pregnancy and early age, heart related disease, Attention deficit hyperactivity disorder, inflammation, metabolism related syndrome, autoimmune diseases, age-related mental diseases, mental abnormalities, and cancer, Alzheimer's disease, child asthma, liver fat, bone and joint health, pain during periods, and skin health.

Anxiety and depression are fairly common disorders across the globe. Depression causes lethargy, sadness, and loss of interest in life[19] whereas anxiety causes nervousness and constant worry.[20] It is interesting to note that the consumption of ω3FA helps in decrease of depression and anxiety. The supplementation to individuals suffering from anxiety showed improvement in symptoms.[19,21] EPA is one of the fatty acids that was shown to have significant effects against depression and was able to work as an antidepressant drug.[22]

The fatty acids can also aid in the improvement of eye health. The DHA is observed as a structural component in the eye, particularly in the retina. The lack of DHA can result in vision problems. The intake of appropriate quantities of ω3FA is related to the reduction in macular degeneration risk which is a main reason of blindness and eye damage across the globe.[23,24]

The use of ω3FA is vital for the development and growth of the in infant's brain. In the brain, 40% of poly-unsaturated fatty acids (PUFA) are attributed to DHA and in the eye, 60% are attributed to DHA.[25,26] Thus, the eyesight of infants that take DHA-fortified formula is better in comparison to those infants that don't take DHA.[27] Omega 3 fatty acids provide several advantages during pregnancy, including improved social and communication abilities, increased IQ, healthy development, a lower chance of autism, cerebral palsy, and attention deficit hyperactivity disorder, and fewer behavioral disorders.[28]

The heart diseases related risks such as stroke, heart attack, which are leading death cause in the world, can also be reduced with the consumption of omega 3 fatty acids. It had been long since the prevalence of these diseases was found to be lower in communities with fish consumption that was further related to consumption of fatty acids.[29] Heart health is related to fatty acids due to many benefits. The omega 3 fatty acids are proved to reduce triglycerides by 15%-30% in individuals. Omega-3 fatty acid ingestion was also found to reduce blood pressure. Moreover, omega 3 fatty acids were shown to increase the level of HDL cholesterol.[30] The effect of omega 3 fatty acid was also observed on the aggregation of platelets that prevents clot formation in the blood.[31,32] The omega 3 fatty acids prevent the damage to arteries and keep them smooth by preventing the formation of plaque that is the major cause of hardening of arteries. Omega 3 fatty acids decrease inflammation and have a significant role in the inflammation related response. In certain individuals, Omega 3 fatty acid's use can reduce LDL cholesterol levels, but the indications provided by studies are mixed in nature.[33,34] Although there are many beneficial effects on the risk factors for heart issues; however, the evidence on the prevention of strokes or heart attacks is still not conclusive with some investigations showing no benefits.[35]

Attention deficit hyperactivity disorder is another disease that has been linked to omega 3 fatty acid usage. The disease is behavioral and is characterized by impulsivity, hyperactivity, and inattention.[36] The children with the disease were observed to exhibit lower levels of omega 3 fatty acid in comparison to healthy peoples. Studies have indicated that supplementation with omega 3 can improve the symptoms of attention deficit hyperactivity disorder, aid in the completion of tasks, improve attention, decrease aggression, restlessness, impulsiveness, and hyperactivity.[37,38] Fish oils have also been shown to be an effective therapy for attention deficit hyperactivity disorder.[39]

Metabolic syndrome is another condition that can benefit from supplementation of omega 3 fatty acid. The metabolic syndrome includes a group of diseases like obesity, insulin resistance, higher level of LDL cholesterol, lower levels of HDL cholesterol and higher blood pressure. They enhance the danger for diabetes and heart diseases and gain a major health concern. The use of omega 3 fatty acid was shown to reduce inflammation, insulin resistance, and risk of

heart problems in individuals with metabolic syndrome.[40]

Inflammation, on the other hand, is the response to damage or infection in humans and is very crucial for the health of an individual. In certain cases, however, it can last for a longer duration without injury or infection. This condition is referred to as long-term or chronic inflammation. Omega-3 fatty acids have been shown to reduce the synthesis of cytokines and eicosanoids, which are associated with inflammation.[41]

Moreover, ω3FA can fight against autoimmune diseases as well, where the body recognizes the own cells as foreign and attacks them. During early life, it is essential to use the ω3FA to fight certain diseases. Availability of ample omega 3 fatty acids at early stages have been linked to lowered disease risk such as multiple sclerosis, type 1 diabetes and autoimmune diabetes. It may also aid in the treatment of Crohn's disease, psoriasis, rheumatoid arthritis, lupus and ulcerative colitis.[42]

Reports have shown that psychotic individuals have reduced amounts of ω3 fatty acids.[21] The supplementation of these has been suggested to lower mood swing frequency and in the case of bipolar disorder and schizophrenia, it can lower relapse in individuals.[21] The violent behavior was also observed to be decreased with the intake of ω3FA.[43]

The brain's function declines as we age. Various studies have related ω3FA with reduction of decline in mental health and risk of Alzheimer's disease.[44,45] An investigation highlighted that supplementation of ω3FA at the time of onset of disease may be beneficial due to mildness of Alzheimer's disease.[46]

In the western world, cancer contributes to the death rates significantly. The claim that ω3FA lower cancer risk was investigated. In cancer related studies, it is highlighted that ω3FA reduces the 55 percent risk of having cancer in the colon.[47] Consuming ω3FA has been linked to lower risks of prostate and breast cancer. These results, however, were not consistent across all studies.[48,49]

Asthma is another health condition that is characterized by wheezing, shortness of breath, and cough and forms a dangerous threat to life. The cause includes swelling and inflammation of lung airways. The frequency of asthma has been rising in past years across the world including the US.[50] The relation of ω3FA with reduced risk of asthma has been reported by various studies on young adults and children.[51]

ω3FA can also cure non-alcoholic fatty liver disease. In the Western world, rising obesity has resulted in an increase in non-alcoholic fatty liver disease.[52] Using ω3FA may decrease inflammation associated with fatty liver and non-alcoholic fatty liver disease.[52]

Skeletal system is defected by two common diseases known as arthritis and osteoporosis. The use of ω3FA has the potential to increase the strength of the bone through calcium boosting in bones that alleviate osteoporosis risk.[53] The individuals on ω3FA show the pain reduction in joints and an increase in strength of the grip.[54]

The quality of life can be significantly altered by the pain radiating in the thighs and lower back from the pelvis and lower abdomen due to menstruation. Research indicates that women with ω3FA have less discomfort during menstruation compared to those without.[55] A research found that using ω3FA instead of ibuprofen was more effective for treating menstruation discomfort.[56]

The skin contains DHA as a structural element. The cell membrane of the skin is maintained by DHA that is a major part. The cell membrane remains moist, soft, supple, and without wrinkle, if it is healthy. The EPA is known to impart several benefits to the skin. These include skin hydration and management of production of oil, prevention of the red bumps observed on upper arms due to follicular hyperkeratinization of hairs, reduction of acne risk, and premature aging. The skin damage from the sun can also be protected by ω3FA. EPA aids in blocking collagen damage observed after exposure to the sun.[57]

Microbial biosynthesis of omega (ω)-3 fatty acids

For lipid production, acetyl-CoA and NADPH are continuously required with an excess of carbon.[58] The generation of acetyl-CoA (Figure) is continually produced in oleaginous organisms through a series of processes initiated by an environment devoid of nutrients and causes the citrate pool to expand in the mitochondria. The dependent enzyme on Adenosine monophosphate (AMP) in the Kreb's cycle is the isocitrate dehydrogenase (IDH) enzyme.

The IDH is responsible for performing oxidative decarboxylation reaction in the oleaginous organisms. The AMP is converted to ammonia and IMP under nitrogen-deficient conditions because of increased AMP deaminase activity in the mitochondria. The consequence of this reaction is the blockage of isocitrate to citrate conversion and citrate translocation to cytoplasm.[59]

ATP: citrate lyase (ACL) enzyme cleaves acetyl-CoA and oxaloacetate (OAA) from citrate, resulting in a constant source of acetyl-CoA for lipid synthesis.[60] Other than acetyl-CoA, NADPH is also an important parameter for fatty acid synthesis. According to reports, malic enzyme's decarboxylation activity is ascribed to the conversion of malate to pyruvate, which processes NADPH supply.[58] In certain microbes such as *Yarrowia lipolytica*, the NADPH regeneration of is possibly catalyzed by malic enzyme. Pentose phosphate pathway also forms an alternative pathway for the production of NADPH.[61] Stearic acid or palmitic acid form the end products for biosynthesis of lipids in microbes.[62] Desaturase and elongase enzymes function sequentially to produce polyunsaturated fatty acids through desaturation and elongation activities. Hence, amount and type of the fatty acid are dependent on elongases and desaturases genes in the microbial genome.[58]

The humans can either take DHA as a dietary component or can use it from the transformation of DPA or EPA in a lesser amount as intermediate. The Δ4-desaturase is required for this elongation step. It has been observed that "Sprecher's shunt" is more likely to produce DHA through C24 intermediate.[63] Microbes including mosses, fungus, and microalgae use a series of enzyme processes to synthesize DHA. These involve two major pathways namely PKS pathway (polyketide synthase pathway) which is anaerobic and elongase/desaturase pathway which is aerobic.

Key Determinants of Microbial Production Efficiency

A variety of variables impact the formation of ω3FA. These include temperature, pH, aeration, media composition, incubation time, stabilization of ω3FA. It was observed, cell growth is favored by high temperature whereas production of fatty acids is favored by lower temperature.[64] Psychrophiles create small amounts of fatty acids at optimum temperatures. Thus, cyanobacteria and bacteria rarely produce fatty acids. The EPA production is favored by low growth temperatures. For instance, the *Mortierella* species are known to produce EPA at high concentrations in 12-15 °C temperature. The change in temperature from 25 to 11 °C was observed as the triggering factor for the production of EPA. But post cultivation for 4 days at 12 °C when 25 °C temperature was maintained, there was no increase in production of EPA.[65] It was shown that 21.5 °C was the optimum temperature for *P. tricornutum* to enhance the production of EPA whereas *M. alipina* showed a rapid decrease in EPA production.[66]

pH is also known to affect the synthesis of fatty acids. The pH is recommended to be maintained optimum as per the microbes used, as the change in pH will require energy to restore it to normal. Fungi produce more fatty acids when the pH of their culture medium rises. For EPA production in algae and fungi, the optimum pH is 6.0-7.6. The pH of 6.0 is required by *Thraustochytrium aureum* whereas *Mortierella* fungi and red alga *Porphyridium* requires 6-7 pH initially.[66] The pH value of 8-9 is required for the production of ARA by *Mortierella* species.[67]

The presence of molecular oxygen in the medium influences the composition of fatty acids. The level of unsaturation is governed by the availability of oxygen, which is required in the desaturation pathway by bacteria. *Gyronidium cohnii* is a dinoflagellate that exhibits an increase in fatty acid content with oxygen level increment.[68]

The components like carbon source, cell density, light intensity, metal ions, media salinity influence the composition of fatty acids. The *Mortierella* is known to use linseed oil for the enhancement of EPA production. Using glucose, maltose, or starch as carbon source, the *Thraustochytrium aureum* can produce an increased amount of DHA.[69]

Incubation period is another element that influences fatty acid synthesis. Extension of time for fermentation can amplify EPA. It was observed that after 20 days of incubation, maximum lipid content and biomass was recovered from fungal species. The EPA concentration was elevated even after 36 days.[70]

The susceptibility of fatty acids towards oxidation is high that can further cause the production of toxic substances like peroxides or volatile substances.[2] There is a complex mechanism that underlies fatty acid oxidation and is dependent on the form of the lipid. The oxidation reaction can be catalyzed by temperature, light, or the trace metals present. Thus, optimum storage, processing, and packaging are required for the microbial oil. There are antioxidants like Ascorbic acid, gallic acid, lactoferrin, ascorbyl palmitate, tocopherols, propyl gallate that can be added.[3] Another approach of microencapsulation has been proposed with the potential to increase stability of oil and control of-flavors.[3]

Aerobic Desaturase and Elongase Pathway

Microalgae, thraustochytrids, fungi, bacteria, mammals, plants, and other microorganisms use an aerobic enzyme encoded route to synthesize ω3FA.[4] The enzymes can be divided into two categories: chain elongation and fatty acid desaturation (Figure). The desaturation process is catalyzed by desaturase enzyme by the introduction of a double bond in the carboxyl end of the substrate fatty acid. In microbes, the front-end desaturase namely, Δ8, Δ6, Δ5, and Δ4 desaturases, and membrane-bound desaturases namely Δ15, Δ12, and Δ3 desaturases are present.[71] The elongation complex is for fatty acid chain elongation and consists of 4 subunits namely, Acyl-CoA elongase, enoyl-CoA reductase, ketoacyl-CoA reductase, hydroxyacyl-CoA dehydratase.[72]

The steps involved in the synthesis of DHA are as follows:

1. Step I: Generation of stearidonic acid (SDA) from Alpha-linolenic acid catalyzed by Δ6 desaturase on 6th carbon.
2. Step II: Generation of eicosatetraenoic acid (ETA) from SDA by action of Δ6 elongase.
3. Step III: Generation of EPA from ETA by the action of Δ5 desaturase on 5th carbon.
4. Step IV: Generation of docosapentaenoic acid (DPA) from EPA by action of Δ5 elongase.
5. Step V: Production of DHA from DPA by the action of Δ4 desaturase on 4th carbon.

Photosynthetic and non-photosynthetic microorganisms produce de novo fatty acids in their plastids and cytoplasm. Endoplasmic reticulum-associated enzymes, on the other hand, are primarily responsible for fatty acid desaturation and elongation.[4] Stearic acid is the end product for fatty acid synthase and is formed by a reaction catalyzed by an enzyme known as acyl carrier protein Δ9-desaturase. The ω3 desaturases (mainly Δ12 and Δ15) are present in microbes, some cyanobacteria and plants for the formation of linoleic acid and ALA. These enzymes, however, are lacking in animals and humans.[4] The microbes thus need the insertion of desaturases and elongases to overcome the deficiency of such enzymes.[4] The use of the genetic engineering approach has been combined with the conventional methods to scale up fatty acid production.

The ω3FA are formed in microbes through 2 convergent and different pathways. These are Δ8-pathway and Δ6-pathway. The Δ6-pathway involves the action of Δ6-desaturase on linoleic acid and ALA. This leads to the formation of SDA and γ-linolenic acid.[73] After this, the ETA and DGLA are generated by Δ6-elongase action. The ETA and Dihomo-γ-linolenic acid are further converted into EPA and ARA by the enzyme Δ5-desaturase. The Δ8-pathway, an alternate mechanism, initiates ω3FA production by Δ9-elongation of substrates, similar to the Δ6-pathway. This is catalyzed by Δ9-elongases that is responsible for the generation of eicosatrienoic acid (ERA) and eicosadienoic acid (EDA). The fatty acids (Δ9-elongated) are catalyzed by desaturase (Δ8) and the reaction is desaturation that leads to the production of ETA and DGLA. Later, ETA and DGLA form EPA and ARA through Δ5-desaturation in a manner similar to that in Δ6-pathway. Biosynthesis of DHA in microbes includes elongation of EPA via Δ5 elongases to form DPA (docosapentaenoic acid) that at last produces DHA by the process of desaturation in Δ4 position. ω3-desaturases convert n-6 PUFAs (e.g., linoleic acid) to n-3 PUFAs (e.g., ALA), thereby linking the n-6 and n-3 pathways.[4]

In eukaryotic organisms, the ω3FA synthesis occurs through conventional 6-pathway. In certain protists like *Euglena gracilis, Acanthamoeba* spp., and *Tetrahymena pyroformis* and microalgae like *Pavlova salina* and *Isochrysis galbana*, ω3FA synthesis takes place via Δ8-pathway. The ω3FA with long-chain were created by cloning the genes that encode for elongation and desaturation process

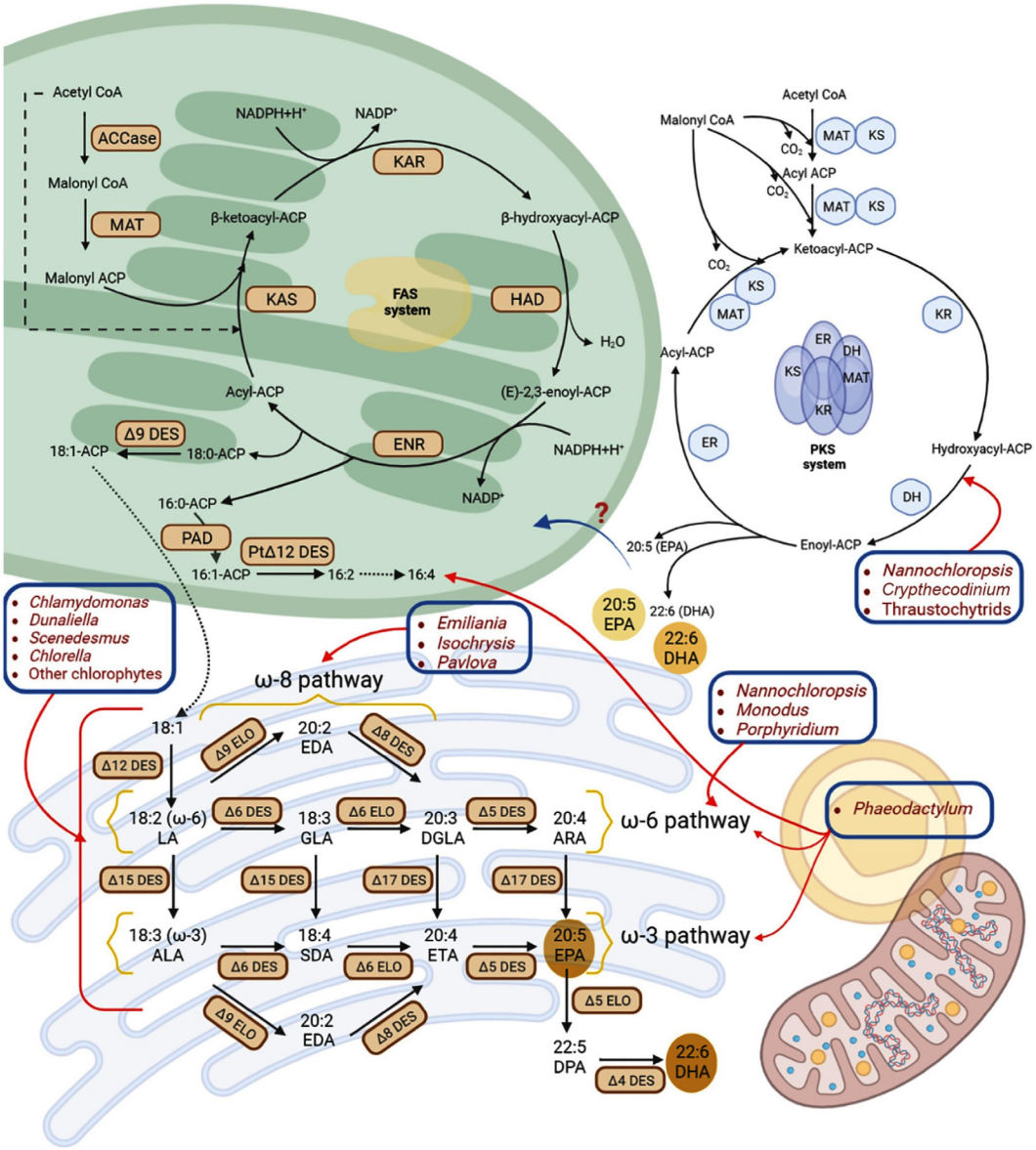

Figure. Pathways of ω (omega)-fatty acid biosynthesis in microorganisms.[106] In microalgae, saturated (C16:0) and monounsaturated (C18:1) fatty acids are initially produced within chloroplast and subsequently transported to endoplasmic reticulum, where further desaturation and elongation occur. The specific desaturation and elongation pathways-namely omega-3, omega-6, or omega-8 differ among species. Additionally, certain microalgae possess a cytoplasmic PKS (polyketide synthase) pathway capable of synthesizing DHA and EPA through iterative chain extension and desaturation. The lines, red in color, highlight the presence of these biosynthetic routes in various algal taxa, blue boxes denote species-specific pathways, dashed arrows represent multi-step enzymatic conversions, and question marks indicate unidentified mechanisms for DHA/EPA incorporation into the endoplasmic reticulam. **Abbreviations used include:** ACCase (acetyl-CoA carboxylase), ACP (acyl carrier protein), ALA (alpha-linolenic acid), ARA (arachidonic acid), DES (desaturase), DGLA (dihomo-gamma-linolenic acid), DPA (docosapentaenoic acid), EDA (eicosadienoic acid), ELO (elongase), ENR/ER (enoyl reductase), ETA (eicosatetraenoic acid), FAS (fatty acid synthase), GLA (gamma-linolenic acid), HAD/DH (hydroxyacyl dehydratase), KAR/KR (ketoacyl reductase), KAS/KS (ketoacyl synthase), LA (linoleic acid), MAT (malonyl/acetyl transferase), PAD (palmitoyl-ACP Δ9 desaturase), PtΔ12 DES (plastidial Δ12 desaturase), and SDA (stearidonic acid)

identified from various organisms. The approach has successfully integrated both standard Δ6 and alternative Δ8 pathways in microorganisms.[4]

Anaerobic polyketide synthase pathway

The fatty acid synthesis also occurs without the use of the elongase/desaturase system through an alternate route called as the anaerobic polyketide synthase pathway (Figure) to form EPA or DHA. The PKS pathway (polyketide synthase pathway-anaerobic) was explained for the first time in *Shewanella pneumatophore* which is a marine bacterium.[74] It was sometime later that de novo synthesis was also discovered in the species for production of C18 and C16 fatty acids. The enzymes in the polyketide synthase pathway (anaerobic) are multifunctional and can undertake EPA synthesis without needing desaturase, elongase, and fatty acid synthase.

The fatty acyl chain extension requires double bond insertion with the help of dehydratase-isomerase module in the anaerobic polyketide synthase pathway. The aerobic desaturase/elongase pathway is distinct and needs oxygen-dependent desaturation steps for the elongation of the fatty acyl chain and insertion of double bonds.[4] In *Thraustochytrium* and *Schizochytrium*, the existence of both systems has been reported. The *Schizochytrium* is known to lack Δ12-desaturase activity in their aerobic desaturase/elongase system and is not competent to utilize FAS (fatty acid synthase) products for long-chain polyunsaturated fatty acids (LCPUFAs) (long chain poly-unsaturated fatty acids) long chain fatty acid synthesis. Gene involved in desaturases has been recognized in anerobic and aerobic pathways of *Thraustochytrium* and *Schizochytrium*. The findings demonstrated the presence of an incomplete elongase/desaturase system in *Schizochytrium* sp. *Thraustochytrium* possesses Δ12-desaturase, which produces long-chain polyunsaturated fatty acids (LCPUFAs) through the desaturase/elongase and polyketide synthase pathways.[4] Thus, microbes have either aerobic desaturase/elongase or anaerobic polyketide synthase pathway and in some cases both the systems for the synthesis of fatty acid.

Metabolic engineering of microorganisms for fatty acid production

Microbial biosynthetic pathway reconstitution

The compositions of fatty acids vary from strains to species to genus in microbes and are based on the conditions of cultivation and stages of growth. The fatty acid synthesis by de novo pathway in photosynthetic cyanobacteria and microalgae, is catalyzed by dissociated set of enzymes that form FAS II (fatty acid synthase type II).[75] In contrast, the yeast-like eukaryotic microorganisms utilizes FAS I (fatty acid synthase type I) for fatty acids synthesis.[76] These microbes have C16 or C18 SFA (saturated fatty acid) and MUFA (mono-unsaturated fatty acids). The desaturation and elongation reactions help some microbes to achieve synthesis of GLA, ALA and LA. Many microbes have the potential to produce C18 monounsaturated fatty acid but are not capable of performing desaturation and elongation reactions. The process of reconstitution in such microbes requires the introduction of polyketide synthase (PKS) pathway which is anaerobic, gene set or assembly of the entire aerobic Δ9-elongase or Δ6-desaturase system. In *E. coli*, attempts were initially made to introduce the anaerobic polyketide synthase-like pathway due to the growth rate and synthesis of fatty acids. Such investigations used heterologous expression polyketide synthase-like pathway and were not optimized for the strain or titer or yield. The DHA and EPA were 0.7%-22% and 0.4%-5.2% of the total fatty acids produced by the genetically modified organism's expression, respectively (Table 1). The gene cluster from the polyketide synthase-like pathway was successful in producing EPA in *E. coli.* There was 6% EPA production by the introduction of 38kb DNA fragment from *S. pneumatophore* strain. The EPA yield was found to be low in the case of gene cluster isolated from *Shewanella oneidensis.*[77] Another research showed that the transgenic strain could generate 0.1%-0.4% DHA and 8%-14% EPA.[78] With the progress in the field, the unnecessary genes were filtered, and the new gene cluster of 20 kb was used for transfection in *E. coli.* The results showed a 3.5-6 fold increase in EPA production.[79] These reports were the first to show the possibility of producing EPA/DHA using

Table 1. The summary of ω3FA production from engineered microorganism strains

Study	Omega 3 fatty acid	Genes	Host strain	Yield (%)	Time (hours)	Temp. (°C)
Orikasa et al.[80]	DHA	DHA gene cluster (pfaA-E) from *Moritella marina* MP-1	*Escherichia coli* DH5a	5.2	96	15
Lee et al.[77]	EPA	EPA gene cluster (pfaA–E) from *Shewanella oneidensis* MR-1	*Escherichia coli* XL1-Blue	0.689	34	20
Orikasa et al.[82]	EPA	EPA gene cluster (pfaA–E) from *Shewanella* sp. SCRC-2738	*Escherichia coli* JM109	22	ND	15
Amiri-Jami et al.[78]	EPA	EPA gene cluster (pfaA–E) from *Shewanella baltica* MAC1	*Escherichia coli* EPI 300T1	14	120	15
Amiri-Jami et al.[78]	DHA	DHA gene cluster (pfaA–E) from *Shewanella baltica* MAC1	*Escherichia coli* EPI 300T1	0.4	120	15
Amiri-Jami et al.[79]	EPA	EPA gene cluster (pfaA–E) from *Shewanella baltica* MAC1	*Lactococcus lactis* subsp. *cremoris*	0.12 mg/gm of dcw	24	15
Amiri-Jami et al.[79]	DHA	DHA gene cluster (pfaA–E) from *Shewanella baltica* MAC1	*Lactococcus lactis* subsp. *cremoris*	1.35 mg/gm of dcw	24	15
Takeyama et al.[81]	EPA	EPA biosynthetic gene cluster from *Shewanella putrefaciens* SCRC-2738	*Synechococcus* sp. NKBG042902	0.5	24	17
Tavares et al.[83]	EPA	Δ6-Desaturase (D6-Des), Δ9-Desaturase (D9-Des), Δ12-Desaturase (D12-Des), ω3-Desaturase (ω3-Des), Δ6-Elongase (D6-Elo)	*Saccharomyces cerevisiae*	0.49	48	30
Xue et al.[16]	EPA	The metabolic process involves a range of enzymes such as Δ9-elongase (D9-Elo), Δ8-desaturase (D8-Des), Δ5-desaturase (D5-Des), Δ17-desaturase (D17-Des), and Δ12-desaturase (D12-Des), as well as carnitine palmitoyltransferase 1 (CPT1)	*Yarrowia lipolytica*	56.6	168	30

Table 1. Cont...

Study	Omega 3 fatty acid	Genes	Host strain	Yield (%)	Time (hours)	Temp. (°C)
Damude et al.[84]	DHA	The enzymes involved include Δ6-desaturase (D6-Des), C18/20-elongase (C18/20-Elo), Δ5-desaturase (D5-Des), Δ17-desaturase (D17-Des), C20/22-elongase (C20/22-Elo), and Δ4-desaturase (D4-Des), all of which play crucial roles in the biosynthetic conversion of essential fatty acids.	*Yarrowia lipolytica*	5.6	120	30
Hamilton et al.[85]	EPA and DHA	D5-Elo, CoA-D6-Des	*Phaeodactylum tricornutum*	18% EPA and 11% DHA	ND	20
Sugihara et al.[86]	DHA	The bacterial genotype includes mutations and markers such as *deoR*, *endA1*, *gyrA96*, *hsdR17* ($rK^- mK^+$), *recA1*, *phoA*, *relA1*, *thi-1*, a deletion of *lacZYA-argF* (*ΔlacZYA-argF*), *U169ϕ80dlacZΔM15*, *F*, *k* , and *supE44*.	*E. coli* DH5α	2%	48	15
Kajikawa et al.[87]	EPA	MpDES6, MpELO1 and MpDES5	*Pichia pastoris*	0.03	72	30
Li et al.[88]	ARA	The coding sequences for Δ5- and Δ6-desaturases along with 6-elongase were identified	*Pichia pastoris* GS115	0.3	-	-
Li et al.[88]	EPA	The gene segments encoding Δ5- and Δ6-desaturase enzymes, as well as Δ6-elongase, were analyzed.	*Pichia pastoris* GS115	1	-	-
Allen et al.[89]	EPA	EPA-overproducing chemical mutant	*Photobacterium Profundum* EA2	25-30	-	-

Abbreviations ND: Not determined; dcw: dry cell weight

Table 2. Specific metabolic engineering steps in FAS pathway for enhanced EPA and DHA production

Step	Target/Enzyme	Modification	Microorganism	Effect	Reference
1	fabH (β-ketoacyl-ACP synthase III)	Deletion	*Escherichia coli* expressing *pfa* genes	Increased DHA production from 7.5% to 17% of total fatty acids	Giner-Robles et al.[107]
2	fabF (β-ketoacyl-ACP synthase II)	Over-expression	*Synechococcus elongatus* PCC7942	Enhanced α-linolenic acid (ALA) production up to 22.6% of total FA	Santos-Merino et al.[108]
3	ACCase (Acetyl-CoA Carboxylase)	Overexpression	*Schizochytrium* sp.	DHA content increased from 36.4% to 37.6% of total FA	Han et al.[109]
4	MCAT (Malonyl-CoA:ACP Transacylase)	Overexpression	*Schizochytrium* sp.	Total lipid content increased by 39.6% and DHA by 53.7% (from 27.9% to 42.9%)	Li et al.[110]
5	ATP-Citrate Lyase	Co-overexpression with ACCase	*Schizochytrium* sp.	Further increased DHA content to 37.9% of all FA	Wang et al.[111]
6	Malic Enzyme	Overexpression	*Schizochytrium* sp.	Enhanced NADPH availability, leading to increased DHA yield from 19.2% to 26.7% of DCW	Wang et al.[111]
7	FAS Pathway Suppression	Downregulation	*Schizochytrium* sp.	Diverted carbon flux towards synthesis of PUFA via PKS pathway, increasing DHA proportion in total fatty acids	Liu et al.[112]
8	Pfa Gene Cluster	Introduction	*Lactococcus lactis* subsp. cremoris MG1363	Enabled de novo DHA and EPA synthesis; DHA production reached to 1.35 mg/gm DCW	Amiri-Jami et al.[79]
9	Pfa Gene Cluster	Introduction	*Escherichia coli* Nissle 1917	Achieved EPA synthesis up to 31.36 mg/gm DCW at 15 °C; DHA was less than 0.2% of total FA	Amiri-Jami et al.[113]
10	Desaturases and Elongases	Introduction of 6, 5, 17 desaturases and C18/20 elongase	*Yarrowia lipolytica*	Produced EPA at 40% of total lipids; further engineering led to 57% EPA in fatty acid methyl esters	Xue et al.[16]
11	Peroxisome Biogenesis Gene (PEX10)	Inactivation	*Yarrowia lipolytica*	Increased EPA yield to 15% of DCW; reduced SFA to less than 5%	Xue et al.[16]
12	FAA1 and GPD1	Overexpression	*Yarrowia lipolytica*	Converted excess free fatty acids into triacylglycerols, increasing EPA production by 18%-110%	Qin et al.[115]
13	TGL3 and TGL4	Deletion	*Yarrowia lipolytica*	Prevented degradation of triacylglycerols into free fatty acids; TGL4 deletion led to a 300% increase in EPA production	Qin et al.[115]

engineered microbes as an alternate to the ω3FA found in the food. The higher DHA levels were also obtained by using DHA biosynthesis genes and expressing them in *E. coli*. 1.3%-5.2% of the fatty acids were found to be DHA in the transgenic *E. coli.*[80] In other studies, cyanobacteria have been used for EPA production. The engineered *E. coli* with EPA synthesis cluster showed a major impact on cultivation temperature and production was found to be 0.12-0.64 mg of EPA/gm dry weight of cell.[81] In general, the yield of DHA and EPA is high with there is low temperatures.

Several researchers have informed the modification of biosynthetic pathways in yeast for the synthesis of ω3FA. In a study, 3 genes named Δ5-desaturase, Δ6-desaturase, and an ELO-like elongase were used for reconstitution of EPA synthesis pathway.[87] Although EPA levels were found to be low in transgenic yeast, they were able to utilize C18 fatty acids for production of EPA. Later diatom *Phaeodactylum tricornutum* was used to increase those genes copy number in *P. pastoris* yeast to increase EPA yield.[88] This discovery revealed that gene expression may not be the only factor influencing EPA synthesis in yeast. In contrast, the introduction of polyketide synthase-like pathway led to rising in DHA production significantly in yeast. 1.3% of total fatty acids (of total lipids) was observed to be DHA in yeast expressing polyketide synthase-like gene clusters. Further, two more modifications were introduced in yeast. These include co-expression of long chain acyl-CoA synthetase and 3-ketoacyl-ACP synthase inhibition. The resultant DHA production was increased to 8% in modified yeast.

The commercial production of EPA has witnessed the engineering of *Yarrowia lipolytica*.[16] The wild strain of *Y. lipolytica* mainly consists of palmitoleic acid (16:1), LA, palmitic acid (16:0), and monounsaturated fatty acid (18:1). The high levels of GLA were found to be accumulating upon engineering Δ6-desaturase pathway in yeast. The accumulation of intermediates in Δ6-desaturase pathway was avoided using assembly of 7 genes from Δ9-pathway namely, Δ17-desaturase, Δ5-desaturase, Δ8-desaturase, Δ9-elongase, Δ12-desaturase, and C16-elongase from various microbes. Subsequently, the wild strain was transformed in 4 different cassettes of expression harboring heterologous genes under the promoter's regulatory control which was present in *Y. lipolytica*. The production of EPA was found to be 9.8% from the resultant strain of yeast. To maximize EPA accumulation further, engineering was performed to conjugate the expression of elongases and desaturases with choline phosphotransferase and *PEX10* deletion that is required for perioxisomal proliferation.[90] The end result of engineering was a strain with 30 copies of 9 heterologous genes with 56.6% EPA production and 30% of lipids from dry cell weight in a high-glucose medium after 6 days.[16] The further genetic analysis highlighted that along with the deletion of *PEX10* gene, disruption of other 3 ORFs SCP2 gene which encoded sterol carrier protein, *LIP1* gene which encoded lipase 1 and nonessential gene ORF also occurred. The functional loss of these genes was associated with the high EPA yield and lipid content observed in the engineered strain of yeast. The biochemical analysis performed in detail revealed that triacylglycerol formed was 85% of the total fatty acids. The triacylglycerol's sn-1 and sn-3 positions were predominantly involved in the production of EPA. The yeast engineering for the accumulation of ω3FA was the first example of its kind and was shown to exceed EPA production in terms of productivity as well as yield in comparison to the previously engineered microbes.

Microbial genetic modification

The production of ω3FA occurs naturally in microbes. The genetic transformation has the potential to boost the output of fatty acids in microorganisms using a technique known as metabolic engineering. The genetic approach in comparison to the reconstitution process of biosynthetic pathway is simpler and involves fewer heterologous genes. The Δ15 and Δ6 desaturase genes transgenic expression in *Synechocystis* sp. which is photosynthetic cyanobacteria, was performed by cloning from *Mortierella alpine, Gibberella fujikuroi,* and *Synechocystis* sp. It was observed 8.9 mg/L ALA production and 4.1 mg/L SDA production at 5-times higher than the wild strain.[91] Engineering using genetic and metabolic approaches also improved the generation of extremely long chain fatty acids. There was 3.5-fold enhancement in the EPA production in *S. baltica* MAC1 mutants generated using transposon Tn5

Table 3. Specific metabolic engineering steps in aerobic Desaturase/Elongase pathway (O_2-dependent) for enhanced EPA and DHA production

Step	Enzyme	Modification	Microorganism	Effect	Reference
1	Δ6-desaturase	Overexpression of endogenous or heterologous gene	*Phaeodactylum tricornutum*, *Yarrowia lipolytica*	Enhances ALA → SDA or LA → GLA	Zhu et al.[116]
2	Elongase (C18→C20)	Expression of 6-elongase	*Schizochytrium*, *Y. lipolytica*	SDA → ETA or GLA → DGLA	Xue et al.[114]
3	Δ5-desaturase	Overexpression from *Ostreococcus tauri*	*Phaeodactylum tricornutum*	Enhances EPA production	Domergue et al.[98]
4	Δ5-elongase	Overexpression	*Y. lipolytica*	Shifts EPA to DPA	Xie et al.[118]
5	Δ4-desaturase	Expression to produce DHA from DPA	*Y. lipolytica*,	Final DHA step	Gemperlein et al.[119]
6	Pathway Optimization	Codon optimization, synthetic operons	*Y. lipolytica*	Balanced flux	Celińska et al.[120]
7	Co-factor supply	↑ NADPH via malic enzyme	*Schizochytrium*, *Mucor circinelloides*	Supports desaturation steps	Zhang et al.[121]
8	Lipid sink	Overexpression of DGAT	*Nannochloropsis oceanica*	↑ EPA in TAGs	Xin et al.[122]
9	Lipase deletion	Knockout of TAG lipase (NoTGL1990)	*Nannochloropsis*	Prevents EPA degradation	Miao et al.[123]

mutagenesis. The mutant was able to yield EPA of 0.3 mg per g dry cell weight whereas the wild strain did not produce any EPA.[92] The marine microalgae were also modified by introducing mutation by ethyl methane sulfonate for EPA production scaling up in *Nannochloropsis oculate*. This method generated two mutated strains that were capable of producing 36.5 and 31.6 mg EPA respectively per gram of dry cell weight.[93] The success in increasing the yield of EPA showed that genetic mutagenesis has the potential to improve strain towards more production of fatty acids with long chain.

A fungus named *M. alpina* has proven to be used for ARA production in the industries. The *Agrobacterium tumefaciens*-mediated transformation for the x3-desaturase gene overexpression has shown 4-fold increment in yield of EPA. The EPA levels were highest in transgenic strains and accounted for 40% of fatty acids.[94] The fungus with mutant Δ12-desaturase could convert ALA to EPA. The mutation was able to achieve EPA content of 20% from the fatty acids produced in 10 days of growth in presence of 3% linseed oil at 20 °C. [95] The *Aurantiochytrium limacinum* is a marine protist and has been used for EPA production through modulation of Δ5-desaturase gene expression under the influence of thraustochytrid ubiquitin promoter. DHA, DPA, and palmitic acid are naturally produced by Thraustochytrids along with low levels of EPA and ARA. The expression of Δ5 desaturase gene in *A. limacinum* extracted from *T. aureum* led to increment in ARA and EPA levels in transgenic thraustochytrids. The levels were elevated by 13.2-fold in comparison to mock transfects.[96] The expression of heterologous genes was also shown to alter the fatty acid content in photosynthetic diatom. Thirty percent of the fatty acids present in diatom *Phaeodactylum tricornutum* is EPA and DHA is only found in trace amounts. However, the picoalga *Ostreococcus tauri* used for acyl-CoA Δ6-desaturase overexpression was not able to show a significant increment in DHA or EPA levels. This shows that Δ6-desaturation is the only rate-limiting bottleneck in fatty acid synthesis. Genetically modified *P. tricornutum* showed a 17.7% and 8.2% decrease in EPA levels at the exponential phase and stationary phase of growth. In transgenic strain, DHA was found to be 10.4% in the stationary phase and this accounted for

Table 4. Specific metabolic engineering steps in anaerobic Poly-Ketide Synthase (PKS) pathway (O, -independent) for enhanced DHA and EPA production

Step	PKS Subunit/Domain	Modification	Microorganism	Effect	Reference
1	PKS_B (AT domain)	Domain swap with EPA-specific AT	*Schizochytrium* sp.	↑ EPA 5-fold	Zhang et al.[124]
2	C16 elongase	Overexpression	*Schizochytrium*	Enhances EPA & DHA	Ma et al.[125]
3	Full PKS cluster	From *Shewanella japonica*	*E. coli*	Enables EPA synthesis	Metz et al.[126]
4	PPTase	Co-expression with PKS	*Y. lipolytica*	PKS activation	Bejenari et al.[127]
5	ACCase	Overexpression → ↑ malonyl-CoA	*Schizochytrium*	Precursor boost	Wang et al.[114]
6	Carbon flux	Overexpression of ME & G6PDH	*Thraustochytrids*	↑ acetyl-CoA & NADPH	Muthu et al.[117]
7	Codon optimization	Gene refactoring for PKS	*Y. lipolytica*	↑ Expression	Schmidt et al.[128]
8	Modular platform	2A peptide for PKS expression	*Aurantiochytrium* sp.	Balanced protein levels	Wang et al.[129]

an 8-fold increment in *P. tricornutum* cells.[85] The metabolic engineering used in this study was the first to report modulation in diatoms for fatty acid content. These instances support the idea that metabolic engineering can boost ω3FA levels.

Summary of strategies taken so far

In a nutshell, the FAS, A desaturase/elongase, and anaerobic PKS pathways detailing the development of EPA and DHA production demonstrate the efforts made towards metabolic engineering. Each pathway has distinct merits: the FAS approach (Table 2) provides the freedom of controlling metabolism of precursor and redox cycling; the desaturase/elongase pathway (Table 3) fine tunes the enzymatic steps of PUFA conversion, and the PKS pathway (Table 4) is a more direct means of producing long-chain PUFAs that does not require oxygen. Precursor amplification, stacking of pathways, redox adjusting, and optimizing sinks lipids have been shown to work with many host systems. The addition of heterologous gene cluster integration silences the sequential control logic of a given organism's genetic backbone, and frame shifting codons with optimization that control expression deepens the influence of synthetic biology on host limitations. All these pieces of research deepen the understanding the engineering of strains, providing a foundation that seeks to render the biosynthesis of ω3FAs economical and widespread amidst their undeniable nutritional importance.

Metabolic bottlenecks

Even though metabolic engineering has been used to reconstitute biosynthetic fatty acid pathways, only a few strains have produced the required outcomes in terms of increased DHA or EPA levels. The modulation and transformation of microbes using various genes is not a hurdle technically; however, the complexity at the metabolic level has led to many bottlenecks in the engineering for high-level production.

The substrate dichotomy is the first bottleneck that is recognized in yeast and plants. In the initial investigations, attempts were made to determine the elongases and desaturases substrates in biosynthetic pathway of fatty acid. The study involved the transformation of yeast *S. cerevisiae* by the introduction of Δ12 desaturases,

Δ5 desaturases, and Δ6 desaturases from other organisms and analysis of composition of fatty acid and acyl-CoA pool in culture. The research highlighted that the desaturases have an affinity for phosphatidylcholine sn-2 position in fungi, lower plants, worms, and algae. Δ12 desaturase was found to use lipid-linked acyl chains and was active on sn-positions of all glycerolipids.[97] The reconstituted ARA pathway in yeast demonstrated the role of acyl carriers with co-expression of Δ5 and Δ6 desaturase from algae and the impact of the yield of ARA was found to be low with 0.4% of fatty acids contributing to ARA. The incorporation of GLA into phosphatidylcholine highlighted that transfer of D6-desaturated product was inefficient from phosphatidylcholine to acyl-CoA pool. This is a bottleneck that limits synthesis of fatty acids in yeast. Research has identified 3 acyl-CoA dependent Δ6 desaturases in microalgae with a 71%-73% of conversion rate which was very high in comparison to other Δ6-desaturases.[98]

The reconstitution of EPA and ARA pathways was successfully achieved with the utilization of ALA as substrates as mentioned before. There was 20 times more production that was achieved using EPA or ARA end products. The comparatively large yields of long-chain fatty acids were attributed to the effectiveness of elongation of desaturated intermediates between phosphatidylcholine and the acyl-CoA pool. In contrast, another enzyme was shown to only transform ALA to SDA in *Mantoniella squamata*. The reconstitution of EPA pathway was also performed using co-expression of D6-elongase (PSE1) with acyl-CoA Δ5-desaturase and MsD6. There was a 2%-3% increase in fatty acids observed from the pathway. Although the yield of EPA was low; however, the interaction between elongase and acyl-CoA desaturases have the ability to overcome acyl-exchange bottleneck. In *Micromonas pusilla*, a marine microalga, identification of acyl-CoA Δ6-desaturase was recently made and it was used for expression in yeast that resulted to the 71% conversion of ALA to SDA.[99] The crucial thing to note here is that, while the substrate specificities of desaturases have been effectively identified in recent investigations, their identification is hampered by the quick activity of acyltransferases, which induce the transfer of acyl groups in several host species, including yeast. *In vivo* investigations may not accurately detect the specificities of acyl carriers. However, the application of acyl-CoA desaturases for engineering in higher plants has shown a high accumulation of fatty acids with long-chain.[100] This shows that employing acyl-CoA desaturases than lipid-linked desaturases can increase the output of ω3 long chain fatty acids.

The competition among other intermediates in fatty acid synthesis may form a second bottleneck. The yeast that has been transformed harbors pools of phosphatidylcholine, phospholipids, and acyl-CoA. These form substrates for many acyltransferases. The transgenic yeast was analyzed in detail highlighted part of the biosynthetic pathway of fatty acid is channeled out for TAG biosynthesis.[97,100] Elongation of intermediates is prematurely ended due to fatty acid flow. Subcellular components and enzymatic activities play critical roles in regulating the fatty acid transit of end products and intermediates, such as TAG pools, phosphatidylcholine and acyl-CoA. The biochemical studies formed the basis for the formulation of a simplified model in yeast for lipid synthesis accumulated TAGs. The engineering of microbes for ideal flux of fatty acid may be achieved by controlling the exchange of acyl intermediates, channeling of fatty acid end products, and minimal flux towards TAG pathway. In most of the fatty acid-producing microalgae, the DHA and EPA are bound to storage TAGs or membrane lipids.[101] The ultimate fatty acids composition in engineered strains is modulated by various acyltransferase activities that use end products as well as fatty acid intermediates.

The acyl group exchange between phosphatidylcholine and acyl-CoA pool is bidirectional and can be catalyzed by acyltransferase in plants, animals, and yeast.[102,103] In yeast, the transfer system is very efficient, and the forward reaction is significantly higher than the reverse activity highlighting the low efficiency of removal of fatty acid from phosphatidylcholine. The enzymatic process in yeast may use a wide spectrum of acyl-CoAs. Although the PC turnover is important in lysophosphatidylcholine acyltransferase, it remains to be explored whether this activity can alleviate the bottleneck that is acyl exchange in recombinant yeast to produce more fatty acids. The characterization and identification

of lysophosphatidylcholine acyltransferase enzymes in microbes has the potential to highlight role of acyl transferases in the fatty acids flux and acyl exchange between acyl-CoA pool and phosphatidylcholine. The intermediates are directly transferred to diacylglycerol to phosphatidylcholine on the sn-2 position. This can be separately catalysed by acyl-CoA enzymes, which eventually combine them into triacylglycerol. The *LRO1* gene encodes for an enzyme in the yeast that also contributes to the triacylglycerol production in cell division.[104] In microalgae and plants, the enzymes for triacylglycerol biosynthesis and lipid turnover have been identified.[105] However, there is very little evidence for phospholipid: diacylglycerol acyltransferase (PDAT) contributing towards the flux of fatty acid intermediates. The redirection of carbon flux is centrally controlled by the membrane-associated Kennedy pathway and that directs the flux to triacylglycerol synthesis. Diacylglycerol acyltransferase (DGAT), glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT) are the three acyltransferases. The DGAT enzyme in comparison to the other two enzymes is crucial for triacylglycerol accumulation in microbes. The DGAT enzymes are part of acyltransferase protein family and are known to exist in membrane-bound and soluble forms. The DGAT2 and DGAT1 are the membrane-bound whereas DGAT3 is mainly soluble. The DGAT2 subfamily is crucial for triacylglycerol production during the stationary phase of growth.[104] The existence of multiple isoforms of DGAT in a single microbial species highlights the complexity of triacylglycerol synthesis via DGAT action.

In various microbes, the triacylglycerol content and composition of fatty acids are the determinants for reactions catalyzed by PDAT and Kennedy pathway activities. The engineered *Y. lipolytica* was analyzed for EPA in triacylglycerols and it was observed that EPA enrichment occurred at positions of sn-1 and sn-3 mainly.[16] The reactions involving desaturase mostly occur at phosphatidylcholine's sn-2 position that may point to the remodeling of lipids and triacylglycerols before the EPA incorporation. The aforementioned acyltransferases may contribute to the remodeling of lipids in strains that have been engineered. The elucidation of the part played by different acyltransferases in the efficient fatty acids flux is essential for the identification of the mechanism underlying acyl exchange in such strains. This in turn will provide highlights for overcoming the various bottlenecks in fatty acid production in engineered microbes. The PKS pathway, unlike desaturase/elongase pathway, is identified to produce lesser intermediates that in various pools of substrate can skip acyl exchange. The polyketide synthase system has substrates common to fatty acid synthase, which signifies the importance of polyketide synthase substrates in the fatty acid yield of microbial hosts. Since the end products of polyketide synthase pathway are free fatty acids, so their transfer from the synthesis site to triacylglycerol may be another bottleneck in the expression and assembly of fatty acid polyketide synthase gene clusters in host cells.

Improvement strategies

Although the microbial engineering has great potential for enhanced production of ω3FA, there are still several bottlenecks that impact the productivity and yield of the strains. These need to be evaluated before the engineering of strains and their utilization for industrial production. Many approaches exist to bypass some of the bottlenecks and enhance the end product yield. These may include acyl exchange reduction between phosphatidylcholine and acyl-CoA pool, improvement of fatty acid flux, increment in the precursor pool, and inhibition of competing pathways.

Future prospects

In the future, metabolic engineering of ω3FAs, particularly DHA and EPA, would be of great interest to meet the world nutritional demands in a sustainable biotechnological approach. Rapid development in synthetic biology and CRISPR/Cas mediated genome editing creates new possibilities to control desaturase/elongase and PKS pathways where titre and selectivity are improved in microbial and algal hosts. In the future, it would be interesting to develop optimal-host chassis such as oleaginous yeasts, thraustochytrids or genetically amenable microalgae, and construct multi-gene pathways, as well as utilize dynamic regulatory systems facilitating flux of pathway. The rational design and optimization of cost-efficient

fermentation processes built on high-throughput screening techniques and systems biology might close the gap between laboratory scale and commercial scale. Environmentally tolerant strains (tolerant to pH, temperature, or salinity) engineered for industrial conditions are expected to be essential for outdoor and heterotrophic production at large scale. Multidisciplinary efforts are needed to adapt molecular advancements to commercial nutraceutical, pharmaceutical, and aquafeed products, making the long-awaited, sustainable, and inexpensive supply of health-promoting ω3FA possible.

CONCLUSION

The growing global demand for omega-3 fatty acids necessitates sustainable alternatives to fish-derived sources. Microbial production, enhanced through metabolic engineering and genetic modification, offers a promising and eco-friendly approach to meet this need. By reconstructing biosynthetic pathways and optimizing enzymatic steps in both aerobic desaturase/elongase and anaerobic PKS systems, microorganisms such as Yarrowia lipolytica, Schizochytrium, and Phaeodactylum tricornutum have demonstrated significantly improved EPA and DHA yields. Continued advances in systems biology, strain optimization, and process engineering are expected to further enhance productivity, paving the way for large-scale, commercially viable microbial omega-3 production that supports both human health and environmental sustainability.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

AD performed supervision. SS performed formal analysis. DS and MK investigated the study. AD and MM wrote the manuscript. CM and AD reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

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