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Nutritional Status, Bioactive Properties, & Therapeutic Efficacy of Omega-3 Polyunsaturated Fatty Acids Obtained by Green-Synthetic Routes

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Abstract

Polyunsaturated fatty acids (PUFAs), particularly omega-3 (n-3) fatty acids, like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are crucial for human health. They support structural brain development, visual acuity, and cardiovascular function, while also exhibiting potent anti-inflammatory, antioxidant, and antimicrobial properties. Historically, marine fish oil has been the primary dietary source of these essential fatty acids. However, escalating concerns regarding ecological environmental impacts, the oceanic bioaccumulation of heavy metal contaminants, and the fundamental limits of global supply have spurred immense interest in the microbial production of PUFAs as a sustainable, land-based, and cost-effective alternative. This review synthesizes current knowledge on the nutritional, therapeutical and biological importance of omega-3 fatty acids and explores the distinct advantages of their microbial biosynthesis. We thoroughly examine the cellular mechanisms of oleaginous microorganisms, including marine microalgae, thraustochytrids, filamentous fungi (such as Mortierella species), and robust yeasts like Yarrowia lipolytica. Furthermore, recent breakthroughs in metabolic engineering and genetic manipulation, specifically the targeted overexpression of desaturase and elongase enzymes to overcome native lipid biosynthetic bottlenecks, have been critically assessed. Crucially, the integration of advanced bioprocess engineering to scale up single-cell oil production through the valorization of agro-industrial and horticultural wastes has been reviewed based on post 2010 scietific data and research papers. Implementing abundant, low-cost substrates, such as sugarcane molasses, corn steep liquor, and fruit processing residues, not only substantially reduces fermentation costs but actively promotes a circular agricultural bioeconomy. We detail fermentation optimization strategies tailored to these complex carbon sources to maximize intracellular lipid accumulation. Finally, while discussing the safety and economic aspects of microbial PUFAs, this review outlines downstream processing metrics and provides a strategic roadmap for translating these vital biotechnological advances into commercial-scale, sustainable production and a potent therapeutic and nutritional source.

Keywords: Anti-inflammatory, antimicrobial, antioxidant, docosahexanoic acid, eicosapentaenoic acid, green synthesis, polyunsaturated fatty acids

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INTRODUCTION

Fatty acids (FAs) are fundamental lipids characterized by a hydrocarbon chain and a carboxyl group, classified based on the presence and number of double bonds into saturated [1–4], monounsaturated (MUFAs), and polyunsaturated (PUFAs) [5, 6]. While saturated and MUFAs can be synthesized by the human body [7], PUFAs are considered essential fatty acids (EFAs) that must be obtained through diet [8]. The two most significant families of PUFAs are omega-6 (n-6) and omega-3 (n-3) [9]. Maintaining an optimal nutritional status

of these crucial lipids is foundational for human health [5], yet the modern Western diet often presents a significant challenge due to a disproportionately high n-6 to n-3 ratio, frequently leading to a suboptimal cellular lipid profile [10, 11]. Key n-3 PUFAs include alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Due to the human body's limited conversion of ALA to EPA and DHA, direct dietary intake of these longer-chain n-3 FAs is crucial [12–19]. Although ALA is relatively abundant in terrestrial plant sources, like flaxseed and chia, human metabolic pathways relying on alternating desaturases and elongases to convert ALA to longer forms are remarkably inefficient and broadly considered inadequate from a medical standpoint [16]. Consequently, securing a robust physiological nutritional status directly relies on the reliable dietary intake of pre-formed EPA and DHA [7, 20–27].

The profound physiological impact of these lipids is fundamentally rooted in their unique bioactive properties and structural mechanisms [24]. Structurally, EPA possesses 20 carbons and five double bonds, whereas DHA contains 22 carbons and six double bonds (Figure 1). Once consumed, these long-chain PUFAs are rapidly esterified and seamlessly integrated into the cellular phospholipid bilayer [23]. This cellular insertion is highly biologically active; the multiple cis-double bonds characteristic of n-3 PUFAs [28–31] introduce distinct structural kinks that significantly enhance membrane fluidity, alter lipid raft dynamics, and profoundly influence cross-membrane signal transduction [32, 33]. By decreasing cholesterol accumulation within the cellular membrane, they optimize the function of embedded protein receptors [34, 35]. Furthermore, EPA and DHA function as indispensable bioactive precursors for specialized pro-resolving mediators (SPMs) [36–44]. These potent autacoids actively orchestrate the resolution phase of cellular inflammation, effectively clearing cellular debris and restoring tissue homeostasis without suppressing necessary host immune defenses [3, 9, 10]. The antiallergic and neuroprotective effects of microbial PUFA have been reported in developing children [1, 2, 5, 11–18].

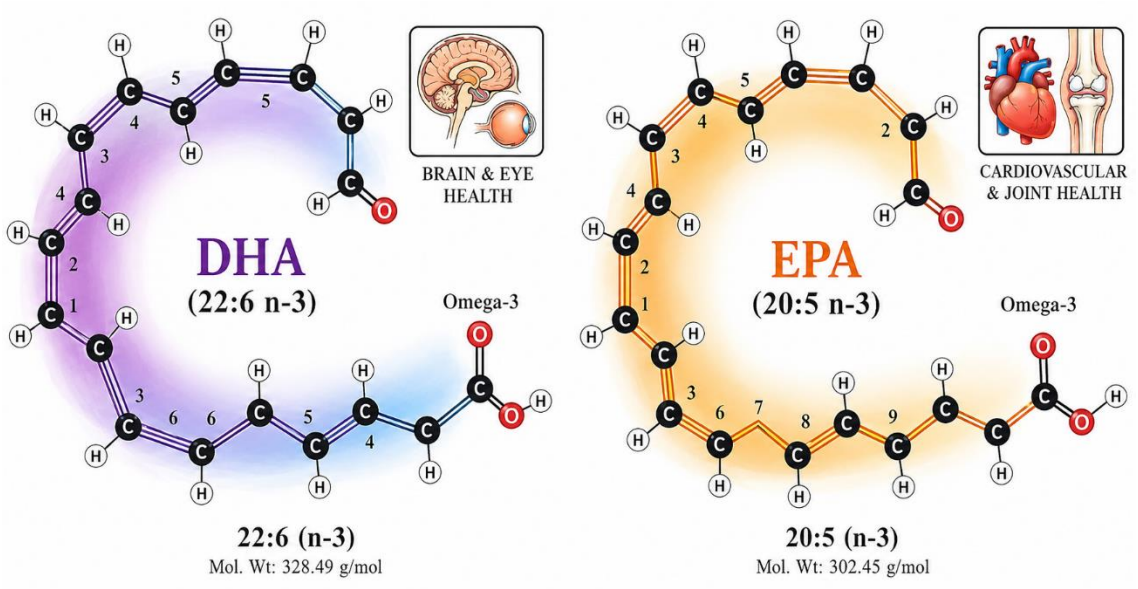

Figure 1. Molecular structure of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

Translating these cellular dynamics into clinical outcomes reveals the vast therapeutic efficacy of EPA and DHA across a multitude of human organ systems [1, 4, 7, 13, 19, 20]. From a cardiovascular perspective [21], routine dietary intake has been definitively shown to decrease the incidence of major cardiovascular events, lower circulating triglycerides, and improve overall vascular endothelial function [15, 22, 23]. Beyond their cardioprotective roles, these compounds are critical for neurological integrity. DHA is a major structural lipid in the human brain and retina for the prevention of cognitive decline in aging populations [11, 18, 24, 25]. Emerging clinical evidence also demonstrates that modulating the

pineal gland's cellular membrane structure with EPA and DHA can enhance melatonin production, thereby regulate human sleep patterns, while concurrently provide measurable relief from anxiety and clinical depression [26]. Additionally, their consumption is linked to a lowered risk of specific cancers, alongside measurable improvements in bone mineralization and muscle preservation [9, 20, 27].

The rising demand for these beneficial compounds, coupled with the limitations of traditional sources, has highlighted the urgent need for alternative and sustainable production methods, with microorganisms emerging as a promising solution [8, 22, 45]. Historically, providing a sufficient supply of these specialized molecules relied almost entirely on marine fish harvesting. However, marine sources face severe existential threats due to global overfishing, environmental accumulation of ocean contaminants, like heavy metals, and climate change-induced temperature elevations that progressively diminish the natural synthesis of oceanic EPA and DHA [28–42, 46–48]. To circumvent these ecological and supply-chain barriers, advanced biotechnological platforms utilizing microbial cell factories have rapidly developed [43, 44, 49–146]. Diverse, sustainable systems employing marine bacteria [2, 142, 143], diatoms [16, 86, 88, 97, 104], algae/microalgae [29, 30, 59, 112, 144], thraustochytrids [4, 59, 81, 111, 136], and metabolically engineered yeasts, such as *Yarrowia lipolytica/ Kluyveromyces /* [22, 23, 130, 131, 141], fungi are now highly optimized via large-scale heterotrophic fermentation [131]. Not only do these microbial factories provide a more consistent, contamination-free, and vegan-friendly source of essential lipids, but they also offer a highly scalable pathway to sustainably meet the surging global nutritional and therapeutic demands [134].

METHODOLOGY

The present review work has been carried out by citing the recent scientific literature and data. The literature was retrieved from PubMed, Google Scholar, Academia Edu, Live-DNA, Wiley-Online, Cochrane database, and manual search from Elsevier, Springer, and Taylor & Francis databases. A total of 370 research articles were pooled, and 146 were selected for the present study using PRISMA guidelines

Biological and Therapeutic Importance of Polyunsaturated Fatty Acids (PUFAs) – Omega-3 Fatty Acids

Omega-3 PUFAs play a pivotal role in maintaining overall human health and development, including functions in the brain, eye, and cardiovascular system.

Health Benefits

- *Nervous System and Brain Health:* DHA is essential for proper neurological and visual development in infants and children. Both EPA and DHA are vital for brain function, memory, and can help prevent neurodegenerative diseases, psychiatric disorders, and improve conditions [57, 58, 65, 75]. They influence neuronal gene expression and maintain cell membrane fluidity and integrity [56].
- *Cardiovascular Health:* Omega-3 FAs are cardioprotective, reducing the risk of cardiovascular diseases (CVDs) by lowering serum triglyceride concentrations, protecting against atherosclerosis, and exhibiting anti-arrhythmic, anti-thrombotic, and anti-atherosclerotic properties [1]. They can also help reduce inflammation and improve blood lipid profiles.
- *Anti-inflammatory and Antioxidant Properties:* EPA and DHA reduce the synthesis of inflammatory eicosanoids and reactive oxygen species, giving rise to anti-inflammatory mediators like resolvins [52, 53, 145]. The anti-inflammatory mechanism involves G protein-coupled receptor 120 (GPR120) as an omega-3 PUFA receptor/sensor. Omega-3 PUFAs also act as antioxidants, protecting cells from oxidative damage [9, 55].
- *Cancer Prevention:* EPA and GLA have shown anti-cancer properties, particularly against certain types of cancer cells [98].
- *Infectious Diseases:* EPA and DHA have demonstrated therapeutic significance against several microbial pathogens, including bacteria, fungi, and viruses [41, 44, 50, 53]. They inhibit bacterial

growth, induce cell death in Plasmodium species, and exert anti-hepatitis C virus activity. Their antimicrobial mechanisms include altering cell membrane properties (hydrophobicity, surface charge, integrity), inhibiting virulence factor gene expression, disrupting cell-to-cell communication, and blocking fatty acid synthesis enzymes like FabI [92, 96, 138]. These bioactives obtained from *Chlamydomonas reinhardtii* have been found to inhibit tuberculosis-causing *Mycobacterium tuberculosis in vivo* [66, 68].

Physiological Role in Microorganisms

PUFAs, particularly EPA, are vital for microorganisms to adapt and survive in extreme environments such as deep seas and polar regions. A high PUFA content in bacterial cell membranes helps maintain membrane fluidity, which is crucial for coping with low temperatures and high hydrostatic pressure. EPA also contributes to cell division, membrane organization, and acts as an antioxidant, scavenging free radicals and protecting against oxidative damage [6, 146]. The presence of EPA in fungal membranes can also enhance resistance against water-soluble antibiotics by improving the biosynthesis of membrane proteins like porins [122].

MICROBIAL PRODUCTION OF OMEGA-3 FATTY ACIDS

Why Microbial PUFAs?

Despite the significant health benefits of omega-3 PUFAs, their traditional sources, primarily fish and certain plants, present several limitations. Plant sources predominantly contain short-chain ALA, with limited EPA and DHA content [54, 99, 107, 108]. Fish oil, while rich in EPA and DHA, is associated with issues, such as undesirable odor and flavor, potential heavy metal and contaminant (mercury, dioxins) contamination, and concerns about the sustainability of fish populations due to overfishing. The global demand for DHA is projected to exceed supply significantly [105].

Microorganisms offer a highly promising and sustainable alternative source for PUFA production [96, 133] due to several advantages, because these can be cultured in controlled bioreactor environments regardless of climate, season, or geographical location, offering a consistent supply [102, 103]. They can utilize a wide variety of low-cost substrates, including agro-industrial wastes [8, 25, 27–29, 40, 45–47, 62, 140], which reduces production costs and offers an environmentally beneficial waste disposal method [65, 69, 118, 119]. Microorganisms can produce specific types of PUFAs, often as a single product rather than a mixture, which simplifies purification and lowers overall production costs and is non-toxic [73, 76, 109]. Microbes have fast growth rates and simpler genetic structures compared to plants and animals, making them ideal models for researching PUFA metabolic pathways and for genetic manipulation to maximize productivity and tailor fatty acid profiles even on waste materials [106, 120], unsterilized substrates, and production conditions [124, 128]. Microbial oils, often referred to as single-cell oils (SCOs), can be produced under sterile and controlled conditions, minimizing contamination risks (Figure 2). They also often contain natural antioxidants, like carotenoids, which protect PUFAs from auto-oxidation and offer a longer shelf-life, and are generally recognized as safe (GRAS) for human consumption.

Types of PUFA-Producing Microorganisms

Oleaginous microorganisms are defined as species that can accumulate lipids exceeding 20% of their total dry biomass weight. This includes bacteria, fungi, and microalgae (Table 1).

Biosynthesis Pathways

Microbes synthesize PUFAs through two primary metabolic mechanisms, the Desaturase/Elongase (Aerobic) Pathway [15, 79], common in eukaryotes and some prokaryotes, which involves a series of desaturase [31] and elongase enzymes [39]. In eukaryotes, these enzymes are typically associated with the endoplasmic reticulum membrane (Figure 2). Second, Polyketide Synthase s(PKS) (Anaerobic) Pathway [42], where some prokaryotes and eukaryotes (e.g., *Shewanella* sp.) and thraustochytrids, utilize a PKS-like multi-functional enzyme complex to synthesize DHA or EPA (26, 32,64). This pathway can produce PUFAs without the direct involvement of fatty acid synthase (FAS, 71-73) or aerobic desaturase/elongase enzymes (Figure 2a). The PKS genes are often clustered, and their presence in diverse species suggests lateral gene transfer.

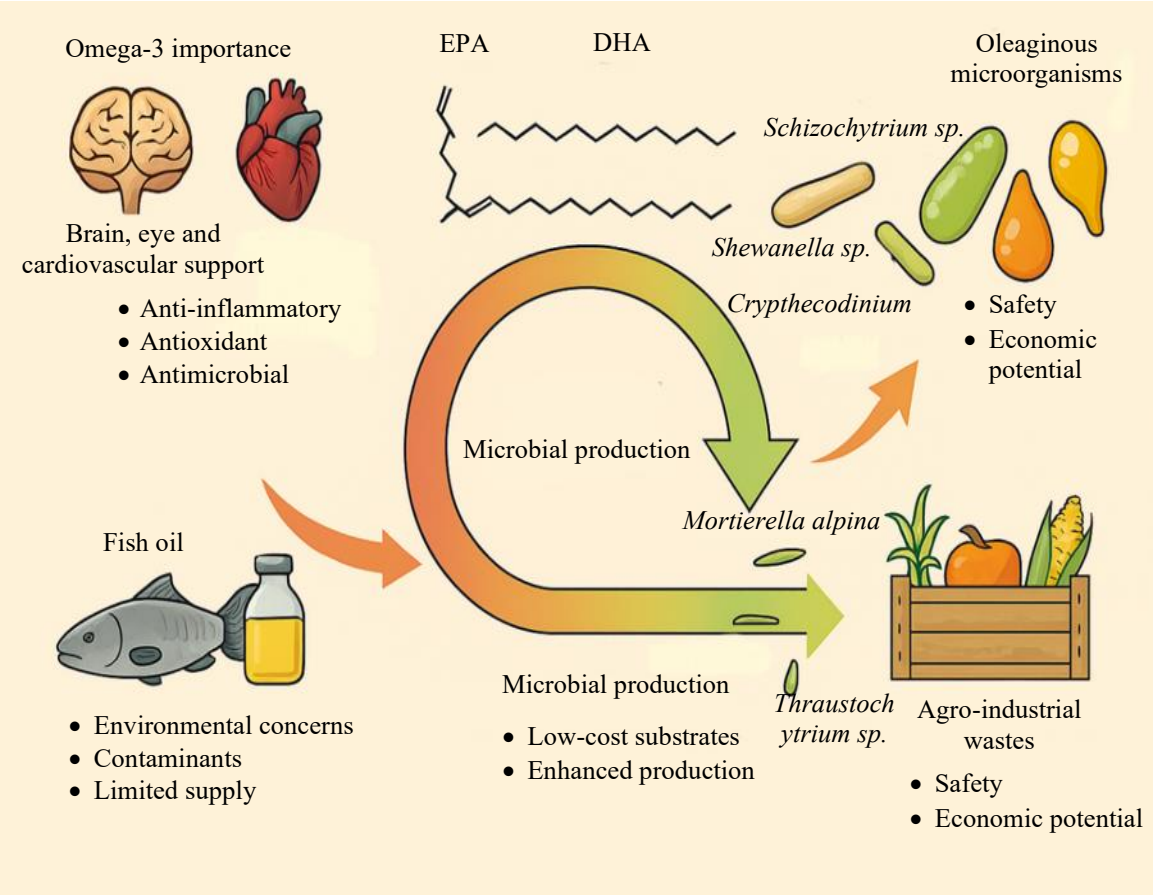

Figure 2. Microbial production of EPA, DHA.

Table 1. Important PUFAs and their microbial sources.

PUFA	Microbial sources
ARA	Fungi *(Mortierella alpina, Entomophthora exitalis, Blastocladiella emersonit, Conidiobolus nanodes);* Algae *(Euglena gracilis, Sargassum salicifolium, Porphyridium cruentum, Heterocapsa tricus, Amphidinium sp., Prorocentrum minimum, Prorocentrum triestinum, Tetraselmis chui);* Bacteria *(Phaselicystis flava).*
DGLA	Fungi *(Conidiobolus nanodes, Mortierella alpina, Saprolegnia ferax)*
DHA	Fungi *(Thraustochytrium roseum, Thraustochytrium aureum, Schyzochytrium aggregatum, Schyzochytrium SR21);* Algae *(Crypthecodinium cohnii, microalgae MK8805, Gyrodinium nelsoni, Gonyaulax polyedra, Amphidinium carteri, Amphidinium sp, Alexandrium sanguinea, Heterocapsa tricuetra, Isochrysis galbana, Pavlova gyrans, Prorocentrum micans, Prorocentrum minimum, Scripsiella trochoidea);* Bacteria *(Rhodopseudomonas spp., Vibrio spp., Colwellia psychrerythraea).*
DPA	Fungi *(Schyzochytrium sp.).*
EPA	Fungi *(Mortierella alpina, M. elongata, Pythium ultimum, P. irregulare);* Algae *(Chlorella minitissima, Amphidinium sp, Alexandrium sanguinea, Asterionella sp, Chlamydomonas sp, Chlorella ellipsoidea, Heterosigma akasiwo, Nannochloropsis sp, Nitschia ovali, Pavlova gyran, Phaedacturum tricornutum, Tribonema sp);* Bacteria *(Shewanella putrefaciens, Shewanella electrodiphila).*
ETA	Fungi *(Mortierella alpina).*
GLA	Fungi *(Mucor circinelloides, Mortierella isabellina, M. mucedo, M. ramanniana, Cunninghamella elegans, C. echinulata, C. japonica, Thamnidium elegans, Rhizopus arrhizus, Gilbertella persicaria* Algae *(Chlorella vulgaris, Spirulina platensis, Amphidinium* sp.*, Dunaliella salina, Isochrysis galbana, Tetraselmis chui, Tetraselmis suecica, P. parvum).*
MA	Fungi *(Mortierella alpina).*
SDA	Fungi *(Mortierella alpina).*

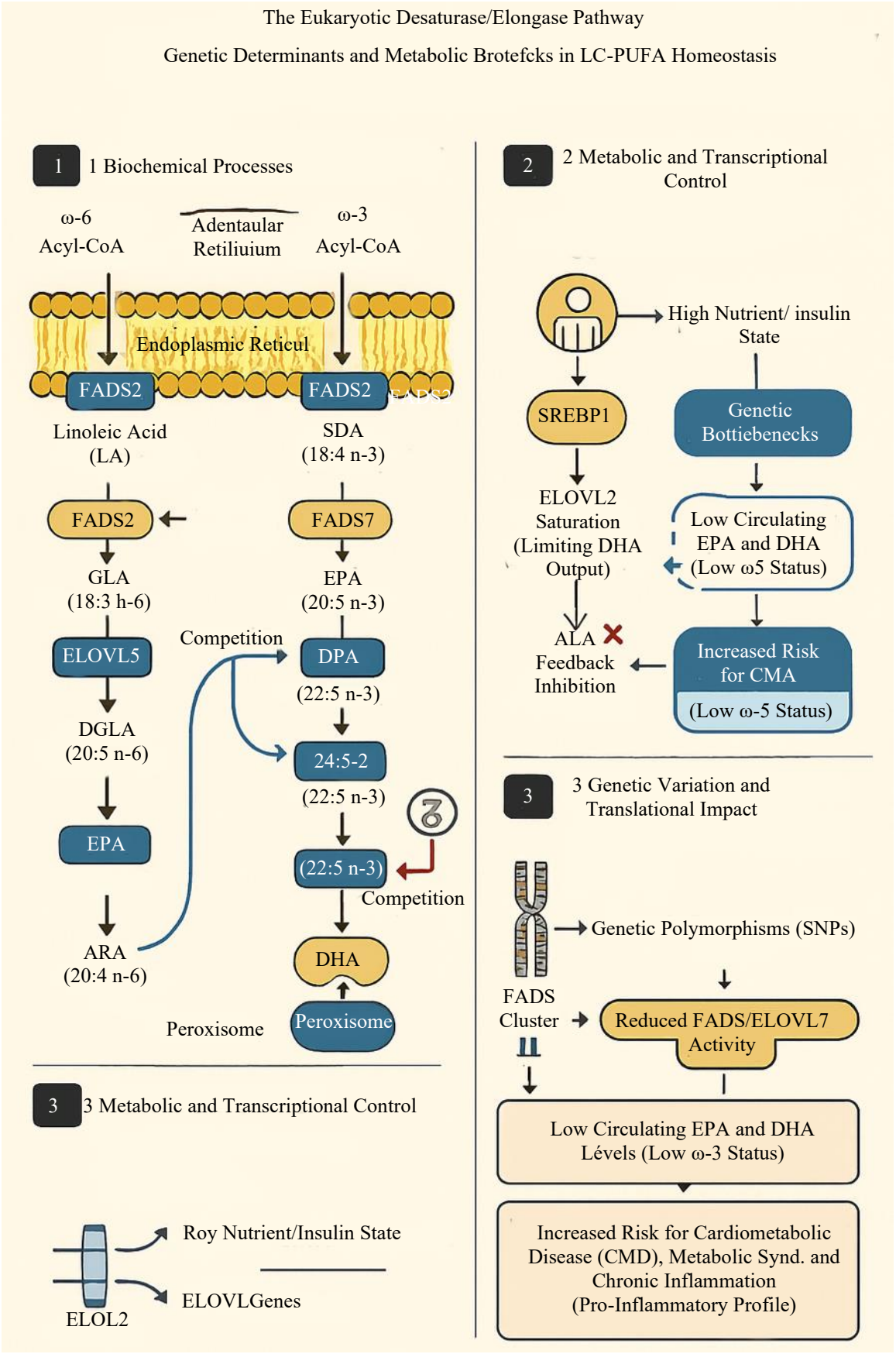

Figure 2(a). Microbial metabolic pathways to produce PUFA.

Table 2. Summary of genetic engineering strategies in microbes for PUFA production.

Genes targeted	Host organism	Resulting PUFAs/effect
DHA synthesis gene cluster (from *M. marina MP-1*); *pfa* gene cluster (Source: *A. fasciculatus*)	*E. coli DH5a, P. putida*	Increased DHA production.
EPA synthesis gene cluster (Source: *Shewanella sp.*)	*E. coli JM109, S. baltica MAC1, C. synechococcus*	Increased EPA production.
EPA/DHA synthesis gene cluster (Source: *Shewanella* sp,)	*L. lactis subsp. cremoris*	EPA/DHA production.
Overexpression of desaturase/ elongase genes (e.g., D6-Des, D9-Des, x3-Des, D6-Elo, D5-Des)	*S. cerevisiae, Y. lipolytica*	Production of EPA, DPA, DHA, GLA, ALA; 56.6% EPA and 18.3% DPA/5.6% DHA in *Y. lipolytica*; Increased GLA in *Y. lipolytica* (20.2% of TFAs).
Overexpression of malic enzyme (malce1), glucose-6-phosphate dehydrogenase (g6pd), leuB	*M. circinelloides*	Increased GLA production (2.5-fold increase in lipid accumulation).
Overexpression of GLA elongase (GLELO), delta-15 desaturase	*M. circinelloides*	Production of DGLA, SDA.
Overexpression of malce1, glelo, D17-desaturase	*M. alpina*	Increased ARA production; conversion of ARA to EPA (boosted EPA to 31.5% of TFAs).
Overexpression of malonyl CoA-acyl carrier protein transacylase (MCAT), glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidate acyltransferase (LPAAT/AGPAT), diacylglycerol acyltransferases (DGATs)	*P. tricornutum, N. oceanica*	Increased total lipid content (31% in *N. oceanica*), increased EPA (8% in *N. oceanica*, 40% in *P. tricornutum*), increased DHA and EPA (1.5-fold in *P. tricornutum*); Increased TAG-associated PUFAs (184%).
Overexpression of fatty acid desaturases (FADs) and elongases (ELOs)	*N. oceanica, T. pseudonana, P. tricornutum*	Increased EPA (25%), increased EPA and DHA (12% and 150%, respectively), accumulation of DHA, EPA, ARA.
RNA interference (RNAi) knockdown of UGPase, chrysolaminarin synthase, TAG lipase, phosphoenolpyruvate carboxykinase (PEPCK)	*P. tricornutum, T. pseudonana*	Increased lipid content (24% in *P. tricornutum*, 2.4-fold in *T. pseudonana*); increased EPA in TAGs (70%); boosted lipid accumulation (40%).
Overexpression of G6PDH, malic enzyme (ME)	*Aurantiochytrium sp. SD116, P. tricornutum*	Enhanced LC-PUFAs (DPA and DHA by 25% and 12.5%, respectively); increased DHA production (up to twofold).

METABOLIC ENGINEERING FOR ENHANCED PUFA PRODUCTION

Metabolic engineering is a powerful approach to modify the lipid composition [67] and enhance PUFA production in oleaginous microbes (Table 2). Strategies include overexpression [84, 85] or blocking/knockout of genes, and knockdown or activation of regulatory elements involved in PUFA synthesis and storage (Table 3).

BIOCONVERSION OF AGRO-INDUSTRIAL WASTES FOR SCO PRODUCTION

A significant advancement in sustainable PUFA production is the bioconversion of low-cost agro-industrial wastes into high-value single-cell oils (SCOs) by oleaginous microorganisms (Table 3). These wastes are rich in minerals, nitrogen, and carbon sources, making them suitable growth substrates.

Oleaginous microbes can utilize both hydrophilic and hydrophobic substrates. "*De novo*" lipid fermentation typically produces large amounts of lipids from simple carbon sources, like sugars, while "*Ex novo*" lipid fermentation transforms existing extracellular or intracellular lipids from complex hydrophobic materials, like waste cooking oils or dairy effluents, into desired fatty acid profiles. Genetic manipulation of microbes to directly convert lignocellulosic biomass or to produce lipases that facilitate hydrophobic substrate utilization further enhances the efficiency of this bioconversion.

Table 3. Bioconversion of agro-industrial wastes into high-value SCO by oleaginous microbes.

Microorganisms	Substrate	PUFAs produced
M. isabelline	Corn stover, douglas fir forest waste, sweet sorghum, rice hull hydrolysates.	GLA.
C. echinulate	Waste from edible oil plants, potato industry starch waste, tomato hydrolysate enriched with glucose, corn steep liquor, soybean oil waste.	GLA.
M. recurves	Hydrolyzed molasses.	GLA.
Mucor circinelloides, *M. isabellina*, *Rhizopus sp.*	Liquid waste from cheese-making process, cooking oil (soybean, sesame, canola, palm).	GLA.
T. elegans, *C. echinulata*	Sugar-based and corn steep lignocellulosic wastes.	GLA.
Schizochytrium sp. *DT3*, *S. Mangrovei*	Cannabis sativa (hemp) biomass, coconut water.	DHA.
P. tricornutum	Birch and spruce biomass.	EPA, DHA.
Trichosporon oleaginosus	Xylose-containing substrates.	ETA, EDA.
Aurantiochytrium sp., *Schizochytrium* sp.	Sugarcane bagasse hydrolysate, orange peel extracts, by-product of palm oil industry.	DHA.
T. kinney, *S. limacinum*, *T. elegans*, *U. isabellina*, *C. echinulata*, *Z. moelleri*, *Mucor.*, *Mortierella* sp.	Crude glycerol.	DHA, GLA, ARA, DGLA.
Navicula sp., *Chlorella sp.*, *Nannochloropsis* sp.,	Phosphate-limited wastewaters from aquaculture stations, shrimp farm wastewaters.	EPA.
Euglena gracilis, *Selenastrum* sp.	Fish farm wastewater.	ALA, ARA, EPA, DHA.
Mortierella sp.	Shrimp shell wastes.	ARA, DGLA.
Mortierella alpina CFR-GV15	Linseed oil and garden cress oil.	ALA, EPA, DHA.

NOVEL SOURCES AND SCREENING APPROACHES FOR MICROBIAL PUFAS

Given that only a small fraction of microbial diversity has been studied, researchers are actively exploring new avenues to discover and develop novel PUFA-producing strains. Underexplored ecosystems, such as hyperarid deserts, high-saline environments, and especially the deep-sea and polar regions, are rich reservoirs of novel microorganisms with unique adaptations and PUFA-producing capabilities. Deep-sea bacteria, like *Shewanella* species, are known for producing significant amounts of EPA and DHA to thrive in psychrophilic (cold-loving) and piezophilic (pressure-loving) conditions. Many microorganisms are difficult to cultivate in laboratories due to specialized growth requirements. Developing tailored media and utilizing signaling molecules can help isolate and grow these rare microbes, potentially uncovering new PUFA sources. HTS approaches, incorporating automated screening and advanced analytical techniques (e.g., GC, LC, MS, NMR, MALDI, FTIR, etc.), enable rapid identification, profiling, and characterization of lipid composition in large numbers of microbial strains [116].

Cultivating two or more microorganisms together (co-culturing) or exposing cells to specific signals ("elicitors") can activate silent gene clusters [129] and induce the production of novel or enhanced metabolites, including PUFAs [75]. Advances in sequencing technology allow for comprehensive genomic analysis, revealing the full metabolic potential of microorganisms, including hidden PUFA biosynthetic gene clusters [95, 114]. Comparative genomics helps identify key genes and pathways associated with high lipid accumulation [126].

Genetic Engineering Method Development in Thraustochytrids

Thraustochytrids, unicellular heterotrophic marine eukaryotes, are highly regarded for their ability to store large amounts of DHA and other ω3-PUFAs [80]. Developing genetic engineering tools for these non-model organisms is crucial but challenging due to strain-specific variations in transformation protocols.

DNA Transformation Methods

The most common methods for delivering DNA into thraustochytrid cells are electroporation, biolistic transformation, and *Agrobacterium*-mediated transformation (AMT).

- *Electroporation:* This is the most frequently used method, involving electric pulses to create temporary pores in cell membranes for DNA uptake. Parameters, such as pulse type (exponential decay, square wave), number of pulses, electric field strength (1.8 to 10 kV/cm), and pulse length (0.65 to 25 ms), are critical and vary by strain. Pre-treatment of cells, especially cell wall disruption (e.g., with dithiothreitol (DTT) or enzymatic treatments), and the use of non-ionic osmotic stabilizers – like sorbitol or sucrose in electroporation solutions – can significantly improve efficiency [129].
- *Biolistic Transformation (Particle Bombardment):* This method uses high-pressure helium to inject DNA-coated metal particles into cells. While less common currently, it has been successful where electroporation failed in some strains.
- *Agrobacterium-Mediated Transformation (AMT):* This cost-effective method utilizes *Agrobacterium tumefaciens* to integrate large DNA fragments (up to 150 kb) into the host genome, typically as a single copy [32]. It has been successfully applied to engineer *Schizochytrium* species [33].

Other Strategies for DNA Transfer

Novel and emerging methods for DNA transfer are also being explored:

- *Conjugation:* This bacterial-based method transfers plasmids between donor *E. coli* and recipient cells via conjugative bridges, eliminating the need for expensive equipment and offering high transformation efficiency [104].
- *Digital Microfluidics Electroporation:* This approach uses tiny oily droplets to encapsulate cells and DNA, significantly reducing required voltages and increasing transformation efficiency by minimizing water electrolysis and improving heat dissipation.
- *Cell-Penetrating Peptides (CPPs):* Small peptides (e.g., TAT peptide, pVEC) can facilitate intracellular transport of biological materials, like DNA or Cas9-gRNA ribonucleoprotein (RNP) complexes across cell membranes, offering a simpler transformation procedure [103].

DNA Properties and Genome Integration

The properties and design of the transforming DNA significantly influence the outcome:

- *Genome Integration Mechanisms:* DNA can be integrated into the host genome either by homologous recombination (HR), utilizing sequence homology, or by non-homologous end joining (NHEJ), which integrates DNA at random double-strand breaks [10]. In most microalgae, random integration is more efficient, but HR allows for targeted gene editing.
- *Homology Arms:* HR efficiency can be enhanced by increasing the length of homology arms flanking the desired gene cassette [13].
- *DNA Structure and Quantity:* Both linear and circular DNA have been used. Linear DNA may be more advantageous for HR. Using higher amounts of DNA (e.g., 1 to 20 µg per electroporation) can increase the number of transformants.

Facilitating HR:

- *CRISPR-Cas System:* This genome-editing technique uses sequence-specific nucleases (e.g., Cas9) and guide RNA to induce DSBs at precise genomic locations, greatly increasing HR efficiency for targeted deletions, insertions, or nucleotide changes [87]. It has been successfully applied in Aurantiochytrium species [102].
- *Interfering with NHEJ:* Downregulating NHEJ-specific enzymes, like DNA ligase type IV, can increase the rate of homologous recombination by reducing the competition from random integration.
- *Extrachromosomal DNAs:* The use of autonomously replicating episomes and plasmids can reduce the potential side effects of random genomic integration and allow for easy removal of the DNA from cells by removing selection pressure.

Gene Expression in Thraustochytrids

Controlling gene expression is crucial for successful genetic engineering:

- *Promoters and Terminators:* The choice of promoter (regulating transcription initiation) and terminator (regulating transcription termination) significantly affects gene expression levels [14].
  - *Constitutive Promoters:* Commonly used promoters include the elongation factor 1-alpha (EF1α) promoter (endogenous and from *Saccharomyces cerevisiae* TEF1) and the glucose-repressible gene (ccg1) promoter of *Neurospora*.
  - *Inducible Promoters:* These allow controlled expression of genes, particularly toxic ones. Examples include the galactose-inducible *GAL1* promoter (*S. cerevisiae*), ethanol-inducible alcohol dehydrogenase I (*AlcA*) promoter (*Aspergillus nidulans*), and methanol-inducible alcohol oxidase I (*AOX1*) promoter (*Pichia pastoris*).
- *Antibiotic Resistance Genes:* Antibiotics are widely used for selecting transformants expressing the resistance gene. Commonly used antibiotics in thraustochytrids include G418, zeocin, and hygromycin (Table 4). The optimal concentrations vary significantly between strains and even within the same strain across different protocols.

Table 4. Antibiotics used for selecting thraustochytrid transformants (in μg/mL).

Strain	Zeocin (μg/mL)	Hygromycin (μg/mL)	G418 (μg/mL)	Blasticidin (μg/mL)	Other (μg/mL)
A. limacinum	2000	500	–	–	–
A. limacinum mh0186	500, 2000	500	1200		500 (neomycin)
A. limacinum OUC168	5				100 (chloramphenicol)
A. limacinum SR21	30, 50, 100	200	500	–	–
Aurantiochytrium sp. KRS101	–	–	–	–	30 (cycloheximide)
Aurantiochytrium sp. MP4	50	–	–	–	–
Aurantiochytrium sp. PKU#SW7	500	500	–	–	–
Aurantiochytrium sp. RH-7A	100	–	–	–	–
Aurantiochytrium sp. SD116	30, 50, 100	500	50	–	100 (Tetracycline)
Parietichytrium sp. TA04Bb	2000	500	800	–	–
Schizochytrium sp. S31	40, 50	100	–	–	50 (bleomycin), 250 (cefotaxime), 50 (paromomycin)
Schizochytrium sp. HX-308	1.5, 20	–	–	–	–
Schizochytrium sp. PKU#Mn4	800	–	–	–	–
Schizochytrium sp. TIO01	–	–	100	–	–
Schizochytrium sp. TIO1101	300	–	–	–	–
Schizochytrium sp. SEK 579	2000	500	–	–	–
Schizochytrium sp. CB15-5	20	–	–	–	–
Thraustochytrid strain 12B	500	–	–	–	–
T. aureum ATCC 34304	2000	1000, 2000	200–400	–	–
Thraustochytrium sp. ONC-T18	250	400	–	–	–

- *Expression of Multiple Genes:* To reduce cassette size and enhance DNA delivery, multiple genes can be linked using *2A linker sequences* (viral-origin peptides that induce polypeptide cleavage during translation), allowing them to be expressed as a single multicistronic transcription unit [4]. Alternatively, genes of interest and antibiotic resistance genes can be on separate DNA molecules and co-transformed.

SAFETY, TOXICITY, AND COST OF MICROBIAL PUFAS

Microbial oils are generally composed of triacyl glycerides and sterols like plant and animal oils. Rigorous safety assessments are required for their use in food [37]. Studies have shown that DHA-rich and EPA-rich algal oils are safe for human consumption, with no observed toxic effects or mutagenicity at high concentrations [1]. Concerns about contaminants found in fish oil are largely absent in microbial oils due to controlled production environments [76]. The cost of microbial oil production is a significant factor, with substrate costs accounting for 60% to 75% of the total price (Table 5), compared with plant oils.

Table 5. Lipid productivity comparison of selected oleaginous microbes and plants.

Source (microbe and plant)	Oil yield (kg/m³/year)	Oil yield (kg/ha/year)
R. toruloides	2120	–
C. curvatus	1154	–
M. isabelline	679	–
C. echinulate	134	–
C. gracilis	525	–
S. limacinum	404	–
Sunflower	–	500–700
Soybean	–	450–500
Palm	–	3000–5000

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

The increasing global demand for essential omega-3 PUFAs, coupled with the limitations of conventional sources, underscores the critical need for sustainable and efficient production methods. Microbial production of PUFAs offers a promising solution, with significant advantages in terms of sustainability, safety, and versatility.

Future research should focus on continuing to explore underexplored microbial habitats and applying advanced HTS and genome-mining techniques to uncover new, high-yielding PUFA producers. Further refining metabolic engineering strategies to maximize PUFA yield and tailor fatty acid profiles in existing and novel oleaginous microbes. This includes a deeper understanding of lipid metabolism, precursor molecule generation, and cofactor production (e.g., NADPH). Developing more robust and universally applicable genetic manipulation protocols for non-model organisms like thraustochytrids, potentially leveraging emerging technologies such as CRISPR-Cas, conjugation, microfluidics, and cell-penetrating peptides. Prioritizing the utilization of diverse low-cost agro-industrial wastes and exploring efficient biomass recovery and lipid extraction procedures to improve the economic viability of microbial PUFAs. Implementing biorefinery concepts for the total exploitation of microbial oils can further address cost challenges.

A multidisciplinary approach involving nutritionists, microbiologists, molecular biologists, and natural product chemists is essential to accelerate the development and industrial scaling of PUFA production from microorganisms. This shift towards microbial sources represents a significant step towards meeting global nutritional needs and promoting environmental sustainability.

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