

Diatoms' Breakthroughs in Biotechnology: *Phaeodactylum tricornutum* as a Model for Producing High-Added Value Molecules

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Abstract

With a world growing in population and nutritional needs, diatoms are considered nowadays as microalgae of a very important potential, thus they are exploited in several fields such as ecology, aquaculture, molecular farming, and pharma nutraceuticals. These coveted microalgae are characterized by their diversity, their high division rates, their complex life cycle, likewise their silicified cell walls named frustules. Thus, diatoms have been used for over a century after proving an efficient production of several molecules including Triacylglycerols (TAGs), H₂, free fatty acids, vitamins, nutraceuticals, amino acids, proteins, terpenoids, alcohols and carbohydrates like starch, glycogen, and sucrose. *Phaeodactylum tricornutum* is the most promising diatom exploited to date, especially as a platform of pharmaceutical production. Herein, we expose diatoms' main features that allowed using them for molecular farming. This review exposes likewise, the metabolism and the post-translational modifications (PTMs) of diatoms as well as current tools and challenges for their molecular and metabolic engineering for a more efficient production of valuable molecules. The knowledge on the biology of the diatoms, the molecular tools, and the various transformation methods available demonstrate the potential in biotechnology of these photosynthetic microorganisms. The widely studied *P. tricornutum*, as a model organism, is a promising diatom for production of valuable metabolites, despite the challenges and issues related to cultivation.

Keywords

Diatoms, Silicified, Frustules, Metabolism, Lipids, Genetic Engineering, Molecules

1. Introduction

Diatoms are defined as a major group of photosynthetic unicellular microalga, which represent the highest diversity in the microalga realm and are considered as the dominant group of primary producers in aquatic systems. They comprise 10,000 to 10,000,000 different species across all habitats [1]. The bulk of diatom species range in size from 10 - 50 μm [2] [3]. During evolution, these microorganisms gained different physiological and biochemical properties which allowed them to exist in different types of habitats. Researchers studied the plasticity of diatoms and their ability to adapt to different conditions of salinity [4] [5], current water, CO_2 ratio [6], nitrogen (N), and phosphorus (P) availability [7] and other environmental factors such as the temperature [8]. Many scientists have also approved that, they are photoautotrophic algae, so they carry out photosynthesis like green plants but with few differences such as in lipid metabolism. Thus, many studies focused on the response of diatoms to different light wavelengths, hence explaining the high adaptability of one specie to shallow water or deep oceans [9] [10] [11]. Thus, this process allows them to fix about 40% of atmospheric CO_2 within the oceans and produce more than 20% of the O_2 generated on earth [12] [13] [14]. In the recent study of Sharma *et al.*, 2020, researchers have exposed *P. tricornutum* strains to different light conditions and different growth stages of the diatom. Under autotrophic conditions, these strains exposed to the redshift (RS) condition (substitution of white light (W) by red light (R)) have significantly increased growth that the biomass and the lipid content compared to the control light condition (W). These findings suggest one strategy among others aiming to increase the lipid accumulation and biomass [9].

Besides autotrophy, rigid silicified cell walls are a second feature making diatoms interesting candidates for molecular farming due to the conferred rigidity and protection. Cell walls or frustules are composed of amorphous silica [15] so they are known for their main role within the biogeochemical cycling of minerals, as it's the case for silicates that can be integrated into their frustules [16]. These important structures can be harnessed to their flexibility of being incorporated into nanodevices as well as for the bio encapsulation and the delivery of therapeutic molecules [17] [18] [19] [20].

As expression systems, diatoms are often used for the production of recombinant proteins or biomaterials as bioplastics [21] [22], but the potential keeps increasing. Very recently, diatoms have proved an increasing and very exceptional interest as a source of a large variety of bioactive substances (Figure 1). Different genetic tools helped efficiently within the progress of exploiting diatom's biotechnological potential. In fact, for the last decade, researchers have succeeded in producing high-value products in diatoms, such as polysaccharides in *T. pseudonana* [23], polyunsaturated fatty acids (PUFAs) [24] [25] and sapogenins [26] in *P. tricornutum* using available genetic tools. Beside protein and bioactive molecules production, diatoms are known for offering an important platform for

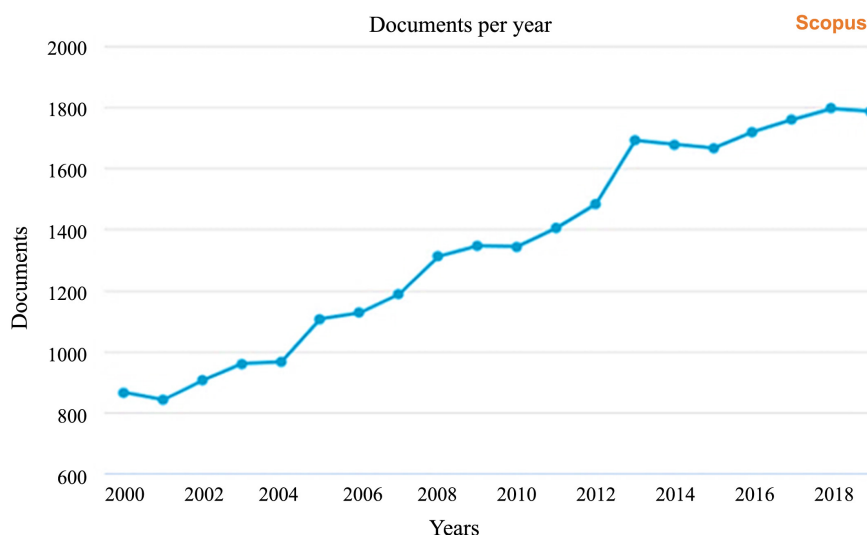


Figure 1. Trending research on diatoms during the recent years presented by the correlation between the number of documents published and the year of publication, data was collected from Scopus: <https://www.scopus.com> (using the keyword diatoms).

the production of diverse and high-value products for the nutraceutical and pharmaceutical industry as well as the production of biofuel due to their great ability of lipid accumulation during their dormancy and under certain stress conditions [13].

Diatoms have also proved their interesting natural accumulation of pigments of high therapeutic and commercial value such as fucoxanthin. Fucoxanthin is produced in considerable amounts in *P. tricornutum* (15.42 - 16.51 mg·g⁻¹) and several tools are optimized for its extraction and purification using high-performance liquid chromatography (HPLC) and spectrophotometry [27]. Recently, fucoxanthin proved its human benefits with anti-inflammatory, anti-tumor, and anti-obesity properties.

These pigments present a challenge for the large-scale production of the biomass and economically valuable co-products in diatoms [28] [29] [30]. Besides, diatoms have a wide range of metabolism pathways that are probably acquired during their evolution [31], so they are often used as a model microorganism for a far better understanding of microalgae metabolism. Hence, they are for major interest in the discovery of novel processes not found in more intensively studied model organisms [32].

Beside the natural production of lipids and pigments, diatoms present a novel and unique platform to produce natural products such as antimicrobial [33] [34] [35] [36] and antifungal molecules [37]. These antimicrobial products are needed as an alternative to chemical and not efficient current products for overcoming different microbial diseases. Nowadays, the pharmaceutical industry is looking for new compounds to be produced on a large scale as a substitute for traditional antibiotics that became worthless with antibiotic-resistant traits of microbes [38]. Recent studies have confirmed that diatoms

such as *P. tricornutum* seem to be of great potential to produce antimicrobial compounds such as eicosapentaenoic acid (EPA), hexadecenoic acid and, hexadecatrienoic acid. These unsaturated fatty acids were identified using analytical techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy [38] [39] [40] [41].

While working on different species of diatoms can offer diversity in platforms, one specie rose to represent a multi-faced molecular platform: *P. tricornutum* is a brown unicellular photosynthetic microorganism, partially silicified and genetically traceable diatom which has become a key organism for diatoms biology studies [39]. This diatom can grow and occur under sterile and easy conditions. To grow photoautotrophically, they just need a carbon source for growth and for gaining energy and a light source to ensure the photosynthesis process [39]. *P. tricornutum* uses CO₂ in oceans as the main carbon source, which explains their role in the biogeochemical cycling of minerals in the aquifer [42]. These species can also grow in mixotrophic conditions based on several nutrients in their environment such as glucose, fructose, and, glycerol which is considered as the most efficient carbon source for the growth and productivity [43].

The choice of this microorganism as a model for microalgae studies and the production of bioactive compounds is justified by their distinguishing features. Indeed, they are characterized by their high division rates generating a fast and important biomass accumulation and they proved the feasibility of cryogenic preservation [44]. Moreover, *P. tricornutum* has a known morphology and a whole-genome fully sequenced over a decade ago [44]. Besides, several genetic tools were developed nowadays for gene transformation of diatoms and a series of genetic sources helped a lot for a better comprehension of translational and post-translational modifications of their genetic material [45]. These resources are mainly genetic transformation [46] [47] [48] [49], Expressed Sequence Tags (ESTs) databases [32] [50], gateway vectors [47] [51] [52], and gene silencing [53] [54]. The selection process of literatures for this review is illustrated in **Figure 2**.

2. Biology of Diatoms and Features of *P. tricornutum* Species

2.1. Diatoms' Life Cycle

Since the beginning of the 19th century, the diatom life cycle has been well known and proved as complex. However, their stages with different morphology, ploidy, and function have been elucidated more recently [55]. Two main phases describe the life cycle of most unicellular microalgae [56] [57] [58]. In diatoms, these phases are distinct but interconnected (**Figure 3**). One phase exhibits successive mitotic division which ends up with a rise within the cell number: the vegetative phase. Another phase is meiosis allowing genetic recombination. During the sexual cell phase, researchers investigated a progressive reduction within the cell size which represents the most peculiar and distinctive characteristic of the diatom life cycle.

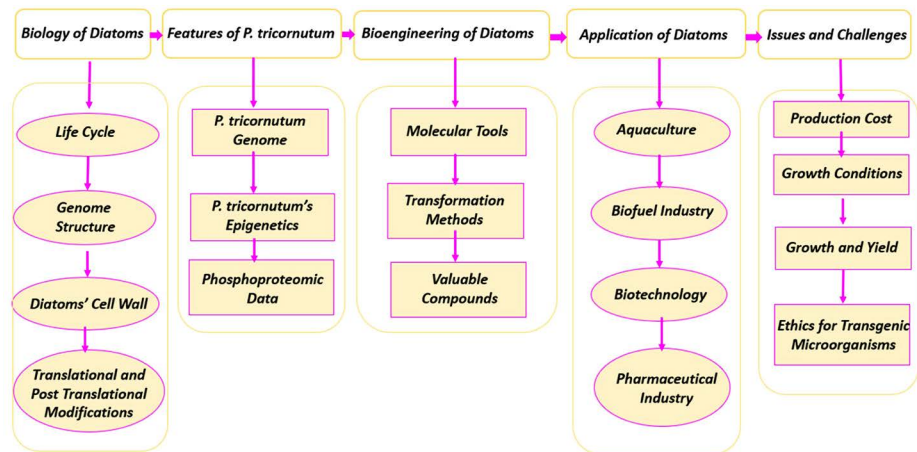


Figure 2. Flow chart describing the selection process of literatures used for this review.

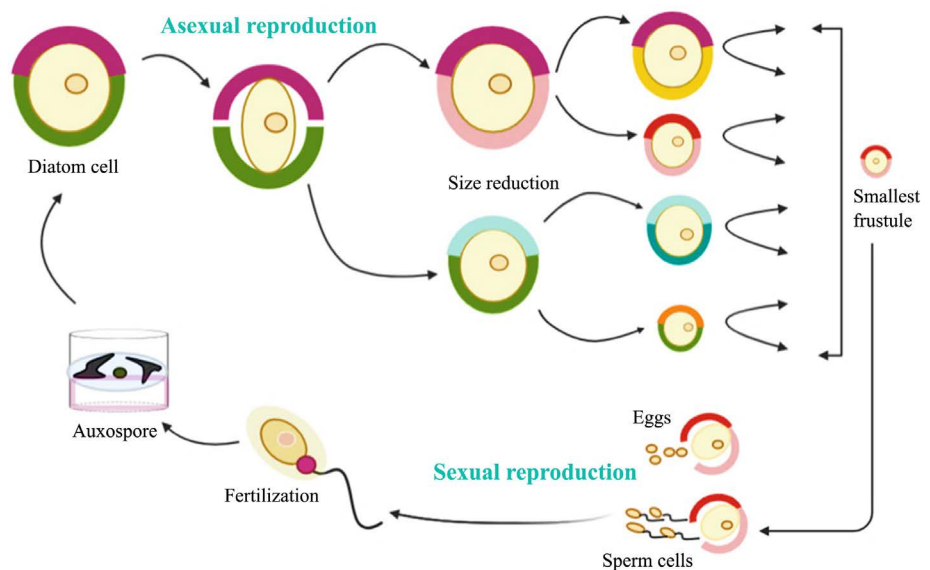


Figure 3. A Schematic summarize of the two main phases of the diatom cell cycle redrawn from Kale and Karthick (2015) [59].

As a first step of the gametes' oogenesis, gametangia differentiate to come-up with two haploid gametes. Then, conjugation continues matching gametes of opposite mating type and produces from gametangia two diploid zygotes. The zygote called at this stage "auxospore" elongates and deposits perizonial bands weakly silicified. As a final step, it generates an extended initial cell that will serve for the next division cycle [59].

Diatoms must reproduce sexually to restore maximal size after their progressive cell size reduction during the asexual phase. Nonetheless, sexual stages are rarely reported in nature, however, there are few reports about the production of the auxospore by planktonic diatoms in the natural environment [60] [61]. The results of those studies suggest that this process could be extremely restricted in time and occur at very low rates. Another hypothesis states that this phenomenon could occur in layers that are extremely difficult to sample from the water

[60]. However, mating or sexual reproduction is not observed in all species of diatoms. Among them, we find the pennate diatom *P. tricornutum* which is classified with asexual species because of the non-detection of neither its sexual cycle nor a meiotic process during its cell cycle to date. In general, meiosis represents a significant clue of the sexual reproduction in which several meiosis-specific genes are involved. Thus, the analysis established in the *P. tricornutum* genome has confirmed the existence of homologous of five of these genes such as the homolog of the SPO11 gene detected previously in *Pseudonitzschia multistriata* and *Seminavis robusta* [62]. These genes and others such as RAD51 are essential for the establishment of the meiosis process. Very recently, the research of Mao *et al.*, 2020, hypothesized that *P. tricornutum* could generate the meiosis process, which can confer to us a better understanding of the genetics of this species and, allow the development of their genetic modification tools [63]. Nevertheless, this still always a hypothesis based only on the presence of homologous meiosis-specific genes which is not robust enough argument to confirm the establishment of this process in *P. tricornutum*.

2.2. Diatoms' Cell Wall Structure

Diatoms' cell walls can be compared to a petri dish. It's composed of two thecae, "epitheca", and "hypotheca" which are overlapped. During cell division, valves which constitute each theca are synthesized. While during the interphase, several girdle bands are synthesized and added to each valve (**Figure 4**) [64].

Thus, this step enables the cell to expand. Cell wall size is of about tens-hundreds of micrometers and is based on SiO₂ (silica), which represents the most interesting features of diatom's structure. A modification of pore sizes and surface area of diatom silica can be generated by changing the salt concentration in the growth media [65]. Studying the silica cell wall, important proteins were revealed silaffins, which is a family of phosphoproteins, and long-chain polyamines (LCPA), are silica associated non-protein components [14]. Literature has evenly mentioned that these components play a crucial role within diatom silica morphogenesis. They also stated that silaffins and LCPA are associated with diatom silica and that is maybe due to physical entrapment with numerous amino groups present in these two components, inside the silica. Another hypothesis describes the interaction as a covalent bond [57]. Data have also mentioned the presence of simple elongated chloroplasts and circular lipid droplets in certain diatom's wall cells [66]. Several studies of this amorphous cell wall confirmed the association of the hydrated SiO₂ (silica) with proteins and polysaccharides [66] [67].

2.3. Diatoms' Genome Structure

Studies on the genome structure of diatom species have increased since the sequencing of the whole genome of two diatoms *P. tricornutum* (27.4 Mb) [45] and *Thalassiosira pseudonana* (32.4 Mb) [31] (**Figure 5**). Their genome contains particularly two nuclei, a macronucleus, and a germinal micronucleus. In the

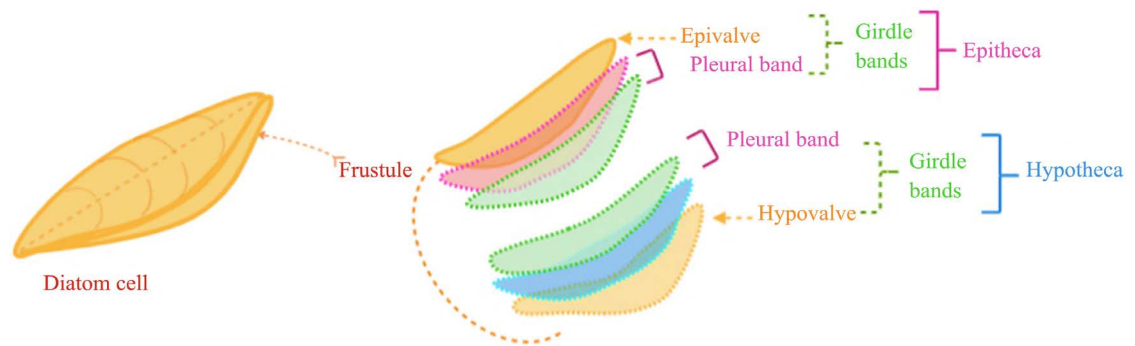


Figure 4. Schematic illustration of a diatom frustule redrawn from Zurzolo and Bowler (2001) [64].

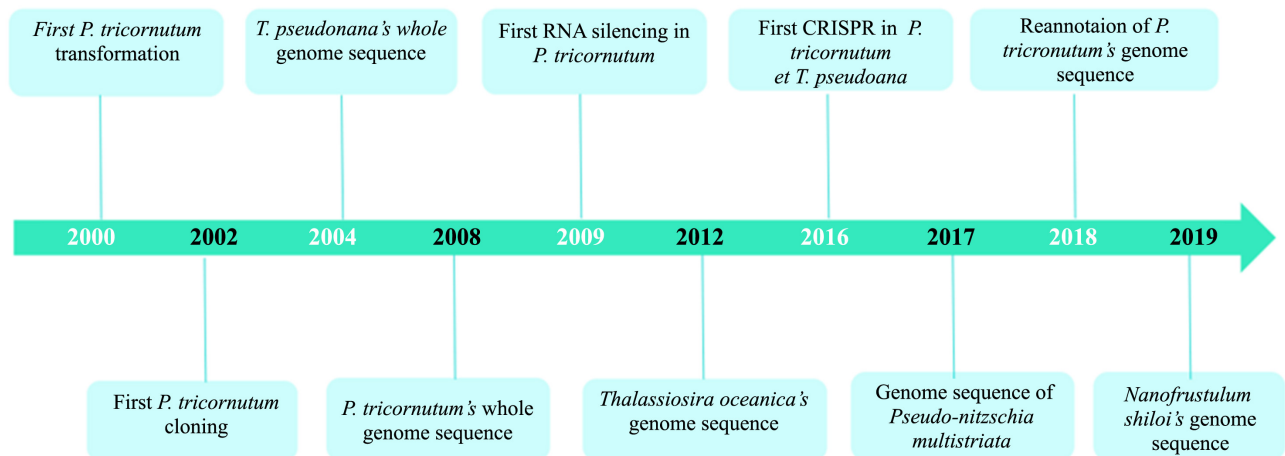


Figure 5. Key events related to diatoms including *P. tricornutum* and *T. pseudonana* species: First *P. tricornutum* transformation [72], First *P. tricornutum* cloning [73], *T. pseudonana*'s whole genome sequence [31], *P. tricornutum*'s whole genome sequence [45], First RNA silencing in *P. tricornutum* [53], *T. oceanica*'s genome sequencing [69], First CRISPR in *P. tricornutum* [74] and *T. pseudoana* [75], Genome sequence of *Pseudo-nitzschia multistriata* [71], Reannotation of *P. tricornutum*'s genome sequence [76], *Nanofrustulum shiloi*'s chloroplast genome sequence [77].

macronucleus, proteins transcription takes place, while the germline micronucleus is silenced. Several researchers suggest that this unusual diatom genome organization could be a result of an endosymbiotic gene transfer between green and red algae while others propose that this may be the fruit of an amount of horizontal gene transfer from bacteria [68].

Other genomes of diatom species are currently available such as the whole genome sequences of *Thalassiosira oceanica* (92.15 Mb) [69], *Fragilariopsis cylindrus* (61.1 Mb) [70], and *Pseudo nitzschia multistriata* (59 Mb) [71] (Figure 5). According to the recent literature, for the diatoms: *Seminavis robusta* and *Fustulifira sp.*, only their chloroplast genomes have been recently sequenced. This increase in genome revelation could only mean more advanced tools for endogenous and heterologous protein production in diatoms.

P. tricornutum's whole genome is approximately 27.4 Mb in size. This genome contains 33 chromosomes and 12,233 predicted genes and is relatively small compared with other diatoms' genomes [39] [45]. More than 130,000 ESTs generated from cells grown under 16 different conditions are available which

made easy the gene identification and functional analysis in this diatom species [45]. Diatoms' low GC content in the centromeres allows foreign DNA with a lower GC content to be maintained in *P. tricornutum* as episomes. In diatoms, this maintenance could be assisted by recruiting specific chromosome maintenance proteins such as histones by the low GC foreign DNA. This hijacking mechanism helps for the acquisition of foreign DNA in diatoms [78].

2.4. Translational Modifications, Post-Translational Modifications (PTMs), and Epigenetic of Histones in Diatoms

Marine diatoms are well-known for their strong adaptation in various biotic and abiotic stress factors, which allows them to live in different aquatic habitats all over the world [79]. This plasticity can be justified by strategies used by these species in response to the various stress factors in their environment. To understand these strategies routed by specific enzymes, proteomic studies of *P. tricornutum* have been established using available technologies to elucidate their PTMs. Recently, researchers succeeded to draw a comprehensive landscape of PTMs in *P. tricornutum*, using high accuracy mass spectrometry (MS), in which different enzyme digests were combined with purified histone preparations and whole-genome maps of five PTMs was also generated by chromatin immunoprecipitation (ChIP) [80]. Thus, revealing changes in response to different nutrients limitations served to demonstrate the dynamic nature of the chromatin code [81]. Recent studies have tested the adaptive responses of marine diatoms by starvation of their growth media of some nutrients such as nitrogen, iron, and phosphorus [82] [83] [84]. Although limited, these studies have provided data helping a lot for a better understanding of the sophisticated mechanisms of diatoms' adaptive responses in the environment.

Several studies of *P. tricornutum*'s epigenetics showed the presence of PTMs, which are conserved in eukaryotes such as acetylation involved in the metabolism of some bacterial strains [85] [86] [87]. Other several signaling pathways, which are common pathways with both plants and animals, have been found also in diatoms [88]. Herein, we talk about the example of Ca^{2+} and nitric oxide (NO) involved in the aldehyde (2E, 4E/Z) decadienal-triggered diatom cell death and the ornithine-urea cycle, which is known as a key PTM in the physiological and the life history adaptations in vertebrates [79]. Data evenly proved that diatom proteomes have undergone changes induced in both, abiotic and biotic stress conditions, which contribute to the adjustment of their metabolism and to enhance their stress tolerance. Recently, studies on the PTMs (phosphoproteomics and redoxome) in diatoms exposed to stress are published [89].

Crucial cellular functions such as signal transduction, cell proliferation, and stress responses are regulated by protein phosphorylation at serine (S), threonine (T), and tyrosine (Y) residues [65] [90]. Indeed, Protein phosphorylation and dephosphorylation are almost involved in all cell signaling processes. Hence, analyzing PTMs in diatoms allows the deduction of their regulatory mechanisms under varied stress conditions. However, data related to the diatom proteomic

PTMs are still limited and to date, since few studies have used proteomic approaches in diatoms. A recent research established in the diatom *T. oceanica* under copper (Cu)-limiting and, sufficient conditions predicted several variable protein PTMs such as deamidation, oxidation, and phosphorylation [91]. Besides, phosphoproteomic analyses of *P. tricornutum* are recently established [89] and were based on the separation strategy of proteins/peptides using titanium dioxide (TiO₂) enrichment and LC-MS/MS analysis [89]. Two major mechanisms in diatoms were suggested regarding their coping with diverse environmental conditions. These mechanisms are; the programmed cell death (PCD) process induced by intracellular reactive oxygen species (ROS)/nitric oxide (NO) and Ca²⁺-dependent and ROS scavenging process, which coincides with TAGs accumulation mediated by the ubiquitin-proteasome pathway and the amino acid degradation pathway. The phosphoproteomic analysis of *P. tricornutum* also suggested new prospects on strategies employed by diatoms into different stress conditions present in the marine environment [89]. Based on an in-depth functional analysis, cellular homeostasis is maintained by the regulation of the redox regulation of metabolic rates. Efficient regulation of the proteome is essential for diatoms to optimally respond to the ever-changing environment [89].

The functional regulation of many eukaryotic proteins involves several lysine acylations, such as acetylation, succinylation, crotonylation, malonylation, propionylation, and butyrylation [92]. Modifications of lysine residues in diatoms shared with plants have been shown in several results such as mono-, di- and tri-methylation, acetylation, and mono-ubiquitination. However, some positions of several modified sites as the ubiquitination of lysine 111 of histone H2B have been changed. Yet, acetylation of lysines 31 and 59 of histone H4, and acetylation of lysines 2, 34, and 107 of H2B are novel unique PTMs identified recently in diatoms by Veluchamy and his team in 2015 [81]. In recent studies, a large conservation of eukaryotic histone modifications was revealed by MS and ChIP-Seq analysis in the diatom model *P. tricornutum*. Besides, acetylation of several lysines on the N-terminal tails of histones H2A, H2B, H3, and H4 and also on mono-, di-, and tri-methylation of lysines 4, 9, 27, and 36 of histone H3, were some of the histone modifications identified as similar to histone modifications detected in plant and mammalian cells. Thus, researchers have suggested their role in transcriptional regulation of many biological processes such as activation of the transcription process [93]. Furthermore, they detected acetylation of lysines 2, 34, and 107 of H2B as well as the ubiquitination of lysine 111 of the same histone. *P. tricornutum* share likewise, the acetylation of lysine 20 of histone H4 present in *Arabidopsis thaliana*. Histone PTMs founded only in humans, yeasts, and *Toxoplasma gondii*, but not in *Arabidopsis* [94], such as acetylation, mono- and di-methylation of lysine 79 of histone H3, were also found in *P. tricornutum*. However, acetylation which contains several gene promoters was found in correlation with transcription activation and with genome studies of several cell species such as yeast, human, and *Arabidopsis* [69].

After the analysis established in the diatom *P. tricornutum* for its combina-

torial histone marks with DNA methylation, three chromatin states have been detected and those results were similar to those of previous studies done in *Arabidopsis*. These three states are active, repressive, or intermediate, and suggest the presence of an epigenetic code added to the histone code in *P. tricornutum*. The chimeric nature of *P. tricornutum*'s genome which combines plant and animal histone's PTMs, suggests a different evolution of its histone PTMs [81]. Yet, several common features with plants and animals have been recently revealed using the genome-wide mapping of some key PTMs in diatoms such as the distribution of acetylation and di-methylation of lysine 4 of histone H3. Moreover, the H3K9me3 profile found in *Arabidopsis* with an active effect on genes is associated with repressive genes in *P. tricornutum* [81] [95]. This profile may underscore the evolutionarily ancient function in transcriptional repression of transposable elements (TEs) in *P. tricornutum* [81] [96].

Several other PTMs such as glycosylation and phosphorylation impact directly the function of the desired protein during the heterologous expression of valuable proteins. Therefore, they are very important for the activity and the efficiency of therapeutic proteins produced by genetic engineering. Especially for human molecules expressed in the host engineered cells, which must express special PTMs to mature the protein of interest and make it active. These PTMs present an essential tool for drug companies therefore they must understand them very well because their absence or presence in the engineered cells determine the expression level of their pharmaceutical products [97]. Researchers have succeeded in the expression of monoclonal antibodies in several expression systems such as mammalian cells (CHO lines) [98], transgenic plants, yeast, and insect cells but with an awfully low expression level. For example, in the study of Hampel *et al.*, 2011, they succeeded to produce the functional human antibody "CL.4mAb" against the anti-Hepatitis B Virus surface protein in the diatom *P. tricornutum* [99]. Thus, the efficient production of these antibodies in diatoms couldn't be without the implication of PTMs of *P. tricornutum* which was mainly the glycosylation. Interestingly, analyzes with the Western Blot technique proved that *P. tricornutum* has established glycosylation in its endoplasmic reticulum for the chains of the antibody protein surface produced [100]. The diversified PTMs in diatoms represent an additional feature encouraging using these creatures to become complex protein production platforms compared to bacteria and yeast that shows a relative lack of PTMs.

2.5. Molecular Tools and Diatoms Bioengineering

To provide an environmentally friendly, sustainable, and renewable platform for the manufacture of valuable molecules, researchers are focusing on deeper understanding and better use of the available tools aiming to produce biomolecules in diatoms. In fact, instead of classically producing bioproducts and biofuels from fossil hydrocarbons, nowadays it is possible to produce all these products and others by diatoms bioengineering. Molecular tools developed and used to

produce biomolecules in bacteria, fungi, and plants have revolutionized the biotechnology and are currently applied to diatoms. Fortunately, several studies on biochemistry and physiology of the microalgae helped to develop the basic and necessary tools such as its cellular structure, resistance to selective agents, and the critical targets to manage metabolic processes of interest [101]. For several diatom strains, the entire genome sequence is now available as mentioned earlier, which allows us to spot heterologous genes in important metabolic pathways for genetically manipulate new diatom strains.

As the genome of diatoms is contained in the nucleus, the chloroplast, and the mitochondrion, introducing actively the nucleic acid presents a real challenge. Indeed, the nucleic acid introduced in the diatom needs to penetrate not just the cell wall and plasma membrane, but also membranes that surround the targeted genome. There are several methods to facilitate the entry of transforming DNA into a diatom cell. In several diatom species, particle gun bombardment using high-velocity microprojectile gold or tungsten particles coated with DNA is used to permit the entry of DNA into the cell [47] [72] [102] [103] [104]. Other methods have been used for the same aim such as glass-bead mediated transformation, where diatom cells are vortexed with the foreign DNA, polyethylene glycol (PEG), and sterile glass beads [105]. In the study of Karas *et al.*, 2015, PEG-mediated transformation was established to integrate p0521 and p0521s plasmids in *P. tricornutum* [105]. To allow successful entry of the transforming DNA into the cell these methods require that the integrity of the diatom cell wall is compromised. Additionally, the progress of genome editing tools such as nucleases, TALEN, zinc-finger, and CRISPR/Cas approaches hold noteworthy promise to advance the genetic engineering of the nuclear genome of diatoms [13] [101]. In the work of Daboussi *et al.*, 2014 [13], authors were able to target and edit 7 important genes involved in the lipid synthesis to improve the oil production in the engineered diatom *P. tricornutum*, which represents a major advance for non-fossil biofuel production.

As a useful part of the molecular toolbox for engineering diatom strains, transcriptomics and gene knockdown by RNA interference (RNAi) allow us to explore the transcribed portions of the genome and help in designing useful organisms. These tools are of great potential because they have simplified both the analysis and manipulation of the complex diatom genomes [13]. The metabolic mapping tools provided by transcriptomics are holding the application of RNAi to improve the production in diatom strains and to increase yields of specific products. The work done on *T. pseudonana* by Trentacoste *et al.*, 2013 [106], based on both transcriptomics and RNAi; represent an example of the utility and the promising results of these tools to increase lipid production in diatom cells. To better genetically improve microalgal strains, several different genomes editing techniques such as TALEN, zinc-finger nucleases, and the CRISPR/Cas9 systems could compete with RNAi. All these molecular tools allow us to generate directed knockouts and gene replacements that might be used to generate improved diatom strains [53] [74] [101] [107] [108].

2.6. Biolistic and Microfluidic Approaches

Currently, the biolistic technique is considered as the most frequently used method of gene delivery in diatoms also referred to as micro-projectile bombardment. This method is based on the use of DNA-coated gold or tungsten micro-particles that are delivered through a particle delivery system with a high velocity into diatoms. This technique allows efficient gene delivery by surpassing the physiological barrier of the cell wall. The success of this transformation with this technique was reported for different diatoms cells such as *P. tricornutum* and *T. pseudonana* [72] [104] [109]. Several protocols aiming to transform several diatoms including *P. tricornutum* and *T. pseudonana* are based on biolistic methods that led to random integration of the transgene which causes its multiple insertions in the cell genome [110]. However, this integration of foreign DNA was proved stable into the chromosomes of the nucleic and plastid genomes in several diatom strains [104] [111] [112] [113]. The introduction and the expression of heterologous genes in diatoms using the biolistic approach seem to be a manageable task. However, this current technique is not enough to generate the highest number of transformants in *P. tricornutum* and require maximal settings of all parameters [109]. Moreover, this method is also limited because of the insertions which may cause the knockout of the essential genes.

Other emerging techniques changed the process of transforming diatoms. One of these techniques is the droplet-based microfluidics, based on the encapsulation of sub-microliters to picolitres of aqueous phase into monodisperse droplets. This approach is especially useful for applications requiring parallel experiments at minimal reagent costs. Moreover, microfluidic technologies enable high-throughput and controlled processes for cell-free, artificial cells, and genetic circuits applications [114]. Furthermore, advances made on microfluidic platforms offer a lower entry price point alternative to robotics and maintain high throughput and reproducibility. A variety of microfluidic systems support synthetic biology applications spanning DNA assembly to single-cell phenotyping [115] [116]. For example, the microfluidic approach helps a lot of the fluorescent analyses which can be established into droplets and could be used for functional genomic analysis.

Synthetic biology or genetic engineering cycle often goes through a critical and essential stage, which is to culture and screen cells for expression of proteins, peptides, or chemicals of interest. Researchers try to measure at the same time with one technique, both the amount and the activity of the expressed chemical or protein. So, integrating fluorescence which is a widely used method with microfluidic platforms remains the ideal solution. The works of Romero *et al.*, 2015 [116] provide examples of including the measurement of the protein amount by expressing a protein with a fluorescent tag (e.g., GFP) and the measurement of enzyme activity by using fluorogenic substrates. Another example of using droplet microfluidics for synthetic biology applications includes cell-free and artificial cell systems [114] [117]. Recently, researchers

have used *in vitro* cell-free systems instead of bacterial, fungal, and mammalian cell systems, in controlled microfluidic environments to investigate biochemical reactions, gene expression, and protein synthesis [114]. In a recent study of Geisler *et al.*, 2019 (unpublished work: Ketrin Geisler, Ziyi Yu, Chris Abell, Alison G. Smith), metabolic engineering of the diatom *P. tricornutum* was combined with a state-of-the-art microfluidic device. This technique allowed them to establish the encapsulation and the growth of the wild-type of the diatom, and then its transformation in microdroplets. Thus, researchers were able to analyze single cells and screen them in a high throughput manner.

3. Molecular Tools, Transformation Methods, and Application of Diatoms

3.1. Diatoms' Transformation Methods

Genetic transformation methods have been established for many decades for both eukaryotic and prokaryotic cells. The purpose of these methods is to introduce an external DNA molecule into a receptor cell, to make it able to express the desired protein. This introduction cannot be established without causing a temporal permeabilization of the cell membrane or the cell wall of the receptor cell. Thus, integration of the exogenous DNA into the genome of the receptor cell mainly occurs by random recombination and can succeed with or without any external assistance [118]. Practically, the embrittlement of the cell membrane or the cell wall of the host cell is not the most exhausting stage of the genetic transformation procedure. However, the most important fact is that the transformed cell affected by the transformation method must survive despite this severe damage and still able to express the desired protein.

The most frequently used transformation method for diatoms is the biolistic delivery system also called gene gun transformation, micro-projectile bombardment, particle gun transformation, or simply biolistic. This method is rapid, generates multiple copies, and presents higher rates of success even in the presence of silencing. Besides, it's used to deliver RNA and protein as well as DNA molecules. Biolistic was used for several diatom cells not only for basic studies on diatoms but also to produce different high added value molecules. Several diatom species were transformed by this method such as *P. tricornutum* [72] [102] [111] [119], *Cyclotella cryptic* [120], *Navicula saprophila* [120], *T. pseudonana* [104], and other diatom species (Table 1). However, the transformation efficiency of this classical method is relatively low, and the material used is very expensive.

Another popular method that has been also used a lot for the transformation of diatom cells is the electroporation transformation method. It is based on the application of large electronic pulses in specially designed electroporation cuvettes containing a mixture of the exogenous DNA molecules and the host cells to be transformed. This pulses-based technique allows the integration of the DNA molecules to pass the phospholipid bilayer of the cell membrane. It can be applied for protoplasts, cell-wall reduced mutants and cells with thin cell walls.

Table 1. Transformation methods used for several diatom species, targeted cell compartments and resistance markers.

Transformation method	Diatom species	Target organelle	Resistance marker	Refs
Electroporation	<i>P. tricornutum</i>	Nucleus	Nitrate reductase	[123]
			Zeocin	[110]
			Phleomycin	[105]
Multipulse electroporation	<i>P. tricornutum</i>	Plastid	Chloramphenicol	[112]
			Zeocin	[122]
			Nourseothricin	[46]
PEG mediated	<i>P. tricornutum</i>	Nucleus	Phleomycin	[105]
Conjugation	<i>P. tricornutum</i>	Nucleus	Phleomycin and nourseothricin	[105]
	<i>T. pseudonana</i>	Nucleus		[111]
Gene Gun (Biolistic approach)	<i>P. tricornutum</i>	Nucleus		[72]
			Zeocin	[127]
				[47]
				[102]
	<i>Seminavis robusta</i>	Nucleus	Phleomycin	[78]
			Nourseothricin	[128]
	<i>Cyclotella cryptica</i>	Nucleus	Geneticin	[120]
	<i>Navicula saprophila</i>	Nucleus		
	<i>Amphora coffeaeformis</i>	Nucleus	Nourseothricin	[129]
	<i>Cylindrotheca fusiformis</i>	Nucleus	Zeocin	[130]
	<i>T. pseudonana</i>	Nucleus		[104]
	<i>Chaetoceros sp.</i>	Nucleus	Nourseothricin	[48]
	<i>Fistulifera sp.</i>	Nucleus		[49]
	<i>Fistulifera solaris</i>	Nucleus	Geneticin	[103]
	<i>P. tricornutum</i>	Chloroplast	DCMU ^a	[113]

a. DCMU = Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea).

This technique was employed successfully with a simple pulse [105] [110] [112] [121] [122] [123] or a multipulse system [122] in several diatom cells such as *P. tricornutum*.

These methods of gene transfer are based on physical forces so they can affect the genome integrity producing the double-strand breaks, repaired by nonhomologous end-joining [124]. Thus, to hinder this issue and other issues of these methods, new vectors have been designed to be transformed by the conjugation method in diatom cells [125]. These vectors are efficient because they contain a yeast-derived sequence that can auto replicate as episomes in diatom cells [105].

These episomes were delivered to *P. tricornutum* and *T. pseudonana* in the work of karas *et al.*, 2015 through bacterial conjugation using *Escherichia coli*. This strategy used for both diatom species *P. tricornutum* and *T. pseudonana* cells remains a more efficient method than previously described methods [105]. Other genetic transformation methods have been employed for diatom cell transformation such as PEG-mediated transformation [105] [126]. As listed in **Table 1**, several methods of transformations have been used for the transformation of *P. tricornutum* presenting different transformation efficiencies.

To successfully transform and induce the expression of a gene of interest, strong promoters are a must. For example, the commonly used promoters in *P. tricornutum* and *T. pseudonana* [104] transformations are mainly *Lhcf1* promoters involved in the light-harvesting process of diatoms, also the nitrate reductase (nr) promoter which acts by shifting the transgenic cells from nitrate-deficient to nitrate-sufficient medium [131]. Strong and/or inducible promoters in diatoms with a higher diversity of choice would be an empowering tool for optimizing diatom transformation in the future.

3.2. Diatoms in Aquaculture

An ever-increasing attention is focused on aquaculture these several years, because of the wild fisheries and the rapid decline in fish stocks, which is illustrated by the shrimp industry [132]. Herein, diatoms are attracting an economic interest in several industries because they contain various classes of lipids and high levels of amino acids and vitamins essential for the food chains [133]. Therefore, the commercial rearing of many aquatic animals was based on using microalgae as the most important and preferred food source and feed additive especially for bivalve mollusk larvae and post-larvae, shrimp, and live food organisms. For this purpose, diatoms have been grown in large scales production for aquaculture diets [134]. Interestingly, after analyzing fatty acids profiles of twelve diatom strains, *P. tricornutum* showed the highest amount of lipids (EPA = 28.4 mg.g⁻¹). This diatom has proved an important potential as a source of fatty acids such as C14:0, C16:0, C16:1, and C20:5 x3, for aquaculture. Furthermore, proteins and carbohydrates of these diatoms will also be tested as a live feed and in formulations [135]. However, the production cost of the microalgal biomass stills the major constraint since it remains higher than traditional large-scale producers' [136].

3.3. Diatoms in the Biofuel Industry

Data confirmed that to have fuels, great amounts of accumulated ancient deposits of organic material stored as fossils are combusted every year around the world which allows society to cover 80% of their daily needs. Therefore, the combustion of 24 tons of ancient plant matter can generate only 1 L of gasoline [137] [138]. Thus, scientists are constantly searching for a renewable, ecological, and sustainable natural resource as an alternative to the available exhaustible re-

sources. Among these resources, they choose the diatom species as an organism owing to the ideal characteristics of an ideal producer of biofuels. These species are well-known of their capacity of accumulating rapidly high levels of lipids such as triacylglycerols (TAGs) when his environment is starved of nitrogen (N) and especially under (Si) limitation. For example, the model marine diatom *P. tricornutum* is evenly able to accumulate a greater quantity of lipids than other algal classes thanks to the photosynthesis process under these special stress conditions [134] [139]. This capacity allows them to produce clean and renewable biofuel [79] [139].

Thereby, diatoms are considered as more efficient biofuel production systems than vascular plants. Diatoms exhibit optimal criteria such as high growth rates, tolerance of harsh environmental conditions, and performance in large scale cultures [134]. Whereas, making their productivity ecologically viable mandatory needs developing novel methods of algal biomass production [140]. Recent studies are trying to boost and to improve biofuels production by modifying the *P. tricornutum* genome, via several genetic and biochemical engineering tools already used for these species. So that, understanding of their molecular and metabolic mechanisms involved in lipid accumulation under different growth conditions and investigation of more efficient molecular tools are always needed [139] [140].

3.4. Diatoms and Biotechnology

Different diatom species have been exploited in several biotechnological applications since the discovery of their main features especially for upgrading the production of pigments naturally produced in these cells such as fucoxanthin from *P. tricornutum* and *Nitzschia microcephala* [27]. Their frustules have been also exploited for their potential as a UV-resistant coating for voltaic cells [141] and their ice-binding proteins (IBPs) which are founded in antarctic sea ice diatom “*Navicula glaciei* Vanheurck” are used for the blood cryopreservation [142]. Lipids are also one of the most important molecules produced in diatom species such as in “*Fistulifera solaris*” which are exploited for biodiesel production thanks to its high countenance of lipids [143].

P. tricornutum is a brown unicellular microalga belonging to the subgroup of the heterokont photosynthetic diatoms which represents nowadays a source of several high added-value molecules. Organic compounds, such as polysaccharides, proteins, and lipids are the main components of its cell slightly silicified wall. This peculiar structure gives *P. tricornutum* moderate stability towards mechanical stress. When growing, these microalgae can reach a length of up to 30 µm and can exist in three different morphologies (fusiform, triradiate, and ovoid). Their different morphologies are depending on the respective strain and various environmental conditions [144]. Recent research, come to confirm the role of *P. tricornutum* in the understanding progress of microalgae metabolism as a great model for biochemical studies [145].

Furthermore, previous researches have demonstrated the feasibility of introducing plant transgenes in *P. tricornutum*, to produce high-value bioactive molecules such as high-value sapogenins [26]. D'Adamo *et al.*, 2019 proved in this work the possibility of introducing a complex triterpenoids plant pathway in *P. tricornutum* that required the expression of the genes corresponding to three different membrane enzymes: a lupeol synthase, a P450 enzyme, and an NADPH reductase. *P. tricornutum* has a large biotechnological potential for producing valuable substances for the feed, food, cosmetics, and pharmacy industries as well as for biotechnological processes [26]. Besides, genetic engineering of *P. tricornutum* has proved the possibility of introducing long metabolic pathways containing eight genes involved in the biosynthesis of the product of interest such as vanillin [146]. *P. tricornutum* has a large biotechnological potential for producing valuable substances for the feed, food, cosmetics, and pharmacy industries as well as for biotechnological processes [42]. **Table 2** contains several natural compounds that were successfully produced in *P. tricornutum* and other diatom species by genetic and metabolic engineering approaches.

P. tricornutum can accumulate proteins when cultivated under nitrogen-rich conditions and is further known for its high carotenoid content. Downstream processing scheme established in a recent study was used for the protein extraction of different microalgae including *P. tricornutum*. In comparison with commercially available milk protein concentrates used as food emulsifiers nowadays, lyophilized soluble proteins expressed in this microorganism were the most promising candidates due to their high stability, moderate pigmentation, and high initial protein content [151]. Thus, we can confirm the promising potential of this miracle organism for the biotechnological production of commercially natural products with proven stability and efficiency. **Figure 6** summarizes several application fields of the high-value added molecules produced in diatoms.

Furthermore, diatoms are well-known for their accumulation of essential fatty acids such as the arachidonic acid (AA, C20:4 x6) eicosapentaenoic acid (EPA,

Table 2. Examples of compounds successfully produced in bio-engineered diatom species (*P. tricornutum* and *T. pseudonana*) for several applications

Diatom species	Modified target/modification	Products	Application	Refs
<i>P. tricornutum</i>	Heterologous $\Delta 5$ -elongase expression	Fatty acids (omega 3)	Biotechnology	[147]
	Pyruvate dehydrogenase kinase (Antisense knockdown)	Lipids (Biofuels)	Biofuel industry	[148]
	Constructions with IgG1 ^a /kappa gene	Antibody (IgG ^a)	Pharmaceutical industry	[149]
	Episome-based approach	Monoterpenoids	Biotechnology	[150]
<i>T. pseudonana</i>	Multifunctional lipase (Knockdown)	Lipids	Biofuel industry	[106]
	Silica forming machinery	3D-nanomaterials	Material engineering	[65]

a. IgG = immunoglobulin G.

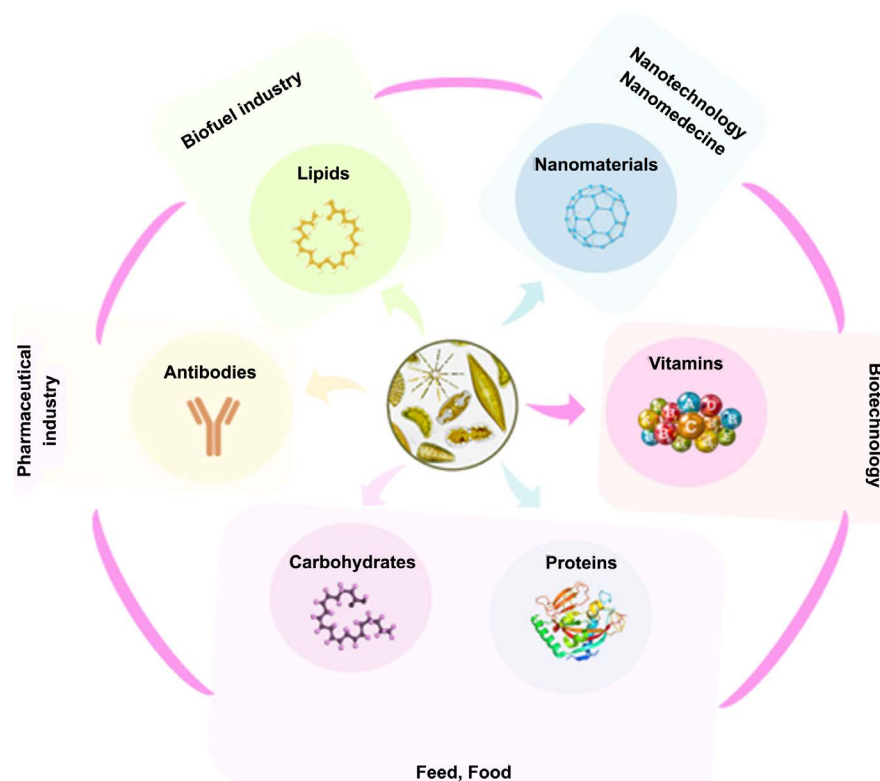


Figure 6. Schematic representation summarizing the several added-valued molecules produced in diatoms and their applications in several fields.

C20:5 x3) and docosahexaenoic acid (DHA, C22:6 x3). These two x3 polyunsaturated fatty acids (PUFA) have proven numerous nutraceutical and pharmaceutical applications [24] [152] [153]. They are often used in the treatment of several diseases like atherosclerosis, cancer, rheumatoid arthritis, psoriasis, Alzheimer's, and age-related macular degeneration [154]. Therefore, modern nutritional theories stated the correlation between maintaining enough levels of x3-PUFAs and health benefits [135].

3.5. Antimicrobial Compounds from Diatoms

Diatoms are increasingly attracting researcher's interest, for many decades, because they have shown huge genetic and chemical diversity and a strong ability to produce natural compounds biotechnologically interesting. For example, antivirals from the diatom species *Haslea ostrearia* [155] and *Navicula directa* [156], antifungals [37] [157] [158], protein inhibitors [159], and toxins [160] [161] [162] can be produced into diatoms, because they are often accumulated into these organisms under special conditions [163]. Like all the organisms coexisting with other communities, these microalgae live in a microbial-rich environment, so they must compete with bacteria for the limited space and resources. This competition is often accompanied with natural protection against competitors and other external conditions such as opportunists and environmental stress. So, to protect itself *P. tricornutum* often produces antimicrobial

natural compounds such as the polyunsaturated fatty acids “oxylipin” molecules [164] produced against its predator crustacean copepods [165]. These molecules reach a great interest of several drug researchers because they have been recently evaluated as anticancer drug candidates [166]. This diatom’s mechanism of defense leads in the most of cases, to the damage or even to the death of the dangerous organisms. Moreover, *P. tricornutum* has been proved as efficient against multi-resistant *Staphylococcus aureus* thanks to its production of antimicrobial compounds such as the polyunsaturated fatty acid, eicosapentaenoic acid (EPA) [34] [167]. These molecules are supposed useful for killing aquatic pathogens so they may present a great prospective potential in controlling diseases in the marine culture industry where the use of conventional antibiotics is undesirable [40] [41]. Thus, the interest in the use of diatoms as an ideal accumulator of fatty acids to produce antimicrobial molecules nowadays is in a great increase [168] [169].

Diatoms have not been employed for expression of any biopharmaceutical proteins, but recently Hempel and collaborators have been the first to report the stable expression of the full-length human monoclonal antibody “CL4mAb” against the Hepatitis B Virus in the diatom *P. tricornutum* [99] [100] [170], then more recently researchers succeeded the production of monoclonal antibodies against the Marburg virus [171]. Another successful production of a biopharmaceutical protein has been established in the work of Carlier Bardor and his team (Unpublished work, Carlier, A., Bardor, M., Lerouge, P., Delavault, P., Saint-Jean, B., Gerard, A., and Cadoret, J.P.). For this research, the authors have confirmed the efficient production of the therapeutic protein murine erythropoietin (mEPO) in the diatom *P. tricornutum*. Importantly, *P. tricornutum* Bohlin’s extracts, containing the polyunsaturated fatty acid eicosapentaenoic acid (EPA), have shown antibacterial effect against numerous bacterial species [40] [41] [172]. Moreover, this diatom has shown industrial potential as it can grow photoautotrophically and stably express functional enzymes using genetic modification tools. Antibodies and biodegradable plastics are also two commercially interesting products that have been produced in *P. tricornutum* [21] [22] [99].

Diatom’s antimicrobial activity was proved in several studies in which, different cell lyses and extracts of various microalgal species were tested [121] [173] [174] [175]. After identifying a few of these antimicrobial compounds, researchers have found fatty acids as one of the major groups proving an antimicrobial effect [174]. Other groups are also identified, such as nucleosides, peptides, and pigment derivatives [176] [177]. Evenly, *P. tricornutum*’s antibiotic activity extracts have been reported by several studies [34] [41] [172] [178] [179], but the identification of their compounds responsible for the antibacterial effect is not yet defined. However, the study of Mendes *et al.*, 2003 [180] has proved that the major fatty acid component of *P. tricornutum* (EPA) exhibits an inhibition effect on the growth of the fish and shellfish pathogen. Microalgal antibacterial com-

pounds identified are classed in different chemical classes: indole, terpenes, phenols, volatile halogenated hydrocarbons, and long-chain unsaturated fatty acid, which all have proved their antibacterial effect against various pathogens. These findings and others offer hope to pharmaceutical industries because, they are always focusing on finding novel useful agents to cure persistent microbial diseases [121] [173] [174] [175] [180].

Humans and society witness the real progress of human medicine, but nowadays there is a need to double efforts for combating infectious diseases which cause the death of people all over the world [40] [41]. These infections are often caused by bacteria, fungi, and viruses and they still present a major menace and a hot topic for public health. Over and above that, microbial pathogen still evolves, thus increasing the level of bacterial resistance. Here, the development of new and more efficient antimicrobial compounds is requested urgently. Fortunately, diatoms such as *P. tricornutum*, *Skeletonema marinoi*, and *Thalassiosira sp.* were confirmed as a good choice for combating antibiotic-resistant bacteria and fungal infection areas [181]. Their extracts can represent a production source of new drugs against resistant pathogens, to be the alternative of available antibiotics [37] [167] [178] [179].

3.6. Issues and Challenges

Due to the rise of the world population, the need of producing high added value molecules (nutraceutical, pharmaceutical, etc.), is becoming the main and the common challenge of industrials all over the world. Therefore, trials of transforming cells such as yeast [182], bacterial [183], mammalian [184], plant cells [185], and microalgae [186] [187] to produce wide quantities of bioactive and efficient molecules, are of a serious expansion. Several artificial synthesized molecules used in medicine, pharmacy as antibiotics, vaccines, and, enzymes are currently produced in bacteria, yeast, or often in mammalian cells. However, these systems present many disadvantages like the risk of toxin contamination, viruses, and increased expenses. Besides, trials to circumvent these disadvantages by using more safely organisms like plants are not the ideal solution, as they present a risk of transgenic pollen, which possibly leads to undesired propagation of genetic mutations [188]. Besides, the main limitations of production at a large scale of transformed cells in bioreactors are light-dependency and low growth.

Thus, the issues related to the use of these cells are not surmounted yet. Therefore, diatoms have the added advantageous potential to compartmentalize the processes introduced by transgenics. In addition to that, as diatoms can be grown asexually and in closed fermenters, they allow us to avoid problems associated with other expression systems [189]. Thereby, diatom cells such as *P. tricornutum* are considered as an effective alternative for producing safe biomolecules, which can contribute to the development of the industrial applications [39] [189] [190]. Available molecular tools, whole genome sequences, and the promotion of un-

derstanding metabolic pathways in diatoms have been investigated for this purpose (<http://genome.jgi.doe.gov/bacillariophyta/bacillariophyta.info.html>). Furthermore, stable mutants of some species of diatoms such as *P. tricornutum* have been obtained in the last years. Recently, an antisense knockdown was established for the enzyme pyruvate dehydrogenase kinase to produce lipids by accumulation in *P. tricornutum* [148].

Besides, in a recent work knockout of the diatom *P. tricornutum* has been established to create mutants by CRISPR-episome assembly. In this study, researchers developed new methodologies for the introduction of CRISPR/Cas9 in *P. tricornutum* cells and, other strategies for the rapid generation and isolation of CRISPR/Cas9 mutants [191]. Moreover, using diatom species in the fermentation approaches for the pilot-scale production seems to be promoting in many industrial fields such as antimicrobial production [133]. Interestingly, these species modified by genetic engineering methods can grow in photoautotrophic conditions using suitable photobioreactors [38]. However, the production cost of the autotrophic diatoms is still rather high. Other limiting factors must also be considered, such as light, temperature, nutrients (iron, nitrogen, and silicon), pH, etc. [192].

Yet, one of the main challenges for increasing biotechnological applications of diatoms is the enhancement of their growth and yield. The growth rate of a diatom population is the result of the balance between actively dividing and dying cells. Two of the most promising routes to progress, in enhancing biomass or interesting molecules production, are the Photosynthetic Regulation Biotechnology (PRB) and the genetic engineering of strains [12]. Another issue is also ethically discussed, which is about transgenic microorganisms and cautious measures that must be taken for ecosystem biosafety and to monitor transgenic microorganisms in nature [12]. The potential advantage of genetically engineered diatoms as a strategy for biotechnological mass cultivation is growing with the need for an optimal recombinant system. When we focus on the increase of the diatoms high-value products generated by the time thanks to the genetic transformation, we must confirm that this tool could revolutionize the blue biotechnology. Nevertheless, the low metabolic rate efficiency of these diatom species limits their applications in biotechnology [38] [193] [194]. Besides, transgenic microorganisms are supposed to be monitored tightly for the safety of the ecosystem. For the latter, to have efficient production of biomolecules in diatoms, we must focus especially on the improvement of culturing conditions, in addition to the development of genetic engineering technologies [195]. Besides genome draft cleaning and microarray studies, an international collaboration was launched in the genetic engineering workshop on the molecular life of diatoms in July 2019 aiming to create a single platform allowing the obtention of all bioinformatic data on diatoms such as *P. tricornutum* and *T. pseudonana*. This platform would operate similarly to Genvestigator *et al.*, 2006 [196], facilitating web-based gene-expression analysis and allowing all the

community to share input on gene expression, proteomics, metabolomics, and lipidomics. The future of diatom synthetic biology is promising higher efficiency of transformation and more steadiness in manipulating metabolic pathways, but this future also relies on the advances made on diatom cell biology and detailed metabolic and transcriptomic investigations. Also, further studies on the animal-like and plant-like process in diatoms would help in the orientation of synthetic strategies by using diatom own features in combination of genetic transformation. Finally, it is important to shed light on the importance of classical diatom studies such as plasticity and interaction under different stress conditions. These classical investigations will continue to add to the diatom physiology knowledge and could help, by combination to molecular tools, the channeling of specific pools of substrates and proteins that could improve diatom high-value molecules production.

Diatoms in general, and *P. tricornutum* in particular, form an excellent platform for expressing recombinant proteins and production of biofuel. But many challenges hinder further achievement and usage of diatoms at large scale biorefineries. For instance, the cultivation of diatoms is relatively easy by ensuring optimal light, temperature and nutrient concentrations, but harvesting becomes critical due to the low number of cells in a given media. Autoflocculation process could be an important alternative of traditional biomass concentration but this approach needs further adjustments and replaces flocculation which requires adding chemicals to the media during the preharvest [39]. But beside the harvesting step, all the other cultivation conditions and montage are well studied in diatoms. It is important to note that the usage of diatoms as cell factories is still shy, compared to yeast, and this could be justified by the cost of diatom cultivation compared to yeast fermenters as well as the amount of extractible in each system. On the molecular level, regardless of the genome annotation and transformation tools multitude, transforming microalgae is still a challenge nowadays. Firstly, not many selection genes are available, only few antibiotic selection markers and knock-out strains are available, limiting the number of serial transformations that could be done. Besides, strong promoters and inducible promoters are not very well studied, Fucoxanthin-chlorophyll binding proteins promoters and N/P starvation induced promoters are among the most redundant choices that are met when designing a transformed diatom. Finally, even though microalgae usage in pharmaceuticals is widely approved, the usage of genetically modified diatoms and CRISPR-Cas systems needs to be regulated. Diatoms are promising candidates for synthetic biology and could replace many synthetic pathways in the near future, regardless of the efforts that need to be achieved on the optimization of cultures and molecular conditioning.

4. Conclusion

Diatoms are important primary producers of the organic matter in the marine ecosystem and are becoming nowadays the most attractive microorganisms to

produce highly valuable molecules. Over the past 20 years, a considerable amount of information related to diatoms is provided. Nowadays, *P. tricornutum*'s genome, physiology, growth conditions, metabolism, and post-translational modifications are more elucidated. *P. tricornutum*'s growth rate and molecular manipulation allow it to become the model species for diatom research and a platform for biomolecules production. Combining available information and molecular tools, attempts for the genetic engineering of *P. tricornutum* are increasing. Nowadays, several stable nuclear transformation methods are previously reported for *P. tricornutum* transformation [47] [72] [102] [111] [122] [123] [127].

Diatoms proved a high ability to produce and overproduce bioproducts such as fatty acids and hydrogen but also engineered recombinant proteins such as antibodies [99] and, pharmaceutical proteins [197]. Yet, several limitations such as the cost strain development and the time consuming of both equipment and specialized process, still delaying the progress of their biotechnological production. On the other hand, such production is driving by energy from the photosynthesis process which leads to several advantages such as fast growth, the low-cost-production, and simple scalable cultivation. Shortly, we envisage an increasing number of experiments testing new tools for an optimal production of high value-added products in *P. tricornutum* [198]. Plastid is considered as an ideal site of storage for the recombinant proteins compared to the cytoplasm, so genetic manipulation of the *P. tricornutum* plastid genome is increasing since it has been sequenced [199]. These systems allowed the production of different biopolymers, therapeutic proteins, and industrial enzymes [113].

Moreover, it has been proved that diatoms double in as little as five hours under laboratory conditions, which enable them to reproduce quickly and generate very large bio volumes of valuable molecules [200]. Thus, developing a commercial-scale diatom bioreactor using genetic and metabolic engineering is a real need for industries to produce recombinant molecules in a large-scale [38] [112] [201]. Interestingly, providing cumulative knowledge of the physiology, metabolism, and biology of diatoms represent the prime key to maximize their potential as lipid and biomass producers. Regardless of all the highlighted gaps in our knowledge on diatoms, we strongly believe that soon, *P. tricornutum* will allow us to produce other valuable metabolites in more reliable and controlled ways, just by using light and carbon dioxide [149] [193] [202].

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Conflicts of Interest

The authors declare no conflict of interest.

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