Diatom-Derived Silica for Biomedical Applications

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Diatoms are unicellular eukaryotic microalgae widely distributed in aquatic environments, possessing a porous silica cell wall known as frustule. Diatom frustules are considered as a sustainable source for several industrial applications because of their high biocompatibility and the easiness of surface functionalisation, which make frustules suitable for regenerative medicine and as drug carriers. Frustules are made of hydrated silica, and can be extracted and purified both from living and fossil diatoms using acid treatments or high temperatures. Biosilica frustules have proved to be suitable for biomedical applications, but, unfortunately, they are not officially recognised as safe by governmental food and medical agencies yet.

biosilica diatom frustule sustainable production drug delivery

1. Introduction

Diatoms are an extremely diverse group of algae, comprising more than 100,000 different species [1]. They are able to colonise a large plethora of aquatic environments, and play a significant role on a global scale in the biogeochemical cycles of carbon and silicon in the water column. Two diatom species, *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, have been employed as model species for studies of gene expression and regulation, since they were the first species for which the whole genome was fully sequenced [2][3]. Subsequently, genomes have been sequenced from a number of diatoms possessing specific metabolic or physiological features, such as oleaginous (*Fistulifera solaris*), psicrophylic (*Fragilariopsis cylindrus*), araphid (*Synedra acus* subsp. *radians*), oceanic (*Thalassiosira oceanica*), biofilm-forming (*Seminavis robusta*), and heterotrophic (*Nitzschia* sp.) species [4][5][6][7][8][9]. Apart from their ecological role, diatoms are also suitable for several biotechnological applications. They can be cultured in the laboratory under sterile conditions and controlled temperatures, light irradiance and nutrient concentrations in order to achieve faster growth rates and to promote the accumulation of specialty products. Diatoms have been employed during the last decades for the production of metabolites exhibiting different biological activities and used as sources for cosmetic ingredients [10], food or feed supplements [11][12][13], fertilizers [14], and sorbents or accumulators for the bioremediation of aquatic environments [15][16]. Microalgae other than diatoms, especially freshwater green algae, also exhibit a great potential in one or more of the abovementioned fields of research.

The true distinctive feature that makes diatoms more suitable than other taxa for biotechnological purposes, is the high proportion of amorphous silica within their cell wall. This natural source of silicon has already shown several advantages, such as its high surface area and biocompatibility, and can be employed for various research fields,

especially for biomedical applications after in vitro or in vivo treatments [17]. Diatom-derived silica is also available in huge amounts in aquatic benthic environments, as a consequence of the sedimentation of dead diatom cells.

Currently, diatom biosilica is considered as a suitable biomaterial for metal removal from aquatic environments, as a catalyst support, in optical devices, as a microsensor, and other kinds of applications [18][19]. Since its presence on the market as a device for aquatic remediation and as food-grade products is a pledge of its effectiveness in these fields, the present review is mainly focused on evaluating the potential of diatom biosilica for biomedical applications.

Diatom biosilica is actually exploited, indeed, for its potential as a drug carrier [20] and as a scaffold for bone tissue regeneration [21]. Biosilica-based processes can be considered as low-cost and environmentally friendly alternatives to processes based on artificial structures. While the production of synthetic materials requires the implementation of specific protocols, biosilica carries the advantage of triggering natural and sophisticated structure formation. For example, the employment of diatom-derived biosilica for the development of optical sensors may turn out to be, in the future, more attractive than using synthetic crystals, since it allows control and manipulation of light in a cost-effective way [22]. Biotemplated-based silica can be synthesized by rapid environmentally sustainable methods (solvent-free procedures), thus avoiding the use of hazardous chemicals, and allowing a good control of condensation rates [23].

2. Diatom Biosilica Sources

Diatom-derived silica can be obtained either from living cultures or fossil diatoms (diatomite, e.g., chalky deposits of skeletal remains). The energy required for diatom growth is sustained by either led-based (i.e., low energy demanding) artificial light or sunlight. Furthermore, the nutrients required for algal growth, such as nitrates, phosphates, silicates, vitamins, and some trace elements, can be purchased for a relatively cheap price or even obtained from wastewaters. To avoid both the costs of artificial illumination and the seasonal variability of sunlight, cells can also be grown heterotrophically [24][25][26][27], although organic substrates are to be supplied in this case. However, only a small number of species are able to grow in the dark [28][29], and organic compounds can promote bacterial growth leading to culture contaminations and to a decrease in cell growth. Biosilica is obtained after cell dewatering (i.e., centrifugation or filtration of the whole culture), followed by a purification process that is usually based on treatments with strong acids and/or high temperatures (see below). Besides, the limited motility of diatoms (due to the lack of flagella) and the “heavy” cell wall (due to the presence of a high silicon amount) enhance the spontaneous sinking of cells, limiting the volume to harvest and, thus, costs of biomass collection.

Diatoms generally exhibit fast growth rates and high lipid and biomass productivities, [30] which can be further enhanced by tuning growth conditions [31][32], making diatoms promising candidates for mass culturing. However, to the best of our knowledge, no diatom-based industrial plants (i.e., indoor or outdoor systems of algal culturing) are focusing on biosilica production as their main activity. Follow-up studies are thus required to lay the foundations for the industrial production of silica-based biomaterials.

The most abundant source of biosilica that does not foresee the induction of living cultures is diatomite, which can be easily crushed into a fine powder to become a marketable product, namely, diatomaceous earth (DE). Diatomite is made of frustules of dead diatom cells, usually found in benthic environments. The harvesting of fossil frustules, which are naturally present in benthic environments, is cost-effective and makes diatomite a promising starter for the industrial production of biosilica. However, the composition of DE is variable and the purity is often lower than that of living culture-derived frustules. The quality and abundance of these impurities vary upon environmental and aging conditions [18]. DE, generally made of ca. 80–90% of silicon and of clay minerals [33], is used as a raw material for different kinds of applications, such as agricultural fertiliser, sorbent for pollutants, and filler in plastics and paints to improve the strength of construction materials. In addition, DE is also employed to filter impurities and as an abrasive agent in cleaning and polishing products.

3. Frustule Cleaning/Purification: Main Techniques and Technical Issues

Frustules can be thus purified from both living culture-derived algal biomass and diatomite stocks. The impurities of diatom frustules mainly consist of organic matters adhered to their surface [34]. In the case of diatomite samples, impurities are present in larger amounts, and can vary in relation to the local environment and aging conditions of these natural stocks [18]. Diatomite impurities typically contain also clay and metallic oxides, such as aluminium and ferric oxides [35]. Before cleaning procedures, diatomite particles usually undergo a first step of pulverization, in which micrometric powder is grinded to nanoparticles by mechanical crushing and sonication. However, apart from a few exceptions, most studies report purification protocols based on raw material derived from living cultures rather than diatomite, which is currently the only diatomic silica-based marketable product.

Organic impurities can be removed from the silica frustule by either a chemical pre-treatment with acids or other oxidative agents, or by exposing the frustules to high temperatures. Some studies, aimed at assessing the efficacy of preliminary hydrochloric acid treatments for organic mass removal, showed that acid concentration greatly influenced both the removal rate of impurities and the state of preservation of the frustule shape, with strong acidic pre-treatments causing frustule erosion [36]. Potassium permanganate can be also used to pre-treat frustules for organic compound removal [37][38]. However, this procedure is essentially limited to remove impurities outside the frustule, and pre-treatments with acidic solutions are usually applied (even if they are not mandatory) when purification protocols do not foresee acid-based cleaning procedures, such as baking-based purifications [39]. Some preliminary oxidations with acid solutions do not exclude the employment of both acids and high temperatures. Treatment of diatom frustules with sodium permanganate and oxalic acid, for example, is followed by perchloric acid treatments at 100 °C [37].

Baking (i.e., strong heating of silica cell walls) of diatom frustules at 400–800 °C is the simplest and least expensive method to remove organic components. However, high-temperature treatments can alter diatom architecture and pore size [40]. Oxygen plasma etching, a procedure consisting of the removal of impurities using ionised gases, was found to be effective to preserve the frustule structure, with a negligible loss of material and without shape alterations [41][42].

The most commonly used procedure for the removal of organic matter and the purification of diatom biosilica is, however, an oxidative washing treatment. Some protocols require the use of 30% [34][43][44][45][46][47] or 15% [48] hydrogen peroxide solutions.

The most common washing solvents used in acid-based treatments of diatom frustules are sulphuric [49][50] and nitric [48][51] acids. Sulphuric acid treatment is rapid (10–30 min) and revealed successful even on small amounts of biosilica [35]. Despite the rapidity of this strong acid-based method, cleaning procedures are time-consuming, since several washes with distilled/deionised water are required for a complete acid removal. However, the effect of acid strength needs to be evaluated in each case, since silica nanostructures can be damaged by the action of acids. For example, frustules from poorly silicified diatom species can be dissolved in strong acid cleaning solutions [50].

To improve the efficiency of biosilica purification, Wang and co-workers [52] set up a vacuum cleaning method in which all the cleaning steps, which are cell extraction, acid treatment and washing, are carried out on polytetrafluoroethylene (PTFE) filter cloths, thus decreasing the processing time. This allows the recycling of the sulphuric acid used for cleaning, decreasing the amount of both the reagent needed for purification and the liquid wastes. The main drawback of the vacuum cleaning method is that it depends on the mechanical properties of the raw material, and cannot be applied on poorly silicified diatoms.

Some purification methods combine the use of both sulphuric acid and hydrogen peroxide in a strong oxidizing agent (2 M H_2SO_4, 10% H_2O_2) called Piranha solution [53][54]. The purification process is relatively fast, while post-treatment washes can be time-consuming. The removal of Piranha solution requires, indeed, an overnight treatment with HCl (5 M, 80 °C) and two further washes with distilled water to eliminate the HCl residuals [20]. The main treatments for frustule separations, the tested diatom silica sources, and the main bottlenecks of each cleaning technique are summarized in Table 1.

Table 1. Pre-treatments and treatments for diatom frustule cleaning and their main advantages and drawbacks.

	Treatment	Principle for Organic Matter Removal	Diatom Species	Diatom Silica Source	Advantages	Drawbacks	Reference(s)
Pre-treatments	HCl	oxidizing washing	*Nitzschia closterium*, *Thalassiosira* sp.	freeze-dried samples	high purity of frustules	possible frustule erosion depending on acid strength	[36]
	$KMnO_4$ + $C_2H_2O_4$	oxidizing washing	*Fragilariopsis cylindrus*, *Fragilariopsis kerguelensis*, *Pseudonitzschia seriata*, *Thalassiosira nordenskioeldii*, *Thalassiosira aestivalis*,	wet pellets washed with sodium lauryl sulfate	no frustule erosion	removal of the only external organic matter	[37][38]

	Treatment	Principle for Organic Matter Removal	Diatom Species	Diatom Silica Source	Advantages	Drawbacks	Reference(s)
			Thalassiosira pseudonana, Thalassiosira weissflogii				
Treatments	baking	high temperature	*Navicula* sp.	APS-fuctionalised diatoms on a mika surface	reduction in hazardous chemicals	possible alterations of pore size, possible post-treatments with acid solutions	[40]
	low-temperature plasma ashing	ionised gas	*Navicula, Amphora, Cocconeis, Planothidium* spp.	desalted drops of cultures, freeze-dried samples	no frustule dissolution	unsuitable for saltwater species, expensive, post-treatments with hazardous chemicals	[41][42]
	H_2O_2	oxidation	DE, *Ni tzschia frustulum, Pinnularia* and *Coscinodiscus* spp., *Thalassiosira pseudonana, Cylindrotheca closterium*	desalted and freeze-dried cultures, diatom composites	less dangerous than strong acids	long incubation, high-temperature post-treatments needful to increase efficiency	[34][42][43][44] [45][46][47]
	H_2SO_4	strong oxidation	*Thalassiosira rotula, Coscinodiscus wailesii*	living cultures	high efficiency in organic matter removal	hazardous chemicals, dissolution of thin frustules, time-consuming post treatments	[49][50]
	H_2SO_4 + PTFE filters	strong oxidation under vacuum	*Nitzschia, Ditylum, Skeletonema, Coscinodiscus*	living cultures on a filter cloth	reduced acid amounts	unsuitable for thin frustules	[52]

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Treatment	Principle for Organic Matter Removal	Diatom Species	Diatom Silica Source	Advantages	Drawbacks	Reference(s)
HNO_3	strong oxidation	*Pinnularia* sp., *Coscinodiscus concinnus*	harvested cells	high efficiency in organic matter removal	high-temperature treatments needful to increase efficiency	[48][51]
Piranha solution (H_2SO_4 + H_2O_2)	strong oxidation	*Thalassiosira pseudonana*	PBS-washed cells	high efficiency in organic matter removal	time-consuming post-treatments	[54]

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4. Silica for Biomedical Applications: Advantages

The main benefits of biosilica for biomedical purposes are as follows: plasticity of frustules for functionalization, biocompatibility, possibility of genetic transformation of living cultures for protein immobilization, and high availability of silica-derived diatoms. The biosilica derived from diatoms requires cheap synthesis processes [53] and is also characterized by chemical inertness, low or null toxicity, thermal stability and high availability [18]. Silica has been widely investigated in drug delivery systems because of its high robustness and versatility compared to other materials [55], and frustules derived from both living cultures and diatomite particles have successfully been employed as drug carriers [54][56].

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4.1. Surface Functionalization for Drug Loading and for Biosensing Chips for Biomedical Applications

Frustule functionalization consists of modifying its surface to enable the formation of stable covalent bonds with proteins or DNA [53][57], by introducing chemically reactive species functioning as cross-linkers. This step is crucial to improve the quality of the resulting material for specific applications. Chemical modification of biosilica can be critical, for example, to regulate the kinetics of drug release, and the high surface-to-volume ratio makes this raw material particularly suitable for drug delivery. Diatom frustules are characterized by precise and species-specific cell morphologies, and both the size and shape can highly differ among distinct diatom taxa. It has been estimated that the surface area ranges between 1.4 and 51 $m^2\ g^{-1}$ [58][59][60][61]. The size and the architecture of the pores are likely to influence drug release [56].

Drug release in biosilica-based systems is usually characterized by the following two phases: a first phase of fast release, due to the detachment of drug molecules weakly bound to the frustule surface, and a slow-releasing phase, due to drug delivery from the internal pore structure of diatom frustules [62]. Chemical modifications of diatom-derived biosilica allow their use as a carrier of both soluble and insoluble drugs.

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demonstrated as studies carried out on DE as fertilizers with Trichoderma viride [47].

organosilanes and phosphonic acids, thus rendering the diatom frustules hydrophilic or hydrophobic, respectively, before drug loading. A sustained release of indomethacin, which has been exploited as a model drug for silica-based devices, was also demonstrated with DE particles functionalised by dopamine-modified iron oxide nanoparticles (DOPA/Fe_3O_4 nanoparticles). Diatom-derived silica was employed, in this case, as a magnetically guided micro-carrier for drug delivery, since dopamine amino groups on the diatom surface allow the attachment of targeting biomolecules [49]. Another kind of functionalization can be obtained by combining the frustule with graphene oxide (GO) sheets through covalent bindings. These nano-hybrid composites are suitable drug microcarriers, GO sheets enhanced, indeed, drug-surface interactions, improving the kinetics of drug release [63].

Silica functionalization was also used to counteract cancer progression, through the delivery of water-insoluble antitumor drugs. A recent study showed that DE particles coated with vitamin B12 allowed better delivery of cisplatin and 5-fluorouracil (5-FU), two anticancer agents effective against colorectal cancer cells [64]. Silicon nanoparticles (SiNPs) were also functionalized with 5-FU and the chemopreventive agent curcumin, and then encapsulated into acid-resistant microspheres to show the effectiveness of oral administration of these chemotherapeutics against colorectal cancer [65].

DE particles were also used as a solid drug-carrier in phospholipid suspensions for new oral formulations of non-anticancer water-insoluble drugs, such as the anticonvulsive carbamazepine [66].

Where, also, cancer cells proliferation is concerned, DE particles were employed as drug-delivery vehicles. Other studies were focused on culture-derived biosilica. Functionalised frustules of the diatom *Nitzschia palea* have been successfully exploited as carriers for the antibacterial complex tyrosine-Zn(II); zinc ions covalently bounded to the frustule surface showed, indeed, a toxic effect on bacteria, thus reducing their concentration [67]. Esfandyari et al. [68] exploited the potential of *Chaetoceros* sp. frustules to detect circulating tumour cells. Diatoms were magnetized with iron oxide nanoparticles, and then conjugated with the monoclonal antibody Trastuzumab; this system was effective in selectively targeting and separating breast cancer cells, SKBR3 cells (HER2 positive cells), from HER2-negative cells under a magnetic field. The optical properties of these diatoms allowed to detect this specific binding ability by fluorescence microscopy, thanks to the optical properties of the silica.

Similar studies on antibody-functionalized nanoparticles deriving from living cultures were already performed more than ten years ago, and they exploited the potential of two modified centric diatoms as photoluminescent biosensors. Functionalization of *Coscinodiscus wailesii* frustules was one of the pioneer studies highlighting antigen recognition from antibodies that had been covalently bound to frustules [68]. Gale and co-workers [70] succeeded in transforming *Cyclotella* sp. frustules with the model rabbit IgG antibody, showing a correlation between the photoluminescence associated with the frustule/antibody complex and the antigen (goat anti-rabbit IgG) concentration. The main types of diatom silica functionalization are summarised in Table 2.

Table 2. Sources, type of functionalization and biomedical applications of diatom-derived biosilica.

Diatom Source	Type of Functionalization	Main Application	Aim	Reference(s)
Coscinodiscus wailesii	Silanization and antibody conjugation	Biosensor	Specific recognition antigen–antibody (murine monoclonal antibody)	[69]
Coscinodiscus wailesii	Silanization and antibody conjugation	Biosensor	Tethering and detecting antibodies (mix of normal rabbit serum and purified Ig-Y)	[44]
Cyclotella sp.	Silanization and antibody conjugation	Biosensor	Selective and label-free photoluminescence-based detection of antigen–antibody (IgG-rabbit) complex formation	[70]
Chaetoceros sp.	Iron oxide nanoparticles and antibody conjugation	Biosensor (with magnetic properties)	Selective targeting of SKBR3 cancer cells through the employment of antibody (Trastuzumab) bioconjugation	[68]
Thalassiosira weissflogii	Nitroxide 2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) conjugation	Drug carrier	Ciprofloxacin delivery in fibroblasts and osteoblasts	[71]
Aulacoseira sp.	Silanization, and oligo (ethylene glycol) methacrylate copolymers addition	Drug carrier	Improvement of levofloxacin delivery	[56]
Nitzschia palea	Amino acid (Tyr-Zn^{II}) conjugation	Drug carrier	Inhibition of bacterial growth	[67]
Diatomaceous earth	Silanization and phosphonic acids conjugation—self-assembling monolayer	Drug carrier	Improvement of indomethacin and gentamicin delivery	[47]
Diatomaceous earth	Silanization and phosphonic acids modifications	Drug carrier	Improvement of indomethacin delivery	[72]
DE mineral rocks	Graphene oxide, silanization	Drug carrier	Improvement of indomethacin delivery	[63]
Diatomaceous earth	Dopamine modified iron-oxide	Drug carrier (with	Improvement of indomethacin delivery	[59]

2011, 26.

Diatom Source	Type of Functionalization	Main Application	Aim	Reference(s)
	nanoparticles ($DOPA/Fe_3O_4$)	magnetic properties)		
Diatomaceous earth	vitamin B12 and ruthenium (II) complex	Drug carrier	Improvement of the anticancer tris-tetraethyl [2,2′-bipyridine]-4,4′-diamine–ruthenium (II) complex delivery (tested on HT-29 and MCF-7 cancer cells)	[64]
Calcined diatomite	Silanization and siRNA conjugation [18][53]	Drug carrier	Vehiculating siRNA into tumour cells to downregulate the expression of cancer-associated genes (tested on murine A20 lymphoma cells)	[73]
Calcined diatomite	Silanization and siRNA conjugation [75]	Drug carrier	Vehiculating siRNA into tumour cells to downregulate the expression of cancer-associated genes (tested on H1355 cancer cells)	[74]

assay. Different concentrations of diatom particles were tested for 24, 48 and 72 h, and the results showed very low cytotoxicity against the abovementioned tumour cells. This feature made functionalized DE particles useful carriers to transport small interfering ribonucleic acid (SiRNA) inside human lung epidermoid carcinoma cells (H1355) [74]. Biocompatibility evaluations of diatom frustules for nanotechnology-derived fibroblast (NDHS) and Saos-2 osteosarcoma cells [71].

The biological compatibility of silica-derived diatoms was also assessed in studies aimed at targeting the antiapoptotic factor B-cell lymphoma/leukemia 2 (Bcl2) with small interfering RNA (siRNA). Specifically, the amino groups of silanized diatom silica were exploited to bind siRNA, paving the way to the development of a nanoporous silica-based sensor for enhanced electrochemical detection of cardiovascular biomarkers proteins [73].

Biocompatibility between functionalized DE particles and breast cancer cells (lines MCF-7 and MDA-MB-231) has also been assessed in studies aiming at targeting the overexpression of tumour-associated proteins, such as the transmembrane glycoprotein CD44, by exploiting hyaluronic acid (HA) [76].

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Most cytotoxicity assays carried out above were performed on human cervical cancer cells (HeLa) and human embryonic kidney cells (HEK-293) and MDA-MB-231 breast cancer cells exposed to syntherized (e.g., fused at high temperature) diatoms. Biocompatibility was tested through viability assays with the dye Calcein-AM (its fluorescence intensity depends on the activity of cellular esterases, and thus of viable cells), and the results confirmed that natural silicon is not toxic. This suggested that fused diatom frustules could be a suitable alternative for synthetic bone graft substitutes [77]. In order to foresee the effects of long-term exposure of silica-based devices on biological systems, Terracciano and co-workers [78] investigated the in vivo impact of diatomite particles on the model organism *Hydra vulgaris*. Untreated specimens and animals exposed to bare frustules and to diatom nanoparticles modified with the cell-penetrating peptide [(aminooxy)acetyl]-Lys-(Arg)9 (to enhance cellular uptake) were monitored for 14 days, and no detrimental effects in terms of growth rates and apoptosis were observed in all conditions.

In our opinion, further studies on living organisms are mandatory to definitely ascertain the lack of toxicity of biosilica, especially in the perspective of concrete biomedical applications for drug loading and as scaffolds for bone regeneration.

4.3. Employment of Genetically Engineered Diatom Frustules for Protein Immobilization

Diatom particles can be considered as useful scaffolds for enzyme immobilization that could enhance protein properties. Genetic engineering represents a viable alternative to in vitro immobilization systems, as it does not require protein purification and is carried out under physiological conditions [65]. Since silaffins and cinguilins are involved in silica condensation becoming part of diatom frustules, the fusion of an exogenous protein to these frustule-associated proteins can result in the strong binding of exogenous proteins to the silica cell wall.

Transformation of diatom genomes with recombinant genes is a useful tool to allow the fusion between enzymes and cell wall proteins. This technology is mentioned in a recent study as living diatom silica immobilization (LiDSI), and has been mostly performed on the model species *T. pseudonana* [79][80]. To our knowledge, the pioneer studies focused on enzymes immobilised on diatom biosilica were aimed at inserting and blocking the bacterial enzyme hydroxylaminobenzene mutase (HabB) on the silaffin tpSil3 of *T. pseudonana* frustule [79]. Aside from the potential of this specific genome modification, this study paved the way for the genetic manipulation of diatom species to enhance protein immobilization on frustules for biomedical purposes.

The genome of *T. pseudonana* has been recently modified with the insertion of exogenous genes encoding the fusion of two enzymes, glucose oxidase and horseradish peroxidase, with cell wall proteins, enabling a regioselective functionalization, and suggesting that silica morphology could influence the effectiveness of the enzymes reactivity [80]. The frustule of this species has been also antibody-functionalised, in order to test its effectiveness in binding large and small antigen molecules [81].

4.4. Availability of Biosilica Feedstocks

In contrast with other synthetic materials, diatom biosilica is already available in huge amounts as diatomite. Moreover, diatom-derived silica feedstock could be easily obtained by culturing these microalgae in open ponds or enclosed systems, and separating them from the organic matter after culture dewatering.

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