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ECOLOGY

Immobilized Microalgae in Biotechnology

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Abstract—Here we present a brief account of current data on immobilization of oxygenic phototrophic microorganisms—cyanobacteria and eukaryotic microalgae—in natural and artificial experimental systems. We emphasize that immobilization e.g. in biofilms is a basic, widespread in nature strategy ensuring the survival of microorganisms. Accordingly, the artificially immobilized microalgal cells might be considered as a special group of biomimetic materials. Special attention is paid to the effect(s) of different immobilization on the physiology of microalgal cells and their stress tolerance as well as productivity of microalgal cultures. A comparison of the advantages and drawbacks of different immobilization techniques and cell carriers is presented. The review concludes with outlook on the possibilities of using of the immobilized phototrophic cells in biotechnology. Specific areas include (but not limited to) the biomass and metabolites production and harvesting, removal of heavy metals, biocapture of nutrients from wastewater and destroying of organic pollutants are explored.

Keywords: immobilization, microalgae, cyanobacteria, biotechnology, biofilms, review

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INTRODUCTION

In nature, many oxygenic phototrophic microorganisms, including cyanobacteria and eukaryotic (referred to below as microalgae, MA) exist in associations with heterotrophic microorganisms and other MA species. Such associations are often apparent as aggregates, clusters, flakes, granules attached or suspended in aqueous media [1] and generally form communities of the cells embedded in a matrix of extracellular biopolymers.

Therefore, the immobilized state can be considered as a universal form of MA existence when the microorganisms mimic, to a certain degree, the functioning of a multicellular organism [1].

The use of immobilized MA in biotechnology shows an upward trend during recent years. Currently, immobilized MA are employed in biomass and metabolite production, bioremediation of wastewaters, specifically, in biocapture of nutrients and heavy metals [2, 3]. The main advantages of immobilized cells as compared with free cell are facilitation of biomass harvesting and increase of cell tolerance to unfavorable factors (e.g. extreme temperatures, acidity, toxicants).

Below, we review the current experimental evidence regarding different methods of MA immobilization, carriers. Special attention is paid to the effect of immobilization on the physiological condition of the cells. Advantages and drawbacks of application of the immobilized MA cells in biotechnological processes are also compared with those of suspended cultures.

Natural Biofilms as the Prototype of Artificially Immobilized Microalgae

Stable algae–bacterial associations in nature exist due to the crucial role played by MA in the formation and maintenance of productivity of the associations. The central role of MA is defined by (i) the presence of highly organized surface structures (mucous capsules, sheathes, colonial mucus) in their cells and (ii) the ability to excrete exometabolites supporting the growth and physiological activity of other community members. The microorganisms embedded in the extracellular biopolymer matrix are effectively confined and hence have a limited mobility resembling in this regard naturally immobilized cells [4].

Microalgae form diverse functional (based on trophic and spatial interactions or mutual protection) links with other community members. The intercellular communication within these associations forms the regulatory foundation of their stability. These communities (associations) are exemplified by stromatolites and cyanobacterial mats—the oldest (3.5 billion years old) and the most evolutionarily successful form of life [5]. The associations (communities) of microorganisms of this type are commonly termed as "biofilm" in the current literature [6].

Formation of Biofilms is a Key Strategy of Microorganism Survival in Nature

About 99% of prokaryotes exist in the form of biofilms and the formation of the latter is a complex and tightly regulated biological process. Microorganisms within in the biofilm are protected against unfavorable physical, chemical and biological environmental factors—extreme temperatures, dehydration, ultraviolet radiation, nutritional deficiency, toxic substances. As a result, they exist under relatively stable conditions [7]. So the formation of biofilm communities is a key survival strategy of microorganisms under natural conditions.

Microorganisms in the biofilm communicate via chemical signals regulating the expression of genes in their cells. The chemical signaling is an important vehicle by which the community members control their structure, morphogenesis and adaptation [8]. The biopolymer matrix is normally a polyanionic hydrogel comprised mainly of polysaccharides and proteins (up to 85%) with small amounts of nucleic acids and lipids [6].

The biofilms implement the principle of cooperative existence, which is at the foundation of evolutionary development [9]. In the microbial biofilm community, the exometabolites of one member(s) usually serve as nutrients for others and microorganisms of similar and different species interact via special signal systems [10].

An illustrious example of MA immobilization in nature is the colonization of transparent gel-like structures on the surface of animals (hydroids, mollusks, nematodes, egg clutches of axolotl). The MA provide the animals with organic nutrients, contribute to the synthesis of the protective mucus, mineralization of external body cover and pigmentation. In return animals provide the MA with suitable habitat, shield them from unfavorable environmental conditions without too much blocking of light [11].

Expectably, immobilization of MA cells in artificial systems should be able to increase tolerance of cells to diverse stresses. These traits are essential for their successful biotechnological application and comprise distinct advantages as compared to suspended MA cells.

The Immobilization Techniques for Microalgae

Immobilization is the process of cell attachment to the surface of a carrier or confinement (entrapment) of the cells within the bulk of the carrier [12]. The entrapment of the cells in polymer beads can increase the local cell concentration as compared to the immobilization on the surface. In addition, the cells inside the polymeric beads are better protected from contamination by external microorganisms [13].

The immobilization technique and carriers should be minimally damaging to the immobilized cells. Most of standard methods designed for microorganisms are applicable to MA as well if they will not limit the light reaching the MA cells.

Material Carriers for Immobilization of Microalgae

Materials for immobilization of microorganisms can be natural and synthetic. The natural materials (wood, wool, minerals) are insoluble carriers often colonized by MA in nature. The advantages of natural carriers are hydrophilicity, biocompatibility, ease of utilization. Their drawbacks are poor stability and high cost. Loofa, sphagnum, peat, glass, plastic, wood, natural polysaccharides (agar-agar, cellulose, alginate, carrageenan, chitosan), synthetic polymers (polyacrylamide, polyurethane, polyvinyl chloride, polypropylene, polysulfone, epoxy resin) are most often used as carriers for MA attachment [14, 15].

The ideal carrier for MA should not disturb the cell functioning, impede the mass transfer in the cultivation system and block light significantly. It must have high mechanical, chemical and biological stability and produceability. The carrier must also be inexpensive, possess a stable affinity to the cells and a high hydrophilicity (for compatibility with aqueous media).

Immobilization Techniques

Current immobilization techniques are divided into two main groups of passive and active immobilization methods. The passive immobilization harnesses the natural capability of microorganisms of attaching to solid and gelatinous surfaces [16].

This is the simplest, mildly stressful way of microorganism immobilization mimicking the cell attachment in nature.

In contrast, the immobilization by active methods does not depend on the natural attachment ability of MA. It is implemented mainly via two approaches:

—Covalent binding of the cells to the carrier surface with cross-linking agent such as glutaraldehyde;

—Entrapment of MA in different gels, e.g. in alginate beads or gels [16].

Passive immobilization. The natural MA attachment to solid and gel surfaces is performed by chemical (covalent binding) or physical (ionic, electrostatic, hydrophobic) mechanisms [13]. The carriers suitable for passive immobilization can be either natural or synthetic. Among the natural carriers, loofa biomass is a widespread one [13] since it is porous, biodegradable, non-toxic and relatively cheap. Travieso and coauthors used a synthetic material such as polyurethane foam cubes of 1 cm3 as carriers for *Scenedesmus quadricauda* cells for wastewater treatment [17]. The same authors [18] proposed the design for a bioreactor with a rotating drum made of polyurethane foam for bioremoval of heavy metals from wastewater. The carriers made from glass, plastic, wood with passively immobi-

lized MA are also used in environmental, ecotoxicological and biotechnological studies [19, 21].

Active immobilization. Both synthetic and natural materials such as chitin or chitosan can be used for covalent binding of MA cells. However, covalent binding with toxic reagents (dialdehydes, diisocyanates) is more suitable for immobilization of dead MA cells [16].

The method of cell entrapment in the bulk volume of natural carriers such as agarose, agaropectin is the most widely used technique for MA immobilization. The agar is also widespread material for this purpose which is nontoxic, has low melting point and the ability to form durable gels at low concentrations [22, 23].

Beads from Ca-alginate also find extensive use for MA immobilization [3]. The beads do not limit growth of the entrapped MA by blocking the light [23] and they are not toxic to MA cells [24–26] but are not stable in sea- and wastewater. Although carrageenans are of inferior stability in aqueous media as compared to alginates, these carriers are also widespread nowadays.

Effect of Immobilization on Microalgal Cells

The capability of microorganisms attachment to different kinds of surfaces depends on the age, growth phase and cultural media composition. The maximal ability for immobilization is recorded in the cells at the exponential growth phase; at the stationary phase it decreases or remains the same [27].

An increase in pigment content, as well as changes in lipid and fatty acid composition as common effects in the immobilized as compared with free (suspended) MA cells [14, 28]. Chlorophyll content of *Chlorella vulgaris* cells immobilized in carrageenan gel was twice higher than in suspended culture [28]. *Botryococcus braunii* and *B. protuberans* entrapped in alginate beads contain more chlorophylls, carotenoids and lipids during the stationary phase of growth in comparison with free cells [29]. Co-immobilized microalgae *Chlorella* spp. and bacteria *Azospirillum brasilense* also showed increased pigment and lipid contents [30].

Immobilization or encapsulation of microorganisms in polymers can be a severe stressor for MA causing the reduction of immobilized cell population as compared to suspended cells although this effect is species-specific [14]. Thus, the cells of *Skeletonema costatum* and *Heterocapsa* sp. ceased to divide in alginate beads whereas the growth rate of other immobilized MA did not decrease as compared to free cells [31].

In some cases, immobilized MA cells showed better growth than free cell cultures, for example when *Chlorella minutissima*, *Pavlova lutheri*, *Haematococcus pluvialis* and *Dunaliella bardawil* were immobilized in 2% carboxymethylcellulose gel [32]. In contrast, toxic carriers exert strong adverse effects on MA growth [33, 34].

As it is mentioned above, immobilization can increase cells tolerance to abrupt changes in pH, temperature or ionic strength of the medium [13]. For example, immobilization in chitosan protected the cells of *Synechococcus* sp. against NaOH toxicity [35]. Immobilized cultures are more tolerant to different toxicants. For example, toxic effect of nickel and chromium ions on cells of a nitrogen-fixing cyanobacterium Aulosira fertilissima was drastically reduced after their immobilization in alginate beads [36].

Immobilization often influences the metabolic activity of cells thereby benefiting biotechnological applications. Immobilization of *Dunaliella salina* in agar-agarsignificantly improved production of glycerol in comparison with free cells [37]. The marine diatom cells *Haslea ostrearia* entrapped in agar gel increase the production of marennin, a blue-green pigment demanded by commercial culturing of oysters [38].

The evidence of alterations in shape and size of immobilized MA cells are also extensively recorded in the literature [39] as well as changes in size of trichomes and MA colonies [2].

In most cases the influence of immobilization on the photosynthetic activity of MA cells is defined by alteration of their illumination environment as compared with free-living cells [39–41]. Photosynthesis rate decreases if the immobilized cells receive insufficient illumination although the carrier may protect cells from photodamage if the illumination is in excess. Photosynthesis of the entrapped MA cells can be also limited by CO_2 shortage. In this case, the co-immobilization of MA with heterotrophic microorganisms is beneficial since the bacteria supply carbon dioxide to MA cells due to respiration [16].

Biotechnological Applications of Immobilized Microalgal Cells

Nowadays immobilized algal cells are used mainly for biomass and value-added metabolite production, obtaining biohydrogen, biocapture of nutrients, metals or organic pollutants from wastewaters. The immobilized MA are also used as biosensors for estimation of waters pollution degree [2, 4, 31]. A serious problem in industrial cultivation of MA is harvesting of the biomass. Currently employed approaches (filtration, centrifugation, flocculation) are energy and/or time-consuming [42]. The using of immobilized cells streamlines considerably the process of biomass harvesting. Other areas of immobilized MA cell application are exemplified in the following sections.

Biomass and Value-Added Metabolite Production

Immobilized MA cells e.g. *Porphyridium cruentum* are used to obtain sulfated polysaccharides [43]. The cyanobacterium, *Aphanocapsa halophytia* MN-11 was immobilized in calcium alginate gel and coated on light-diffusing optical fibers for sulfated polysaccharide production [44]. Immobilized in alginate cyanobacterium *Spirulina platensis* converted morphine to the alkaloid codeine [45]. Immobilization of the nitrogen-fixing cyanobacteria *Anabaena azollae* in polyurethane foam increased NH_3 production in a photobioreactor. The possibility of using this immobilized culture as a bio-fertilizer on rice fields are investigated [46].

Biohydrogen Production

There is a growing interest in renewable energy sources such as biohydrogen produced by MA cells. Certain MA species are able to produce hydrogen under stressful conditions (e.g. in the absence of sulphur in the cultivating medium). Sulfur starvation prevents the synthesis of photosystem II-specific proteins, causes an inhibition of photosynthetic processes and eventually induces the synthesis of hydrogenase and hydrogen production [47]. *Chlamydomonas reindhartii* seems to be one of the most promising organisms for biohydrogen production on an industrial scale [48, 49]. The most promising cyanobacterial species are nitrogen-fixing strains evolving H_2 as a by-product of their nitrogenase activity. Significantly, nitrogenase is protected from the inhibitory effect of oxygen in heterocysts [48]. It was shown [50], that the hydrogen productivity of cyanobacterium *Anabaena* N-7363 immobilized in 2% carrageenan gel was 2.4 times higher than that of free algal cells (up to 3.24 mmol per hour per 1 g dry gel) as compared to free cells.

Immobilization of *C. reinhardtii* cells in alginate beads leads to decreasing of oxygen inactivation of hydrogenase, since the layer of alginate limits oxygen penetration into the beads. As a result, the cells entrapped in alginate produce more hydrogen as compared to free cells [48]. The rate of hydrogen production by free and immobilized on glass fibers *C. reinhardtii* cells does not differ, but the period of active hydrogen evolution was longer for the immobilized cells [49].

Nutrient Biocapture from Wastewater

Biological treatment with MA seems to be among the most promising biotechnological methods for wastewater remediation (including agricultural wastewater). Such treatment is more economical and environment friendly as compared to currently used technologies [51]. Cultivation of MA in wastewater rich with nutrients simultaneously allows to remove effectively nitrogen and phosphorus from wastewater and obtain biomass enriched with these elements [2]. Fertilizer production is among the most promising methods of nutrient-enriched MA biomass utilization.

There are two approaches for efficient nutrient removal by immobilized MA cells: (i) their entrapment in natural or synthetic polymer gels [2] and (ii) immobilization on the surface of polymer materials [52]. The enhancement of nutrient removal by immobilized MA cells depends on enhancement of their photosynthetic activity as compared to free cells. At the same time, some polymers adsorb the nutrients as well. For example, the matrix of carrageenan adsorbs ammonium cations, while chitosan adsorbs anions (phosphate, nitrate and nitrite) [27]. *Chlorella* vulgaris immobilized in alginate removed 80% of ammonium and 70% of phosphate from wastewater [53]. Immobilized on polyurethane or polyvinyl carriers cyanobacterium *Phormidium laminosum* also successfully removed nitrates from the medium [34]. The cells of Phormidium sp. immobilized on the surface of chitosan utilized up to 95% of inorganic nitrogen and 87% of phosphate within 24 hours (60% of orthophosphate are absorbed by a carrier within 4–6 hours) [54].

At low concentrations of the nutrients their consumption by MA cells entrapped into polymer beads can be limited, possibly due to limitation of the nutrient diffusion through the immobilizing matrix. Thus, it was found [55] that *C. reindhartii* immobilized in Ca-alginate did not consume nitrate when its concentration was below 0.14 mM, while free cells almost fully consumed it.

Despite the limited growth of MA cells in beads, the metabolic activity of immobilized cells may be higher than that of free cells. This can be important to attain higher rate and efficiency of wastewater treatment. Thus, within 3 days, more than 95% of ammonium and 99% of phosphate were used by immobilized *C. vulgaris* cells while the efficiency of free cells was two times lower [28].

If the nutrient biocapture should be accomplished at high temperatures (>30°C), the use of thermophile strains can benefit the treatment process. Removal of nitrate and phosphate ions with the thermophilic cyanobacterium *Phormidium laminosum*, immobilized on hollow cellulose fibers in a tubular photobioreactor at 43°C can serve as an example [56].

Removal of Heavy Metals

The problem of heavy metals (HM) pollution is becoming increasingly important nowadays. MA cells can accumulate many elements including HM in high concentrations, so they are widely used for metal removal from wastewater [57].

Metal ion adsorption on the surface of MA cells accounts for HM binding to cell wall and/or cytoplasmic membrane, surface structures and extracellular

compounds. Efficiency of HM adsorption depends on the total MA cell surface area, so concentrating the cells by immobilization results in significant increasing of HM bioremoval from wastewater [58]. Both natural (carrageenan, alginates, chitosan, agarose) and synthetic (polyacrylamide, polypropylene, polysulfone, various copolymers) carriers are widely used for immobilization of MA for bioremoval of HM. The biomass of sea algae *Sargassum* sp. and *Ulva* sp. was used for microalgae *Tetraselmis chuii* and cyanobacterium *Spirulina maxima* immobilization [59].

Even dead cells of MA can be very efficient at accumulation of HM. The biomass of cyanobacterium *Phormidium laminosum* immobilized in polysulphone and epoxy resin beads was used for Cu, Fe, Ni and Zn sequestering [33]. It was found that the amount of biosorbed metal increased with biomass and the amount of metal available. The effective adsorption of HM was maintained during 10 cycles of biosorption–acid-mediated desorption. Copper is selectively adsorbed by alginates [60]. For enhancing copper recovery from the media, sodium poly-styrenesulphonate (NaPSS) was added to alinate gels, but the addition of *Microcystis* sp. cells resulted in even better result [61].

Reportedly [17, 62] , recovery of nickel, cadmium, chromium and lead from industrial effluents was fulfilled with *C. sorokiniana* immobilized on loofa sponges. The maximum adsorption capacity for Cd и Ni was about 192 mg and 71 mg g^{-1} immobilized biomass, correspondingly. Adsorption was always higher in immobilized systems than that measured for free cells (loofa sponges without immobilized cells adsorbed a small amount of this metal). The maximum amount of lead was absorbed in 5 min at pH 5 and adsorption efficiency was 96%.

Microalgae *Chlamydomonas reinhardtii* immobilized in Ca-alginate beads was successfully used for Hg, Cd and Pb removal from aqueous solutions [63]. At pH 5.0–6.0 adsorption of Hg, Cd and Pb ions by immobilized microalgae was 89.5, 66.5, 253.3 mg g-1 dry weight correspondingly.

The efficient removal of uranium ions from both freshwater and seawater samples was achieved using cells of *Chlorella* sp. immobilized in polyacrylamide gel [64] It was also reported that this immobilized system can be effectively used through several cycles of adsorption-desorption process.

Cells of MA are currently used for separation and concentration of Pd, Pt, Pb, Cu, Cd and Au [31]. The selective biosorption of palladium and platinum by *C. vulgaris* cells immobilized on anion exchange resin Cellex-T was studied in highly acidic media (pH < 2) [65].

CONCLUSION

The results of many experimental studies summarized in this review suggest that immobilization can be beneficial for industrial cultivation of MA. Immobilization facilitates harvesting of MA biomass, increases the stress-tolerance of cultures and provides flexibility to design photobioreactor. All these advantages contribute to the higher productivity of MA in terms of biomass and value-added products and enhance their efficiency in wastewater treatment. Currently, the special biofilm photobioreactors (PBR) are being developed for generation of value-added biomass and metabolites as well as for wastewater treatment. Finally, it should be emphasized that successful application of immobilized MA under real-life conditions entirely depends on the informed choice of the organism, optimal carrier and immobilization technique.

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