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Micro algal Immobilization Techniques

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Abstract:

Immobilization of microalgae, as part of a global trend of immobilizing microorganisms in an assortment of matrices, is used for a wide variety of biotechnological applications that started over 40 years ago. The types of immobilization can be grouped as passive (natural or synthetic) and active (flocculant agents, chemical attachment, and gel encapsulation). Adsorbents (carriers) for passive immobilization can be natural or synthetic. Attachment of periphyton to different surfaces, used in ecology studies was discussed. The use of flocculant agents, chemical attachment and gel entrapment methods should be differentiated in active immobilization techniques. Gel entrapment in natural polysaccharide matrices is the most widely used immobilization technique for microalgae in particular. Carrageenan, agar and alginate are the most employed among them. These systems produce no health hazards, are environmental friendly, produce no secondary pollution. There are still a number of technical aspects of this technology that could be developed. A general perspective of the field with known examples from common literature was presented in this review.

Keywords: Adsorbents, Carrageenan, Encapsulation, Immobilization, Microalgae.

Introduction:

The use of microalgae in biotechnology has been increased in recent years, these organisms being implicated in food, cosmetic, aquaculture and pharmaceutical industries (Borowitzka & Borowitzka, 1988.), but small size of single cells implies a problem in the application of biotechnological processes to those organisms. In order to solve those problems, cell immobilization techniques have been prospered (Jyothi, 2016). An immobilized cell is defined as a living cell that, by natural or artificial means, is prevented from moving independently from its original location to all parts of an aqueous phase of a system (Ramakrishna and Prakasham, 1999; Tampion and Tampion, 1987). The concealed concept is that immobilized microalgae in matrices may assist the required biotechnological applications from the mass culture of the microalgae, either a specific metabolite or removal of pollutants. This concept evolved from the basic nature of its components, the microalgae and the immobilizing matrix (Luz and Yoav, 2010). Most of the general immobilization techniques for microorganisms can be easily modified and applied to microalgae, adding a design factor that these are photosynthetic microorganisms that require light. Six different immobilization types have been defined: covalent coupling, affinity immobilization, adsorption, confinement in liquid–liquid emulsion, capture behind semi-permeable membrane, and entrapment in polymers (Mallick, 2002). These types of immobilation can be grouped as ‘‘passive” (using the natural tendency of microorganisms to attach to surfaces – natural or synthetic – and grow on them) and ‘‘active” (flocculant agents, chemical attachment, and gel encapsulation) (Cohen, 2001; Moreno-Garrido, 2008).

Passive immobilization

Adsorption

Many microorganisms (including some groups of microalgae) have a natural tendency to attach to surfaces and grow on them (Robinson et al., 1986). This characteristic can be exploited in order to immobilize cells on carriers of different types (Codd, 1987). These processes are easily reversible. Adsorbent materials (carriers) for passive immobilization can be natural or synthetic. With respect to natural carriers, efforts have been made involving loofa biomass. Loofa sponges are the fibrous support of the fruit of different species from the genus Luffa (*L. cylindrical: L. aegyptiaca, L. operculata, L. acutangula*). This carrier is non-toxic, non-reactive, cheap, mechanically strong and stable in long-term cultures (Liu et al., 1998). Akhtar et al. (2004) used loofa sponge biomass in order to immobilize cells of *Chlorella sorokiniana*, to remove nickel (II) from aqueous solutions. This immobilized system demonstrated to accumulate 25% more nickel than free cells after 20 min exposition. Ogbonn et al. (1996) reported the possibility of the co-use of chitosan in order to increase the flocculating process of free cells over the loofa surfaces, for cells that have no natural trend to attach this type of support. A problem on designing research involving loofa sponge biomass is repeatability. Structure of the skeleton of fruits varies from a plant to another in function of culturing conditions,

each loofa sponge has different structure (Liu et al., 1998). Passive immobilization of algal cells on loofa sponges seems to be a very promising field for industry or commercial purposes.

Synthetic materials are also widely used in passive immobilization experiments. Urrutia et al. (1995) immobilized *Scenedesmus obliquus* cells in polyvinyl and polyurethane in order to remove nitrate from water and also compared the survival of adsorbed cells with entrapped cells by mixing concentrated cells with one of the pre-polymers. Cellular growth was found to be higher for adsorbed cells than for entrapped cells, possibly due to the toxicity of the pre-polymers. Yamaguchi et al. (1999) achieved a noticeable degradation of hydrocarbons by the colorless hydrophobic microalgae *Prototheca zopfii*, adsorbed to 8 mm-cubes of polyurethane foam in a bubble reactor. Huang et al., 2003, immobilized *Chlorella pyrenoidosa* in polyvinyl acetate (PVA) sulfate for removing nitrate and phosphate, showed that the microalgae reproduced rapidly inside a PVA gel carrier under pH ranging from 5 to 10. The immobilized system had higher removal efficiency in the water when close to neutral pH and efficiency to remove nitrate reached 80%; meanwhile, the highest efficiency for removing phosphate was 88%, but decreased over time to 56% (Huang et al., 2003). Similarly *Chlorella vulgaris* and *Scenedesmus acutus* had been immobilisesd by Travieso et al. (1999) in Polyurethane foam for removal of chromium. Kannaiyan et al., 1997, immobilized the nitrogen-fixing cyanobacteria *Anabaena azollae* in polyurethane foam. Foam-immobilized cyanobacteria treated with fungicides stimulate nitrogenase activity and increase ammonia production at significantly higher rates. Inoculation of rice field with this formulation significantly increases ammonia that the cyanobacteria excrete into the flood water of the rice fields, increases chlorophyll content of the plants, and eventually increases rice grain and straw yields. The cyanobacterium *Phormidium laminosum,* immobilized on polymer foams, was demonstrated to have potential value for removing nitrates in a continuous-flow system (Garbisu et al., 1993). *Spirulina maxima* immobilized in polymers enhanced removed more than 90% of ammonium from waste water (Canizares et al. 1993). Attachment of periphyton to different surfaces can also be used in ecology studies. Admiraal et al. (1999) used microbenthic algae colonizing glass discs to measure different levels of metal pollution. Danilov and Ekelund (2001) compare settlement patterns of periphyton on glass, wood and plastic in different lakes, noticed glass is the most preffered material to attach on than wood and plastic. Diatoms attachment to glass slides and population growth on this type of surfaces has been studied by Brandini et al. (2001) in a subtropical estuary a whole year, comparing the differences caused by depth, light, temperature and grazing pressure. Nayar et al. (2005) also studied the settlement of diatom species (*Skeletonema costatum* and *Thalassiosira rotula*) together with cyanophytes (Synechococcus sp.,) in glasses in a tropical estuary of Singapore. Robinson et al., 1986 have been used glass-immobilized filaments of *Anabaena* sp., in hydrogen production experiments. These support materials can be used with species showing natural tendency to attach and aggregate (diatoms and cyanophytes).

Active immobilization

The use of flocculant agents, chemical attachment and gel entrapment methods should be differentiated in active immobilization techniques

Flocculant agents

In order to avoid tediuos centrifugation when algae are intended to be removed from a liquid medium flocculant agents were used. Chitosan has been the most widely used among the flocculants. Chitosan is a linear amino polysaccharide of b-D-glucosamine (2-amino-2-deoxy-b-D-glucan) units joined by (1, 4)-linkages (Oungbho and Muller, 1997). It is obtained through chitin (obtained from exoskeletons of crustaceans) alkaline deacetylation. This polysaccharide posess positive-charged amino groups, providing sites for adsorbing negative charged particles and it is proved to be useful for a huge number of microalgal species (Lubian, 1989). As it is biodegradable, it can be used in harvesting of algae for nutritional purposes (Gualtieri et al., 1988). The inconvenience of chitosan in immobilizing techniques is its weak stability. High viscosity chitosan gels (2% p/v) showed a higher chemical stability in the experiments. Chitosan can interfere in the growth of immobilized algae. Moreira et al. (2006) found low growth rates of *Phaeodactylum tricornutum* immobilized in alginate treated with chitosan as additional hardener, while cells immobilized in alginate beads hardened with $CaCl_2$ or $SrCl_2$ showed increasing rates of 30 and 76 times, respectively. *Scenedesmus sp*. when immobilized in chitosan, showed higher growth rate and accomplished a 70% nitrate and 94% phosphate removal within 12 h of incubation (Fierro et al., 2008).

Chemical attachment

Chemical interaction causes damages in cellular surface and reduces viability of cells hence it is not recommended when living cells are intended to be immobilized. The effectiveness of Ion attraction depends on pH and ionic strength of the surrounding media (Codd, 1987). Neverthless, some experiments do not require active metabolism of cells, and non-living organisms are used in chemical attachment immobilization techniques. The adsorption capacity of two floc-type biosorbents (milk casein floccules and glutaraldehyde) was compared in order to remove a *Heterosigma akashiwo* a marine microalga that can accumulate Cd and Pb from aqueous solution (Seki and Suzuki, 2002).

Gel entrapment

It is the widely used technique for algal immobilization. Gel entrapment can be performed by the use of synthetic polymers (acrylamide, photocrosslinkable resins, polyurethanes), proteins (gelatine, collagen or egg white) and natural polysaccharides (agars, carrageenans or alginates) (Codd ,1987).

Synthetic polymers for gel entrapment.

Microalgae are immobilized in various polymers for different biotechnological purposes, such as morphology studies, production of fine chemicals, energy production, and wastewater treatment (Lebeau and Robert, 2006; Ignacio, 2008). In polymeric immobilization systems, similar to other biofiltration systems, there is physical separation between the microorganisms and the treated wastewater. The microorganisms are immobilized (trapped) alive within the polymer because its pores are smaller than the microorganisms, while the fluid flows through it and sustains their metabolism and eventual growth (Cohen, 2001). Immobilization in polymers is especifically important in wastewater treament because it solves the inherent problem of biomass produced by suspended microalgae in the wastewater (Travieso et al., 1992; Valderrama et al., 2002). Several synthetic (acrylamide, polyurethane, polyvinyl, resins) and natural polymer derivatives of algal polysaccharides (alginate, carrageenan, agar, agarose), and chitosan, an amino polysaccharide derived from chitin, has been experimentally used. Regardless of the polymers used, the material must be hydrophilic, allowing wastewater to diffuse into the bead. The most commonly used natural polymers are alginate and carrageenan (Bashan, 1998; Ignacio ,2008). Some natural polymers can dissolve in highly contaminated wastewater, while synthetic polymers do not. Also, natural polymers are more vulnerable to environmental degradation by microbes. However, diffusivity is higher in natural polymers and they are less hazardous (Leenen et al., 1996).

The microbial suspension is mixed with the polymer and then, the mixture is solidified to produce a polymeric gel, by linking the monomers to each other to form a polymer with as little interaction with the living microorganisms, leaving the microbes inside the matrix intact. Polymerization can be achieved by several physical and chemical treatments. Solidification can be done by linking the monomers with di- and multi-valent cations. The rate of solidification can be increased or decreased by changing the temperature and different chemical and photochemical reactions (Cohen, 2001).The mechanical strength of the final polymer increases with the increase in concentration of the monomers and the cross-linking agents used. Spherical beads are made by slowly dropping the mixture through a small orifice, such as a syringe or specific equipment designed for that purpose. Once produced, the beads can be used directly or with secondary multiplication in a growth medium to increase the number of microorganisms within the bead (Bashan et al., 2002). Beads also can be dried, as for agricultural inoculants (Bashan et al., 2002) or when used with dead biomass. Immobilised microalgae showed several improvements in the metabolism, function, and behavior of the microalgae. Immobilization in alginate beads of the hydrocarbon-rich microalgae, *Botryococcus braunii* and *Botryococcus protuberans*, yielded a significant increase in chlorophyll, carotenoids, dry weight, and lipids during the stationary and resting growth phases, compared to free-living cells. Photosynthesis in both species was enhanced, relative to free cells with delayed senescence (Singh, 2003). Immobilization in chitosan protected the cell walls of *Synechococcus sp.* against NaOH toxicity, showed better growth than free cell cultures (Aguilar-May et al., 2007). Blanco et al. (1999) entrapped *Phormidium laminosum* in Polysulphone (a thermoplastic material) and epoxy resin (bisphenol and epichlorohydrin) to check the biosorption capacity of Cu(II), Fe(II), Ni(II) and Zn(II). Epoxy resins are not suitable for entrapping living cells because of their toxicity of the components. Thepenier et al. (1985) has been immobilized *Porphyridium cruentum* cells in polyurethane foam for production of polysaccharides. Jeon et al. (2002) , described the technique of immobilizing in polyvinyl alcohols and glutaraldehyde. Silica gels can be used for immobilizing microalgal cells. Rangasayatorn et al. (2004) checked the cadmium adsorption by immobilized *Spirulina platensis* in alginate and silica gel. Cadmium

adsorption of silica gel entrapping cells is as high as the alginate entrapped cells. Stark and Rayson (2000) used immobilized polysilicate materials (Chlorella vulgaris cells) in comparing metal ion binding capacities.

Natural polysaccharides for gel entrapment

Gel entrapment in natural polysaccharide matrices is the most widely used immobilization technique for microalgae in particular. Carrageenan, agar and alginate are the most employed among them. Carrageenan is a term for polysaccharides prepared from some Rhodophyceae (from the families Gigartinacieae and Solieriaceae) by water alkaline extraction. Carrageenan consists of alternating 3-linked-b-D-galactopyranose and 4-linked-a-D-galactopiranose units. It precipitates as a gel in the presence of cationic compounds such as metal ions, amines, amino acid derivates and water-miscible organic solvents (Tosa et al., 1979). Different isomeric forms of carrageenan (i, j and k) are primarily produced by *Aghardhiella subulata* and *Chondrus crispus* (Burdin and Bird, 1994). Hardening processes in order to increase the mechanical stability of carrageenan-based matrixes have also been designed (Chamy et al., 1990). Travieso et al. (1996) compared the nutrient removal capacity of three microalgal species (*C.vulgaris, Chlorella kessleri* and *Scenedesmus quadricauda*) immobilized in different matrixes including j-carrageenan. Agar is a sulphated galactan obtained from some species of red algae (from the genus *Gelidium, Pterocladia* or *Gracilaria*) (Burdin and Bird, 1994). The major gel forming component of agar (agarose) consists of a linear chain of sequences of (1–3) linked-b-D-galactopyranosyl units and (1–4)-linkages to 3,6-anhydro-a-D-galactopyranosyl units. Agar is a thermoreversible gel. This polymer is best suitable for immobilizing microalgal cells (Codd, 1987 and Papageorgiou,1987). Agar melts around 85^0C and solidifies between 35- 40^0C. Species able to resist a short thermal shock of this level should be selected for agar immobilization. Temperatures over 30^0C could damage a wide variety of marine microalgae (except cyanophytes and some species from the genus *Dunaliella, Nannochloropsis* or *Tetraselmis)*. Agarose has been used as an immobilization matrix type fixed-bed for C. vulgaris in experiments of Cu (II) biosorption (Aksu et al., 1998). They reported that metal adsorption capacity of agarose–microalgae system was lower than that reported for Ca-alginate in the same conditions. Immobilization of *Dunaliella salina* in agar significantly improved production of glycerol, in comparison with free-living cells (Thakur and Kumar, 1999).

The most widely used polysaccharide gel for entrapping living cells is alginate. Alginates constitute a family of unbranched binary copolymers of 1–4-linked-b-D-mannuronic acid and a-L-guluronic acid (Smidsrod and Skja k-Braek, 1990). Commercial alginates are extracted from brown algae, mainly from the genus *Laminaria (L. hyperborea, L. digitata, L. japonica*), the species *Macrocystis pyrifera, Ascophyllum nodosum, Lesonia negrescens* or species of the genus *Sargassum,* although all brown algae contain alginate in different proportions reaching up to 40% of dry weight (Ertesvag and Valla, 1998). A major advantage of alginate gel entrapment is that immobilized cells do not suffer extreme physical–chemical condition changes during the immobilization process (Araujo and Andrade, 1996). The polymer is soluble in cold water and forms thermostable gels. Hertzberg and Jensen (1989) described a protocol for immobilizing marine microalgae in alginate beads. Pane et al. (1998) immobilized *Tetraselmis suecica* in Ca-alginate beads and compared the cellular growth of immobilized and free cells, finding that the chlorophyll content per cell in immobilized cells was higher. Moreno-Garrido et al. (2005) checked the growth of immobilized cells and Ca-alginate bead stability involving 11 marine microalgal species, finding that the stability of beads can also depend on the immobilized species. Immobilization of the wallless marine microalga Dunaliella tertiolecta in alginate in hypersaline medium produce significant amounts of glycerol (Grizeau and Navarro, 1986).

Microencapsulation has also been developed by co-use of alginate and polylysine (Jen et al., 1996). Other microencapsulation strategy for microalgae is described by Joo et al. (2001), a mixture of 2% sodium carboxymethyl cellulose, 2% $CaCl_2$ and microalgae is stirred in 0.8% sodium alginate till the capsules are formed. After washing, the capsules are submerged in 2% $CaCl_2$ for 20 min for the purpose of hardening.

Conclusion

Currently, there are major advantages to ‘greener’ technologies. Immobilization technology for microalgae is a prime candidate as a green technology. From a practical view, microalgal systems use solar energy and need relatively small amounts of other inputs for operation. They are relatively easy to handle on a large scale because they have been used by compound producing industries for a very long time. These systems produce no health hazards, are environmental friendly, produce no secondary pollution, and their end products can be converted to additional by-products (like fertilizers or biofuel) that may further reduce costs. The compactness of these systems produces less sludge and is smaller and simpler to maintain than large fluidized beds. There are still a number of

technical aspects of this technology that could be developed, such as improvement of the polymers themselves to create ‘‘alloys” of organic polymers by mixing different polymer types to improve diffusion of effluents, development of less stressing immobilization procedures for microalgae, and optimizing selection of the proper polymer and the changes in metabolism of the microalgae that it induces.

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