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Review

Biotechnological Potential Uses of Immobilized Algae

MOHAMED SAYED ABDEL HAMEED[1] AND OLA HAMMOUDA EBRAHIM
Botany Department, Faculty of Science, Beni Suef University, Beni Suef, Egypt
[1]Corresponding author's e-mail: drmohamedsa@yahoo.com

ABSTRACT

The purpose of this review is to evaluate the different uses of immobilized algae. Details of the techniques of immobilization and the effects of immobilization on cell function are included. Special concern to the use of immobilized algae for wastewater treatment and heavy metals removal has been taken into consideration. The use of immobilized algae in these processes is efficient and offers significant advantages in bioreactors. Toxicity tests and long-term storage of immobilized algae are also considered. The future prospects of this area of algal biotechnology are considered.

Key Words: Immobilized algae; Biotechnology; Wastewater treatment; Heavy metal removal; Toxicity

INTRODUCTION

In the past 20 years, the use of immobilized enzymes or cell components for the production of a series of metabolites has become a branch of biotechnology of rapidly growing importance. Although in the initial stage most of the research work on immobilization dealt with systems designed for the release of products, synthesized by enzymes or multi-enzyme complexes, a more recent development focuses on the immobilization of complete cells or cell agglomerates (Becker, 1995). An immobilized cell is defined as a cell that by natural or artificial means is prevented from moving independently of its neighbours to all parts of the aqueous phase of the system under study (Tampion & Tampion, 1987).

To a certain extent these systems resemble natural environmental conditions as many microorganisms grow in a biotype, where they are also immobilized by encapsulation in slimes or as a partner of symbiotic systems. Although the pioneering work with immobilized cells mostly employed heterotrophic organisms, a number of scientific reports today deal with studies on plant cells, algae, cyanobacteria and photosynthetic bacteria. These phototrophic organisms offer several prospects for use in immobilization techniques, because they can use sunlight as their sole, or major, energy source to make products from the substrates of photosynthesis.

To date, there have been a considerable number of papers concerning immobilized algae. However, since these papers have been published in a great diversity of journals and conference proceedings, the task of keeping up to date with published material proved to be difficult for workers interested in this growing field of cell biotechnology. The purpose of this review is to sum up the work being carried out on algal cell immobilization, including studies on both eukaryotic algae and prokaryotic Cyanobacteria.

Immobilization techniques. In principal, six different types of immobilization methods can be distinguished. They are covalent coupling, affinity immobilization, adsorption, confinement in liquid-liquid emulsion, capture behind semi-permeable membrane and entrapment (Mallick, 2002). Entrapment is the most frequently used method in laboratory experiments. Entrapment methods are based on the confinement of the cells in a three dimensional gel lattice. The cells are free within their compartments and the pores in the material allow substrates and products to diffuse to and form the cells. Several synthetic (acrylamide, polyurethane, polyvinyl, etc.) and natural polymers (collagen, agar, agarose, cellulose, alginate, carrageenan, etc.) are used for this purpose. However, for algal immobilization the most frequently used natural gels are alginate and carrageenan.

Effects of immobilization on growth and physiology of immobilized algal cells. Though growth rates of immobilized cells are generally found to be lower than those of corresponding free cell cultures (Bailliez *et al*., 1985; Robinson *et al*., 1985; Abdel Hameed, 2002). An opposite trend was demonstrated by Chevalier and de la Noue (1985a). They reported that the maximum growth rate observed during the exponential phase was essentially the same for immobilized and free cells. Similar observations were reported (Tam *et al*., 1994; Lau *et al*., 1998a & b; Kobbai *et al*., 2000). However, Rai and Mallick (1992) reported a higher final yield for alginate immobilized *Anabaena* and *Chlorella* after 15 days in growth medium compared to free living cells. Gonzalez and Bashan (2000) also, reported increased growth of the microalga *Chlorella vulgaris* when co-immobilized and co-cultured in alginate beads with the growth-promoting bacteria *Azospirillum brasilense.*

The chlorophyll content of immobilized cells generally has been found to be higher than that of free cells (Robinson

et al., 1985; Bailliez *et al*., 1986; Abdel Hameed, 2002) probably, because self-shading and subsequent reduction in incident light in the immobilized state results in a promotion of photosynthetic pigment synthesis. So far, studies using chlorophyll as an index of biomass (Musgrave *et al*., 1982; Day & Codd, 1985; Grizeau & Navarro, 1986) have not taken this phenomenon into account; they would therefore probably over-estimate the numbers of immobilized cells and hence biomass and thus under-estimate mean cellular activity e.g., production of ammonia (Musgrave *et al*., 1982), glycollate (Day & Codd, 1985) or glycerol (Grizeau & Navarro, 1986).

Various studies have been made of photosynthetic oxygen evolution by direct comparing the activities of immobilized and free cells (Jeanfils & Collard, 1983; Robinson *et al*., 1986a; Bailliez *et al*., 1986). No difference in oxygen evolution was observed between free and immobilized *Chlorella* cells (Adlercreutz & Mattiasson, 1982; Robinson *et al*., 1985; Day & Codd, 1985). Other studies found that oxygen evolution was greater in the immobilized state, suggesting a fundamental change of metabolism (Bailliez *et al*., 1988; Garnham *et al*., 1992; Abdel Hameed, 2002). Immobilization enhances the stability of protein-chlorophyll complexes in *Euglena* (Tamponnet *et al*., 1985) and *Botryococcus* (Bailliez *et al*., 1986). De-Bashan *et al*. (2002a) also reported increases in pigment and lipid content, lipid variety and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense*.

There have been few studies on the respiratory rate of immobilized algae. Robinson *et al*. (1985) found that the mean respiratory rate of entrapped *Chlorella* to be lower than that of free cells, with both the size of immobilized algal particle and cell stocking density having effects on the mean rate. They suggested that only a proportion of the immobilized cells in the beads were metabolically active. In another study Tam *et al*. (1994) found that the cellular metabolic activity (indicated by respiratory rate per cell) of cells immobilized at low density was similar to the free suspended *Chlorella emersonii* cells and the cellular activity of immobilized cells would be reduced as stocking density increased (Robinson *et al*., 1986a).

Various microscopic studies have been carried out on the growth and morphology of immobilized algal cells and most have confirmed that immobilization slightly alters morphology of unicellular (Brouers *et al*., 1982; Chevalier & de la Noue, 1985a) and multicellular algae (i.e., small chain, Musgrave *et al*., 1983). Immobilized colonies of *Botryococcus* have been found to be more regular in shape and have a 2.5-fold higher mean size than free cell controls (Bailliez *et al*., 1985). Also, calcium alginate entrapped *Chlorella*, (a unicellular organism) tend to form small colonies (8 - 30 cells) when released from the immobilized gel (Trevan & Mak, 1988). Electron microscope studies on alginate immobilized *Chlorella* have found cell growth to be largely restricted to the periphery of the beads (Day & Codd, 1985; Robinson *et al*. 1986a). Further work has shown that this is a result of carbon dioxide limitation within the matrix (Robinson *et al*., 1986a). Cytological studies on *Euglena* (Tamponnet *et al*., 1985), using transmission electron microscopy, demonstrated that cells immobilized in calcium alginate and stored in darkness at 4˚C in 0.1 m $CaCl_2$ were fixed in the same cellular state as when the immobilization occurred.

Current uses of immobilized algae. Details of the algae, which have been successfully immobilized for the production of various materials are summarized in Table I.

Using the biomass for production of modern energy carriers such as electricity has a wide range of other environmental, social and economic benefits. Direct generation of electricity has been demonstrated by immobilizing the cyanobacterial species *Mastigocladus laminosus* (Ochiai *et al*., 1980) and *Phormidium* (Ochiai *et al*., 1983) onto SnO_2 optically transparent electrode with calcium alginate, functioned as an anodic photoelectrode on continuous illumination for periods of time adequate for use in a conventional electrochemical cell. This "living electrode" shows promise of use as a long-lived photoconverter of solar radiant energy to electric energy and as a suitable replacement for un-stable chloroplast systems. Such "living electrodes" have been used to generate photocurrents of up to 2.5 mA $mgChl^{-1}$ cm^{-1} in preliminary studies and have been operated for 20 days or more (Ochiai *et al*., 1980).

The use of immobilized algae for *de novo* biosynthesis of hydrogen has attracted much interest (Lambert *et al*., 1979; Muallem *et al*., 1983; Markov *et al*., 1993). Weetall and Krampitz (1980) reported that hydrogen gas has been produced on a continuous basis using two immobilized microorganisms. One organism, the cyanobacterium *Anacystis nidulans*, oxidizes water, producing molecular oxygen and reduces exogenous NADP. The second organism, *Rhodospirillum rubrum*, reoxidizes NADPH and produces molecular hydrogen. Thus the complete system oxidizes water and produces oxygen and hydrogen, while recycling NADP. Kayano *et al*. (1981) co-immobilized whole cells of *Chlorella vulgaris* and *Clostridium butyricum* in 2% agar gel. NADP was suitable as an electron carrier. The rate of hydrogen evolution increased with increasing NADP concentration. The optimum conditions for hydrogen evolution were pH 7.0 and 37˚C. The immobilized *C. vulgaris*-NADP-immobilized *Cl. butyricum* system continuously evolved hydrogen at a rate of 0.29 - 1.34 µmol/h per mg Chl for 6 days. On the other hand, the system without NADP evolved only a trace amount of hydrogen. Markov *et al*. (1995) have reported that high rates of hydrogen production are made possible by using immobilized cyanobacteria on hollow fibers. Guan *et al*. (2003) used the immobilized marine green alga *Platymonas subcordiformis* in a two-stage photo-biological hydrogen production. Antal and Lindblad (2005) immobilized the

unicellular cyanobacteria *Gleocapsa olpicola* and *Synechocystis* sp. PCC 6803 for hydrogen production under sulphur starvation conditions, which enhanced the rate of hydrogen production. Laurinavichene *et al*. (2006) used the immobilized *Chamydomonas reinhardtii* for hydrogen photoproduction. They indicated that sulphur deprivation is necessary for hydrogen photoproduction by immobilized cultures. They reported maximum total volume of hydrogen produced by the system (160 mL of reactor volume) was 380 mL over 23 days.

Nitrogen fixing *Anabaena* sp. have been immobilized in calcium alginate beads and films, polyvinyl foam and polyurethane foam for the photoproduction of ammonia (Musgrave *et al*., 1983; Kerby *et al*., 1983; Brouers *et al.*, 1989; Wang *et al*., 1991). The strategy employed has been to first grow cultures under N_2-fixing conditions to maximize N_2-fixing capacity, then to immobilize and maintain the biocatalyst under N-fixing conditions and to prevent the assimilation of the fixed N_2. This has been achieved by the incorporation of the chemical inhibitor of the main NH_4-assimilating enzyme glutamine synthase, methionine sulphoximine, in the medium (Musgrave *et al*., 1982, 83) and the use of mutants deficient in glutamine synthase (Kerby *et al*., 1983). Kannaiyan *et al*. (1994) immobilized *Anabaena azollae* in polyvinyl foam for the production of ammonia, which was produced continuously and in significant amounts. Extracellular amino acid production by immobilized N_2-fixing *Anabaena*, N_2-fixing *Anabaena* mutants have also been obtained (Kerby *et al*., 1987).

Immobilized phototrophs can be used to convert CO_2 into extracellular organic carbon. Gudin and Thepenier (1986) have made extensive studies on sulphated polysaccharide excretion by immobilized *Porphyridium cruentum*. Entrapment in polyurethane foams has enabled extracellular polysaccharide release to be obtained from a red-batch fluidized bed for 3 years (Gudin & Thepenier, 1986). Glycerol excretion and glycollate excretion have been obtained with immobilized *Dunaliella parva* and *D. tertiolecta* (Grizeau & Navarro, 1986) and *Chlorella emersonii* (Day & Codd, 1985). Hydrocarbon production and transformation has also been obtained with immobilized *Botryococcus braunii* (Bailliez *et al*., 1985, 88). Matsunaga *et al*. (1996) immobilized the cyanobacterium *Aphanocapsa* MN-11 in calcium alginate gel and coated on light-diffusing optical fibres for sulfated extracellular polysaccharide production. Their results indicated that sulfated extracellualr polysaccharide production depends on the number of immobilized cells and the light intensity.

Thakur and Kumar (2004) used different types of polymers as matrices for immobilization of *Dunaiella salina* for glycerol production. The maximum glycerol production of 9.2 µM/mg chl *a* was recorded in agar-agar immobilized algae. Gonzalez *et al*. (2005) used immobilized filaments of the Cyanobacterium *Anabaena* sp. for exopolysaccharide production.

As regards the different uses of immobilized algae, various studies have shown the feasibility of using the immobilization technique for long term storage of algae. Tamponnet *et al*. (1985) reported that immobilized *Euglena gracilis* alga was kept for more than two years in alginate-beads. Faafeng *et al.* (1994) reported that, immobilized algae when stored at low temperature (4ºC) in darkness, can resume normal growth after more than 12 months of immobilization. Romo and Martinez (1997) immobilized the Cyanobacterium *Pseudanabaena gateata* in alginate beads and stored them for a long time. The green alga *Scenedesmus quadricauda* was cultivated and entrapped into alginate beads for long term storage. The entrapped cells were alive and maintained their physiological activities after three years of storage in absolute darkness (Yean-Chang, 2001). *Isochrysis galbana* immobilized cells maintained their physiological activities after one year storage in absolute darkness (Yean-Chang, 2003). Similar results on immobilized *Chlorella vulgaris* cells were recorded by Abdel Hameed (2005) and Nowack *et al*. (2005).

Immobilized algae for wastewater treatment. Immobilized algae have been investigated for their potential use for the accumulation of waste materials, specifically for the uptake of nitrogen and phosphorus (Table II). The algal cells immobilized in carrageenan and alginate beads had the same efficiency to remove wastewater nitrogen and phosphorus as the free suspended cells (Chevalier & de la Noue, 1985a; Proulx & de la Noue, 1988; Garbisu *et al*., 1992; Garbisu & Hall, 1993; Tam & Wong, 2000). Immobilized *Scenedesmus* was found to be capable of removing 90% of the ammonium (within four hours) and 100% of phosphate (within two hours) from a typical effluent (Chevalier & de la Noue, 1985a), suggesting possible uses in the tertiary treatment of wastewaters. Travieso *et al*. (1992, 96) used immobilized *Chlorella vulgaris* for secondary wastewater treatment in a laboratory scale for 6 months. Taking into account the results of their experiments, their conclusion is: immobilized cells of *Chlorella vulgaris* could be used for sewage treatment; the down-flow fluidized column appears to be the better reactor for sewage treatment, the efficiency of the fluidized columns was not sensibly affected by changes in the range of photosynthetically active radiation studied. Tam *et al*. (1994) used *Chlorella vulgaris* cells immobilized in alginate beads for removing N and P from wastewater. They achieved significant reductions in wastewater ammonia and phosphate especially in reactors containing algal beads of high density. Their results suggested that immobilized *Chlorella vulgaris* can be used as secondary treatment process for domestic wastewater. Lee *et al*. (1995 & 96) applied the immobilization technology for the removal of nitrogen from wastewater. Lau *et al*. (1997 & 98a) studied the removal of nutrients from wastewater by algae immobilized in carragennan. Sawayama *et al*. (1998) used *Phormidium laminosum* immobilized on hollow fibres to

Table I. Summary of algal taxa successfully immobilized and techniques adopted for the production of electricity, hydrogen, ammonia, polysaccharides and glycerol.

Algal taxa	Immobilization matrix	Production of	References
Mastigocladus laminosus	Alginate	Electricity	Ochiai, *et al*. 1980
Phormidium sp.	Alginate	Electricity	Ochiai, *et al*. 1983
Anabaena azollae	Alginate and polyurethane foam	Hydrogen	Rao &Hall (1984)
A. cylindrica	Polyurethane foam	Hydrogen	Jeanfils & Loudeche, R. (1986)
A. cylindrica CCAP 1403/2a	Alginate	Hydrogen	Muallem *et al*. (1983))
A. cylindrica UTEX B629	Polyurethane foam	Hydrogen	Lambert *et al*. (1979)
A. sp. N-7363	Glass beads	Hydrogen	Kayano *et al*. (1981)
A. variabilis	Agar	Hydrogen	Markov *et al*. (1995)
Chamydomonas reinhardtii	Hollow fibers	Hydrogen	Laurinavichene, *et al*. (2006)
Chlorogloea fritschii	Fiber glass matrix	Hydrogen	Muallem *et al*. (1983)
Gleocapsa olpicola	Polyurethane foam	Hydrogen	Antal & Lindblad (2005)
Mastigocladus laminosus	Agar	Hydrogen	Rao &Hall (1984)
M. laminosus	Alginate/agar	Hydrogen	Muallem *et al*. (1983)
Nostoc muscorum	Polyurethane foam	Hydrogen	Rao &Hall (1984)
N. muscorum	Agar	Hydrogen	Rao &Hall (1984)
Oscillatoria lemnitica	Polyurethane foam	Hydrogen	Muallem *et al*. (1983)
Phormidium laminosum	Polyurethane foam	Hydrogen	Muallem *et al*. (1983)
Platymonas subcordiformis	Polyurethane foam	Hydrogen	Guan *et al*. (2003)
Porphyridium purpureum	Polyurethane	Hydrogen	Brouers *et al*. (1983)
Scenedesmus obliquus	Polyurethane	Hydrogen	Brouers *et al*. (1983)
Synechocystis sp. PCC 6803	Polyurethane/alginate	Hydrogen	Antal & Lindblad (2005)
Anabaena azllae	Alginate	Ammonia	Kannaiyan *et al*. (1994)
A. cylindrical	Polyvinyl foam	Ammonia	Jeanfils & Loudeche (1986)
A. sp	Alginate	Ammonia	Kerby, *et al*. (1983)
*A.*sp ATCC 27893	Alginate	Ammonia	Musgrave, *et al*. (1982)
Aphanocapsa MN-11	Alginate	Polysaccharide	Matsunaga, *et al*. (1996)
*A.*sp.	Alginate	Polysaccharide	Gonzalez, *et al*. (2005)
Porphyridium cruentum	Polyurethane	Polysaccharide	Gudin&Thepenier (1986)
Chlorella emersonii	Alginate	Glycerol&glycolate	Day and Codd, (1985)
Dunaliella parva	Alginate	Glycerol&glycolate	Grizeau & Navarro, (1986)
D. tertiolecta	Alginate	Glycerol&glycolate	Grizeau & Navarro, (1986)
D. salina	Agar, agarose, alginate, carrageenan	Glycerol	Thakur and Kumar (2004)

remove nitrate and phosphate ions from water. They concluded that treatment with immobilized *P. laminosum* appears to be an appropriate means for the removal of inorganic nitrogen and phosphorus from treated wastewater. Tam and Wong (2000) studied the effect of the immobilized algal bead concentration on the removal of nutrients from wastewater. Kobbai *et al*. (2000) applied the immobilization technique for two green fresh water microalgae *Scenedesmus obliquus* and *Chlorella vulgaris* for wastewater treatment. They reported 86 and 81% phosphorus removal and 100 and 98.4% ammonia removal in the two reactors, respectively after 7 days of treatment. De-Bashan *et al*. (2002b) used the microalga *Chlorella vulgaris* coimmobilized in alginate beads with the microalga growth-promoting bacterium *Azospirillum brasilense* for the removal of ammonium and phosphorus ions from synthetic wastewater. Wang and Huang (2003) co-immobilized *Chlorella pyrenoidosa* and activated sludge for nitrate and phosphate removal. They reported 80% nitrate removal in all experimental periods, meanwhile the highest removal efficiency of phosphate was 88%, but decreased in later experiments. Perez *et al*. (2004) used immobilized *Scenedesmus intermedius* Chod. and *Nannochloris* sp. for phosphorus and nitrogen uptake from wastewater.

Heavy metals removal by immobilized algae. One of the main interests for microalgae in biotechnology is focused on their use for heavy metals removal from effluents and wastewater (Mallik, 2002).

Immobilized algal systems have been tested by many workers for their efficiency in heavy metals removal. Immobilization generally tends to increase metal accumulation by biomass (Darnell *et al*., 1986; Aksu, 1998). Immobilized cells accumulate more metals than free cells (Khummongkol *et al*., 1982; Brouers *et al*., 1989). Immobilization of living biomass also provides protection to cells from metal toxicity (Bozeman *et al*., 1989). On the contrary, some reports show a higher metal sorbing efficiency of free cells compared to immobilized cells (Wong & Pak, 1992; Rangsayatorn *et al*., 2004). Size of immobilized bead is a crucial factor for use of immobilized biomass in bio-sorption process (Mehta & Gaur, 2005). It is recommended that beads should be in the size range between 0.7 and 1.5 mm, corresponding to the size of commercial resins meant for removing metal ions (Volesky, 2001).

Data in Table III revealed that more than 14 algal species have already been studied for their potential in heavy metals removal. *Chlorella vulgaris* cells immobilized in alginate supply a good system to remove a wide range of heavy metals (Tam *et al*., 1998; Ilangovan *et al*., 1998; Lau

Table II. Summary of algal taxa successfully immobilized for the removal of nitrogen and phosphorus.

Algal taxa	Immobilization matrix	Removal of	References
Anabaena CH_3	Alginate	Nitrogen	Lee *et al*. (1995)
Anabaena doliolum & Chlorella vulgaris	Alginate	Nit.& phos.	Rai & Mallick (1992)
Anabaena doliolum & Chlorella vulgaris	Alginate	Nit.& phos.	Rai & Mallick (1993)
Anabaena doliolum & Chlorella vulgaris	Alginate, agar, chitosan, carrageenan	Nit.& phos.	Rai & Mallick (1994)
Chlamydomonas reinhardti	Alginate	Nitrogen	Vilchez & Vega (1994)
Chlamydomonas reinhardtii	Alginate	Nitrogen	Vilchez & Vega (1995)
Chlamydomonas reinhardtii	Alginate	Nit. & phos.	Garbayo *et al*. (1996).
Chlorella emersonii	Alginate	Phosphprus	Robinson *et al*. (1988)
Chlorella emersonii	Alginate	Phosphorus	Robinson *et al*. (1989)
Chlorella emersonii	Alginate, agarose	Phosphorus	Robinson &Wilkinson (1994)
Chlorella emersonii	Alginate	Phosphorus	Robinson (1995)
Chlorella emersonii	Alginate	Phosphorus	Robinson (1998)
Chlorella kessleri &Chlorella vulgaris	Carrageenan, alginate, polyurethane, polystyrene	Nit. & phos.	Travieso *et al*. (1996).
Chlorella pyrenoidosa	Alginate	Nit. & phos.	Wang and Huang (2003)
Chlorella vulgaris	Alginate	Nit. & phos.	Tam *et al*. (1994)
Chlorella vulgaris	Carrageenan , alginate	Nit. & phos.	Lau *et al*. (1997)
Chlorella vulgaris	Carrageenan	Nit. & phos.	Lau *et al*. (1998b)
Chlorella vulgaris	Alginate	Nit. & phos.	Tam & Wong (2000)
Chlorella vulgaris	Alginate	Nit. & phos.	De Bashan *et al*. (2002b)
Chlorella vulgaris & Chlorella kessleri	Alginate	Nit. & phos.	Travieso *et al*. (1992)
Chlorella vulgaris &Scenedesmus bijugatus	Alginate	Nit. & phos.	Megharaj *et al*. (1992)
Chlorella vulgaris & Scenedesmus opliquus	Alginate	Nit. & phos	Kobbai *et al*. (2000)
Chlorella vulgaris & Scenedesmus quadricauda	Alginate , polyurethane	Nit. & phos.	Cordoba *et al*. (1995)
Dunaliella salina	Alginate	Nit. & phos.	Thakur & Kumar (1999)
Phormidium laminosum	Polyurethane	Nit. & phos.	Garbisu *et al*. (1991)
Phormidium laminosum	Polyvinyl	Nit. & phos.	Garbisu *et al*. (1992)
Phormidium laminosum	Polyvinyl	Phosphorus	Garbisu *et al*. (1993)
Phormidium laminosum	Cellulose	Nit. & phos.	Sawayama *et al*. (1998)
Phormidium sp.	Chitosan	Nit. & phos.	de la Noue &Proulx (1988a&b)
Pormidium uncinatum	Polyvinyl	Nitrogen	Gil & Serra (1993)
Scenedesmus bicellularis	Alginate	Nit. & phos.	Kaya *et al*. (1995)
Scenedesmus bicellularis	Alginate	Nit. & phos.	Kaya & Picard (1995)
Scenedesmus bicellularis	Alginate	Nit. & phos.	Kaya *et al*. (1996)
Scenedesmus bicellularis	Chitosan	Nit. & phos.	Kaya & Picard (1996)
Scenedesmus intermedius	Alginate	Nit. & phos.	Perez *et al*. (2004)
Scenedesmus obliquus	Carrageenan	Nit. & phos.	Chevalier & de la Noue (1985a)
Scenedesmus obliquus	Alginate	Nitrogen	Jeanfils & Thomas (1986)
Scenedesmus obliquus	Polyurethane, polyvinyl	Nitrogen	Urrutia *et al*. (1995)
Scenedesmus quadricauda & Sc. Acutus	Carrageenan	Nit. & phos.	Chevalier & de la Noue (1985b)
Spirulina maxima	Carrageenan	Nit. & phos.	Canizares *et al*. (1993,1994)

et al., 1998b; Abdel Hameed, 2002; Martinez *et al*., 2006). Significant accumulation of Co, Zn and Mn was also recorded for *Chlorella salina* cells immobilized in alginate (Garnham *et al*., 1992). Rai and Mallick (1992) and Mallick and Rai (1993, 94) also demonstrated a greater potential of immobilized *Chlorella vulgaris* and *Anabaena doliolum* in accumulating heavy metal ions, (Cu, Ni, Fe). Abdel Hameed (2002) reported that *Chlorella vulgaris* beads were more efficient in heavy metals removal from sewage than free cells. The efficiency in iron, nickel and zinc removal was higher in the immobilized cells than the free cells by 27, 23 and 25%, respectively. Travieso *et al*. (2002) designed a bio-alga reactor, which consisted of a pilot scale model that was operated with synthetic wastewater with an initial concentration of 3000 µG/l of cobalt ion. *Scenedesmus obliquus* was immobilized in the reactor, which was operated in a batch mode. They recorded that the maximum removal of cobalt ion of 94.5% was reached after 10 days. Accumulation of mercury by free and immobilized algal systems (*Chlorella emersonii*) was studied by Wilkinson *et al*. (1990). About 90% recovery was recorded after 12 days and the immobilized cell system was found to accumulate more mercury than free cell system. It was suggested that levels of mercury volatilization could be reduced by using agarose rather than alginate as the immobilization matrix (Robinson &Wilkinson, 1994).

Akhtar *et al*. (2004) used loofa sponge as a matrix for immobilization. The unicellular green microalga, *Chlorella sorokiniana* was immobilized on loofa (*Luffa cylindrical*) sponge and successfully used as a new bio-sorption system for the removal of lead (II) ions from aqueous solutions. The bio-sorption kinetics, were found to be fast with 96% of adsorption within the first 5 min. They found that the bio-sorption capacities were dependent on the pH of the solution. The loofa sponge-immobilized *C. sorokiniana* biomass could be regenerated using 0.1 m HCl, with up to 99% recovery. The desorbed biomass was used in five bio-sorption-de-sorption cycles, with no noticeable loss in the bio-sorption capacity. They concluded that loofa sponge-immobilized biomass of *C. sorokiniana* could be used as an efficient bio-sorbent for the treatment of lead (II) containing wastewater. Moreno-Garrido *et al*. (2005) used the marine microalga *Tetraselmis chui*, (Prasinophyceae) to perform a short term heavy metal accumulation experiment. Beads of

Table III. Summary of algal taxa successfully immobilized for heavy metal removal.

Algal taxa	Immobilization matrix	Removal of	References
Aulosira fertilissima	Glass beads	Ni, Cr	Banerjee *et al*. (2004)
Ascophyllum nodosum	Alginate	Cd	Volesky & Prasetyo (1994)
Chlorella	Alginate	Ni, Zn, Cd	Awasthi & Rai (2004)
Chlorella vulgaris & Anabaena doliolum	Alginate	Cu, Ni, Fe	Rai & Mallick (1992); Mallick & Rai (1993, 1994)
Chlorella emersonii	Alginate	Hg	Wilkinson *et al*. (1990)
Chlorella emersonii	Alginate, agarose	Hg	Robinson &Wilkinson (1994)
Chlorella homoshaera	Alginate	Cd, Zn, Au	De costa & Leite (1991)
Chlorella salina	Alginate	Co, Zn, Mn	Granham *et al*. (1992)
Chlorella sorokiniana	Loofa sponge	Pb	Akhtar *et al*. (2004)
Chlorella vulgaris	Polyacrylamide	Cu, Pb, Zn	Darnall *et al*. (1986 a&b)
Chlorella vulgaris	Alginate	Cu, Ni	Lau *et al*. (1998b)
Chlorella vulgaris	Alginate	Cu	Tam *et al*. (1998)
Chlorella vulgaris	Alginate	Cd, Zn	Ilangovan *et al*. (1998)
Chlorella vulgaris	Alginate	Fe, Ni, Zn	Abdel Hameed (2002)
Chlorella vulgaris	Alginate	Pb	Abdel Hameed (2006)
Chlorella vulgaris	Silica gel	Hg	Martinez *et al*. (2006)
Chlorella ellipsoida, Scenedesmus quadricauda & Navicula canalis.	Alginate	Cd, Co, Cu, Cr, Fe, Hg, Mn, Ni, Pb & Zn	Azab (2002)
Nannochloropsis gaditana	Alginate	Cu, Zn	Moreno-Garrido, *et al*. (2002)
Scenedesmus acutus & Chlorella vulgaris	Polyurethane, carrageenan	Cd, Cr, Zn	Travieso *et al*. (1999)
Scenedesmus obliquus	Alginate	Co	Travieso *et al*. (2002)
Spirulina platensis	Silica gel	Cd	Rangsayatorn *et al*. (2004)
Tetraselmis chui	Alginate	Cu, Cd	Moreno-Garrido, *et al*. (2005)

calcium alginate containing (or not) the algal cells were exposed to 820 μG L^{-1} Cu and 870 μG L^{-1} Cd during a 24 h period. They reported that practically all Cu was removed by the beads, beads with immobilized algae removed around 20% of total Cd, while beads without algae removed half of that percentage. Martinez *et al*. (2006) developed a method for mercury speciation in water using columns packed with *Chlorella vulgaris* immobilized on silica gel. This method was applied to the analysis of spiked tap, sea and wastewater samples.

Maintaining a viable organism during metal recovery can be very difficult. Processing complications caused by using living microorganisms or biomass can be avoided by using inactive or dead biomass, because non-living biomass does not need to be provided with nutrients and can be used for many process cycles. In addition, non-living biomass can be immobilized in a granular or polymeric matrix and used in conventional ion exchange equipment (Tsezos, 1985; Darnall *et al*., 1986b; Volesky, 1990). The advantages of using inactive or dead biomass over ion exchange resins can include lower cost, improved selectivity for specific metal interest, easy regeneration and in some cases higher capacity (Tsezos, 1985; Greene *et al*., 1986; Darnall *et al*., 1986b; Volesky, 1990; Maranon & Sastre, 1991).

These considerations prompted researchers to develop immobilized biomass beads (Ferguson *et al*., 1989). These beads contain *Sphagnum* peat moss immobilized in porous polysulfone and have been successfully used to remove zinc, manganese and cadmium from acid mine drainage waters. Greene and Bedell (1990) reported that immobilized, non-living algae are capable of reversibly binding a number of heavy metal ions. Another study by Spinti *et al*. (1995) proved that immobilized biomass beads, effectively remove heavy metals from wastewater under appropriate conditions. Tan *et al*. (2002) investigated the bio-sorption of copper by inactivated biomass of the brown seaweed *Sargassum baccularia*, immobilized into polyvinyl alcohol gel beads. They reported that the robustness and stability of the immobilized biomass beads could lead to the development of an efficient and cost-effective bio-remediation technology for the removal and recovery of toxic metals from aqueous solutions.

Toxicity assessments. Several studies on toxicity of metals on algae confirmed the deleterious effect of metals to biological macromolecules (Tripathi *et al*., 2004; Awasthi, 2004, 05; Awasthi & Das, 2005). Bozeman *et al*. (1989) used free and immobilized *Selenastrum capricornutum* in algal toxicity assays. They investigated cadmium, copper, Glyphosate, Hydrothol, Paraquat, pentachlorophenol and sodium dodecyl sulfate.

A simple microplate technique was adopted for toxicity assessment of a number of pesticides to immobilized cultures of the green alga *Selenastrum capricornumtum* (Abdel-Hamid, 1996). Frense *et al*. (1998) developed an optical biosensor for the determination of environmental impurities of water based on immobilized living algal cells. Naessens *et al*. (2000) constructed a new biosensor for the detection of some herbicides based on kinetic measurements of chlorophyll-a fluorescence in immobilized *Chlorella vulgaris* cells. The detection of 0.1 μG.L^{-1} of a single herbicide as is required by European Community legelisation for drinking water is possible with this algal biosensor especially for atrazine, simazine and diuron. Naessens and Tran-Minh (2000) made a biosensor using *Chlorella* microalgae immobilized on the membrane of an oxygen electrode to determine volatile organic compounds in form of aerosols by measuring the oxygen production during the algae photosynthetic process. Durrieu and Tran-Minh (2002) constructed a biosensor to detect heavy metals from inhibition of alkaline phosphatase

present on external membrane of *Chlorella vulgaris* microalgae. They immobilized the microalgal cells on removable membranes and was cultivated in the laboratory. They reported that its alkaline phosphatase activity is strongly inhibited in the presence of heavy metals. This property has been used for the determination of those toxic compounds. Banerjee *et al*. (2004) investigated the effect of nickel and chromium stress on the immobilized nitrogen fixing cyanobacterium *Aulosira fertilissima*. They reported that cell immobilization could protect the organism's growth against the toxicity of both heavy metals at LC_{50} as compared to lethal concentrations. Awasthi (2004) studied the effect of heavy metal toxicity on nitrate reductase (NR) activity. He reported a highly significant increase in NR activity in the immobilized *Chlorella vulgaris* over free cells when supplemented with nickel. A toxicity biosensor based on immobilized *Anabaena torulosa* for the determination of copper toxicity was developed by Chia *et al.* (2005). The live cyanobacteria cells were immobilized in a poly (2-hydroxyl ethyl methacrylate) (pHEMA) membrane and interfaced with an oxygen electrode. The analytical device allows the monitoring of cell inhibition due to copper toxicity through the changes of photosynthetic oxygen release. Slijkerman *et al*. (2005) studied the potential use of an in situ assay with immobilized *Chlorella vulgaris* as an indicator of effects on ecosystem functioning with regard to primary production. They used the herbicide linuron to induce direct effects on primary producers. They monitored direct and indirect changes in structure and function within outdoor models. Awasthi and Rai (2005) used immobilized *Scenedesmus quadricauda* to access the toxicity of nickel, zinc and cadmium on nitrate uptake. Shitanda *et al*. (2005) used the immobilized *Chlorella vulgaris* cells to access four toxic compounds in water. Chouteau *et al*. (2005) used a conductometric biosensor using immobilized *Chlorella vulgaris* microalgae as bio-receptors as a bi-enzymatic biosensor for heavy metal ions and pesticides detection in water samples. Luan *et al*. (2006) studied the removal and degradation of tributyltin (TBT) at 10, 50 and 100 μG Sn L^{-1} contamination levels by alginate immobilized *Chlorella vulgaris* beads during six consecutive cycles (4 days each). Their results suggested that the alginate immobilized alga *C. vulgaris* was able to continuously detoxify TBT into DBT and MBT for six consecutive cycles even at the highest TBT contamination level.

CONCLUSION

Generally, it has been claimed that immobilization results in better bio-catalyst stability and increased productivity over free systems. In case of wastewater treatment studies, the results were good. Immobilized systems were efficient in heavy metals removal and toxicity assessment tests. Therefore, immobilized algal systems currently offer various advantages over free systems.

Future prospects. The biotechnology of algal immobilization is still an open area for various researches in different fields. Screening for new products is processing in an increased rapid rate using the immobilized algae. The introduction of new techniques such as permeabilization of the algal cell wall may enable future utilization to be extended to both extra-and intra-cellular algal products (Robinson *et al*., 1986b). Genetic manipulation is another field of growing interest, which will increase our understanding of algal genetics and manipulation. The technology of immobilized algae proved to be very efficient in remediation, heavy metals removal and toxicity assessment. One of the most promising areas of research is using this technology to reduce environmental pollutions through bio-sorption and biodegradation of many harmful compounds.

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