Review

Immobilization of microbial cells: A promising tool for treatment of toxic pollutants in industrial wastewater

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The review articles on cell immobilization have been published since 1980 and reflect the general interest in this topic. Immobilized microbial cells create opportunities in a wide range of sectors including environmental pollution control. Compared with suspended microorganism technology, cell immobilization shows many advantages, such as resistance to toxic chemicals. This review presents the potential of immobilized microbial cells for treatment of toxic pollutants in industrial wastewater, the fundamentals, history and advantages of immobilized cells compared with suspended cells, characteristics of support materials and the principal methods of immobilization, with special emphasis for natural immobilization by cell adsorption.

Key words: Cell immobilization, microorganisms, adsorption, toxic pollutants, wastewater.

INTRODUCTION

Large-scale production of wastewater is an inevitable consequence of all contemporary societies. Most wastewaters are usually hazardous to human populations and the environment and must be treated prior to disposal into streams, lakes, seas, and land surfaces (Zhou et al., 2008; Bashan and Bashan, 2010). Although, the field of environmental biotechnology has been around for decades, starting in the early 20th century, the introduction of new technologies has enabled engineers and scientists to tackle the more contemporary environ-ment problems such as detoxification of hazar-dous wastes through the use of living organisms (Chen et al., 2005).

Traditional biological treatment processes can eliminate a large fraction of biodegradable organic compounds existed in wastewater. Moreover, the biological treatment cost is much lower than that of physical and chemical methods (Kumar et al., 2011). However, many hazardous compounds are poorly removed in conventional biological processes due to their toxicity. Furthermore, they also have adverse impact on the composition and activities of microorganism communities in activated sludge flocs, thus reducing the overall performance of these facilities. The removal of these compounds is a real challenge for waste treatment engineers and scientists (Wang et al., 2002).

Immobilization of microbial cells has received increasing interest in the field of waste treatment (Winnicki et al., 1982; Westmeier and Rehm, 1987; Heitkamp et al., 1990; Hallas et al., 1992; Cohen, 2001; Ahmad et al., 2012). Compared with conventional sus-pension system, the immobilized microorganism tech-nology offer a multitude of advantages, such as high bio-mass, high metabolic activity and strong resistance to

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toxic chemicals (Cassidy et al., 1996; Freeman and Lilly, 1998; Velankar and Heble, 2003; Junter and Jouenne, 2004; Wang et al., 2005; Wang et al., 2006; Zhou et al., 2008; Cai et al., 2011; Liu et al., 2012). Moreover, immobilized microorganisms could be cost effective since they can be used several times without significant loss of activity (Rhee et al., 1996; Devi and Sridhar, 2000). Therefore, immobilized microorganism technology has been explored as promising for wastewater treatment in the past few decades and in the near future (Zhou et al., 2008).

CELL IMMOBILIZATION

Immobilization is a general term describing a wide variety of the cell or the particle attachment or entrapment (Lopez et al., 1997). It can be applied to basically all types of biocatalysts including enzymes, cellular organelles, animal and plant cells. Currently, different kinds of immobilization have found wide applications not only in the field of biotechnology, but also in pharmaceutical, environmental, food and biosensor industries (Peinado et al., 2005).

The cell immobilization emerged as an alternative for enzyme immobilization (Cheetham et al., 1979; Parascandola and Scardi, 1980; Woodward, 1988). Immobilization of cells containing specific enzymes has further advantages such as elimination of long and expensive procedures for enzymes separation and purification and it is vital to expand their application by enabling easy separation and purification of products from reaction mixtures and efficient recovery of catalyst (Junter and Jouene, 2004; Stolarzewicz et al., 2011). In comparison with immobilized enzymes, immobilized cells provide new possibilities since they can be used as natural, water-insoluble carriers of required enzyme activities (Vojtisek and Jirku, 1983).

In the case of the immobilization of microbial cells, their field of application spreads from industrial to environmental process. Microorganisms retained on a carrier can be used in continuous and semi-continuous production processes allowing for significant cost decrease, as the biocatalyst does not need to be refilled (Wada et al., 1979; Park and Chang, 2000; Mrudula and Shyam, 2012).

Cell immobilization has been defined as the phy-sical confinement or localization of viable microbial cells to a certain defined region of space in such a way as to limit their free migration and exhibit hydrodynamic characteristic which differ from those of the surrounding environment while retaining their catalytic activities for repeated and continuous use (Dervakos and Webb, 1991; Freeman and Lilly, 1998; Covizzi et al., 2007; Amim et al., 2010).

Since the early 70s, when Chibata's group announced successful operation of continuous fermentation of L-aspartic acid (Coughlan and Kierstan, 1988), numerous research groups have attempted various microbial applications with immobilized cells (Ramakrishna and Prakasham, 1999). Environmental applications of immobilized microbial cells are reported by Bettmann and Rehm (1984), Anselmo et al. (1985), Sahasrabudhe et al. (1988), Oreilly and Crawford (1989), Beunink and Rehm (1990), Balfanz and Rehm (1991), Stormo and Crawford (1992), Cassidy et al. (1996), Wang et al. (1997), Wang et al. (2002), Wang et al. (2007), Zhang et al. (2007), Zhou et al. (2008), Bazot and Lebeau (2009), Wang et al. (2010), Ahmad et al. (2012) and Nickzad et al. (2012).

SUPPORT MATERIALS

The support selection is one of the crucial decisions to be made in the course of preparation of the immobilization process (Zacheus et al., 2000). For treatment of wastewater, support materials need to meet the following criteria: insoluble, non-biodegradable, non-toxic, non-polluting, light weight; flexibility in overall shape, high mechanical and chemical stability, high diffusivity, simple immobilization procedure, high biomass retention, minimal attachment of other organisms and preferably a low cost price (Leenen et al., 1996; Zacheus et al., 2000). Other criteria, such as physical characteristics (porosity, swelling, compression, material and mean particle behavior), as well as, possibility for microbial growth and solubility, are more application specific (Górecka and Jastrzębska, 2011).

The carriers are classified as inorganic material (zeolite, clay, anthracite, porous glass, activated charcoal, and ceramics) and organic polymers. Inorganic carriers were selected to immobilize microorganisms because they can resist microbial degradation and are thermostable (Cassidy et al., 1996; Verma et al., 2006). The organic polymeric carriers are more abundant than inorganic carriers and can be natural and synthetic polymeric carriers (Cassidy et al., 1996).

Several syn-thetic (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. The most commonly used polymers are the natural polymers alginate and carrageenan but these natural polymers are less stable in wastewater than synthetic polymers (Bashan, 1998; Arica et al., 2004; Moreno-Garrido, 2008; Stolarzewicz et al., 2011).

Alginates (polymers made of different proportions and sequences of mannuronic and guluronic acids extracted from brown algae) are easy to handle, nontoxic to humans, the environment, and the entrapped microorganisms, legally safe for human use, available in large quantities, and inexpensive. From a physiological pers-pective, a major advantage of alginate is that immobilized

cells do not suffer extreme changes in physicochemical condition during the procedure of immobilization and the gel is transparent and permeable (Bashan and Bashan, 2010). However, this substance cannot be maintained for a long period in aqueous solution because the encapsulation immobilized microorganism can easily be broken during the operation (Cassidy et al., 1996).

Chitosan is inexpensive, non-toxic property and possesses potentially reactive amino functional groups which can enhance the affinity of the carrier with the microorganisms. However, the mechanical stability of the carrier would decrease because of the biodegradability in the course of usage.

Other natural gels, such as agar, collagen andagarose, also can be used as microbial encapsulation carriers (Zhou et al., 2008). Some natural polymers are more vulnerable to environmental degra-dation by microbes. However, diffusivity is higher in natural polymers and they are less hazardous to produce (Leenen et al., 1996; Cassidy et al., 1996).

Synthetic polymeric supports are not easily biode-gradable and have much better mechanical performance compared with nature carrier. Materials, such as poly-acrylamide (PAM), polyvinyl alcohol (PVA), polyethylene-glycol (PEG) and polycarbamoyl sulphonate (PCS) were synthesized as encapsulation carriers (Leenen et al., 1996).

In order to improve the stability of gel carrier, various synthetic plastics, for example polypropylene (PP), polyethylene (PE), polyvinylchloride (PVC), poly-urethane (PU) and polyacrylonitrile (PAN) have been explored extensively as immobilized microorganism carriers more recently (Zacheus et al., 2000).

Among the various extensively used plastics carriers, polyurethane (PU) is one kind of outstanding carrier for entrapping microorganisms in piloted applications in practical wastewater treatment (Guimarães et al., 2002). Martins et al. (2012) reported potential of the Gram-negative bacterium *Serratia marcescens* and the yeast *Candida rugosa* to immobilization on polyurethane foam.

METHODS FOR IMMOBILIZATION OF MICROBIAL CELLS

Immobilization of microbial cells in biological processes can occur either as a natural phenomenon or through artificial process (Ramakrishna and Prakasham, 1999). Different immobilization types have been defined: covalent coupling/cross linking, capture behind semi-permeable membrane or encapsulation, entrapment and adsorption (Mallick, 2002). The types of immobilization 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) (Cassidy et al., 1996; Cohen, 2001; Moreno-Garrido, 2008).

Covalent bonding/Cross linking

The mechanism involved in this method is based on covalent bond formation between activated inorganic support and cell in the presence of a binding (crosslinking) agent. For covalent linking, chemical modification of the surface is necessary. Covalent attachment and cross-linking are effective and durable to enzymes, but it is rarely applied for immobilization of cells. It is caused mainly by the fact that agents used for covalent bonds formation are usually cytotoxic and it is difficult to find conditions when cells can be immobilized without any damage (Ramakrishna and Prakasham, 1999).

There are few reports of successful covalent binding of the cells and most of them concern yeast. Navarro and Durand (1977) published an article describing a successful way of covalent binding of *Saccharomyces carlsbergensis* on porous silica beads. Two years later, there was another publication concerning yeast (*Saccharmyces cerevisiae*, *Saccharomyces amurcea*) immobilization with this method on borosilicate glass and zirconia ceramics (Messing et al., 1979).

Entrapment

Entrapment is an irreversible method, where immobilized cells are entrapped in a support matrix or inside fibers. This technique creates a protective barrier around the immobilized microbes, ensuring their prolonged viability during not only processing but also storage.in polymers (Górecka and Jastrzębska, 2011). Entrapment is the most method extensively studied in cell immobilization. The matrices used are agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, epoxy resin, photo cross-linkable resins, polyacrylamide, polyester, polystyrene and polyurethane (Lopez et al., 1997; Ramakrishna and Prakasham, 1999).

Entrapment of the microorganisms in porous polymer carrier was often used to capture the microorganisms from suspended solution and then obtain the immobilized microorganisms. The polymer matrix used in this method confining microorganisms has porous structure, and thus the pollutant and various metabolic products could easily diffuse through into the matrix. In this method, a lot of porous polymers can entrap microorganisms under ambient conditions (Verma et al., 2006).

As a rule, the entrapment methods are based on the inclusion of cells within a rigid network to prevent the cells from diffusing into surrounding medium while still allowing penetration of substrate. Entrapment of cells in alginate gel is popular because of the requirement for mild conditions and the simplicity of the used procedure. Several reports are available employing alginate gel (Kierstan and Bucke, 1977).

Entrapment allows high mechanical strength, but contains some disadvantages, such as, cell leakage,

costs of immobilization, diffusion limitations, and deactivation during immobilization and abrasion of support material during usage. Another disadvantage is low loading capacity as biocatalysts have to be incorporated into the support matrix (Krekeler et al., 1991; Song et al., 2005; Gao et al., 2010; Stolarzewicz et al., 2011).

Encapsulation

Encapsulation is another irreversible immobilization method, similar to entrapment. In this process, biocatalysts are restricted by the membrane walls (usually in a form of a capsule), but free-floating within the core space (Górecka and Jastrzębska, 2011). The membrane itself is semi-permeable, allowing for free flow of substrates and nutrients (when cells are used as a biocatalyst), yet keeping the biocatalyst inside. The factor determining this phenomenon is the proper pore size of the membrane, attuned to the size of core material. This limited access to the microcapsule interior is one of the main advantages of microencapsulation, for it protects the biocatalyst from the harsh environmental conditions. As most immobilization method, it prevents biocatalyst leakage, increasing the process efficiency as a result (Park and Chang, 2000).

The encapsulation method was used to enclose the microorganisms in a polymer-gel by Jen et al. (1996) and is one of the most frequently used in laboratory experiment up to now and there is far away engineering application for wastewater treatment (Lozinsky and Plieva, 1998).

However, even though in encapsulation, high cell loading can be achieved, but the capsules are still very weak (Song et al., 2005). The diffusion limitation is one of the inevitable drawbacks associated with encapsulation method (Lozinsky and Plieva, 1998).

Adsorption

The immobilization passive or adsorption natural of microorganisms onto porous and inert support materials is similar to the adsorption of colloid particles (Araujo et al., 2010). Apparently, it is the first example of cell immobilization and probably is the simplest method of reversible immobilization (Monsan et al., 1987; Klein and Ziehr, 1990).

This technique is based on the physical interaction between the microorganism and the carrier surfaces, while frequently reversible is simple, cheap and effective. The immobilization of microorganisms on properly chosen adsorbents stimulates microbial metabolism, protects cells from unfavorable agents, and preserves their physiological activity (Nikovskaya, 1989; Kozlyak et al., 1991, 1993).

Different from the inherent problems associated with cell entrapment, cell immobilization through adsorption provides a direct contact between nutrients and the immobilized cells thus, eliminating such concerns (Braschler et al., 2005). This cell immobilization technique involves the transport of the cells from the bulk phase to the surface of support (porous and inert support materials), followed by the adhesion of cells, and subsequent colonization of the support surface (Kilonzo and Bergougnou, 2012).

Adsorption is based on weak forces, however, still enabling an efficient binding process. Usually in bonds formation, several forces are involved: van der Waals forces, ionic and hydrophobic interactions and hydrogen bonds. Both electrostatic and hydrophobic interactions govern the cell-support adhesion, which is the key step in controlling the cell immobilization on the support (Hsu et al., 2004, Górecka and Jastrzębska, 2011).

In contrast to ceramics, wood chips and straw, fibrous matrices provide adequate supporting surfaces for cell adsorption (Talabardon et al., 2000; Chu et al., 2009) due to their high specific surface area, void volume, mechanical and permeability, low pressure drop, diffusion problems and toxicity, maximum loading, biodegradability and durability and low cost and high availability (Huang and Yang, 1998). Their natural configuration also allows them to trap more cells than other materials (Yang and Shu, 1996; Yang and Lo, 1998)

Polyurethanes foams for immobilization by adsorption

Polyurethanes (PU) are one of the most versatile materials in the world today. They are known for being a perfect material for footwear, machinery industry, coatings and paints, rigid insulation, elastic fiber, soft flexible foam, medical devices (Romaškevič et al., 2006). Some time ago PU was found to be applicable in the biochemical and biotechnological fields and flexible polyurethane foams have gained relevance as microbial carriers for their good mechanical properties, high porosity, large adsorption surface, resistance to organic solvents and microbial attack, easy handling, regenerability and cost effectiveness (Patil et al., 2006). In general, the high rates of sorption of positive charge and hydrophobic character of the polyurethane foam, allow interaction with most microbial cell surfaces (Afghan et al., 1984; Wang et al., 2009). They are inexpensive and easily regenerated by extraction or washing with solvents (Belyakova and Schevchenko, 1986).

The microbial immobilization in polyurethane, combined with the use of bioreactors improved significantly the biodegradation process of phenols and derivatives (Pai et al., 1995). The highest efficiency in the degradation of o-phthalate by cells *Bacillus*-spp. immobilized in polyurethane foam, in relation to alginate was reported by

Table 1. Factors affecting the microbial cell adsorption.

Material support	Environmental factor	Microbial cell surface
	Flow velocity	
Texture or roughness	pH	Hidrofobicity
Hydrophobicity	Temperature	Extracelular appendices
Surface charge	Cations	Extracellular polymeric substances
	Antimicrobial agents	

Source: Donlan (2002) and Kilonzo and Bergougnou (2012).

Patil et al. (2006). Chanthamalee; Luepromchai (2012) described the efficiency of the *Gordonia* sp immobilized in polyurethane foam in removing lubricants boats, while Silva et al. (2006) have described that the immobilization of bacteria in polyurethane foam increased resistance to high concentrations of sulphate.

Factors affecting microbial cell adsorption

There are many factors (such as the age and the physiological state of cells) that influence the sorption of microbial cells. The surface structures of bacterial cells (flagella and other appendages), superficial charges and hydrophobicity also play an important part in the cell adherence to solid surfaces (Donlan, 2002; Chae et al., 2006; Oulahal, et al., 2008). The composition of the medium, its pH, and environmental conditions considerably influence the adsorption of cells by changing their electrokinetic potential (Stanley, 1983; Fletcher and Pringle, 1986; Kilonzo and Bergougnou, 2012).

The surface properties of adsorbents also affect the process of cell immobilization (Busalmen and Sanchez, 2001, Ubbink and Schar-Zammaretti, 2007). The degree of cell immobilization depends on the structure and the size of adsorbent pores (Arinbasarova et al., 1982). The nature of adsorbents is also important. Organic adsorbents are chemically stable and show a great variety of surface properties and pore structures, whereas inorganic adsorbents are resistant to biological degradation are affordable, and can be easily regenerated. The disadvantage of inorganic adsorbents is that they are soluble in alkaline solutions (Samonin and Elikova, 2004). The principal factors affecting the microbial cell adsorption are presented in Table 1.

CONCLUSIONS

Immobilized microbial systems currently offer various advantages over free systems. One of the most promising areas of research is using this technology to reduce environmental pollutions through biodegradation of many harmful compounds. The application of immobilization technology to environmental area is in its preliminary stages, but the results seen so far are promising.

The immobilized cells will be useful to treat the waste to convert the toxicant into nutrient, biomass and CO_2 via biodegradation through their intermediates. Better biodegradation rate was observed in immobilized cells due to absence of internal and external mass transfer resistance. An immobilized cell is one of the approaches for incorporating fungal biomass into an engineering process. The advantage of the process based on immobilized biomass include enhancing microbial cell stability, allowing continuous process operation and avoiding the biomass - liquid separation requirement.

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