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MICROBIAL CELLULOSE AS SUPPORT MATERIAL FOR THE IMMOBILIZATION OF DENITRIFYING BACTERIA

Abbas Rezaee[1*], Hatam Godini [2], Hossein Bakhtou [1]

[1]*Environmental Health Dept., Faculty of Medical Sciences,Tarbiat Modares University,Tehran, Iran*
[2]*Department of Environmental Health, Lorestan university of medical sciences, Khoramabbad, Iran*
Iran

Abstract

The purpose of this study was to investigate the immobilization of denitrifying bacteria on microbial cellulose (MC) for biological denitrification. A novel denitrifying bacterium, *Pseudomonas stutzeri*, was immobilized in microbial cellulose and introduced into an up flow packed bed reactor in order to remove nitrate from synthetic influent. The MC presented the high biomass concentration throughout the experiment, achieving 3.4 mg biomass/g support. The efficiency of the system for denitrification was tested under different running conditions. Complete biological denitrification of the synthetic effluent was achieved at low hydraulic residence times, less than 4 h, and high nitrate concentration (200 mg NO_3-N/L). The immobilization of the bacterium in MC increased the adsorption capacity, decreased the cell leakage from the beads, resulted in higher activity of the immobilized cells, and allowed better operational control.

Keywords: Acetobacter xylinum, biological denitrification, immobilization, microbial cellulose

1. Introduction

The application of cell immobilization techniques to the wastewater treatment process has recently gained much attention (Isaka et al., 2007). These techniques not only offer a high cell concentration in the reactor tank for increasing efficiency, but also facilitate the separation of liquids and solids in the settling tank (Chen et al., 2000). The conventional treatment processes generally require a long residence time to retain slow growing organisms such as denitrifyers in the system; moreover, a relatively large volume of reaction is necessary to obtain a high reactor capacity. The reactor capacity can be improved by increasing the biomass retention time using an immobilized cell system (Chen et al., 2000). Nitrate is a common water contaminant that can cause health problems in humans. Also, eutrophication or groundwater contaminations by nitrate, which cause serious social and economical problems, are related to an increase of nitrate concentration in the aquatic environment (Foglar et al., 2005).

Biological denitrification has proved to be one of the most feasible, advanced, selective, and cost effective processes for removing nitrate by dissimilatory reduction (Song et al., 2005), which transforms it into nitrogen gas using biodegradable carbon compounds as the energy source (Ovez et al., 2006). Denitrification can be achieved either in suspended or attached growth systems. Since the 1980s, biological denitrification has been performed using immobilized cells (Nakano et al., 2007; Zala et al., 2004). Since then, many studies of denitrification using immobilized cells have been undertaken (Li and Logan 2004). The treatment of wastewater in packed bed bioreactors using immobilized cells is attracting increasing interest and has prompted the examination of different immobilization methods and a variety of carriers (Kariminiaae-Hamedaani et al., 2003; Hsu et al., 1996). This process has been applied to nitrate removal of wastewater and contaminated groundwater, with the technology achieving a high

* Author to whom all correspondence should be addressed: Fax: +98 21 82883575, Email: abbasrezaee@yahoo.com

removal rate per volume (Cao et al., 2004; Peres et al., 1999). The type of support media used for anoxic biomass immobilization can affect the efficiency of a bioreactor, since the number of cells adhering to the support may vary from one support to another. Several natural materials (agar, agarose, collagen, alginates and chitosan) and synthetic polymer materials (polyacrylamide, polyurethane, polyethylene glycol and polyvinyl alcohol) have been applied to the immobilization (Fang et al., 2004; Jianlong et al., 1998).

Among the various matrixes that are available, the MC has been chosen for its ease of use, low economic cost, low toxicity, and high operational stability (Rezaee et al., 2005; Son et al., 2003). The MC synthesized by *Acetobacter xylinum* is identical to that made by plants in respect to molecular structure. The secreted polysaccharide is free of lignin, pectin, and hemi celluloses, as well as biogenic products, which are associated with plant cellulose (Hong et al., 2001). This cellulose is highly crystalline, has high water absorption capacity, and has mechanical strength in the wet state, ultra fine network structure (Astley et al., 2001). Based of these features, there is an increasing interest in the development of new fields of application (Bae et al., 2004). The objective of this study was to investigate the immobilization of denitrifying bacteria on MC and performance evaluation of a pack bed reactor with MC as support media for biological denitrification.

2. Materials and methods

2.1. The microbial cellulose production

Acetobacter xylinum (ATCC 23768) was used in this study. It was grown in SH medium at 28 ºC under static culture conditions. SH medium was composed of 2% (W/V) glucose, 0.5% (W/V) yeast extract, 0.5% peptone, 0.27% (W/V) Na_2HPO_4 and 0.115% (W/V) citric acid (Kimura et al., 2001). Preinoculum for all experiments was prepared by transferring a single colony grown on SH agar medium into a 50 mL Erlenmeyer flask filled with liquid SH medium. After 5 days of cultivation at 28°C, the cellulose pellicle formed on the surface of the culture broth. Ten milliliters of the cell suspension was introduced into a 500 mL Erlenmeyer flask containing 100 mL of a fresh SH medium. The culture was carried out statically for 72 h and the cell suspension derived from the synthesized cellulose pellicle was used as the inoculum for further cultures. The stationary cultures in Erlenmeyer flasks filled with different volumes of the medium were maintained for seven days. The cellulose sheets were removed after cultivation and rinsed with distilled water and purged of from bacterial and medium residues by 2% sodium dodecyl sulfate (SDS) and 4 % NaOH solutions in a bath of water at a temperature of 90°C. They were cut into 5-10 mm pieces and used for cell immobilization and bioreactor media.

2.2. Denitrifyer and culture conditions

The *Pseudomonas stuzeri* used in the current study was isolated from an up flow anaerobic sludge blanket wastewater treatment plant, Tehran, Iran. The bacterium was isolated in basal salt medium (BSM) contained per Liter of distilled water: K_2HPO_4, 0.9 g; KH_2PO_4, 0.45 g; NH_4Cl, 0.45 g; $MgSO_4$, 0.2 g; $CaCl_2{\cdot}2H_2O$, 0.02 g; $FeCl_3$, 0.005 g; and trace elements solution, 1 mL containing (mg/L): $ZnSO_4{\cdot}7H_2O$, 400; $CoCl_2$, 50; $NiCl_2{\cdot}6H_2O$, 200; $NaB_2Mo_4{\cdot}2H_2O$, 300; $CuSO_4{\cdot}5H_2O$, 10; $MnSO_4{\cdot}H_2O$, 500. The culture plates were incubated at 37°C for 48 h, and the colonies were then transferred to 500 mL Erlenmeyer flasks containing 250 mL of liquid culture medium with the synthetic wastewater containing: 0.1 g $MgSO_4.7H_2O$, 1g KH_2PO_4, 2.5g K_2HPO_4, 0.17 g $CaCl_2.2H_2O$, 5g NaCl. Incubation was carried out with 120 rpm rotary shaking at 37 °C for 48 h. The cells were harvested from the culture medium by centrifugation (2000 × *g*, 10 min, 4 °C) and later used for the immobilization procedures.

2.3. Cell immobilization

The surface of the MC was modified by SDS and NaOH. After this treatment the MC were washed with distilled water before further use. The cell suspension of *Pseudomonas stutzeri* (OD 650 nm 0.5, unless stated otherwise) and MC were shaken together for 96 h on a rotary shaker (120 rpm) and subsequently washed twice with BSM to remove free cells. To determine the effectiveness of the immobilized cell mass, the method of Chen et al. (1996) was modified and a biomass estimation method based on the determination of cell protein content in the MC was established.

2.4. Pilot plant

The pilot scale plant used in this study consisted of a Plexiglass Column [90 cm . 21 cm (ID)], which was packed with immobilized MC (up to 60 cm) and closed at the top with a rubber stopper and at the bottom with glass wool. Inlet and outlet points were set at 2 cm from the bottom and top of column, respectively. The immobilized MC was suspended with the synthetic wastewater.

The column was initially filled with influent, and after complete removal of nitrate, the continuous process was started by running the influent through the reactor at different flow rates. The denitrification rate of the continuous bioreactor with immobilized cells was determined by Eq. 1.

$$\text{Denitrification rate } (N\text{-}NO_3 L/d) = [N\text{-}NO_3]_{in}[M\text{-}NO_3]_{out}(R/V) \quad (1)$$

where R is the waste water flow rate, $[NO_3\text{-}N]_{in}$ and $[NO_3\text{-}N]_{out}$ is influent and effluent NO_3 concentration (g $N\text{-}NO_3$ L/d) respectively, and V the reactor

volume. All the experiments were conducted at a temperature of 25°C and a pH of 7.0.

2.5. Analytical methods

Samples of influent and effluent were collected and filtered through membranes of 0.45µm pore size. Filtrations were analyzed for nitrate, nitrite and COD. The analyses were performed according to standard methods (APHA, 2005). All data reported in our study refer to steady state conditions.

The specific surface area of the MC was determined using the multiple BET method (Micromeritics, Gemini) with nitrogen gas as the adsorbate. Scanning electron microscopy (SEM) and protein assays were used to identify the *Pseudomonas stutzeri* immobilized on MC surface. Biofilm morphology was studied by SEM using standard procedures (Tuscaloosa, 2003). Briefly, the immobilized bacteria were fixed on the surface of MC biofilm with 2.5% (w/v) glutaraldehyde overnight.

Fixed samples were dehydrated by successive treatment with 30, 50, and 75% (v/v) ethanol (each step for 15 min) and finally with pure ethanol for 1 h. Dehydrated MC beads were dried in a CO_2 atmosphere. The surface of particles was examined using a XL30 Philips model SEM operated at 20 kV.

2.6. Biomass determination

The MC was placed on a clear glass plate and was cut into several fine pieces with a sharp surgical knife. The crushed cellulose pieces were collected in a test tube. SDS solution was added (10 %) and sonic treatment was performed for 20 min to extract cell protein in ice water bath. After centrifugation, the cell protein of the MC was measured according to the Bradford method; thereafter, the biomass of the sample was estimated from the standard curve of biomass versus protein concentration.

2.7. Mass loss

Before the reactors were filled, the MC was oven dried at 90 ºC for a minimum of 24 h. The dried MC placed into each reactor were weighed and recorded. Upon the completion of the denitrification tests, the masses of the MC were measured again. The MC was thoroughly washed until minimal bacteria were attached to the MC. Then, the media were oven dried, following the same procedure as before, and weighed.

The loss of mass provides an objective measure of the degradation of the MC. The appearance of the MC was also observed and recorded during the course of the study as a subjective measure of degradation. This measure included changes in color, and the height of the MC in each column.

3. Results and discussion

3.1 Characterization of microbial cellulose

The thick, gelatinous membrane formed in static culture conditions. Characteristics of the MC used in the study are listed in Table 1. The cellulose produced in the form of a gelatinous membrane can be molded into any shape and size during its synthesis, depending on the cultivation technique and conditions used. *Acetobacter xylinum* is a simple gram negative bacterium which has an ability to synthesize a large quantity of high-quality cellulose organized as twisting ribbons of microfibrillar bundles.

Table 1. Main characteristics of the MC used in the experiments as support media

Characteristics	***Amount***	***Unit***
Moisture	96.28	%
Apparent density	990	g/L
Iodine number	210	-
BET	632.015	Sqm/g

During the process of actual biosynthesis, various carbon compounds of the nutrition medium are utilized by the bacteria, then polymerized into single, linear β-1-4-glucan chains and finally secreted outside the cells through a linear row of pores located on their outer membrane. This structure, which is not found in plant cellulose, results in high cellulose crystallinity (60–80%) and an enormous mechanical strength. Particularly impressive is the fact that the size of MC fibrils is about 100 times smaller than that of plant cellulose (Shirai et al., 1997). This unique nano morphology results in a large surface area that can hold a large amount of water and, at the same time, displays great elasticity, high wet strength, and conformability.

The small size of microbial fibrils seems to be a key factor that determines its remarkable performance as an effective adsorbent. Unlike celluloses of plant origin, microbial cellulose is entirely free of lignin and hemicelluloses (Lynd et al., 2002). A vigorous treatment with strong bases at high temperatures allows the removal of cells embedded in the cellulose net, and it is possible to achieve a high performance absorbent. The specific surface area of the biofilm determined by the BET method was 650 ± 0.08 m^2/g. The mass loss in the MC as a result of alkaline treatment was found to be around 15–20%, which is attributed to the loss of protein and nucleic acid contents. The loss in mass of the MC is plotted against operation time (Fig.1).

The most important loss of mass can be observed in the first 40 h. The percent loss obtained has been calculated based on the MC total mass. The losses of total mass following 72 h of shaking became as low as 0.35%. It is evident that such losses are not expected to affect the process in bioreactor.

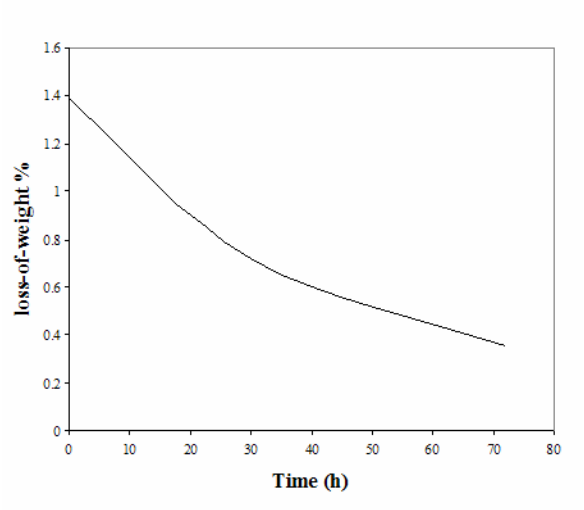

Fig.1. The loss in mass of the MC in the reactor

3.2. Immobilization of Pseudomonas stutzeri

The amount of cells immobilized on the MC was measured as a function of optical density (OD 650) of the cell suspension used for immobilization. Preliminary experiments have shown that adsorption on MC was completed after 72 h (Fig.2). The current data show that the amount of cells immobilized on the surface depended on the density of the bacterial cell suspension. The results indicate that the MC matrixes provided the best special adhesion (3.39 mg biomass/g support). However, the reactors with MC presented the highest absolute biomass retention in the reactor.

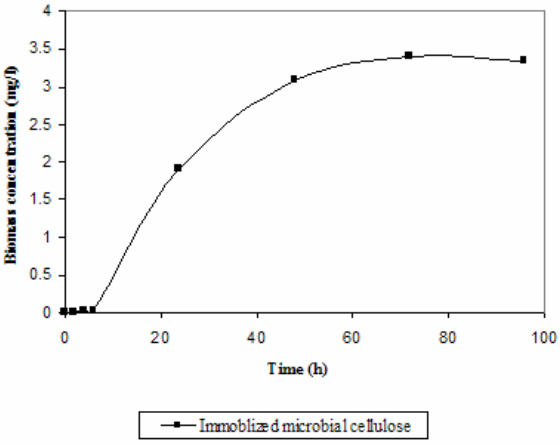

Fig.2. Amount of immobilized bacteria into the MC

This effect could be explained by increased attraction of negatively charged bacterial cells to positive groups. Micrographs of SEM illustrate the adhesion of the *Pseudomonas stutzeri* (quantified as biomass) on support material throughout the experiment (Fig .3). The immobilization of viable cells is a versatile tool that serves to increase the stability of a microbial system, allowing its application under extreme environmental conditions, its reuse and the development of continuous bioprocesses. Advances in biotechnology using immobilization technology have shown that conditions can be modified to enhance the activity of specific microorganisms performing a desirable process. The application of immobilization for wastewater treatment has been developed and tested, and there are currently several full scale wastewater treatment plants using this technology (Tanaka et al., 1991).

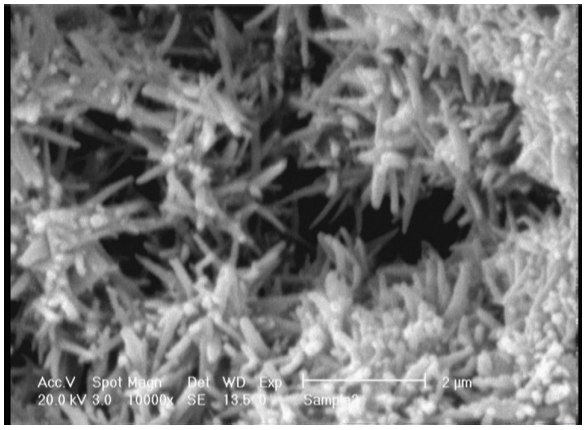

Fig.3. *Pseudomonas stutzeri* adhering to the surface of the MC (original magnification, x10,000).

Through the immobilization process, the denitrifying bacteria are provided with a very suitable environment to perform with maximum effectiveness. This is important when assessing the application of denitrification technologies for treatment plants because construction costs can be limiting factors.

3.3. Assay of biological denitrification

To measure the biological denitrification activity of immobilized MC, a lab-scale bioreactor was used for testing the reduction of nitrate in a continuous mode. In the long term operation test, the synthetic waste water was fed applied with the following conditions: 200 mg NO_3-N/L, 600 mg/L ethanol as the carbon source, and an adjusted pH of 7.2. The performance of continuous denitrification for a period of 20 days was illustrated in Fig.4. The denitrification ability of the bioreactor at the optimum carbon source (3:1 ratio) proved stable under relatively low HRT (3 h) and at a loading rate of 1.61 kg NO_3-N/m^3.d. The nitrate content of the effluent (up to 7.53 mg/L NO_3 –N) was less than the proposed WHO limit (10 mg/L NO3-N) (WHO, 2004). The average denitrification rate with MC as support media was 1.6±.02 kg NO_3-N/m^3.d.

The denitrification rate for wood chips and wheat straw as alternative biofilter media was reported to be 1.36 kg NO_3-N/m^3.d using an anoxic filter (Saliling et al., 2007). A maximum biological denitrification rate for 60 mg/L nitrate was reported as .88 kg NO_3-N/m^3.d with HRT 3 h in a continuous flow pilot bioreactor containing immobilized

Pseudomonas butanovora cells (Peter et al., 2003). Complete nitrate removal efficiency was achieved with HRTs of 9, using a bench-scale anoxic filter. The MC lost 2.3 ± .17% of its initial dry mass over a period of 20 days. There was no observed height loss in the MC reactor.

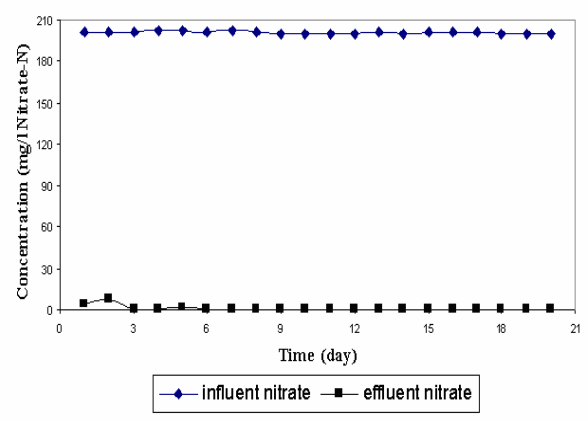

Fig.4. The time course denitrification with continuous packed bed reactor using cellulose as support for immobilization

Wheat straw and wood chips as carriers lost 37.7 ± 2.7% (60.4 ± 4.3 g reduction) of their initial dry mass compared to only 16.2 ± 5.2% (117 ± 43 g reduction) for wood chips over a period of 140 days (Saliling et al., 2007). Soares and Abeliovich (1998) reported that when wheat straw was used as the carbon source for biological denitrification all the water soluble components and a significant portion of the cellulose and hemicellulose had been lost by the end of the experiment, while lignin and mineral components remained unchanged.

4. Conclusions

In this study, *Pseudomonas stutzeri* was immobilized on treated MC with SDS and NaOH. The amount of bacteria attached to the MC support was to a certain extent dependent on the optical density (650 nm) of cell suspension during the immobilization process. Experimentally, it was confirmed that the immobilized bacterium on MC could achieve a nitrate removal efficiency of 99% at an initial nitrate concentration of 200 mg NO_3-N/L under continuous flow conditions. The MC offers an interesting alternative to other types of media such as plastic and ceramic. For better effectiveness, it would be useful to co-immobilize more microbial strains with complementary biodegradation activity. However, the use of MC as support media in the field scale requires more studies.

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