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Micrococcus luteus Strain in Free and Immobilized Form is Capable of Simultaneous Utilization of Aromatic and Aliphatic Xenobiotics

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Abstract

Aliphatic and aromatic xenobiotics can cause serious ecological problems due to their widespread occurrence and toxicity. The investigated strain *Micrococcus luteus* BN56 appeared to be capable of degrading both aromatic and aliphatic xenobiotics simultaneously as carbon and energy sources. Adaptation of the investigated strain was carried out with repeated additions of xenobiotic to the medium. The cells showed the highest capacity for biodegradation after 10 passages simultaneously degrading both xenobiotics - 10 g/l (100%) phenol and 10 g/l (100%) n-hexadecane, in 20 active cycles of operation (duration of 12 - 36 h). The simultaneous biodegradation was not hindered by either of them. By applying an *in situ* immobilization procedure using poly(acrylamide) (PAAam) and poly(ethylene oxide) (PEO) cryogel matrices, these processes were intensified, stabilized and reached 40 cycles of repeated operation of successful simultaneous biodegradation of the aromatic and the aliphatic xenobiotics (20g/l + 20g/l). This capability can be further implemented in different real-world bioremediation processes.

Keywords: *Micrococcus luteus*, biodegradation, immobilization, cryogel, phenol, n-hexadecane

Резюме

Алифатните и ароматните ксенобиотици могат да причинят сериозни екологични проблеми поради широкото им разпространение, наличност и токсичност. Изследваният щам *Micrococcus luteus* BN56 се оказа способен да разгражда едновременно ароматни и алифатни ксенобиотици като въглеродни и енергийни източници. Адаптация на изследвания щам е извършена с многократно добавяне на ксенобиотик към средата. Клетките показаха най-висок капацитет за биоразграждане след 10 пасажи, реализирайки едновременно разграждане и на двата ксенобиотика - 10 g/l (100%) фенол и 10 g/l (100%) n-хексадекан, разградени в 20 активни цикъла на работа с продължителност 12 - 36 часа. Едновременната биодеградация не е възпрепятствана от никой от тях. Прилагайки *in situ* процедура за имобилизация, с използване на матрици от поли (акриламид) (PAAam) и поли (етилен оксид) (PEO), тези процеси бяха интензифицирани, стабилизирани и достигнаха 40 цикъла на повтарящо се успешно едновременно биоразграждане на ароматния и алифатния ксенобиотик (20 г/л + 20 г/л). Тази способност може да бъде приложена в последствие в различни реални процеси на биоремедиация.

Introduction

Aliphatic alkanes and aromatic compounds can be found in nature due to the discharge of a broad variety of harmful chemicals resulting from many anthropogenic activities. Such xenobiotics prevail in various contaminated environments and can cause harmful disturbances to human health (Dawson *et al*., 2007). With the development of modern civilization, humanity is undoubtedly faced with the problem of environmental protection (Kumar and Kuma, 2004). Pollution due to phenol and petroleum oil, with alkanes being the major fraction, is a prevalent ecological hazard (Singh *et al*., 2009). One of the most reliable approaches to solving these problems is to use the natural abilities of different types of microorganisms to break down

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and detoxify xenobiotic substances (Abbasian *et al*., 2015; Lockington *et al*., 2015; Khraisheh *et al*., 2020; Al-Saadi and Mohammad Ali, 2020). The simultaneous biodegradation of these pollutants has become a very important challenge. The application of physicochemical processes for bioremediation of contaminated environments can cause secondary problems in the resultant effluents, therefore, biological treatments are preferred. Some microorganisms possess the catabolic capability of utilizing xenobiotic compounds, producing innocuous end products. Reports exist in literature that *Pseudomonas stutzeri* (Tambekar *et al*., 2012), *Nocardia hydrocarbonoxydans* NCIM 2386 (Shetty *et al*., 2008), *Micrococcus varians* EPRIS14, *Bacillus subtilis*-EPRS12, *Pseudomonas alcaligens* (Safari *et al*., 2019), *Bacillus licheniformis*–EPRIS21, *Bacillus laterosporus*-EPRIS41, *Pseudomonas putida*-DAF1, *Bacillus firmus*-EPRIS22 and *Acientobacter* sp. EPRIS32 (Shourian *et al*., 2009, Bayoumi and Abul-Hamd, 2010, Saraireh *et al*., 2020), are able to perform phenol degradation. n-Hexadecane can be utilized by *P. putida, Rhodococcus erythroplolis* and *Bacillus thermoleovorans* isolated from contaminated soils (Abdel-Megeed *et al*., 2010), as well as by members of *Achromobacter*, *Acinetobacter*, *Alcolizenes, Arthrobacter, Flavobacterium, Nocardia, Corynebacterium, Gordonia*, etc. (Pozdniakova *et al*., 2008; Binazadeh *et al*., 2009; Silva *et al*., 2019). Therefore, many bacterial strains have been isolated with the ability to degrade n-hexadecane and phenol separately, which are often used to represent aliphatic and aromatic pollutants. However, few strains have been reported to have the dual ability (Colombo *et al*., 1996; Sun *et al.,* 2012).

Employment of immobilization procedures in biotechnological processes raises biotechnology to a qualitatively new level. It should be noted that in the case of immobilized cells, all the advantages arise not as a result of changes in the properties of microorganisms, but as a result of significant changes in the conditions for biotechnological processes and proper selection of suitable immobilization matrices (Baron and Willaert, 2004). In this way performing bioremediation processes by immobilized cells favors the realization of faster, more stable, more effective processes, not disturbed by the toxicity of xenobiotics, that can be repeated many times for a long period of time (Hutchinson *et al.,* 2020).

The aim of the present work was to achieve higher simultaneous biodegradation of aromatic and aliphatic xenobiotics by *Micrococcus luteus* cells, applying the immobilization technique.

Materials and Methods

Microorganism, media and cultivation

The strain employed in this study was isolated from hydrocarbon-contaminated soil, followed by adaptive selection on a nutrient medium with the following mineral composition (g/l): $K_2HPO_4.3H_2O$, 4.8; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.2; trace elements (mg/l): $CaCl_2.2H_2O$, 2.0; $MnCl_2.4H_2O$, 0.4; $NiCl_2.6H_2O$, 0.4; $ZnSO_4.7H_2O$, 0.4; $FeCl_3.6H_2O$, 0.2; $Na_2MoO_4.2H_2O$, 0.2 and by standard enrichment techniques. In the course of the experiments aliphatic and aromatic hydrocarbons were used as sole carbon sources. The pH of the medium was adjusted to 7.0. The minimal salt medium (MSM) was solidified as MSM phenol agar by addition of 1.8% agar. Cultures grown on MSM agar supplemented with 500 mg/l phenol were used to inoculate 500 ml Erlenmayer flasks containing 100 ml liquid MSM. Cultures were incubated during long-term semi-continuous biodegradation processes with shaking at 120 rpm at 28°C. Adaptation procedures were carried out by multiple passages on MSM agar with phenol.

Assays

Bacterial growth was assessed by determination of the optical density (OD_{610} nm) of the culture.

Analytical methods included assay of phenol concentrations (in mg/l) by colorimetrical determination with 4-aminoantipyrine according to APHA (American Public Health Association, 1999).

Biodegradation of hexadecane was measured by the substrate depletion. Whole cultures were extracted with equal volumes of n-hexane and residual n-hexadecane was quantified by gas chromatography using a Hewlett-Packard model 5859 instrument equipped with a flame ionization detector.

Immobilization

Immobilization of the bacterial cells of the investigated strain was carried out to select the most appropriate carrier. Two types of matrices were tested. *In situ* immobilization into polymer cryogels was accomplished. Cells were harvested by centrifugation at 8000 x g and afterwards were re-suspended in a phosphate buffer solution (0.06 M, pH 7.0 at 20°C). Cell density of $60x10^9$ g^{-1} carrier material was applied. Then, 0.12 g of 10^6 g mol^{-1} poly (ethylene oxide) (PEO), Union Carbide Corporation, Danbury, USA) was added to 5 ml of the cell suspension thus obtained under stirring

at 20°C. It was kept overnight to ensure complete dissolution of the polymer. Then, 0.006 g of cross-linking agent (N, N'-methylene bisacrylamide; Merck, Darmstadt, Germany) and 0.006 g photoinitiator [(4-benzoylbenzyl) trimethylammonium chloride; Sigma-Aldrich, Schnelldorf, Germany], dissolved in 1 ml water were added. The resulting mixture was poured into Teflon dishes (each portion consisted of 1 ml), in 5 dishes with a diameter of 20 mm, forming a 4 mm thick layer. The samples were frozen at –20°C for 2 h and irradiated with the full spectrum of UV-Vis light from a 400-W metal halide flood lamp (Dymax 5000-EC; Dymax Corporation, USA) for 5 min at a dose of $2.85x10^5$ J m^{-2} and an input power of 930 W m^{-2}.

The cells were immobilized in polyacrylamide (PAAm) cryogels following the same procedure, except that 0.3 g of acrylamide (Merck) and 0.03 g of N, N'-methylene bisacrylamide were dissolved in 1 ml of water and mixed with 5 ml of cell suspension. Finally, 0.075 ml initiator (30 vol. % H_2O_2; Merck), were added prior to freezing. The diameter of each matrix disc was 1.6 – 1.7 cm and its weight was 1.0 – 1.1 g for the PAAm and PEO carriers, respectively.

Scanning Electon Microscopy (SEM) observations

SEM observations were carried out to evaluate the immobilization processes. Preparation was performed using cryogel disks with and without cells which were frozen in a freezer at -20°C, then fractured and freeze dried in an Alpha 1–2 Freeze Drier (Martin Christ) at –55°C and 0.02 mbar for 24 h. Afterwards, the gel specimens were fixed on a glass substrate and coated with a thin layer of gold for 60 sec. The interior and surface morphology of the gels and immobilized bacteria were studied using a JEOL JSM-5300 SEM operating at 10 kV. Magnification varied from 150 to 1000.

Results and Discussion

The adaptation capabilities of the bacterium in a semi-continuous process with repeated additions of xenobiotic were studied. The preliminary contact of the microorganism with the toxic compound is the way to increase its biodegradation potential. The catabolic ability of the investigated strain was followed to prove its potential for bioremediation. Multiple passages on solid media (10 passages) with phenol as a sole carbon and energy source were carried out. It could be assumed that the prolonged exposure to phenol probably led to catabolic changes in the cells, leading to better adaptation and survival (Baptiste *et al*., 2019). The adaptability of cells of *M. luteus* BN 56 was clearly demonstrated by the increased number of active cycles of operation for xenobiotic degradation leading to full depletion. Studies of the biodegradation processes in the presence of aromatic and aliphatic toxic substances in the medium were further conducted in a semi-continuous mode of cultivation. The hydrophilic (phenol) and hydrophobic (n-hexadecane) xenobiotic substances were supplied in equal concentrations. Each new cycle started after the depletion of xenobiotics and with the addition of fresh quantities of phenol and n-hexadecane (500 mg/l each). Substrate concentration of 500 was fully degraded in both cases so it was further used in our investigations. The curves describing the biodegradation process followed by measurement of the residual phenol and n-hexadecane concentration revealed that the process rate was constant for the first 10 cycles. A 12-28-hour period was required for complete degradation of the xenobiotics. A certain increase in the time needed in the next 10 cycles was observed when the biodegradation of n-hexadecane biodegradation was complete in 14 h and of phenol in 36 h. These 20 cycles with a total of 10g/l phenol quantity added and another 10g/l of n-hexadecane demonstrated not only a high rate of degradation, but also good resistance to the xenobiotics (Figure 1). The results presented in Fig. 1 reveal the strong catabolic potential of *M. luteus* BN 56. The twenty active cycles of biodegradation took place at a high rate and after 12-36 h residual quantities of phenol and n-hexadecane were not found. The total amount of phenol and n-hexadecane degraded was 10 g/l each. An important condition proved was that the addition of the second xenobiotic did not inhibit simultaneous biodegradation.

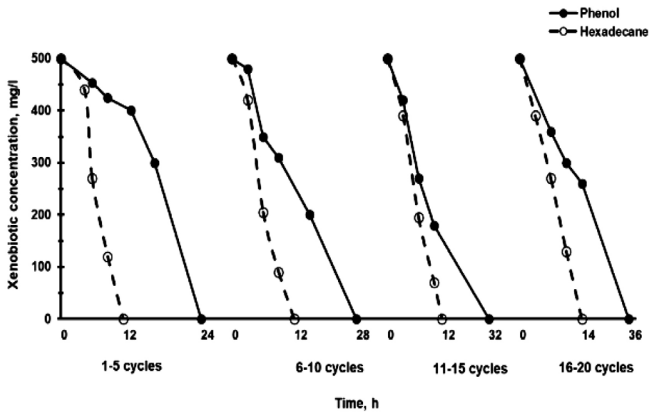

Fig. 1. Simultaneous biodegradation of phenol and n-hexadecane from the adapted strain of *M. luteus* BN56 under conditions of semi-continuous cultivation, presented as arithmetic mean values ($x_{av.} \pm \sigma$)

Having followed the growth of the culture before and after adaptation, it was evident that after a short lag-period the logarithmic phase began, which lasted about five days for the adapted cells. In the fifth supply of xenobiotic in a concentration of 500 mg/l, i.e. the fifth cycle, the stationary phase of growth was reached, which lasted until a slight delay in the catabolic processes (Fig. 2).

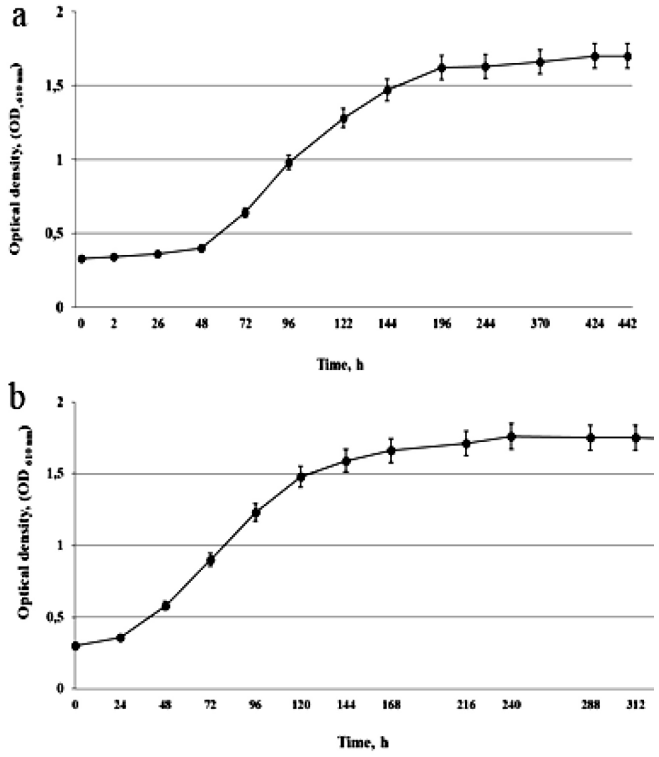

Fig. 2. Growth curve of non-adapted (a) and adapted cells of *M. luteus* BN56 strain (b) under conditions of semi-continuous cultivation, presented as arithmetic mean values ($x_{av.} \pm \sigma$).

In both cases the curves describing the growth of *M. luteus* BN56 cells were similar. It is essential that the cycles of biodegradation took place also during the stationary phase of growth. The growth of the adapted cells included a shorter lag-period and a logarithmic phase lasting until the fifth supply of both xenobiotics.

Investigating the catabolic activity of some microbial strains during the simultaneous biodegradation of several xenobiotics represents a new approach, as such a model is very close to the parameters found in different wastewaters or soils, where toxic compounds do not appear separately but in mixtures. For example, Gallego *et al*. (2003), presented their investigations on the simultaneous biodegradation of 2-chlor-phenol and m-cresol under continuous process conditions. Degradation of petroleum hydrocarbons (both aliphatic and aromatic) by thermophilic microorganisms (*R. rhodochrous* DSM6263) has been found (Nzila, 2018), together with biodegradation of phenol and n-hexadecane by an Acinetobacter strain isolated from river waters during a single active cycle (Sun *et al*., 2012).

In the present study, an attempt was made at simultaneous biodegradation of aliphatic and aromatic xenobiotics, i. e. hydrophobic and hydrophilic toxic substances, which are known to be degraded by various catabolic pathways (Kolvenbach *et al*., 2014). The active simultaneous biodegradation could be explained by the presence of two membrane-bound enzymes (phenol hydroxylase and alkane monooxygenase) located at different sites in the cells, and the use of different channels for the transfer of the hydrophilic and hydrophobic xenobiotic into the cells (van Beilen and Funhoff, 2007, Ullrich and Hofrichter, 2007).

In our next experiments successful simultaneous biodegradation was accomplished of the aromatic xenobiotic phenol and the aliphatic xenobiotic n-hexadecane by applying the immobilization approach. Cells of the investigated strain were immobilized *in situ* in the biocompatible PEO and PAAm cryogels. First, the cells were mixed with the reagents in aqueous media and then the polymer network was formed by UV light induced crosslinking in the frozen state, according to the established mechanism of Lozinsky (2002). During freezing, the major portion of water formed large polycrystals. Cells, polymer (monomer), photoinitiator, crosslinking agent, and bound water (non-freezable solvent) accumulated in a non-frozen liquid microphase, where the formation of a network took place. After thawing, the cryogels consisted of smooth polymer walls resulting from the microphase, which were surrounded by interconnected pores filled mainly with free water from the melted ice crystals (Fig. 4a and 5a). Probably this water is one of the reasons why cells do not lose their catalytic activity and survive being entrapped. During repeated batch experiments with free cells, a decrease in the biodegradation capability was recorded after 10 cycles.

When comparing the two types of matrices, the PEO matrix appeared to be more suitable in terms of number of active cycles of simultaneous biodegradation of phenol and hexadecane (40). It should be noted that the rate of n-hexadecane biodegradation was higher (12 h) compared to phenol (18-28 h) (Fig. 3).

However, when immobilization was performed with the use of PEO cryogel matrices, very active biodegradation of n-hexadecane and phenol was observed after the first addition (first cycle). For forty cycles, the catabolic potential of the cells remained very active (Fig. 3). The selected method

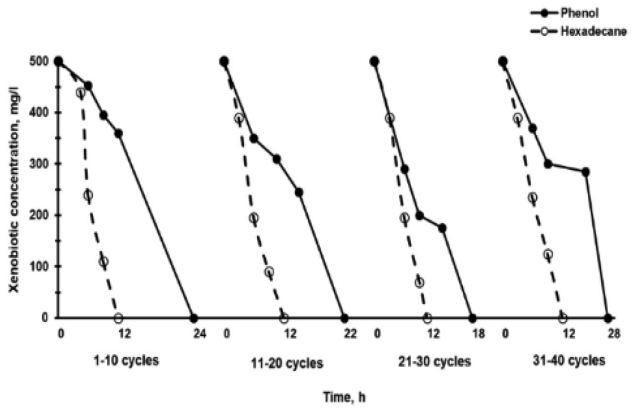

Fig. 3. Processes of semi-continuous biodegradation of phenol and n-hexadecane from PEO immobilized cells of *M. luteus* BN56, presented as arithmetic mean values (xav. ± σ) of residual phenol and n-hexadecane for every ten cycles.

of immobilization proved to be successful and efficient for a long period of time. At the end of each of the first twenty cycles, the measured optical density ($OD_{610\ nm}$) was 0.1. Over the next twenty cycles, the amount of desorbed cells increased slightly to 0.2 ($OD_{610\ nm}$) compared to the optical density of free cells 1.6 ($OD_{610\ nm}$). This fact clearly indicates a negligible leakage of cells and, accordingly, their good retention in the volume of the gel. The walls of the cryogel are partially closed and with interconnected pores containing the cells. This is how they prevent cells from leaking out.

Scanning electron microscopy observations confirmed that the cells preserved their shape and their regular distribution beneath the matrix surface along the formed channels and appeared partly embedded in the matrix pores after many cycles of use.

Immobilization of living cells into the super-macro porous supports offers enormous advantages for their reusability and stability in continuous operation. Several attempts have been made with this purpose in mind. Thus, Wilson and Bradley (1996) used a suspension of free cells and immobilized cells of *P. fluorescence* on a commercial bio-support as bioremediation agents in an aqueous system with petrol as the carbon source. Quek *et al*. (2006) reported immobilization of *Rhodococcus* sp. F92 on polyurethane foam in the bioremediation of petroleum hydrocarbons, as immobilized cells can degrade a variety of petroleum products. In another study, *R. corynebacterioides* QBT immobilized on chitin and chitosan flakes significantly increased the biodegradation of crude oil (Gentili *et al*., 2006). The application of immobilization techniques can protect cells from phenolic toxicity and increase their ability to degrade (Gouda, 2007). Immobilization not only simplifies the separation and recovery of used cells, but also makes their application multiple, which reduces the total cost (Cunningham *et al*., 2004).

Conclusions

With the help of some adaptation and optimization procedures, full depletion of both aromatic and aliphatic xenobiotics tested was successfully achieved. In the course of the experiments, 10 g/l (100%) phenol and 10 g/l (100%) n-hexadecane were degraded for 20 active cycles of operation (duration of 12 - 36 h) by the free cells of *M. luteus* BN56. By means of an *in situ* immobilization procedure using PEO cryogel matrices, these processes were intensified, stabilized and reached 40 cycles of operation of successful simultaneous biodegradation of aromatic and aliphatic xenobiotics (20 g/l + 20 g/l). The catabolic ability of the investigated strain immobilized in PEO cryogel matrices can be applied in the bioremediation of wastewaters and soils containing such pollutants.

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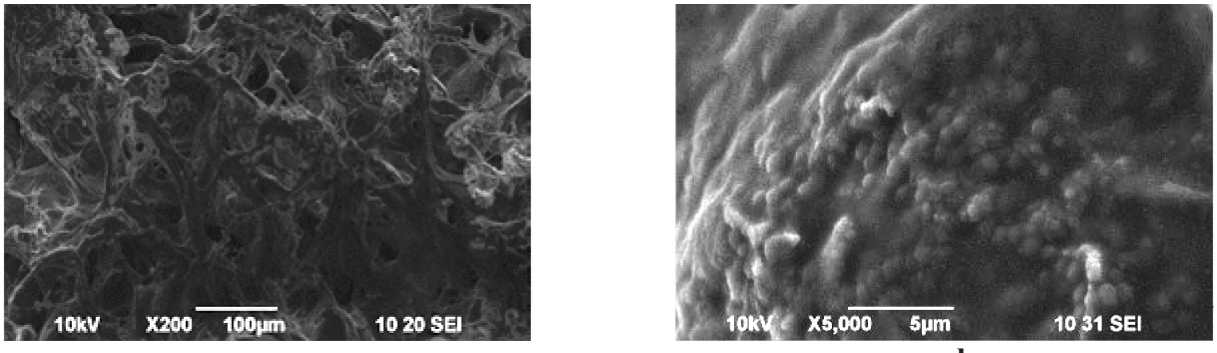
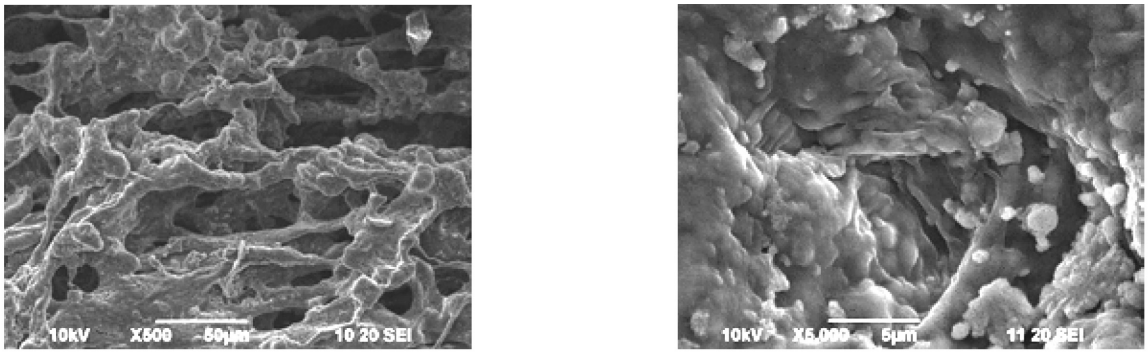


a **b**

Fig. 4. Scanning electron micrographs: PEO matrix only- a); cells entrapped in PEO- b)



a **b**

Fig. 5. Scanning electron micrographs: PAAm matrix only- a); cells entrapped in PAAm- b)

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