The relationship of some polyurethane biocomposites against *Penicillium chrysogenum* and *Aspergillus brasiliensis*

D. MACOCINSCHI*, D. FILIP, C. TANASE[a], S. VLAD, A. OPREA[b], T. BALAES[a]
"Petru Poni" Institute of Macromolecular Chemistry, Aleea Gr. Ghica Voda 41A, Iasi-700487, Romania
[a]*"Al I. Cuza" University, Faculty of Biology, Bd. Carol I nr. 11, Iasi-700505, Romania*
[b]*Biological Research Institute, Str. Lascar Catargi. 47, Iasi-700107, Romania*

Biopolyurethane composites based on poly(ester urethane), and extracellular matrix components (hydrolyzed collagen, elastin, chondroitin sulfate and hyaluronic acid) have been prepared. The biological material used is a very wide spread aggressive fungal species in all media for life and is represented by *Aspergillus brasiliensis* (Trichocomaceae, Eurotiales, Eurotiomycetidae, Eurotiomycetes, Ascomycota, Fungi) and *Penicillium chrysogenum* (Trichocomaceae, Eurotiales, Eurotiomycetidae, Eurotiomycetes, Ascomycota, Fungi). Evaluation of the biologically active potential of these biocomposites against these fungi has been done through inoculation onto Sabouraud–agar nutrient medium. Fungal growth was monitored over 60 days. Visual observations of the fungal growth show that these fungi were aggressive and caused rapid grow after 72 h of inoculation onto Sabouraud-agar nutrient medium, but the biopolyurethane composites samples relatively to the fungal attack particularly showed a different behaviour.

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1. Introduction

Polymeric materials have come into our life and are widely applied in different areas. There has been an increasing interest in using natural based composite materials for a lot of functional applications in diverse areas such as medicine, agriculture, industry, ecological reconstruction, etc. These research activities require the knowledge of the polymer characteristics and contamination/degradation action of microbiotics under various environmental factors.

Polyurethanes can adopt various forms (from soft to hard) depending on the chemical structures of the polyisocyanates and polyols and are suitable for extremely different practical applications as fibers, paints, foams, resins, elastomers, and many others [1-6].

Moreover, this class of polymers has been extensively used and tested in different biomedical applications [7-10]. They have gained great popularity in endotracheal tubes, vascular prostheses, components of the artificial hearts, membranes for dialysis, adhesives for bone tissue and materials for dental recovering, due to their fairly good biocompatibility and antithrombogenicity characteristics [5, 11, 12]. The blends between biopolymers and synthetic polymers are of particular significance because they can combine biocompatibility with good processability and mechanical resistance and can be used as biomedical and biodegradable materials [13]. In order to overcome the biological deficiencies of synthetic polymers and to enhance the mechanical characteristics of the materials used in contact with blood and tissues for long periods, natural polymers like collagen, fibrin and glycosaminoglycans were more often used than other natural products [14-17]. While the elastin matrix is proven a relatively anti-thrombogenic structure, the collagen matrix remains a necessary component for the construction of vascular prostheses and it was shown that elastin represents a better contact surface than collagen due to its antithrombogenic properties [18].

The biodegradable component is mainly the natural polymer. A composite material consists of two or more different materials, natural and/or synthetic that when are combined become superior to individual materials. The polymer scaffold in tissue engineering design represents the critical element, capable to mimic the role of the extracellular matrixes found in tissues. Extracellular matrices composed of proteins and glycosaminoglycans bring cells together and control the tissue structure, regulate the function of the cells, and allow the diffusion of nutrients, metabolites, and growth factors [19-21]. The medical devices should remain neutral and the materials that are being used need to be stable and undegradable by the actions of microorganisms. Traditionally polyurethanes materials have been singled out as being problematic concerning their long-term in vivo biostability in tissues [22].

The introduction of biodegradable polymers into a synthetic polymer matrix restricts the action of a fungal, microbial or enzymatic attack [23-25]. Poly(ester urethanes), largely applied as biomaterials for medical devices, can be hydrolyzed chemically and enzymatically in the aliphatic ester linkage [26]. Such limitations appear even when the biodegradable component occurs as a continuous phase in the composite material. Studies on the

degradations of different types of polyurethanes materials and urethane compounds are multiple. It has been shown that some organisms like bacteria and fungi can degrade polyurethanes materials or affect their properties [27, 28]. Particularly, fungi are capable of deteriorating urethane based materials.

Aspergillus brasiliensis and *Penicilliuym chrysogeum* are common species in all media of life. These species have been reported as degrading a variety of polymers, among them being polyurethane [29]. Also, [30] reported degradations of some polymers and bacterial cellulose composites by *Aspergillus brasiliensis* strains.

In our previous work we demonstrated that the polyurethane mixed with extracellular matrix molecules (hydrolyzed collagen, elastin, chondroitin sulfate and hyaluronic acid) allowed cell attachment and growth over the culture period and did not interfere with morphological and functional characteristics of the cells evidencing a high biocompatibility [14, 15].

The tested biological material is a very wide spread fungal species in all media for life, which is known as ordinary fungus [31].

In a previous article [32] we evaluated the effects of interactions between *Mucor mucedo*'s mycelium and the surfaces of these polyurethane composite materials.

The aim of the present study is to investigate the relationship between *Aspergillus brasiliensis* and *Penicillium chrysogenum* and new polyurethane composites because it is important for the assessment of their potential application for biomedical devices.

2. Experimental

2.1. Materials

Synthesis of polyurethane (PU) The PU used in this study was synthesized in our laboratory and the synthesis is described in [32] and has the structure: [MDI-PEA-$(MDI\text{-}EG)_4]_n$ and composition determined from elemental analyses: 60.01 wt % C; 4.23 wt. % N; 6.16 wt % H; 29.60 wt % O. The weight-average molecular weight of PU is $M_w \approx 150\ 000$; the PU obtained is soluble in DMF, dimethyl sulfoxide and it is a film-forming polymer.

Hydrolyzed collagen (HC) was prepared in the laboratories of the National Institute of Research and Development for Biological Sciences (Bucharest); *elastin (KEL), hyaluronic acid (HA)* and *chondroitin sulfate (CS)* were purchased from Sigma.

Preparation of polyurethane composite matrices is presented in the same reference [32]. Four polyurethane composite matrices were prepared as presented in Table 1.

Table 1. Polyurethane composite matrices.

Polyurethane composite matrices	Weight ratio
PU/ HC	90.9/9.1
PU/HC/KEL	90.1/9/0.9
PU/HC/KEL/CS	90/9/0.9/0.1
PU/HC/KEL/HA	90/9/0.9/0.1

The biological material is represented by *Aspergillus brasiliensis* [33] (Trichocomaceae, Eurotiales, Eurotiomycetidae, Eurotiomycetes, Ascomycota, Fungi) and *Penicillium chrysogenum* (Trichocomaceae, Eurotiales, Eurotiomycetidae, Eurotiomycetes, Ascomycota, Fungi), which were isolated from air, by placing sterile Petri dishes (15×100 mm) with added Sabouraud-agar medium (glucose-10 g; peptone-10 g; agar-15 g; distilled water-1000 mL), for 30 minutes. Pure cultures (100 %) of *Aspergillus brasiliensis* and *Penicillium chrysogenum* were obtained by repeated isolations in sterile box and they belong to the fungal collection of Laboratory of Mycology-Faculty of Biology, Iasi. Inoculum was seeded by puncturing with a spore-laden inoculation needle into Sabouraud-agar medium, uniformly distributed in sterile Petri dishes with polymer fragments (PU, PU/HC, PU/HC/KEL, PU/HC/KEL/CS, PU/HC/KEL/HA), (10×10 mm), placed in the middle. Control samples for each experiment are polymer fragments with dimensions 10×10 mm, which were seeded by puncturing with *Aspergillus brasiliensis* and *Penicillium chrysogenum* inoculum and placed into sterile Petri dishes, without added Sabouraud-agar medium. Two replicates of Petri dishes were incubated at 25^{o}C, in the absence of light for 14 days, and afterwards the action of the fungi onto the selected polymer composites was monitored during 60 days. Fungal growth was measured after 72 h, 96 h, 120 h, 168 h and 60 days. The photographs were collected with a photo camera Canon Power Shot A530. The photomicrographs for detail visualisations were collected with a Trinocular Stereo Microscope Nikon SMZ800.

3. Results and discussion

Aspergillus brasiliensis (syn. *Aspergillus niger* sensu auct. pre 2007) is a cosmopolitan species, found in soil, on decomposing vegetal wastes, on tubers, roots, seeds and fruits, which are usually stored in basements or fabrication halls.

Colony develops rapidly, presents a velvety mycelium, white and yellowish. Following sporulation, colony becomes dark-brown towards black, and gain a granular pulverulent aspect. Reverse of colony can be hyaline or coloured in light yellow. The margin of colony is white or yellowish. Onto Czapek–agar medium, at 25 ^{o}C, colonies can reach after 7 days diameters of 4–5 cm. This species sporulates abundantly on malt extract agar medium.

Mycelium is septate, hyaline and develops both into the substrate of culture medium and in air. Conidiophores are nonsepted and usually develop directly from substrate, reach the heights of 200–400 µm and diameters of 7–10 µm. The surface of conidiophores is smooth, on basis are hyaline and yellow or brown to apex. In some cases are present atypical conidiophores, with conidial head of columnar shape and a low number of phialides. Conidial head has diameters of 300–1000 µm. Vesicle is large, spherical and globular with diameters of 20–60 µm, hyaline or brown-yellowish. Phialides develop radially on

the whole surface of vesicle and are disposed in two rows: primary phialides brown, dimensions of 20–30 x 6–8 µm and secondary phialides shorter, dimensions of 6–10 x 2–3 µm. Phialospores develop in long chains, basipetal from secondary phialides. They have a spherical or globular shape of 2,5–5 µm, initially they are flat, and at maturity becomes rough and present unregulated superficial striations, dark brown, red brown or black.

This species can release enzymes: *amylases, glucoamylases, glucoxidases, cellulases, pectinases* and *proteases.* It can synthesize lipids, and mycotoxines. By contamination in air, it can be a pathogen agent for otomycosis, pulmonary aspergylosis and allergic bronchitis [34].

Penicillium chrysogenum is a cosmopolitan species, can be isolated from different substrates: soil, air, paper, foods, nutriments, seeds, fruits, vegetables etc. This species generates the rot of vegetal products (nutriments, seeds, fruits, vegetables). This species synthesize the well-known antibiotic penicillin.

It can be cultured on Czapek–agar and MEA 2% (malt extract agar), at 25 °C, and the colonies can reach after 10 days diameter of 4–5 cm. Surface of colony is velvety and pulverulent. Mycelium is fast growing, white grey, and the sporifer bodies are blue-green or green-yellow which gradually becomes grey. Reverse of colony can be yellow to brown-yellowish sometimes brown-red. Exudate is present on the surface of colony under drops light yellow towards dark yellow in colour. Conidiophores are of dimensions 200–1000 × 3.0–4.5 µm, with divergent branches, metulae are grouped 3 to 5 of dimensions 8–15 x 2,5–4 µm. Phialides are grouped 4–7, cylindrical, de 8–12 × 2 µm, and phialospores are catenate (disposed in chains) monocellular, globular, of dimensions of 2.5–4 × 2.2–3.8 µm.

Under suitable conditions microorganisms such as fungi which inhabit soil, water, and air can develop and proliferate on polymer materials. Polymer materials made from natural polymers are generally more susceptible to biodeterioration than are the synthetic ones. Microorganisms may attack the entire substrate or they may attack only one component of the substrate. The material is attacked chemically by the action of extracellular enzymes produced by the microorganisms for the purpose of reaching nutrients. Microbial activity can be minimized by keeping susceptible materials dry, as surface growth only occur when the relative humidity is high.

The images of the control sample and fungal growth after 72 h of inoculation are presented in Fig. 1.

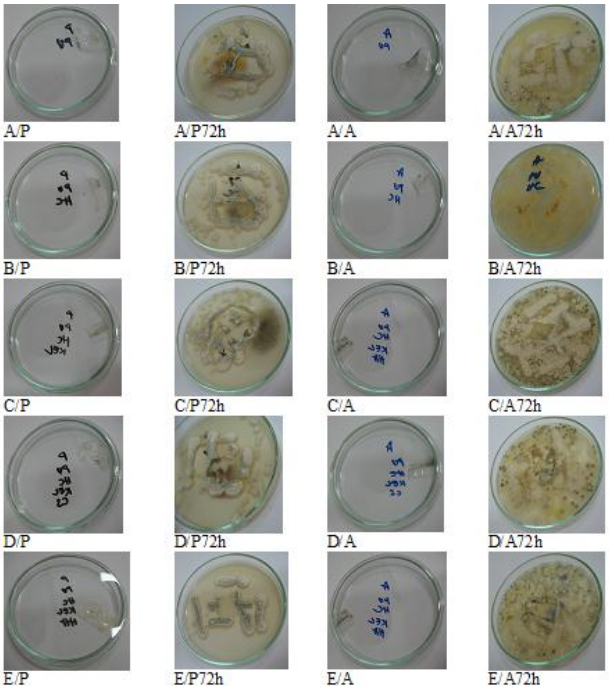

Fig. 1. The images of the control sample (A-E/P for Penicillium chrysogenum colonies, A-E/A for Aspergillus brasiliensis) and fungal growth (A-E/P72h and A-E/A72h) after 72 h of inoculation: A-PU; B–PU/HC; C-U/HC/KEL; D-PU/HC/KEL/CS; E-PU/HC/KEL/HA.

Environmental factors not only influence the polymer to be degraded, they also have a crucial influence on the microbial population and on the activity of the different microorganisms themselves. Parameters such as humidity, temperature, pH, salinity, the presence or absence of oxygen and the supply of different nutrients have important effects on the microbial degradation of polymers. Another complicating factor in this attack is the complexity of the materials with regard to their possible structures and compositions. These materials do not consist simply of only one chemical homogeneous component, but contain different polymers (composites); with one polymer itself different structural elements can also be present. These different structures of a polymer, despite having the same overall composition, can directly influence accessibility of the material to the enzyme-catalyzed polymer chain cleavage, and also have a crucial impact on higher-ordered structures of the polymers (crystallinity) which have been shown predominantly to control the degradation behaviours of many polymers. Numerous published accounts report that polyester polyurethane is susceptible to biodegradation by fungi, bacteria and enzymes whereas polyether polyurethane has been found to be relatively more resistant to biodegradation. It has been reported that the biodegradation of polyester polyurethane is carried out by the esterase enzyme associated with both bacteria and fungi. This biodegradation is achieved mainly by the enzymatic attack on the ester bond in the polyol segment.

Along with the ester bond the urethane bond has also been shown to undergo biodegradation by esterase enzyme in low molecular weight polyester polyurethane. In an effort to improve the adhesion and retention of cells to polymer scaffolds researches typically coated with various extracellular matrix proteins. These studies highlight that extracellular proteins played an important role in attachment and spreading of cells to surface, where specific domains on cell membrane bind directly with extracellular matrix molecules via integrins [35]. Adhesion of microorganisms to host cells and tissues represents a critical step in the process of infection. The colonization of a host by a pathogenic microorganism depends on its ability to adhere to and, in some cases invade host cells and tissues. The development of a fungal infection is thought to be dependent on the adhesion of conidia to host cells and/or to the extracellular matrix. Extracellular matrix proteins have been implicated in the attachment of a variety of pathogens to both host tissues and cells. The interaction of specific molecules on the fungal surface with carbohydrates or proteins of the extracellular matrix components enables fungi to colonize and invade tissue. Some glycosaminoglycans molecules were used to show significant inhibitory effect on *Aspergillus* conidial adhesion [35]. It was suggested that glycosaminoglycans may be a target for conidial adhesion. Glycosaminoglycans draw large amounts of water into their structures, permits migration of cells, nutrients and other substances. Most glycosaminoglycans are bound to a protein core to form proteoglycans. An exception is hyaluronic acid which does not form a proteoglycan. Glycosaminoglycans interact with proteins, anchoring them at specific locations and affect their biological activity [36, 37].

The images of fungal growth after 96h, 120h, 168h and 60days of inoculation for *Penicillium chrysogenum* and *Aspergillus brasiliensis* are presented in Fig. 2 and Fig. 3, respectively.

In general, one can observe that *Aspergillus brasiliensis* is more aggresive than *Penicillium chrysogenum* after inoculation onto Sabouraud-agar nutrient medium, and the biopolyurethane composite samples relatively to the fungal attack show different behaviour.

The investigation of the inoculated samples on nutrient Sabouraud–agar in which were placed polymer fragments, points to the following results:

- Absence of *Penicillium chrysogenum* colonies on the polymeric fragment samples employed in the experiments: PU/HC/KEL and PU/HC/KEL/HA;

- Presence of isolated colonies, punctiform, sporulated, stationary of *Aspergillus brasiliensis* on PU fragments, from 72 h up to 60 days;

- Presence of isolated colonies, punctiform, sporulated, stationary of *Aspergillus brasiliensis* on PU/HC fragments, from 96 h up to 60 days;

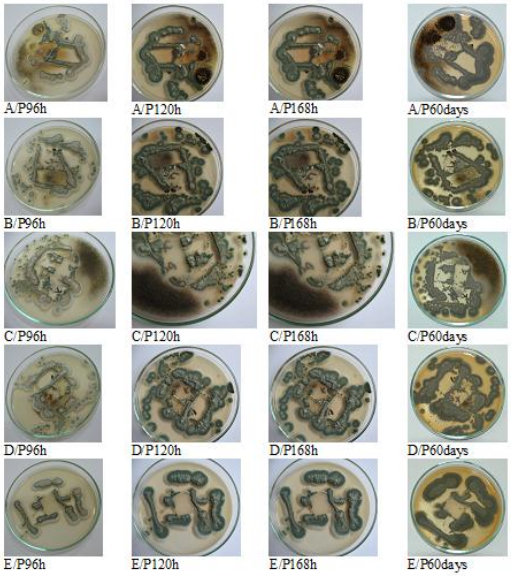

Fig. 2. The images of fungal growth for Penicillium chrysogenum colonies after 96 h, 120 h, 168 h and 60 days of inoculation: A-PU; B– PU/HC; C-PU/HC/KEL; D-PU/HC/KEL/CS; E-PU/HC/KEL/HA.

Fig. 3. The images of fungal growth for Aspergillus brasiliensis after 96 h, 120 h, 168 h and 60 days of inoculation: A - PU; B – PU/HC; C - PU/HC/KEL; D-PU/HC/KEL/CS; E-PU/HC/KEL/HA.

- Presence of isolated colonies, punctiform, sporulated, stationary of *Aspergillus brasiliensis* on PU/HC/KEL fragments, from 120 h up to 60 days;

- Presence of isolated colonies, punctiform, sporulated, stationary of *Aspergillus brasiliensis* on PU/HC/KEL/CS fragments, from 72 h up to 60 days;

- Presence of isolated colonies, punctiform, sporulated, stationary of *Aspergillus brasiliensis* on PU/HC/KEL/HA fragments, from 72 h up to 60 days.

During the experiments inoculum remained stationary in case of all control samples following incubation.

4. Conclusions

Aspergillus brasiliensis and *Penicillium chrysogenum* species were isolated from air and cultivated on Sabouraud–agar medium, in which were immersed polymeric samples for 60 days.

In function of analysed support it was constatated the absence of colonies of *Penicillium chrysogenum* over the whole duration of experiment, on the investigated polymeric fragments: PU/HC/KEL and PU/HC/KEL/HA.

We remark the presence of *Aspergillus brasiliensis* colonies, isolated, punctiform, sporulated, stationary on PU fragments starting with 72 h up to 60 days; from 96 h to 60 days on PU/HC fragments; from 120 h up to 60 days on PU/HC/KEL fragments; starting with 72 h up to 60 days on PU/HC/KEL/CS fragments; starting with 72 h up to 60 days on PU/HC/KEL/HA fragments.

For control samples for whole period after incubation (from 72 h up to 60 days) inoculum remained stationary.

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* Corresponding author: eradro2002@yahoo.com