Towards microfluidics for mycology – material and technological studies on LOCs as new tools ensuring investigation of microscopic fungi and soil organisms

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Abstract. This paper presents material and technological studies on lab-on-chip (LOC) devices as a first step towards biocompatible and reliable research on microscopic fungi and soil organisms on a microscale. This approach is intended to respond to the growing need for environmental control and protection, by means of modern, miniaturized, portable and dependable microfluidics instrumentation. The authors have presented herein long-term, successful cultivation of different fungi representatives (with emphasis put on *Cladosporium macrocarpum*) in specially fabricated all-glass LOCs. Notable differences were noted in the development of these creatures on polymer, polydimethylosiloxane (PDMS) cultivation substrates, revealing the uncommon morphological character of the fungi mycelium. The utility of all-glass LOCs was verified for other fungi representatives as well – *Fusarium culmorum* and *Pencilium expansum*, showing technical correspondence and biocompatibility of the devices. On that basis, other future applications of the solution are possible, covering, e.g. investigation of additional, environmentally relevant fungi species. Further development of the LOC instrumentation is also taken into consideration, which could be used for cultivation of other soil organisms and study of their mutual relationships within the integrated microfluidic device.

Key words: microfluidics; lab-chip; biomaterials; cell cultivation; mycology.

1. Introduction

Environmental changes which we have been experiencing for nearly 20 years now cannot fail to have an impact on our surroundings. Human-related activities, with a view to overpopulation and pollution, are mostly what directly influences the quality of air, water and soil. As indicated in papers [1–3], the functioning of the whole ecosystem draws on the mutual relationships between diverse organisms, i.e. Monera, Protista, Fungi, Plantae and Animalia. On that basis, the problem of, for instance, soil disfunction, influences both the kingdom of Fungi and Plantae, being typically in strong symbiosis or parasitism relations [4–6].

With a view to papers [7–8], within the Whittaker's classification, the kingdom of fungal organisms is considered the one of greatest importance, with notable diversity in morphology and habitat, reaching at least 70,000 of recognized species. Fungi widely occur in air, water, land and soil, so that they constitute distinguished bioindicators of any environmental changes. Nevertheless, currently employed laboratory-based techniques of environmental studies are not able to directly reflect the organismal habitat and local diversities [9–10].

Therefore, a tendency towards novel, microfluidic approaches to provide new insights into soil-based biological objects has arisen, covering the subject of lab-on-chip (LOC) technology [11–13].

For nearly three decades now, the field of microfluidics has been developed to bring a variety of solutions for chemical analysis, DNA investigation, cell cultures, tissue engineering, etc. [14–16]. Apart from the capabilities of reagents' minimization or high throughput performance and sensitivity, the possibility of single-object confinement, controlled triggering and real-time screening within the independent microfluidic device seems to constitute substantial improvement as compared with classical macroscale methods, e.g. Petri dishes or multiwell plates (Fig. 1) [17–18].

Currently, the biotechnology market is dominated by multiwell plates, also finding their use in mycological and environmental studies. Small, multiple "wells", that the plate is built of, can be used mainly as test tubes. Their tiny scale makes them more practical than regular test tubes in laboratory usage. In mycological practice, they are mostly used for detection of antimicrobial activity, optical detection, species identification (genetical, enzyme-linked and photometrical assays) and storage [19–23]. Unified size encourages the industry to produce a strictly specialized device to support the studies being conducted and to make them more time-efficient. Nevertheless, multiwell plates are not free of disadvantages. The unified shape and size, being one of the largest advantages, also leaves them

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- ➤ Stationary culture,
- ➤ No single-cell insight,
- ➤ No external stimulation (media refreshment, chemical gradients),
- ➤ …

Macroscale Petri Dish

- ➤ Flow culture,
- ➤ Single-cell insight,
- ➤ Controlled, contamination-free on-chip stimulation
- ➤ …

Miniaturized LOC device

Microchamber

Microchannel   Via hole

Fig. 1. Scheme illustrating a comparison of the classical Petri dish culture with a lab-on-chip device

unable to modulate depending on individual research properties. LOCs in turn have the potential to be used as a fully individualized tool, according to the requirements of the currently employed research method. The prevalent method of multiwell plates is also unable to link with microfluidic flow for many wells (chambers) simultaneously, which is an important issue in automatization and reaching sterile, repeatable input of substances into working system, decreasing probability of contamination. Possibility of easy adaptation of LOC's shape and geometry along with the small input, via holes, give promising prospects to prevent cross-contamination between LOCs, even during vigorous shaking, which is considered the most critical aspect of microbial growth in microtiter plates. The important thing here is also the flat character of the microchamber, which allows for observation of the growth of mycelium (or other cells) in real time under the light microscope, facing no overgrowth. Moreover, LOCs made of glass would be reusable and, hence, more environmentally friendly. In the near future, LOCs could become a promising substitute or supporting tool for widely used multiwell plates, as new research methods in environmental laboratories develop.

The application of LOCs for environmental studies may provide new opportunities in this emerging field, especially covering microbial ecology, plant science and toxicology. Imitation of natural microenvironment on-chip could provide better understanding of ecosystem functioning, with special attention paid to the influence of environmental change on local diversity, species behaviorism and composition [24–26]. On that basis, herein, we present the material and technological studies on microfluidic device fabrication, as a preliminary step towards research on soil organisms – fungi. Major focus was laid on the applicability of the LOC technique, ensuring fully biocompatible and long-term culturing of selected fungi representatives.

With a view to currently published reports describing investigation of microscopic fungi with the use of microflu-

idic-based tools, very few articles can be found in the literature [27–30]. Herein, strong emphasis has been put on behavioral analyses, specifically – directional memory of fungi and utilizing microfluidic microconfinements in mazelike structures [29–30]. All of the aforementioned solutions are based on polydimethylosiloxane (PDMS) substrates, fabricated by means of replica molding techniques. Authors of these papers use mainly "open" structures, with solid agar nourishment applied as a growth medium. Thus, those cannot be considered fully autonomous and portable devices, ensuring well defined atmosphere within the LOC and confinement of undesired stimulus. To the best knowledge of the authors, only one paper found presents a PDMS-based closed-type structure of the LOC, in which *Neurospora crassa* was investigated [30]. Still, there is a lack of other competing solutions, pointing at new approaches in the field. The research on cultivation of fungi in miniaturized instruments, fabricated out of other materials which are typically employed for laboratory macroscale techniques, e.g. glass, could prove very interesting and significant for the development of mutual relations between engineers and microbiologists. Scaling down of the solutions should be performed in close cooperation between the key representatives of the branch, in order not to lose the functional performance of the device at an early stage of the design. For this reason, in this article, two approaches to LOC development being dedicated to fungi cultivation were presented. The first one was based on a polymer material currently popular with microfluidics, i.e. PDMS [31–32], while the second one utilized well-known and standardized borosilicate glass substrates [33–34]. In addition, the authors decided to use liquid fungi nourishment (Czapek-Dox), making a significant step towards future flow character analyses of soil organisms cultivation. Based on the notable differences in fungi development observed on PDMS and glass substrates (which was a decisive factor here), a selection of appropriate construction materials for the final LOC device was implemented, and for the first time in this paper, successful cultivation of the selected fungi representatives (*Cladosporium macrocarpum*) was performed in an all-glass microfluidic chip. These and other results of the experiments are described in the following sections of this paper.

2. Materials and methods

2.1. Object of the study. *Cladosporium* is a cosmopolitan, ubiquitous genus, which occurs in diverse environments, commonly encountered as saprotrophs on debris, soil, food, paint, textiles and other organic matter. However, understanding of *Cladosporium spp.*'s role in the environment presents multiple difficulties. Generally, the occurrence of this genus may indicate well-developed decomposition on damaged plant tissues or in soil [35–39]. Even though this group of fungi is classified as common decomposers, individual representatives of this genus are frequently noticed among major fractions of the phylloplane, spermosphere and endophytic mycobiota [40–44].

Under specific conditions some of *Cladosporium* species constitute a threat to other organisms such as plants, animals and

humans. Some of them are dangerous plant pathogens, causing economically important plant diseases, although the majority occur on plant tissues as a secondary pathogen [45–46]. Only particular species, such as for example *Cladosporium cucumerinum*, *Cladosporium fulvum* and *Cladosporium herbarum,* are seen as a cause of considerable, yearly losses in the farming industry [47]. However, in the same group fungicolous species occur, considered a potential biopesticide used to control *Melampsora ciliate* rust [48].

Spores of this genus are amongst the most common airborne fungi, considered one of the most allergenic fungal factors [38, 49–50]. Rarely, some individuals from this wide group of fungi may cause opportunistic infections, related mainly to people with compromised immune systems [47].

Under specific conditions, high activity of *Cladosporium* may cause biological corrosion of natural geological forms, historical artifacts and marbles, metal and glass, thus impacting natural and cultural heritage [51–52]. This process affects materials' surfaces by means of organic acids, produced by fungi, which react with elements, causing demineralization, salt production or the deposition of insoluble salts, forming a layer on surfaces. The losses caused by fungi are often irreparable, especially when they directly concern craft legacy (like prehistoric paintings and remains of human activity) or natural form of great cultural significance. In caves and underground environments concentration of fungal spores in the air (CFU – colony forming units) is one of the factors measured to identify the risk of biological corrosion and level of ecological disturbance already caused by fungi. That renders *Cladosporium* species one of the natural indicators of caves deterioration, caused by human activity and increased mass tourist traffic [51–52]. Another specific manner of biodegradation caused by *Cladosporium* is based on slow decomposition of polymers and other organic materials, encountered mostly on petrochemicals such as oils, fuels, rubber or isolation materials, using them as a source of carbon and energy [53]. Activity of this genus can lead to a reduction of the octane number in fuels, through one-way chemical decomposition pathways [54]. The degradation pace is usually low and takes several months or years, but causes severe economic losses, especially due to damage to stored fuel and isolations systems [55].

One of the most widespread and common species of *Cladosporium* is *Cladosporium macrocarpum*. According to the current division, *C. macrocarpum* belongs to one of the cosmopolitan air-borne fungal taxa – the *C. herbarum* complex, as a closely related species [45]. It is a saprotrophic species, mostly occurring on dead debris and woody plants, especially abundant in temperate regions [45–46]. Pace of growth of the species is moderate, and after 14 days in 25°C colonies it reaches 30–43 mm on PDA and 31–50 mm on MEA. In these conditions, the colony produces unbranched or loosely branched mycelium, 1–4.5(–5) μm wide, producing solitary, terminally arising, verrucose conidiophores. Macronematous conidiophores are erect, straight to somewhat flexuous, unbranched, sometimes branched 12–260×(3–)4–6 μm. Conidia are produced in short chains, 0–3 septate, thick-walled, densely verrucose, 9–28×5–13 μm [45].

A fungus strain of *C. macrocarpum* (*Dothideomycetes* class) was used as the object of the study. A particular strain was collected from seeds' surfaces of *Spiraea tomentosa* in a Lower Silesian Forest (SW Poland) in 2018 and stored on the PDA medium in a fridge (7°C, dry conditions). Before experiments, the fungal colony was renewed, moved to a fresh PDA medium and cultivated at room temperature under dark conditions for 1 week. Identification was made according to morphological features, based on keys and publications [37, 38, 45, 46, 56]. Experiments were conducted on the Czapek-Dox medium (liquid) in the temperature of 24°C and in absolute darkness.

2.2. Selection of substrates. As mentioned before in this article, the authors have studied two diverse materials as the substrates for the microfluidic device fabrication – polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, USA) and borosilicate glass (Borofloat 3.3, Schott, Germany).

All-glass substrates were prepared utilizing standard glass micromachining processes – xurography and wet chemical etching in the solution of 40% HF: 69% HNO3 (10:1 V/V) [57–58]. As a result of the experiments, cavities of different depth – 50 μm, 100 μm, 150 μm and 200 μm were fabricated in the centers of the substrates, in which fungi could be inoculated and cultivated (Fig. 2).
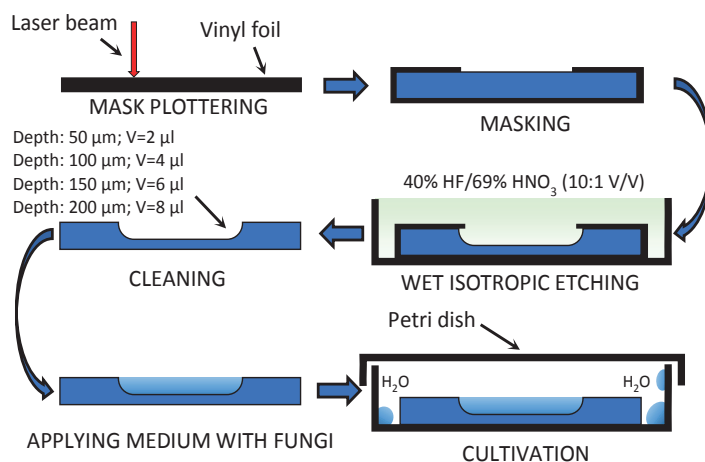

Fig. 2. Fabrication of glass substrates for cultivation of fungi – technology flow

PDMS substrates were fabricated utilizing molding and 3D printing technique (printer model: ProJet 3510, 3D Systems, USA). At first, PDMS prepolymer was mixed with its cross-linking agent at the ratio of 10:1 (m/m). Afterwards, it was poured into 3D printed molds equipped with the posts of height corresponding to the depth of glass cavities. i.e. 50 μm, 100 μm, 150 μm and 200 μm (Fig. 3). Polymerization of the PDMS structures was enhanced by heating the molds in 60°C for circa 1 hr. After this step, the substrates were removed with a laboratory scalpel, sterilized, and used for further biological analyzes (Fig. 4).
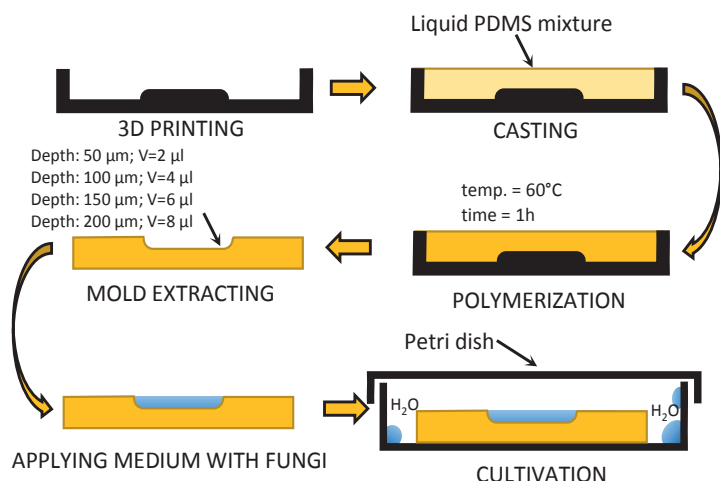
Fig. 3. Fabrication of PDMS substrates for cultivation of fungi – technology flow
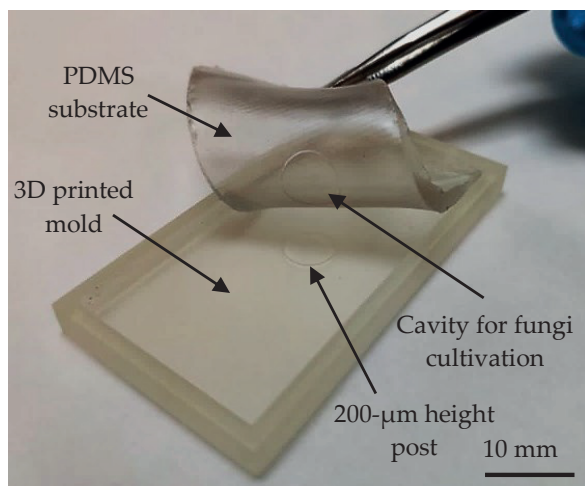

Fig. 4. Removal of PDMS substrate from 3D printed mold

In order to verify fungi development in the prepared glass and PDMS substrates, a portion of the culturing medium containing *C. macrocarpum* was applied to every cavity. Next, all the samples were placed in specially protected Petri dishes with deionized water pipetted around, preventing premature drying of the medium. The samples were incubated in ambient temperature, according to the macroscale laboratory standards. After 7 days of the experiment, comparative analysis of the cultures established on PDMS and glass substrates was conducted.

As a result, the development of *C. macrocarpum* could be observed in both PDMS and glass structures (Fig. 5). However, significant differences in fungi morphology were noted, indicating the uncommon character of mycelium growth in each of the PDMS substrates. In these samples, the mycelium was characterized by rather compact structure, with short and straightforward hyphae of the fungi. In contrast, samples cultured on the glass substrates were found to be developed appro-

priately. A fusiform shape of the mycelium, imitating a cobweb, could be observed, being a typically occurring pattern for that kind of fungi [56]. The depth of the cavities, i.e. 50 μm, 100 μm, 150 μm and 200 μm, had no significant influence on the development of *C. macrocarpum*, with the difference that the culturing medium evaporated faster in the substrates with shallow structures (50 μm, 100 μm).

The reason why *C. macrocarpum* growth on the PDMS substrates differed from that observed on glass may be related to the porous and hydrophobic surface of PDMS [59–61]. Based on that, fungi mycelium could not entirely adhere to the structure, thus solely point-type development was observed here. An additional factor which could influence unphysiological growth of the cultured species may refer to the replica mold utilized itself and to the limited resolution of the 3D printing technique, causing structuring of the surface. However, with a view to authors' previous tests on PDMS and its applicability for cell culturing LOCs [62–64], it can be stated that in comparison to microorganisms colony growth [63] or culture of adherent cancer cells (data unpublished), the results on fungi development on this substrate were not optimum. Certainly, this aspect should be investigated more specifically to achieve substantial data and formulate broader conclusions in this matter.

3.3. Fabrication of LOC. With regard to the aforementioned studies on material and technological biocompatibility of the substrates, borosilicate glass rather than PDMS has been selected for the final fabrication of LOC. The design of the device assumed preparation of structures equipped with microchamber (s) (for fungi cultivation) and via holes ensuring sample input/output [65–67].

Based on previously implemented several works of the authors regarding the process of cell cultivations on-chip and different biological objects management [62–64, 68–70], it has been indicated that availability of oxygen within the microbials colony is a key factor ensuring appropriate development of microorganisms. For this reason, special dependence on surface area of the microchamber vs the size of holes allowing for gaseous media diffusivity in all-glass LOCs has been determined. In order to do so, the authors proposed three different versions of the structures, which were modelled towards oxygen distribution in *Comsol Multyphysics* software. The main difference was related to hole diameter and its placement in
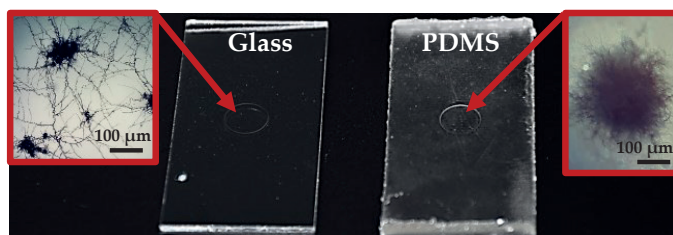

Fig. 5. Glass (left) and PDMS (right) substrates in which cultivation of *C. macrocarpum* was conducted. Magnified views of fungi growth are shown
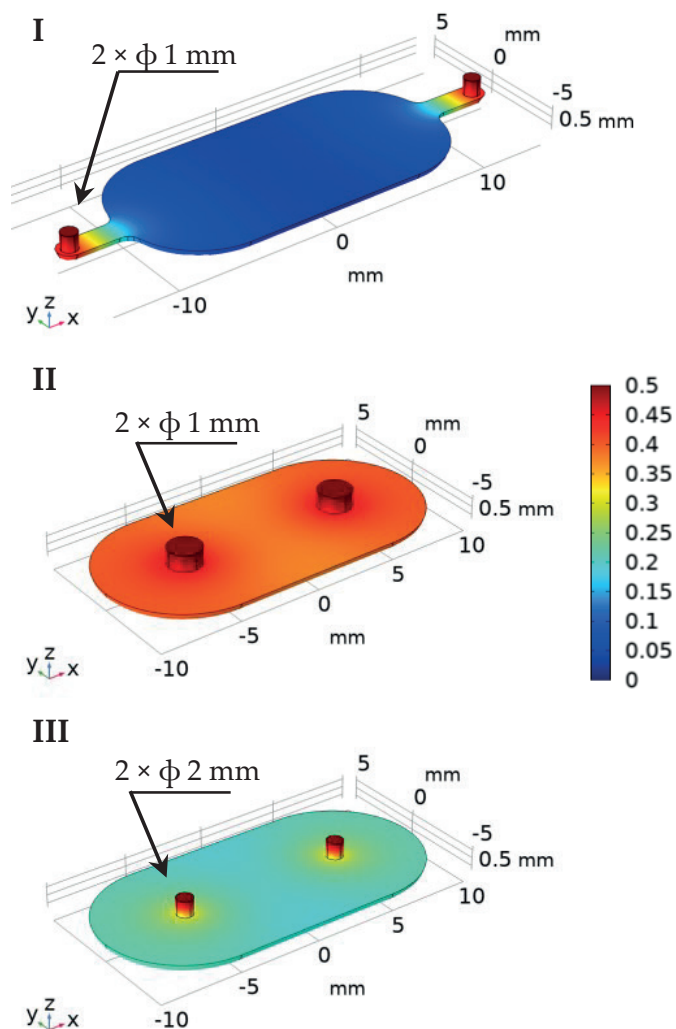
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Fig. 6. Proposed geometries of LOCs – visible microchambers and holes of different placement and diameters (1 mm/2 mm)
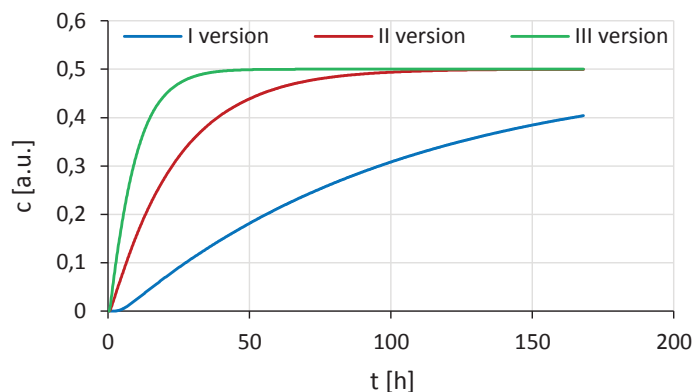

Fig. 7. Graph representing oxygen distribution in time, depending on the version I version II version III

exchange between the culturing medium containing fungi and the atmosphere, and should not be smaller.

For this reason, it has been decided that the third version of the LOC will be used for further tests. Its scheme with a cross sectional view is presented in Fig. 8.
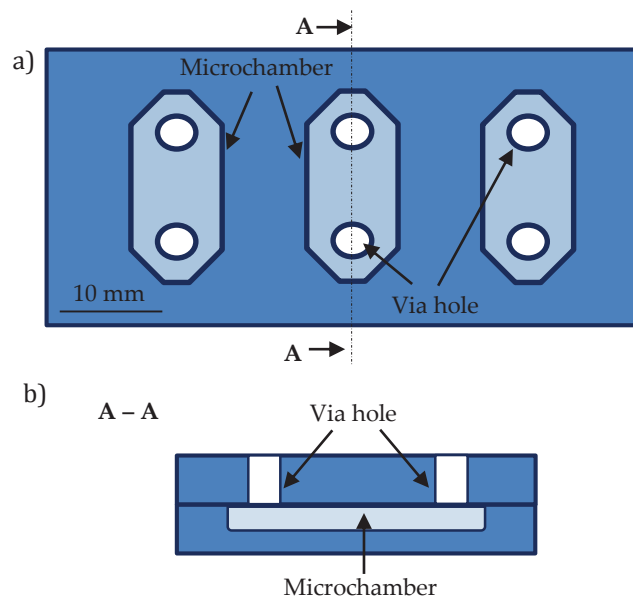

Fig. 8. Schemes of the final all-glass LOC for cultivation of *C. macrocarpum*: a) top view, b) cross-section

the microchamber area (Fig. 6). In each microchamber, similar depth was applied, which is also an important growth factor – the deeper the microchamber, the better the fungi development, as much space for mycelium expansion is provided. Moreover, evaporation of the culturing medium between the culturing medium containing fungi and the atmosphere can be minimized, being essential in the case of long-term cultivation experiments. Eventually, the depth of LOC microchambers has been defined as 400 µm, so that its value has been doubled with a view to the first studies proceeded on glass/PDMS substrates.

As a result of the simulation, it could be observed that the fastest increase in oxygen concentration within the microchamber area occurred in the third version of LOC (Fig. 7). Maximum oxygen concentration [71] was achieved here in the first 48 hours, being the most important period for fungi, since it is a germination time, which determines further appropriate growth of the mycelium. Based on that, it may seem that the chosen hole diameter (2 mm), constituting circa 20% of the microchamber area, fits the requirements for proper gas

Fabrication of the LOC encompassed standard glass micromachining processes, i.e. xurography, wet chemical etching and high temperature bonding, Fig. 9 [68, 70]. At first, two laboratory autoclaved borosilicate glass substrates (Borofloat 3.3, Schott, Germany, $50 \times 25 \times 1.1$ mm^3) were masked with HF-resistive vinyl foil (Avery Dennison Graphics Solutions, USA) with CNC laser cut patterns. Next, the substrates were submerged in the etching solution of 40% HF: 69% HNO3 (10:1 V/V) for a pre-defined time, in order to achieve 400 µm depth of the microchambers (etching rate $\sim$3 µm/min).

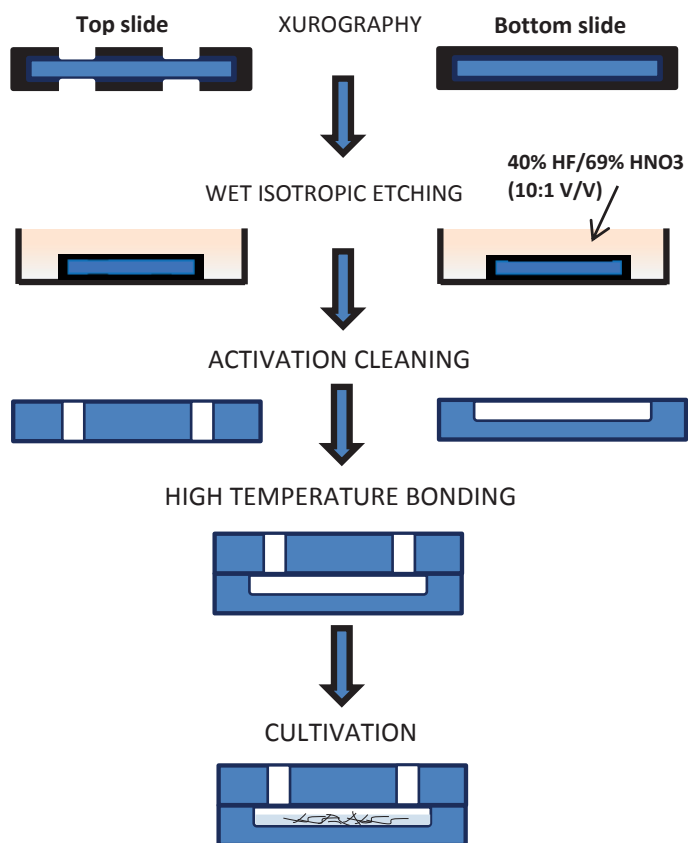A. Podwin, T. Janisz, K. Patejuk, P. Szyszka, R. Walczak, and J. Dziuban

Fig. 9. Fabrication of all-glass LOCs – technology flow

The same etching solution was used to fabricate holes in the upper substrate of the LOC. Afterwards, special cleaning procedures were employed prior to bonding – washing with IPA and ethanol as well as submersion in a piranha solution (98% H_2SO_4 : 30% H_2O_2, 3:1 V: V) for $\sim$15 minutes. After these steps, the substrates were rinsed with DI water, temporarily joined and placed in a furnace for thermal bonding (650°C). As a result, two tightly bonded structures were fabricated, ready for further cultivation experiments (Fig. 10).
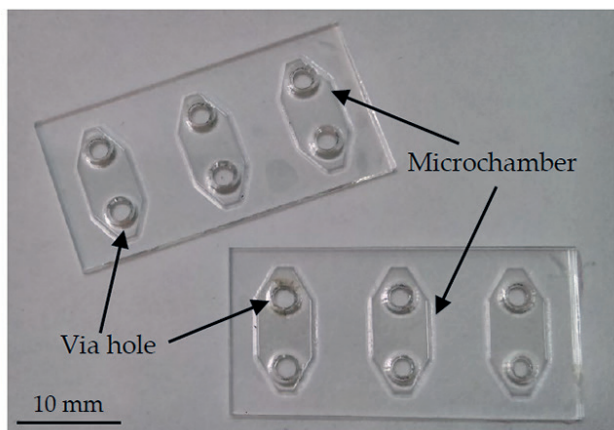

Fig. 10. All-glass LOCs for cultivation of fungi species – *Cladosporium macrocarpum*

3. Results and discussion

Utilizing a sterilized swab stick, a portion of fungal spores (*C. macrocarpum*) was harvested from the macroscale culture, mixed with the CzapekDox medium and centrifuged (Fig. 11a). Next, circa 50 μL of the biological sample was entered into each microchamber of the LOCs using a standard laboratory pipette (the LOCs were autoclaved before usage). In order to protect the cultures – maintain appropriate humidity and simultaneously, gas diffusivity on-chip – the LOCs were carefully covered with parafilm foil during the whole experimentation time (Fig. 11b). The cultivation of fungi was performed for 7 days, under conditions of absolute darkness and ambient temperature. Control of the nourishment content in LOCs and microscopic observations were conducted every 24 hours.
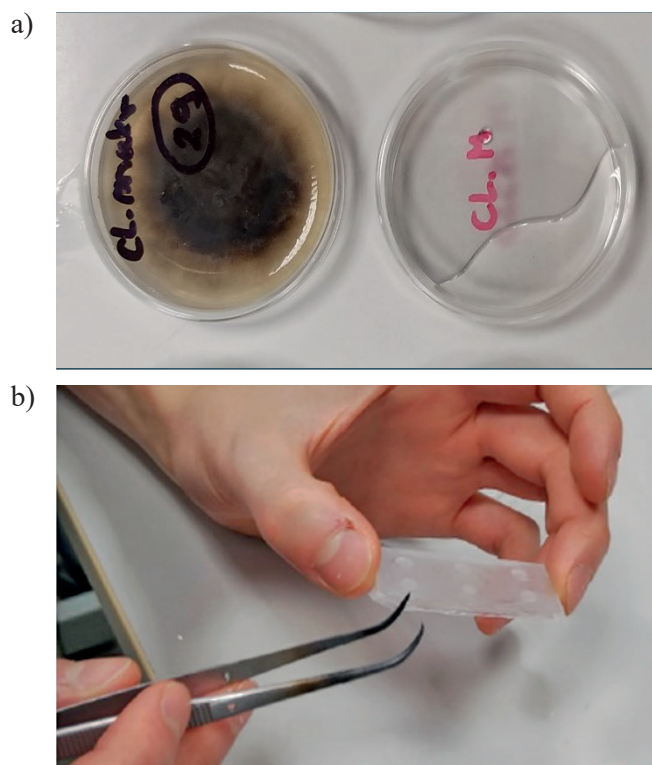

Fig. 11. Preparation of fungi cultivation in LOCs: a) view of the macroscale culture, b) LOC covered with parafilm foil after sample inoculation

After 48 hours of cultivation, noticeable germination of spores was observed, being in strong correspondence with literature reports concerning fungi behavior in classical, macroscale cultures [56]. At the end of the culturing process, spores and mycelium expanded through the whole microchamber area (Fig. 12), which was also visible to the naked eye.

The aforementioned experiment was repeated three times utilizing the LOC device and similar, appropriate results on *C. macrocarpum* development were achieved. In addition, the cultivation process of other cosmopolitan fungi – *Pencilium expansum* and *Fusarium culmorum* – was verified in the same

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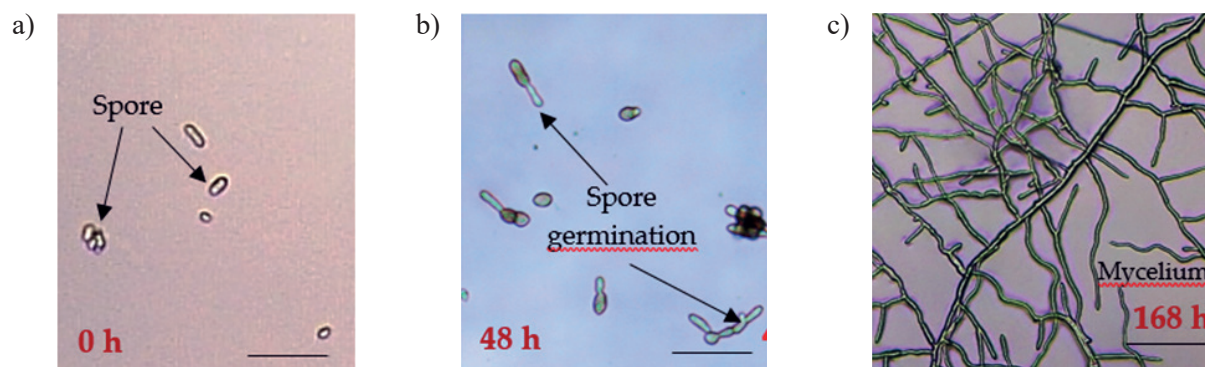
Fig. 12. Microscopic images of *C. macrocarpum* cultivation on-chip: a) at the beginning of the experiment (preliminary sample centrifugation allowed for pure spores introduction, without larger fragments of mycelium), b) at germination time, c) after 7 days of the test. Scale bar – 50 μm
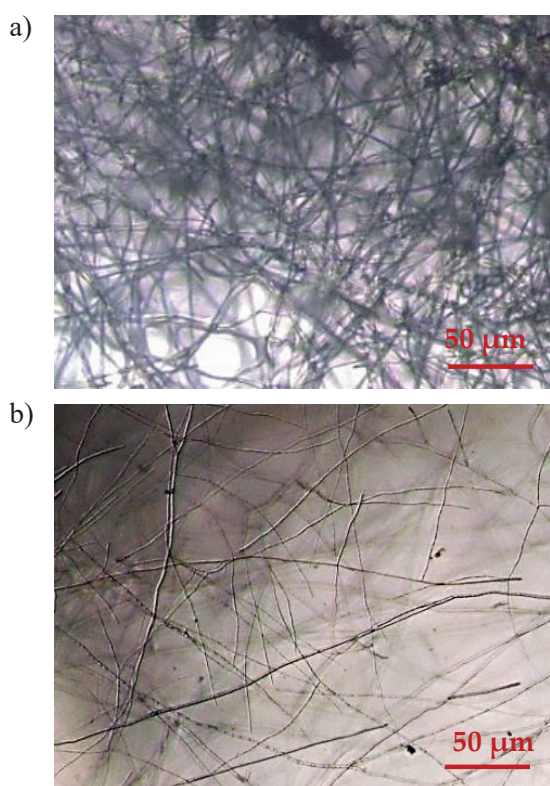

Fig. 13. Microscopic images of expanded mycelium of fungi as a result of 7-day cultivation process in LOC: a) *Penicilium expansum*, b) *Fusarium culmorum*

LOC structures. Each of the species revealed notable spores germination and expansion of the mycelium in a 1-week culturing period (Fig. 13).

It should also be emphasized that the LOCs fabricated herein, after careful cleaning[1], were used for the cultivation experiments many times. The reusability of all-glass LOCs,

mentioned above, is very convenient and constitutes a substantial improvement over popular, mainly polymer-based microfluidic devices, which are mostly disposable due their noticeable degradation in time [72]. In this case, no negative impact on the culturing parameters was observed during repetition of the experiments utilizing the same all-glass structures. Moreover, as a consequence of the microscale solution applied, carefully prepared biological samples of the circa 60 μL range did not face the overgrowth and the cultivation process was fully controlled here.

4. Conclusion

In the article, research towards LOC applicability for cultivation of soil microorganisms – fungi – has been shown. First and foremost, the authors have investigated two different materials as substrates for LOC fabrication – borosilicate glass and polydimethylosiloxane (PDMS). Based on the culturing experiments conducted on the species of *Cladosporium macrocarpum*, a significant difference in growth of its spores and mycelium could be observed, pointing to appropriate/uncommon development on glass/PDMS substrates. The final, biologically compatible and reusable all-glass LOC structures allowed for repeatable and reliable cultivation of *C. macrocarpum* over a 1-week period. Moreover, universality of the solution has been verified for cultivation of other cosmopolitan and ubiquistic fungi – *Fusarium culmorum* and *Pencilium expansum*, showing their appropriate development and physiological character. These and other results form a good base for further in-depth investigation of these interesting species on-chip. In the next step, it is planned to integrate the LOC device with the dedicated set-up, ensuring investigation of the influence of the environmental factors on fungi cultivation. Here, a co-culture of a few soil species, with a mutual dependencies study within a single microfluidic device, could also be performed.

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[1] Cleaning procedure encompasses rinsing of LOCs in IPA with application of UHF, hydrogen peroxide and deionized water. The LOCs are autoclaved before every colony inoculation.

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