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Evaluation of Immobilization of Selected Peat-Isolated Yeast Strains of the Species *Candida albicans* and *Candida subhashii* on the Surface of Artificial Support Materials Used for Biotrickling Filtration

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Abstract: The paper describes the process of *n*-butanol abatement by unicellular fungi, able to deplete n-butanol content in gas, by using *n*-butanol as source of carbon. Isolated and identified fungi species *Candida albicans* and *Candida subhashii* were subjected to a viability process via assimilation of carbon from hydrophilic and hydrophobic compounds. The isolates, which exhibited the ability to assimilate carbon, were immobilized on four different types of artificial support materials used for biotrickling filtration. Application of optical microscopy, flow cytometry and the tests employing propidium iodide and annexin V revealed viability of the fungi isolated on support materials' surfaces at the average level of 95%. The proposed method of immobilization and its evaluation appeared to be effective, cheap and fast. Based on performed comparative analyses, it was shown that polyurethane foam and Bialecki rings (25 × 25) could be attractive support materials in biotrickling filtration.

Keywords: biotrickling filtration; fungi; polyurethane foam; Pall rings; Bialecki rings; *n*-butanol; cyclohexane; flow cytometry

1. Introduction

One of the most common techniques of biological gas treatment is biofiltration. The process of biofiltration consists in passing a polluted stream of gas through a filter bed, inhabited by microorganisms (bacteria, fungi), belonging to different species. The impurities diffuse from the gas phase to the biofilm forming on the surface of elements of the packed bed. The compounds adsorbed on the surface of elements or absorbed in the biofilm undergo biodegradation, and the air free of odorous compounds leaves the biofilter. The most popular apparatus for biofiltration includes conventional biofilters, biotrickling filters and bioscrubbers [1–3].

Microorganisms and type of packing are crucial elements for biological air purification processes. The selection of microorganisms colonizing the biofilter located on the packing is pivotal for the effective degradation of specific groups of chemical compounds. Biofilters can be populated with a single strain of microorganisms or their consortia [3].

A literature review of available techniques for removal of odorous volatile organic compounds (VOCs) from air revealed that biotrickling filtration was a highly effective method for the removal of contaminants from gaseous streams [1]. The most important element of the biofiltration apparatus is a column packing that is colonized by the microorganisms responsible for VOC removal, the compounds of odor nuisance. Usually, ambiguous term like fungi or microbial consortium is used,

without clear indication of the species present in a biofilter bed [4–9]. At present, especially bacterial strains used in biofiltration are a subject of extensive studies [10,11]. In the case of fungi, especially those used in conventional biofiltration are under investigation [12]. Table 1 presents typical fungi and types of packing material employed in the conventional biofilters and biotrickling filters. Considering the technical and operational differences between conventional biofiltration and biotrickling filtration [2,13], the beds of these biofilters differ in the types of fungal strains present in a packing material. Depending on the mode of biofiltration, the process makes use of the microorganisms either naturally inhabiting the packing material (e.g., bark, leaves or peat in conventional biofilters, straw, wood shavings, compost, cones) [3,14–17] or those intentionally inoculated on the filter bed elements (e.g., inoculation of polyurethane foam or ceramic materials for biotrickling filter, alginate beads, ceramic saddles, Pall rings, Raschig rings, tri-Pack) [18–21]. Accordingly, assuring of high efficiency of malodorous compounds removal requires not only selection of suitable microorganisms but also fitting of such biofilter packing, which is easily colonized by selected microorganisms and allows their growth during the process allows maintenance of proper biomass concentration on the filter bed. Cultivation of the microorganisms occurs both in interstitial spaces as well as in micropores, the size of which allows penetration of the cells of microorganisms forming so-called sites protected from the impact of adverse shearing forces due to water flow. The biofilter's packing material can play the role of not only a support material for biological film development but also an adsorbent, an ion exchanger, a nutrient medium and a substance buffering the biochemical reaction environment [22]. Apart from the effectiveness of microorganism colonization, the price should also be taken into account while selecting the biofilter's packing. Hence, the following features of the packing became a target: low price, effective colonization with the microorganisms and stimulation of growth of the microorganisms capable of malodorous compounds removal. Such material should be characterized by a significant specific surface area but it should not influence on biofilm formation [23–25].

Regarding the fungi which are to colonize the biofilm substrate, a big surface area of thread-like structure of a fungi's hyphae allows easy assimilation of carbon from organic compounds present in a surrounding gas phase [26]. Wu et al. revealed that fungi could be as effective as bacteria in the removal of organic contaminants from air [27] and some research indicate that fungi can assimilate carbon with higher efficiency as compared to bacteria [28,29]. The information about VOCs assimilation by the fungi isolated from pine needles can be found in literature [30]. As opposed to bacteria, fungi are more resistant to low humidity and high acidity. It is especially beneficial for the removal of hydrophobic compounds [31]. Moreover, fungi can be applied in acidic gas streams with high concentration of organic contaminants [32]. Abovementioned properties of fungi make them an interesting subject for further investigation.

In microbiological processes it is necessary to provide carbon, nitrogen and phosphorus for microorganisms. The last two can be easily accessed through ammonium salts, nitrates or phosphates. The problem of carbon assimilation is far more complex. Despite the fact that there is about 30 times more carbon in the environment as compared to nitrogen, the multiplicity of its chemical connections causes that carbon containing compounds can affect microorganisms in different ways e.g., enhance as well as inhibit their growth. The form of carbon most suitable for assimilation are carbohydrates, preferably glucose. However, in the absence of carbohydrates, microorganisms can obtain carbon from other sources e.g., volatile organic compounds present in air. To improve the ability of microorganism to decompose VOCs in biotrickling filtration processes, it is desirable to provide the surface of biofilm i.e., packing elements covered with biofilm as densely as possible, to increase the contact area between the biofilm and gas phase. In practice, it is realized by immobilization of the microorganisms on a support material. A literature review revealed a variety of different supports e.g., expanded clay aggregate [33], tuff [34], activated carbon [35], perlite [36], alginate beads [37], polyurethane foam [38], ceramic saddles [39], Pall rings [40–42], and Raschig rings [43].

Table 1. Examples of fungi employed for gas purification in conventional biofiltration and biotrickling filtration processes.

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Predominate species	Contaminant	Packing material	Reference
Conventional Biofilter—microorganisms naturally inhabiting the packing material			
Paecilomyces variotii	toluene	ceramic rings	[44]
Phanerochaete chrysosporium	butanol	straw	[45]
Trametes versicolor	aniline	peat	
Pichia pastoris	methanol	perlite	[46]
Cladosporium sphaerospermum	xylene	wood shavings	[47]
Ophiostoma sp.	α-pinene	volcanic rock	[48]
Aspergillus niger	*n*-hexane	kermesite	[49]
Candida utilis	ethanol	sugarcane bagasse	[50]
Scedosporium apiospermum	toluene	GAC:vermiculite (15:85)	[51]
		perlite	[52]
Paecilomyces variotii	toluene	perlite	
Sporothrix veriecibatus	styrene	perlite	[53]
Exophiala jeanselmei	styrene	perlite	[54]
Exophiala sp.	BTEX	perlite	[55]
Fusarium solani	*n*-hexane	perlite	[56]
Biotrickling Filter—microorganisms intentionally inoculated on the filter bed elements			
Sporothrix variecibatus	styrene	ceramic monolith	[57]
Fusarium solani	*n*-hexane	perlite	[58]
Candida palmileophila strain MA-M11	styrene	ceramic Raschig rings	[59]
Cladosporium sphaerospermum	methyl propyl ketone, MEK, toluene, and *n*-butyl acetate	polyurethane foam	[60–63]
Candida boidinii, *Ophiostoma stenoceras*	α-pinene	polyurethane foam	[64]

*GAC—Granular Activated Carbon, BTEX—Benzene, Toluene, Ethylbenzene and Xylenes, MEK—Methyl Ethyl Ketone.

Flow cytometry can be used to check the effectiveness of fungi colonization in a quantitative as well as qualitative manner [65]. Flow cytometry is a rapid and sensitive technique with important applications in biology and medicine [66,67]. This technique allows analysis of the microorganisms involved in technological processes as well as characterization of the starter cultures aimed at precise determination of their species composition. Monitoring of film damage can provide fast identification of microorganism failure or their death. Flow cytometry has already been utilized for *Candida albicans* [68,69], however, up to our best knowledge, it has not been implemented for investigation of fungi viability during biofiltration processes.

The paper presents the investigation on isolation of fungi from peat as a natural type of packing used in the classic biofilters. The study concerns immobilization of selected fungi (*Candida albicans* and *Candida subhashii)* on the surface of typical support materials of the biotrickling filters i.e., polyurethane foam, Pall rings and Bialecki rings. Effectiveness of their colonization was measured and compared using flow cytometry and optical microscopy. Moreover, the investigations aimed at determination of viability and ability to assimilate carbon from such compounds as *n*-butanol and cyclohexane, representing hydrophilic and hydrophobic compounds removed by the biotrickling filters, were carried out for the aforementioned fungal isolates. The obtained information can be employed for proper design of the process and maintenance of the optimum operation conditions. Additionally, correctly selected support material with respect to fungi types determines achievement of assumed deodorization effectiveness in the biotrickling filters.

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2. Materials and Methods

2.1. Chemicals and Reagents

N-butanol was purchased from POCH (Gliwice, Poland) and was used in the biofiltration experiments as a carbon source for the microorganisms. The composition of the used trickling medium is as follows: $Na_2HPO_4{\cdot}2H_2O$ (15.2 g/dm^{-3}), KH_2PO_4 (3 g/dm^{-3}), NaCl (0.5 g/dm^{-3}) and NH_4Cl (1 g/dm^{-3}). All materials were purchased from POCH (Gliwice, Poland). Cyclohexane was purchased from Merck (Darmstadt, Germany).

The primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) [70] were purchased from Genomed (Warsaw, Poland). The High GC PCR mix was purchased from A&A Biotechnology, Gdynia, Poland.

2.2. Support Materials

Characteristics of the materials employed as a surface for immobilization of selected fungi is presented in Table 2. The shape and general appearance of utilized types of support materials are illustrated in Figure 1.

Table 2. Characteristics of investigated materials used for immobilization of fungi [71].

Type of material characteristics	Polyurethane foam	Bialecki rings (25 × 25)	Pall rings (50 × 50)	Bialecki rings (50 × 50)
material	polyurethane	polypropylene	polypropylene admixed with chalk	polypropylene
color/transparency	blue color, low transparency	milky color, high transparency	milky color, medium transparency	dark green color, no transparency
surface	600 m^2/m^3	245 m^2/m^3 (in bulk); 0.0047 m^2/piece (in bulk)	110 m^2/m^3 (in bulk); 0.017 m^2/piece (in bulk)	155 m^2/m^3 (in bulk); 0.024 m^2/piece (in bulk)
number	6369 pieces/m^3 (arranged)	52000 pieces/m^3 (in bulk)	6500 pieces/ m^3 (in bulk); 8980 pieces/m^3 (arranged)	6500 pieces/ m^3 (in bulk); 8980 pieces/m^3 (arranged)

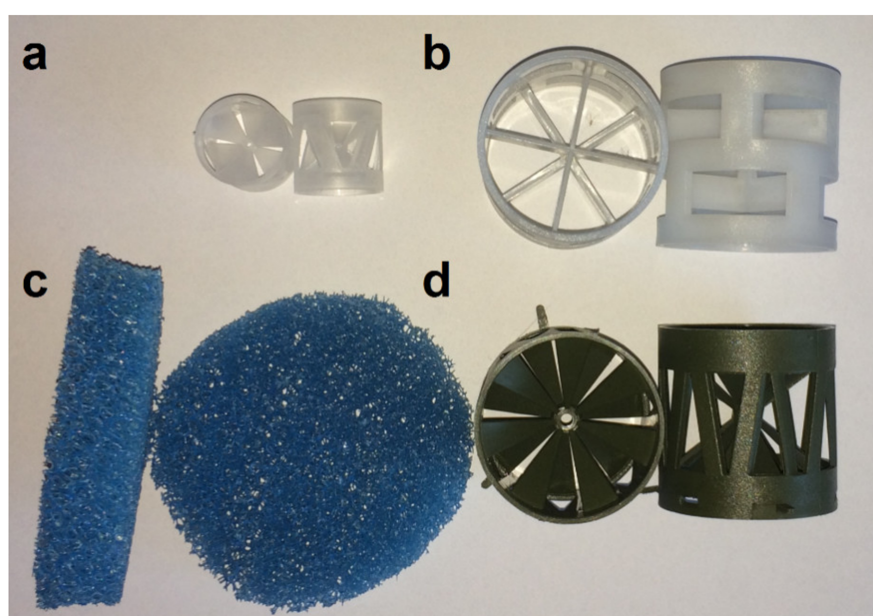

Figure 1. Selected examined support materials. (**a**) Bialecki rings (25 × 25), (**b**) Pall rings, (**c**) polyurethane foams, (**d**) Bialecki rings (50 × 50).

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2.3. Isolation of Fungi Species Present in Peat and Their Species Identification

For the experiment, the peat samples were taken from a biofilter column and cultivated in Sabouraud agar, which is selective for fungal growth. The isolation and preservation of pure culture was carried out as follows: the peat specimens were collected at day 20 of the process from a biofilter removing *n*-butanol. This approach allowed easy selection of the strains being able to survive in the *n*-butanol environment (hydrophilic compound). Then, several (up to 20) streak plates were prepared to obtain pure colonies. Next, the pure isolates were kept frozen at –80 °C (glycerol/water 1/3). After obtaining the isolates, the identification by means of PCR (Polymerase Chain Reaction) and ITS sequencing (Genomed, Warszawa, Poland) was performed. In order to isolate DNA, a short method developed by Brillowska-Dąbrowska [72,73] was used. The PCR mixture was prepared for each of the isolates as indicated in Table 3. The conditions of PCR are described in [3].

Table 3. Composition of the reaction mixture for the PCR (Polymerase Chain Reaction) reaction.

Reagent	Volume (μL)
Water	6.0
2xPCR Mix Plus High GC	10.0
Starter ITS1 (10 mM)	1.0
Starter ITS4 (10 mM)	1.0
Matrix	2.0
Final volume	20.0

2.4. Selection of Fungal Isolates Able to Utilize Hydrophilic and Hydrophobic Compounds as a Carbon Source

After identification (by ITS region sequencing), the isolates were taken for the measurement of capability of *n*-butanol, cyclohexane and their mixture (50/50) biodegradation. Determination of their ability to assimilate carbon for tested compounds was performed as follows: the incubation of all samples was carried out at 24 °C for 16 hours and the optical density was measured at 595 nm wavelength [74–76]. This wavelength is applied for optical density measurement of fungi cultures. *n*-butanol, cyclohexane and selected 2-component mixtures (50/50 (v/v)) at the concentrations of 10%, 1% and 0.1%, were added to the cultures at 24, 48, 72 and 96 hours. Observation of increasing optical density (OD) value over time for specific concentration was a sign of an increase in tested isolates, and thus the ability to assimilate carbon from the tested compounds.

2.5. Immobilization of Selected Fungal Isolates on the Support Materials (Polyurethane Foam, Pall Rings and 2 Types of Bialecki Rings)

The examined support materials were polyurethane foam (Ultramare, Warsaw, Poland) cut into approximately 100 mm diameter and 20 mm high discs, Pall rings (50 × 50) and 2 types of Bialecki rings (25 × 25 and 50 × 50) (LCS, Cracow, Poland). The immobilization of fungi on the support particles was carried out in 500 ml sterile beakers containing single type of the substrate and 100 mL growth Sabouraud medium with 10% (v/v) inoculum, under orbital agitation at 100 rpm and at 24 °C (Figure 1a,b). After 24 hours, 50% of the volume of medium was replaced with the same volume of the minimal medium: $Na_2HPO_4{\cdot}2H_2O$ (15.2 g/dm^{-3}), KH_2PO_4 (3 g/dm^{-3}), NaCl (0.5 g/dm^{-3}) and NH_4Cl (1 g/dm^{-3}) (Figure 1c). The beakers with appropriate contents were shaken throughout the whole immobilization. The next day, all medium was poured out, replacing it with the minimal medium (Figure 1d). The procedure was repeated after one day with the difference that the medium spilled was replaced with the Sabouraud medium (Figure 1e). After the same time interval the support materials were completely colonized (Figure 1f). The samples were routinely taken for analysis (optical density measurement). After 5 days from the beginning, the polyurethane foam material was completely colonized.

Due to lower porosity and the specific surface of Pall rings and 2 types of Bialecki rings as compared to polyurethane foam, and therefore greater difficulty in immobilizing the fungus on the

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surface of the support material, the process has been extended over time. In order to immobilize fungi on these materials, all the above mentioned stages must be carried out, except that all incubation times of the cultures should be extended from 24 to 48 h. Therefore, after 9 days from the beginning, the supporting materials were completely colonized. The process of fungi immobilization on the aforementioned support materials is schematically presented in Figure 2.

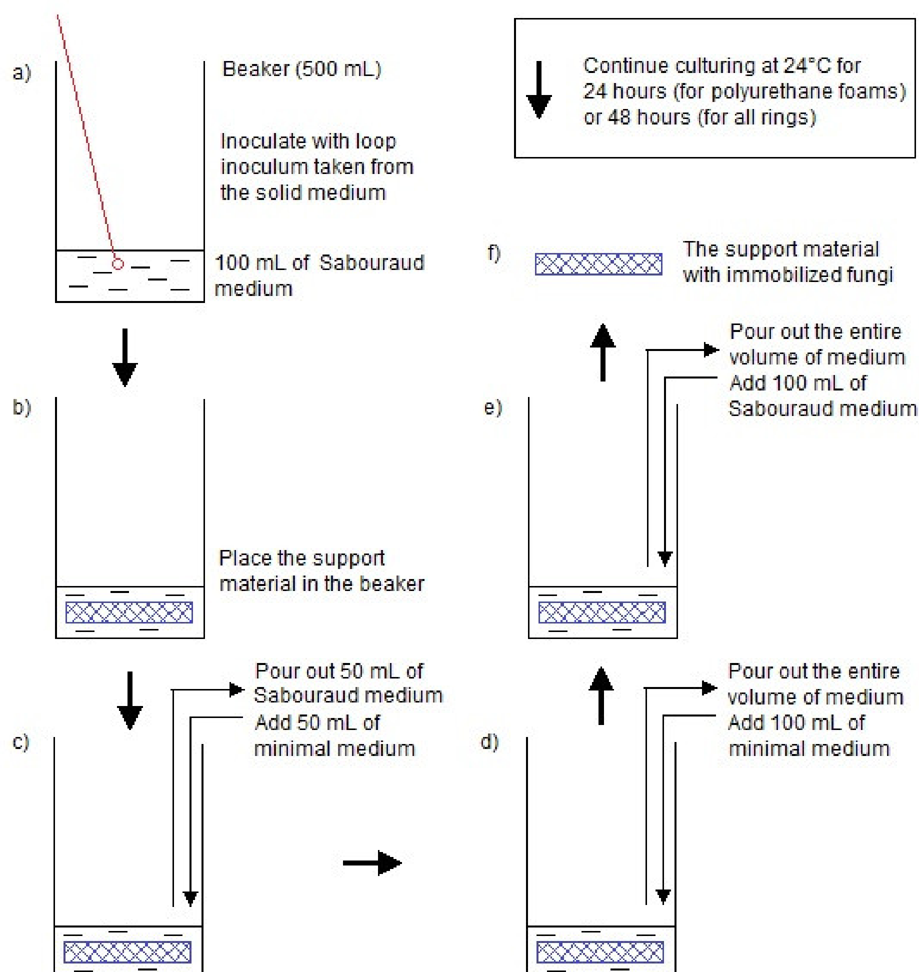

Figure 2. Scheme showing immobilization of selected fungi on the support materials.

2.6. Microscopic Observations

Viability staining technique was used for evaluation of vitality of investigated fungi. Pictures of immobilized fungi were made using transmitted light, employing an optical microscope with a 10× long working distance lens (LAB 40 Series Optical Microscope, Katowice, Poland).

2.7. Preparation of Fungal Cells to Cytometric Analysis

A fragment of the investigated support material was placed in 40 mL of physiological salt solution buffered with phosphate (pH 7,6, 0,01 M) and then shaken in an ultrasonic washer (100 W) for 15 s. Next, the vessel was placed in ice for 15 s. In order to remove contaminants, cell suspension was filtered through a nylon with 400-mesh. The residue was washed twice with PBS and suspended in 1 ml of PBS after centrifugation at 6000 rpm and at 4 °C (Eppendorf Centrifuge 5415 R, Hamburg, Germany) for 10 min. The number of cells in the examined sample was determined using a flow cytometer. The volume corresponding to 1 million of fungal cells was utilized in further investigations.

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2.7.1. Cytometric Analysis of Distribution in the Life Cycle of Fungi after Immobilization

Cytometric analysis of distribution in the life cycle of cells is based on detection of increased permeability of cell membrane for propidium iodide (PI), which binds with DNA in a stoichiometric way. This phenomenon and the knowledge that the amount of bound propidium iodide is proportional to cell DNA make it possible to determine the cell population in a given phase of the life cycle. PI is also capable of binding with RNA, which can lead to erroneous results. Hence, RNase A is added to the staining solution.

Then, 300 μL of PBS (Phosphate Buffered Saline), 300 μL of sodium deoxycholate (25 mM) (Sigma Aldrich, Darmstadt, Germany) and 0.3 μL of PI (2 mg/mL) (Sigma Aldrich, Darmstadt, Germany) were added to the pellet. The final concentration of PI in the tubes was 1 mg/ml. Staining was carried out for 30 min at 24 °C in the dark. Measure the fluorescence intensity emitted by DNA-bound propidium iodide using a flow cytometer (Merck Millipore guava easyCyte 8, Darmstadt, Germany). The excitation wavelength for propidium iodide is 488 nm, emissions are 617 nm. Each flow cytometric susceptibility test analyzed 10,000 events or yeast cells. The threshold between permeable and impermeable membranes was determined based on publication [77]. In order to obtain reliable results, the number of cells from 3 tested elements of each type were measured, settled in the same way, under the same conditions. The technical replicates were not included in the study.

2.7.2. Microbial Populations Viability State after Immobilization (Annexin V Test)

Annexin V test is aimed at investigation of the changes in asymmetry and integrity of cytoplasmic membrane of cells. A method consisting in double staining with annexin V marked with fluorescein and propidium iodide, which allows identification of the cells subjected to apoptosis or necrosis, was utilized in order to detect changes of cytoplasmic membrane after immobilization of fungal cells on the surface of selected materials.

100 μL of annexin V binding buffer (BD Pharmingen, San Diego, CA, USA), 0.5 μL of FITC Annexin V (Annexin V fluorescein conjugate; Introgen by Thermo Fisher, Eugene, OR, USA) and 0.25 μL of 7-aminoactinomycin D (Sigma, Dorset, UK) were added to the pellet. Staining was carried out for 15 min at 24 °C in the dark. These two dye conjugates can emit red and green fluorescence excited by 488 nm wavelength and can be detected by a fluorescence detector of the flow cytometry. In order to obtain reliable results the number of cells from 3 tested elements of each type were measured, settled in the same way, under the same conditions. The technical replicates were not included in the study.

3. Results and Discussion

3.1. Measurement of Viability and Carbon Assimilation from Hydrophilic and Hydrophobic Compounds

Fungi were isolated from the samples of peat collected from the biofilter intended for removal of *n*-butanol vapors from air. Five isolates were identified as *Candida albicans* and one as *Candida subhashii*. Next, evaluation of the microorganisms able to assimilate carbon from selected volatile organic compounds (*n*-butanol (B), cyclohexane (C) and their mixture of 50/50) was performed. For the liquid culture of the test isolates after 24, 48, 72 and 96 hours, the test compound solutions and their mixture at the concentrations of 10%, 1% and 0.1% were successively added. Every 24 hours after the addition of the test compound solution, the optical density of the culture was measured at 595 nm wavelength.

The obtained optical density values (Figure 3) showed that *C. albicans* MG2, *C. albicans* MG4, *C. albicans* MG5 and *C. subhashii* MG6 in concentrations of tested compounds grew with time (an increase in optical density value). *C. albicans* MG2 has higher OD value when fungi grow in the media “B 0.1%”, “C + B 10%”, “C + B 0.1%” and the OD is increased by ~130%, 130% and 97%, respectively. In addition, *C. albicans* MG3 grows better in “C 1%” supplemented media than *C. albicans* MG2. 50% OD increase and can use “C + B 0.1%” as a source of carbon, however in lower yield than *C. albicans* MG2 (70% OD increase). Remarkably, *C. albicans* MG4 is able to assimilate carbon from the medium

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in broad range "C + B 10%" at the same extent as *C. albicans* MG2 (~130% OD increase). On the other hand, *C. albicans* MG5 is able to grow in the media "B 0.1%", "C + B 1%", "C + B 0.1%" owing to the following OD: ~190, ~100 and ~60%. Finally, *C. subhashii* MG6 is able to use cyclohexane and *n*-butanol from the media complemented as "B 10%", "B 0.1%" and "C + B 0.1%", the OD increase is ~200, ~140 and ~70%, respectively. *C. subhashii* MG6 is able to grow only in "B 10%" medium, other isolates require at least mixture of cyclohexane and *n*-butanol to show higher OD after 72 h of incubation. On the other hand, *C. albicans* MG1 and *C. albicans* MG3 did not increase their optical density, so they cannot be used as a source of carbon in tested compound. The studies have shown that the strains isolated from peat, i.e., the isolates of *Candida albicans* and *Candida subhashii*, have a promising future regarding the use of volatile organic compounds as a source of carbon (Figure 3).

Based on the results presented in Figure 3, 2 isolates (*C. albicans* MG2 and *C. subhashii* MG6) exhibiting the highest rate of carbon assimilation from the tested compounds were selected. They were utilized in the further stage of investigation for immobilization on the surface of selected materials employed in biotrickling filtration.

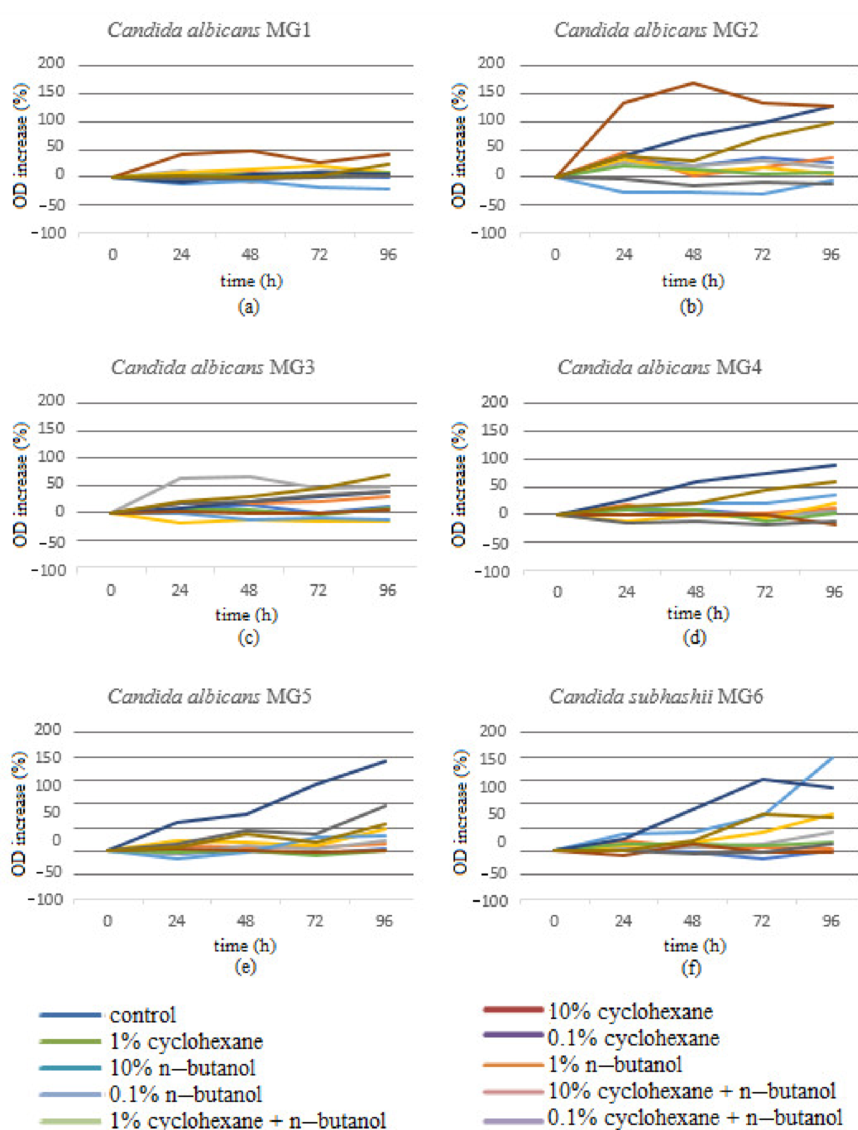

Figure 3. The growth curves of examined strains cultured in the presence of cyclohexane, *n*-butanol and their selected mixtures. (**a**) *Candida albicans* MG1. (**b**) *Candida albicans* MG2. (**c**) *Candida albicans* MG3. (**d**) *Candida albicans* MG4. (**e**) *Candida albicans* MG5. (**f**) *Candida subhashii* MG6. OD—optical density.

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3.2. Evaluation of Immobilization of Fungal Isolates on Support Materials Using Microscopic and Cytometric Measurements

Microscopic observations of the cells of *Candida albicans* and *Candida subhashii* on polyurethane foam reveal that after assumed incubation time selected fungi species are able to form biofilms of relatively comparable thickness on the surface of the foam (Figure 4). The optical microscope image confirmed colonization of the microorganisms on the foam's surface manifested by a presence of numerous stained fungal cells (Figure 4). The colonization was also confirmed by the investigation carried out with a flow cytometer. Obtained information (Table 4) shows that the highest colonization effectiveness (from a quantitative standpoint) on the polyurethane foam occurs for *C. subhashii* 67 ± 0.4 million cells, whereas for *C. albicans* it amounts to 53.5 ± 0.6 million cells.

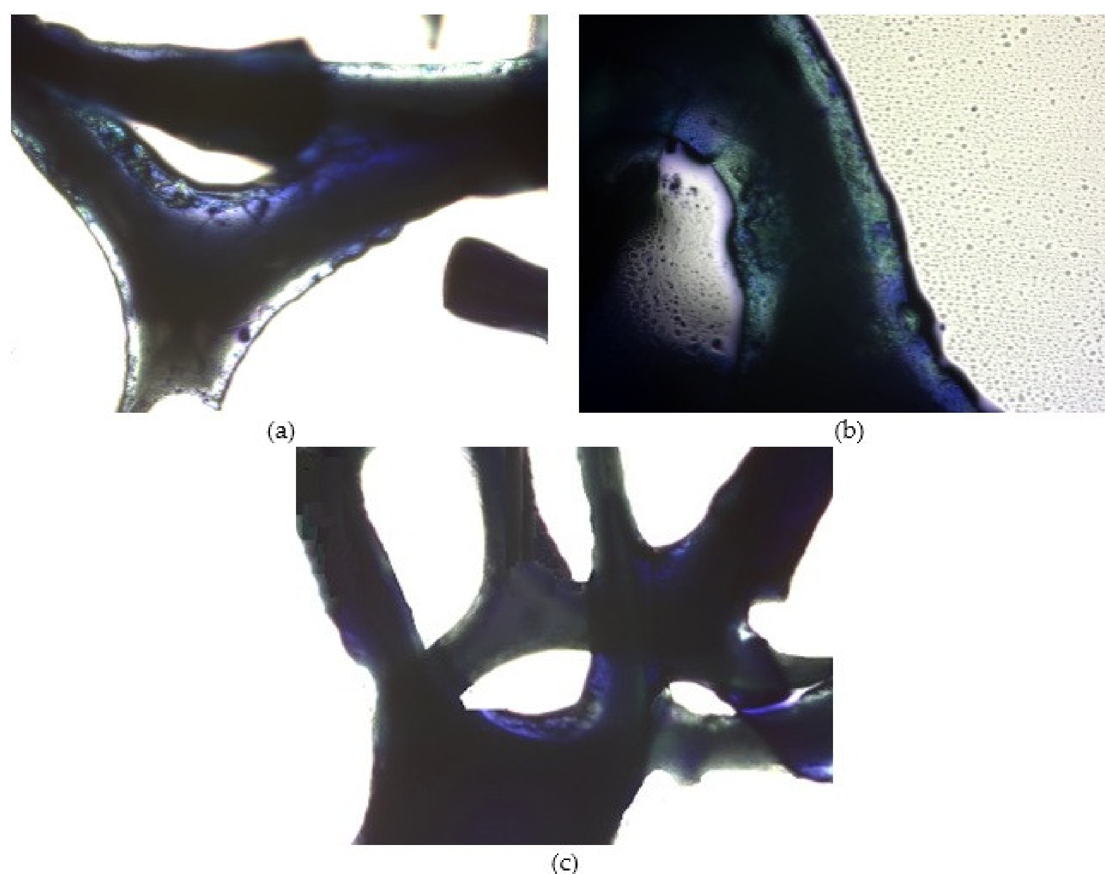

Figure 4. Optical microscope biofilm images. (**a**) The optical microscope images (10×) of a biofilm produced by *C. albicans* on polyurethane foam. (**b**) The optical microscope images (10×) of a biofilm produced by *C. subhashii* on polyurethane foam. (**c**) Control image: clean polyurethane foam.

The microscope images presenting the effect of immobilization of two different fungal isolates on the surface of Bialecki rings (25 × 25) illustrate 2 various ways of the same surface colonization by different fungal isolates (Figure 5). The isolate of *C. albicans* colonizes the surface in form of the clusters. The *C. subhashii* isolate colonized of the surface in a non-uniform way, small clusters of fungal cells were present. The fact that fungi did not occupy the entire available surface is a convenient phenomenon because it enables population growth during running of the processes, in which they will be utilized. According to the microscopic observation and the analyses of fungal cells count using the cytometer (Table 4), the most effective colonization (from a quantitative standpoint) on the Bialecki rings' surface occurs for the isolate *C. subhashii* (1.21 million per 1 cm^2). The smaller numerous colonization was found for *C. albicans* (1.06 million per 1 cm^2).

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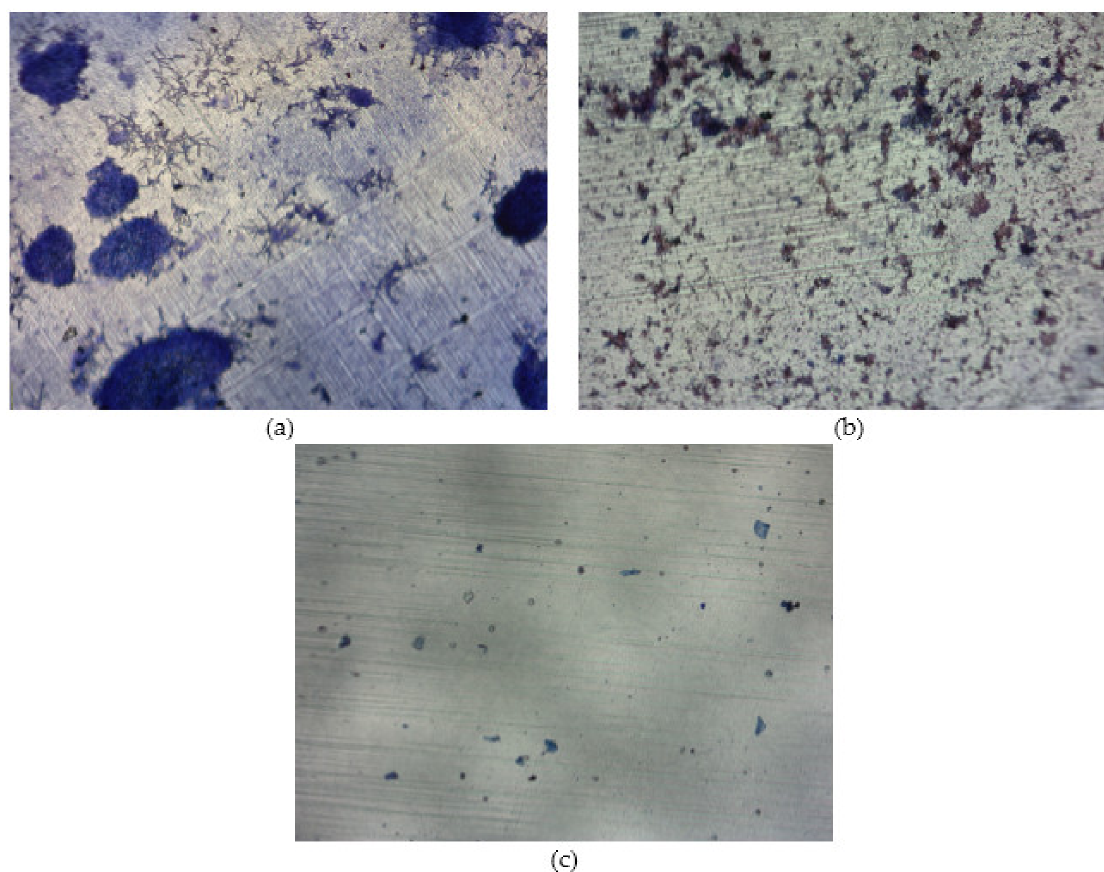

Figure 5. Optical microscope biofilm images. (**a**) The optical microscope image (10×) of a biofilm produced by *C. albicans* on Bialecki ring (25 × 25). (**b**) The optical microscope image (10×) of a biofilm produced by *C. subhashii* on Bialecki ring (25 × 25). (**c**) Control image: clean Bialecki ring (25 × 25).

Analyzing the microscope images showing the effect of colonization of Pall rings with fungi, which have different surface than previously examined Bialecki rings (Table 2). *C. albicans* and the isolate of *C. subhashii* were immobilized on Pall rings' surface (Figure 6) in a similar manner, however different than in the case of Bialecki rings (25 × 25). The cells covered the surface uniformly, locally the cell clusters are also visible. Comparing these two isolates, in the case of *C. subhashii* the average size of the cell clusters is bigger than for of *C. albicans* which translates into the occupied surface by them. Looking at the results of the fungal cell count using the cytometer (Table 4), the biggest number of cells immobilized on the Pall ring occurs for *C. albicans* (0.45 million per 1 cm^2), then for *C. subhashii* (0.42 million per 1 cm^2).

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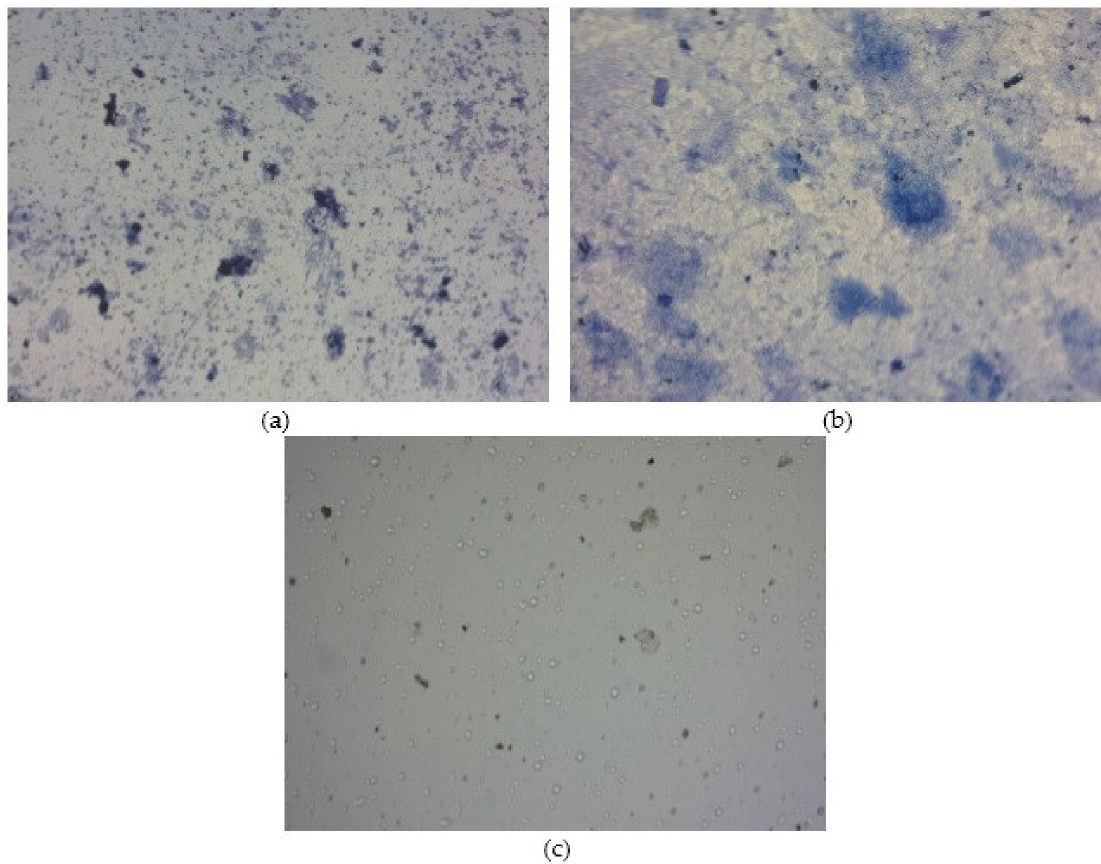

Figure 6. Optical microscope biofilm images. (**a**) The optical microscope image (10×) of a biofilm produced by *C. albicans* on Pall ring. (**b**) The optical microscope image (10×) of a biofilm produced by *C. subhashii* on Pall ring (**c**) Control image: clean Pall ring.

Due to the fact that the third type of the investigated rings—Bialecki rings (50 × 50)—were manufactured from dark green, completely opaque polypropylene, it was not possible to perform observations with the optical microscope. Nevertheless, these rings are made of the same material (the only difference is in color and transparency) (Table 2) and by the same producer as previously analyzed Bialecki rings (25 × 25). Thus, surface of these rings is colonized in a similar way, so the investigation (comparison) should concern only structure of the entire ring and efficiency of its colonization. Looking at the results of fungal cells count using the cytometer (Table 4), the biggest number of cells immobilized on Pall ring occurs for *C. subhashii* (0.34 million per 1 cm^2), then for *C. albicans* (0.29 million per 1 cm^2).

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Table 4. Cytometric count of selected fungal cells after detaching them from the support materials.

	Polyurethane foam		**Bialecki ring (25 × 25)**		**Pall ring (50 × 50)**		**Bialecki ring (50 × 50)**	
	C. albicans	*C. subhashii*	*C. albicans*	*C. subhashii*	*C. albicans*	*C. subhashii*	*C. albicans*	*C. subhashii*
Number of cells from one element of the support material	$(53.5 \pm 0.6) \times 10^6$	$(67.0 \pm 0.4) \times 10^6$	$(49.9 \pm 0.5) \times 10^6$	$(56.8 \pm 0.4) \times 10^6$	$(77.2 \pm 0.6) \times 10^6$	$(72.2 \pm 0.3) \times 10^6$	$(69.6 \pm 0.7) \times 10^6$	$(82.4 \pm 0.5) \times 10^6$
Number of cells from one element of the support material per 1 cm^2	ca. 0.55×10^6	ca. 0.69×10^6	ca. 1.06×10^6	ca. 1.21×10^6	ca. 0.45×10^6	ca. 0.42×10^6	ca. 0.2×910^6	ca. 0.34×10^6
Volume of one element of the support material	157 cm^3		12.27 cm^3		98.13 cm^3		98.13 cm^3	

* ± standard deviation, ca.—circa.

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Comparing the obtained results regarding application of particular support materials in the biotrickling filter, it can be noticed that the best operational parameters are exhibited by Bialecki ring (25 × 25). The biggest number of fungal cells (per 1 cm^2) are colonized on its surface as well as 1 m^3 of this support material also contains the biggest number of fungal cells. Another carrier material characterized by the largest colonization of fungi on the surface is occupied by polyurethane foam. Additionally, it is characterized by the biggest specific surface area, which provides excellent contact of the gas under purification with the biofilm surface on the support material. It should be noted that counting of microorganisms on polyurethane foam requires an exceptional method as the results obtained with application of standard procedure are not realistic. This is due to the fact that the method used to detach fungal cells from the surface does not allow to detach cells from the inside of the foam disk without damaging them. The structure of foam is the reason of retaining the cells inside the disk—both those colonizing and already detached. To perform the examination of the cells presence inside the cells, the disc was cut into 1–2 mm slices stained with methylene blue. Microscopic observation of stained cells allowed for examination the level of their detaching. Such examination exhibited that the number of cells indicated by cytometry is false in case of polyurethane foam discs. Polyurethane foam offers maintenance of constant microbiological activity of biofilm and uniform distribution of biofilm surface over the entire cross-section. It seems that these two types of carrier materials can be successfully used for the immobilization of fungi and used for biotrickling filtration.

3.3. Measurement of Viability of Fungal Isolates Immobilized on Support Material Surface

Membrane permeability after immobilization on the support materials was measured by flow cytometry using propidium iodide, a nucleic acid-binding fluorochrome largely excluded by the intact cell membrane. The results of these studies show that the flow cytometry provides a rapid and sensitive in vitro method for antifungal susceptibility testing of *Candida albicans* and *Candida subhashii.* After staining of all selected fungal isolates with PI, it was determined that viability remained at a very high level (above 95%). Constant percentages of fungi with permeable membranes were observed and they were on average 4.5–5% for selected fungal isolates from polyurethane foams, 1–3% for Bialecki rings (25 × 25), 1.5–2% Bialecki rings (50 × 50) and 1.25–3% for Pall rings (Figure 7).

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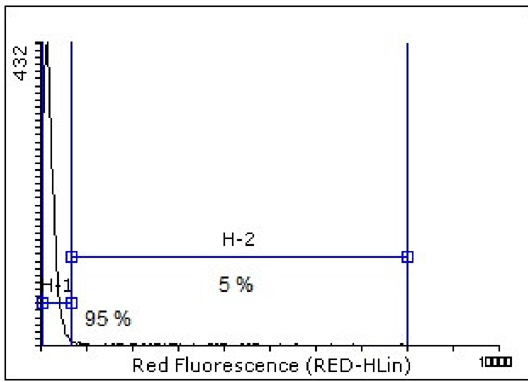

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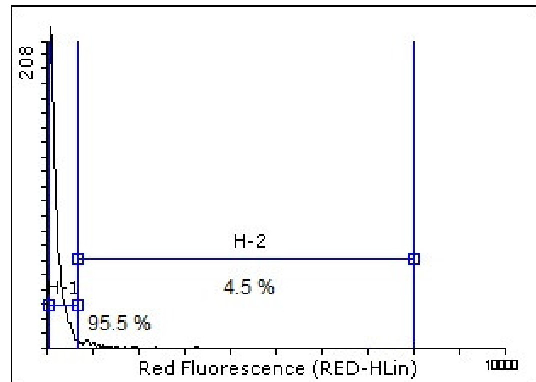

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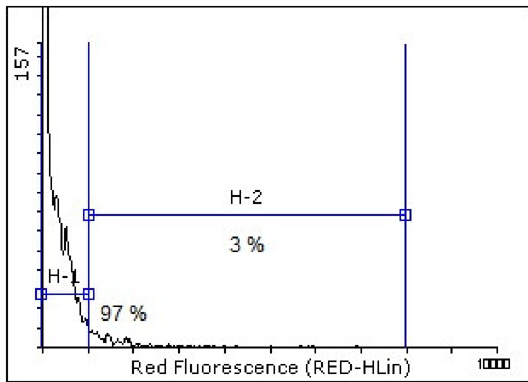

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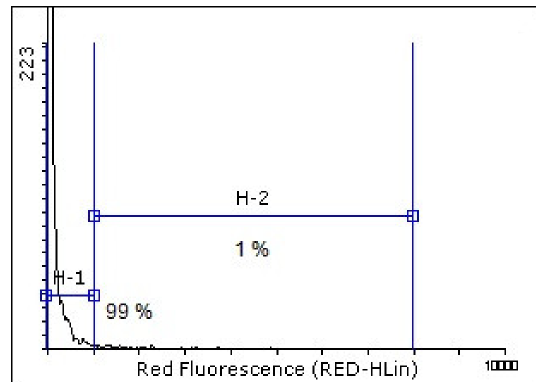

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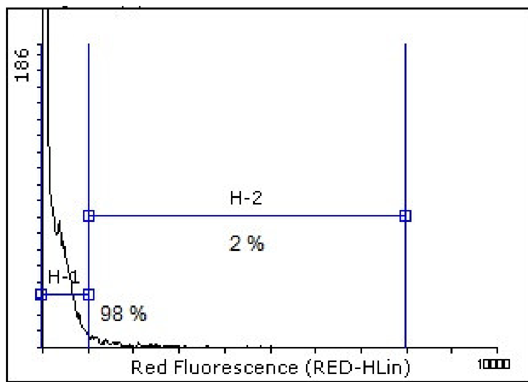

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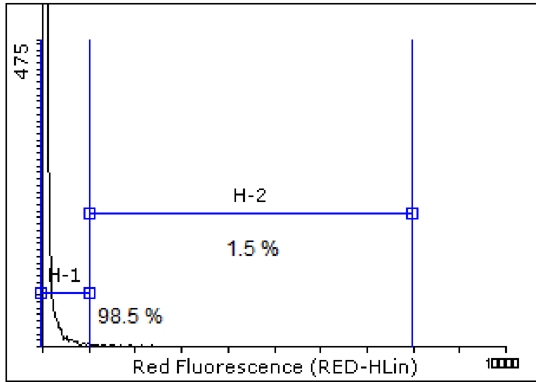

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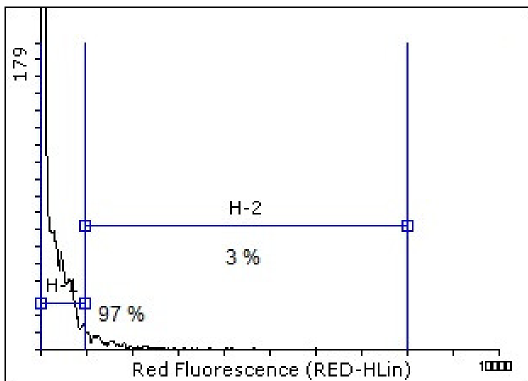

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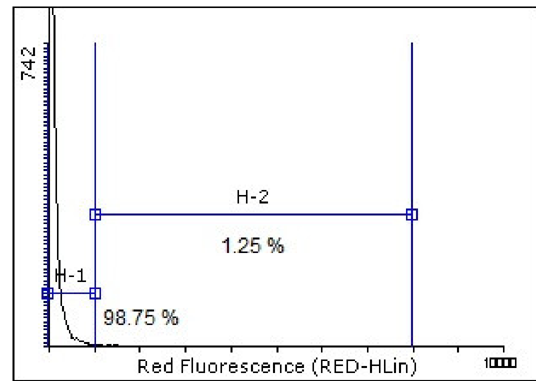

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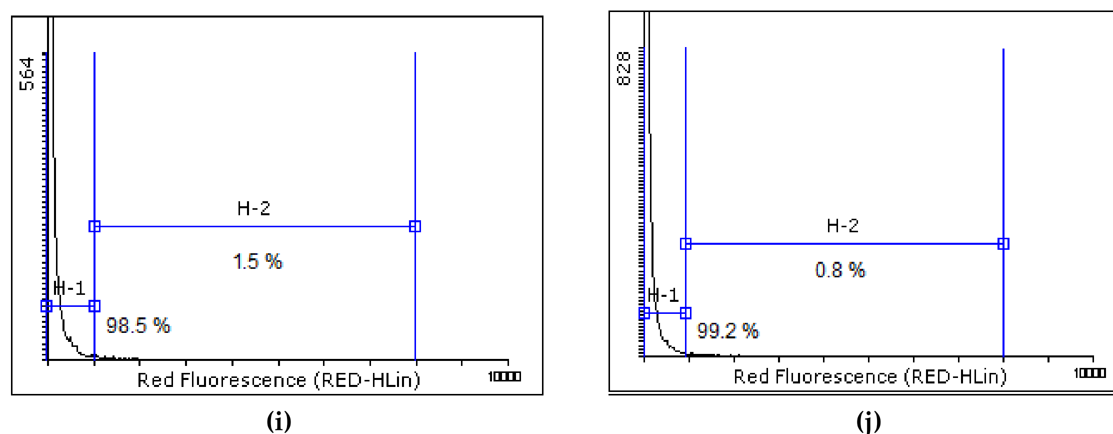

(i) (j)

Figure 7. Analysis of viability determined by flow cytometry for PI-stained selected fungal isolates. (**a**) *C. albicans* from polyurethane foam. (**b**) *C. subhashii* from polyurethane foam. (**c**) *C. albicans* from Bialecki ring (25 × 25). (**d**) *C. subhashii* from Bialecki ring (25 × 25). (**e**) *C. albicans* from Pall ring. (**f**) *C. subhashii* from Pall ring. (**g**) *C. albicans* from Bialecki ring (50 × 50). (**h**) *C. subhashii* from Bialecki ring (50 × 50). The regions H-2 indicated by the bars represent the percentage of fungi with permeable membranes, whereas H-1 are living cells. (**i**) *C. albicans*—control with cells not immobilized. (**j**) *C. subhashii*—control with cells not immobilized.

Immobilization processes can change the microbial population's viability state on the support materials, which includes intact cells, apoptosis-like decayed cells, necrotic cells, and mechanically damaged cells. In this study, the Annexin V binding assay is used to detect microbial population's viability state by the flow cytometry [78–80].

Simultaneous supply of propidium iodide and annexin to the cells allows differentiation between intact cells, necrotic cells as well as early and late apoptosis cells. Annexin V is a protein, which possesses high affinity to phosphatidylserine and thus makes it possible to detect apoptosis-like decayed cells. Propidium iodide binds to cell DNA; it must be emphasized that at low concentrations it can penetrate only through damaged cell membranes, which lost their integrity. The results presented in Figures 8 a-h can be interpreted in the following way: intact cells, which are not penetrated by propidium iodide and not connected with annexin V, are located in the lower left quarter. One of the features characteristic for apoptosis-like decayed cells is phosphatidylserine translocation to the external layer of a lipid membrane. In normal living cells this lipid is present only in the internal layer of the membrane. The upper left quarter is occupied by necrotic cells absorbing propidium iodide, which possess phosphatidylserine on the cytosol side of the membrane, so they do not bind annexin V. The lower right quarter corresponds to early apoptosis cells binding annexin V, the plasmalemma of which is tight and impermeable for iodide. In the upper right quarter there are medium and late apoptosis cells with permeable membrane, which bind annexin and absorb propidium iodide.

Analyzing the changes in structure of the cytoplasmic membrane of all investigated fungi strains on all tested support materials, it can be noticed that in every case the majority of cells remained alive (Figure 8). It is also clear that population distribution in the figures does not differ significantly within a single isolate. Relatively highest decay occurs for the cells, which were immobilized on Bialecki rings 50 × 50. Annexin V test confirmed the results obtained in the experiment on analysis of viability determined by the flow cytometry for PI-stained, which show that the cell immobilization process as well as in principle the dangerous process of cell detachment from the support material's surface are not serious issues here (Figure 8). Cell detachment from the support material imposes a risk of cell membrane damage, which can result even in cell decay. This phenomenon is especially adverse in this type of studies because membrane permeability was employed as a measure of fungal cells viability. Hence, if the results of cytometric investigations had revealed high cell mortality, it would not have been possible to identify the cause of their low viability without additional examinations. It could result from both imperfect method of cell immobilization on the support material as well as

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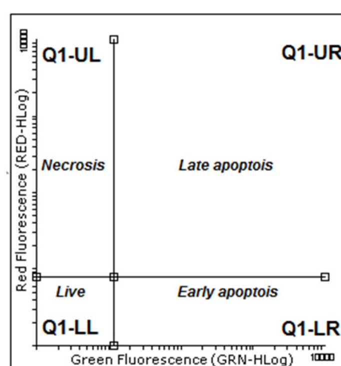
from improper cell detachment. Favorably, both cytometric analyses revealed high viability of all isolates on all support materials, so they can be recommended for future investigations on viability of fungal cells immobilized on the materials utilized in the biotrickling filters. Analyzing Figure 9, regularity can be found; the bigger diameter of the pellet, the bigger number of damaged cells. It results from the fact that for bigger pellets it is more difficult to detach cells from its entire surface. The investigations presented in this paper confirm high effectiveness of the method of colonization of polyurethane and polypropylene materials by fungal (yeast) cells, which, besides ascomycetes, are the fungi species most frequently applied to removal of malodorous compounds from air. Proposed method of cell detachment from the support materials' surface combined with cytometric investigations constitutes a full methodology allowing control over efficiency of colonization of particular support material. The authors are sure that the proposed methodology can be also successfully implemented to control viability of microorganisms during biotrickling filtration processes.



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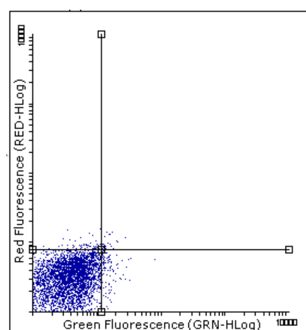

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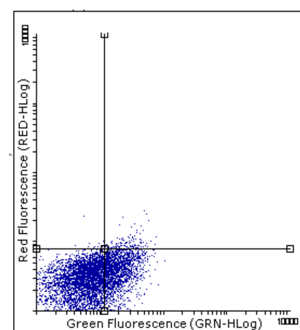

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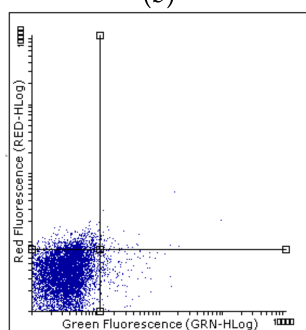

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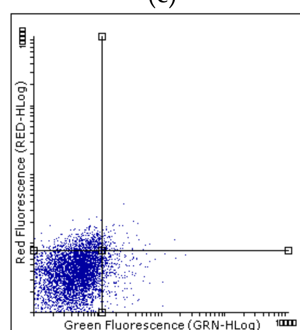

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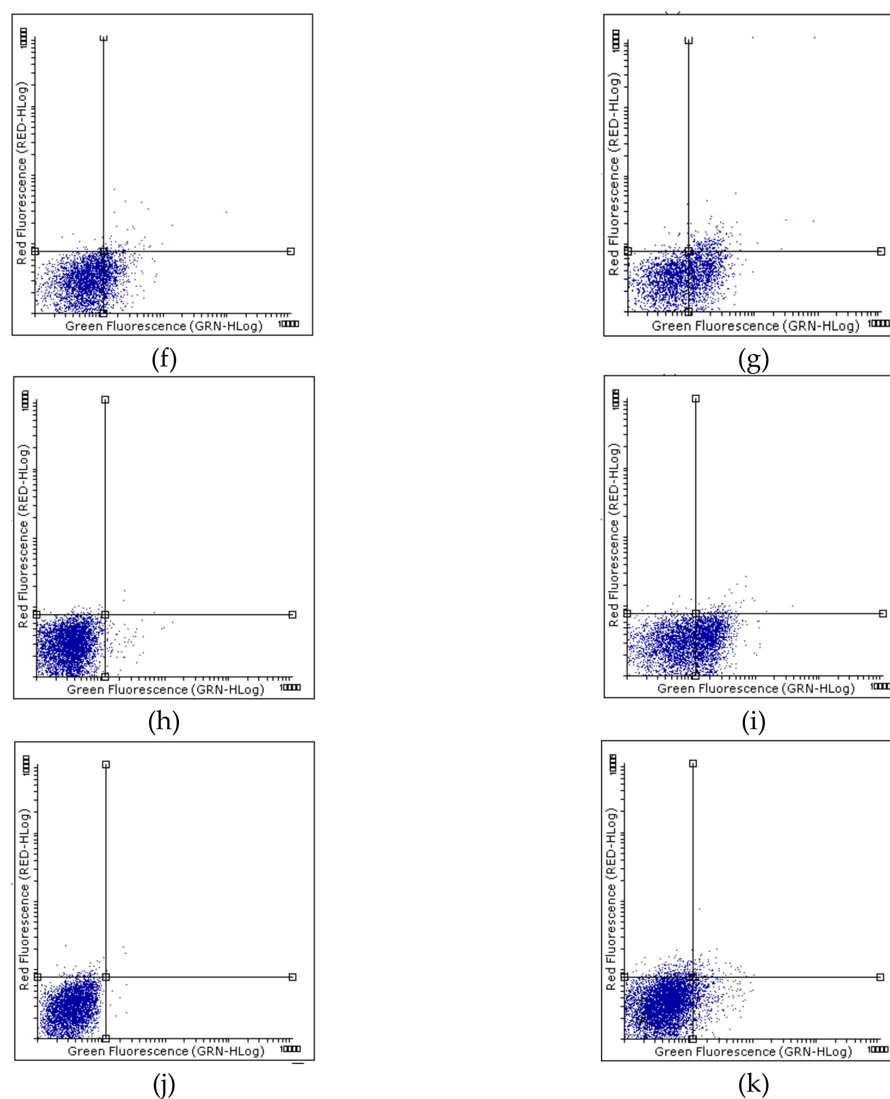

Figure 8. Flow cytometry cytograms of cell viability (intact cells (Q1-LL), early apoptosis cells (Q1-LR), late apoptosis cells (Q1-UR), necrotic cells (Q1-UL)) (**a**) and (**b**) *C. albicans* from polyurethane foam. (**c**) *C. subhashii* from polyurethane foam. (**d**) *C. albicans* from Bialecki ring (25 × 25). (**e**) *C. subhashii* from Bialecki ring (25 × 25). (**f**) *C. albicans* from Pall ring. (**g**) *C. subhashii* from Pall ring. (**h**) *C. albicans* from Bialecki ring (50 × 50). (**i**) *C. subhashii* from Bialecki ring (50 × 50). (**j**) *C. albicans*—control with cells not immobilized. (**k**) *C. subhashii*—control with cells not immobilized.

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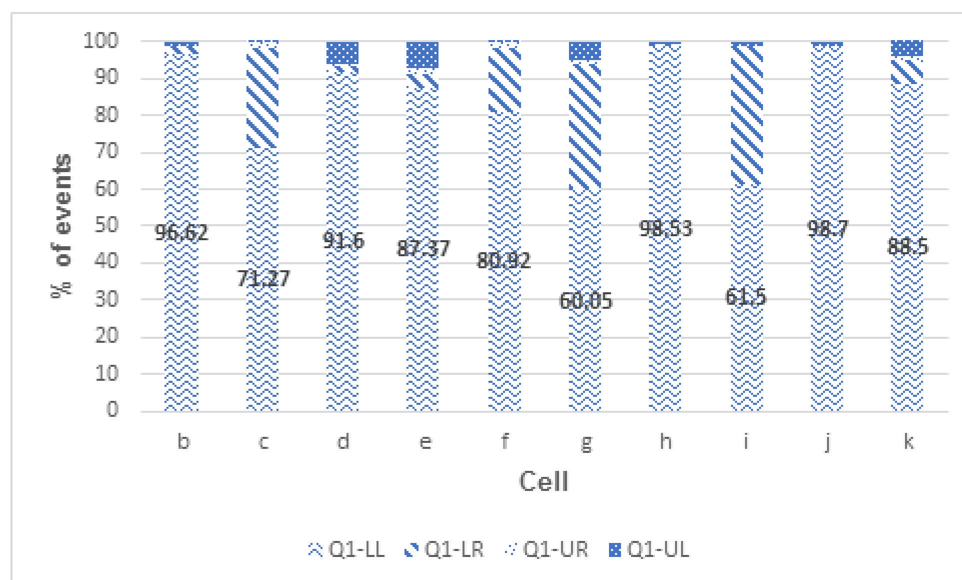

Figure 9. Percentage distribution of cells of selected fungal isolates showing changes in the structure of the cytoplasmic membrane. The diagrams take into account the percentage of cells in the cytograms' quarters (Figure 8): (intact cells (Q1-LL), early apoptosis cells (Q1-LR), late apoptosis cells (Q1-UR), necrotic cells (Q1-UL)). (**b**) *C. albicans* from polyurethane foam. (**c**) *C. subhashii* from polyurethane foam. (**d**) *C. albicans* from Bialecki ring (25 × 25). (**e**) *C. subhashii* from Bialecki ring (25 × 25). (**f**) *C. albicans* from Pall ring. (**g**) *C. subhashii* from Pall ring. (**h**) *C. albicans* from Bialecki ring (50 × 50). (**i**) *C. subhashii* from Bialecki ring (50 × 50). (**j**) *C. albicans*—control with cells not immobilized. (**k**) *C. subhashii*—control with cells not immobilized.

4. Conclusions

Strains of species of unicellular fungi from the peat samples collected from the operating biofilter intended for removal of *n*-butanol vapors from air streams were isolated and recognized. Identified fungi species included: 5 isolates of *C. albicans* (*C. albicans* MG1, *C. albicans* MG2, *C. albicans* MG3, *C. albicans* MG4, *C. albicans* MG5) and *C. subhashii* MG6. Optical density measurements revealed that some fungal isolates were able to assimilate carbon directly from the compounds subjected to removal. The investigations employed *n*-butanol as hydrophilic compound and cyclohexane as hydrophobic one. The purpose of such an approach was to show that fungi inoculated on artificial support material of the biotrickling filter are able to survive and additionally to take part in air purification from malodorous compounds via carbon assimilation from removed compounds. The immobilization process of selected fungal isolates was conducted on four popular artificial support materials utilized in biotrickling filtration, namely polyurethane foam, Bialecki rings (25 × 25) and (50 × 50) as well as Pall rings (50 × 50). Optical and cytometric measurements showed that the immobilization was successful. Propidium iodide and annexin V tests revealed fungal isolates viability at average level of 95%. Moreover, proposed method of cell detachment from the support materials' surface combined with cytometric investigations constitutes a full methodology allowing control over efficiency of colonization of particular support material. Additionally, it can be cheap, fast and highly efficient methodology, which could be a starting point for the biofiltration process aimed at removal of malodorous compounds by selected fungi species. Based on performed comparative analyses, it was found that two types of the support materials, polyurethane foam and Bialecki rings (25 × 25), could be attractive candidates for biotrickling filtration. Polyurethane foam offers maintenance of constant microbiological activity of biofilm and uniform distribution of biofilm surface over the entire cross-section. Small-size Bialecki rings provide unrivalled high colonization of fungal cells per 1 cm^2.

The obtained results could be useful for improving biofiltration processes and provide comprehensive knowledge about the types of microorganisms existing inside the biofilter bed,

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allowing to enhance its performance through isolation of a specific microbial strain and artificial inoculation in the biofilter bed.

Author Contributions: Writing—original draft, M.M. and J.G.; Formal analysis, M.M. and J.G.; Investigation, M.M.; Methodology, M.M. and A.B.-D.; Supervision, A.B.D. and J.G.; Validation, M.M. and J.G.; Visualization, M.M.; Writing—review & editing, M.M., A.B.-D. and J.G. All authors have read and agreed to the published version of the manuscript.

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