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Engineering Microbial Biofilms for Improved Productivity of Biochemicals Important in Restoration of Degraded Ecosystems

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Abstract

Biofilms are being engineered *in-vitro* to produce numerous commodities like biofertilizers, pharmaceuticals, biofuels and electricity, the efficacies of which rely on the biochemicals secreted by the biofilms *i.e.* extracellular polymeric substances (EPS). It has been shown that once EPS-biochemicals of developed biofilms are applied to an ecosystem, they can restore degraded complex ecosystem networks for improved ecosystem functioning and sustainability. Identification of the EPS biochemicals and understanding their contributions to the network interactions in particular, are at initial stage. In the present study, using *Aspergillus niger*, *Nostoc* sp., and gram (−) *Stenotrophomonas maltophilia* & gram (+) *Bacillus subtilis* as test fungal (F), cyanobacterial (C), and bacterial (B) counterparts, respectively we analyzed morphology and biochemical parameters of fungal-bacterial (FBBs), fungal-cyanobacterial (FCBs), cyanobacterial-bacterial (CBBs), and fungal-cyanobacterial-bacterial biofilms (FCBBs). Results revealed that the FCBBs produced the highest concentrations of lipids, proteins, and polysaccharides whereas FBBs generated the highest diversity of biochemicals. Bacterial type (*i.e.* gram + or −) and microbial composition in the biofilm affected the biochemical production. Ecologically and industrially important diverse biochemicals which are used individually as medicines, bioremediating agents and industrial chemicals in human society with certain adverse and beneficial effects were detected in the biofilm-EPS. However, in the nature, simultaneous action of those diverse biochemicals applied as biofertilizers has already shown a huge potential to restore the entire agroecosystems degraded due to farmers' detrimental practices. This striking difference in utilization of the biochemicals and their enhanced effect when they act simultaneously needs further investigations for their better applications.

Keywords

Biochemicals, Biofilms, Ecosystem Restoration, Extracellular Polymeric Substances

1. Introduction

Microbes live mainly in two modes of lifestyles viz. planktonic (freely swimming) and surface-attached biofilm, enabling their endurance in a range of environments including extreme settings. Metabolic changes have been recognized between these two phenotypes [1]. Comparative transcriptome investigations of biofilms versus planktonic cells have confirmed that biofilm cells illustrate divergent metabolic activity with significant up-regulation of genes responsible for survival, persistence and growth in a biofilm environment [2]. This leads the biofilms to produce and secrete a wider range of biochemicals required for ecologically important processes than planktonic cells. Those biochemicals can be found in the EPS which are secreted by the biofilm itself. The EPS is now considered as the "dark matter of biofilm", because their composition has not been fully recognized yet. And also molecular interactions of the biochemicals are still to be defined [3] [4]. Here, we hypothesize that the identification and understanding of specific EPS biochemicals and their contribution to the complex interactive ecosystem networks are important to improve the productivity of the newly developed biofilms and their utilization for various biotechnologies. The knowledge gained would also be beneficial for further improvement of innovative concepts like biofilm biofertilizers [5], which are being practiced successfully in agriculture now [6], and also newly proposed notion of biofilm medicines [7], both of which are engaged in restoration of degraded agroecosystems and human body ecosystem, respectively.

The EPS biochemicals primarily consist of lipids, proteins, and polysaccharides [8]. The productivity of biofilms mainly depends on the metabolic cooperation of their resident microbes. For example, the effective microorganism (EM) technology [9] developed in Japan has shown that the mutually compatible co-existing microorganisms have excellent metabolic cooperation leading to self-sufficiency which helps its stability and a wide range of activities in the environment [10].

In 2003, *in-vitro* engineering of eco-friendly beneficial microbial biofilms, specifically fungal-bacterial biofilms was first introduced for biotechnological applications [10]. Later, the same concept has been reintroduced using a different terminology, *i.e.* synthetic microbial consortia [11]. By now, scientists have developed biofilms to produce numerous commodities like medicines, agrochemicals, bioremediating agents and industrial chemicals because of their inherent characteristics of self-immobilization, high resistance to reactants, and long-term activity, all of which facilitate continuous processing [5] [12]. Howev-

er, optimizing biofilms to achieve desired products is a compulsion to maintain their productivity on an industrial scale.

Therefore, this study was designed to characterize the biofilm-EPS during the growth and maturation of different biofilm complexes in order to develop more productive biofilms, which would be beneficial to innovations in this field of research.

2. Materials and Methods

2.1. Biofilm Formation

To develop monoculture and mixed culture biofilms, *A. niger*, *Nostoc* sp., and *S. maltophilia*/*B. subtilis* were used to represent fungi, cyanobacteria, and bacteria, respectively. From each, one loopful of microbes was introduced to 250 mL CCM [13] broth to produce monocultures. Appropriate volumes of each monoculture was taken and inoculated to have two contrasting ratios [F:B:C 1:3:12 (α), and 12:4:1 (β)] in to 15 ml sterilized centrifuge tubes containing 10 ml of CCM medium to develop different biofilms; FBBs, FCBs, BCBs and FCBBs. In this manner, 14 different biofilms were developed; FBSα, FBBα, CBSα, CBBα, FCBSα, FCBBα, FBSβ, FBBβ, CBSβ, CBBβ, FCBSβ, FCBBβ, FCα and FCβ [C = *Nostoc sp.*, F = *A. niger*, BS = gram (−) *S. maltophilia*, BB = gram (+) *B. subtilis*]. The cultures were incubated for seven days to form biofilms. Completely randomized design was employed with three replicates for each biofilm type.

The developed biofilms were observed under low and high power of the light microscope by staining with lacto-phenol cotton blue to characterize morphological differences among the different types of biofilms.

2.2. Extraction of the EPS

The extraction of EPS was performed by combining physical and chemical methods [14]. A NaCl solution was prepared by dissolving 5 g of NaCl in 100 mL of sterilized distilled water. Ten microliters of the solution was added to 15 ml centrifuge tubes that contained the developed biofilms. Then, they were subjected to ultra-sonication for 10 minutes, followed by centrifugation at 5000 rpm for 10 minutes. Finally, the supernatant in the centrifuge tube was taken for further analyses.

2.3. Characterization and Identification of Biochemicals in the Biofilm-EPS

2.3.1. Attenuated Total Reflection–Fourier Transform Infrared (ATR-FTIR) Spectroscopy

The ATR-FTIR was carried out on a Nicolet is 50 FTIR system (Thermo Fisher Scientific) in the range of 600 - 4000 cm^{-1}.

The production of different classes of biological macromolecules was quantified based on the diagnostic bands for carbohydrates (960 - 1130 cm^{-1}), proteins (1580 - 1700 cm^{-1}) and lipids (1710 - 1765 cm^{-1}) [15].

2.3.2. Liquid Chromatography–Mass Spectrometry (LC-MS) of EPS

Each sample was separately diluted with HPLC grade MeOH to get a 1:1 solution. Then, the samples were filtered through 25 mm nylon mesh and then 0.45 μm filters prior toinjection. The samples were subjected to LC-MS analysis using Thermo scientific DIONEX UltiMate 3000 UHPLC system having a reversed-phase Supelco C-18 analytical column (15 cm, 4.6 mm, 3 μm). UHPLC system included a high-pressure pump, an automatic sample injector, a column thermostat, and a photodiode array detector. The column temperature was 28˚C. The mobile phase consisted of 100% HPLC grade MeOH (A) and 0.001% (v/v) analytical grade formic acid in ultrapure water (B). The separation was carried out in gradient elution as follows: until 2 min 90% of B, at 28 min 2% of B, at 30 min 2% of B, at 32 min 90% of B, and 90% of B until 35 min with a flow rate of 0.4 mL/min. The injection volume was 10 μL. Spectral data from all peaks were accumulated in the range 200 - 400 nm and UV-visible chromatograms were recorded at 224, 254, 280, and 360 nm with 1 mm bandwidth. The data collection rate was 5 Hz. Response time was 2.000 s.

Thermo Scientific LCQ Fleet mass spectrometer, operated with an electrospray (ESI) ion source was coupled to the UHPLC system. Nitrogen generated from pressurized air by F-DGSi Alliance V350 laboratory gas generator was used as a nebulizer gas and drying gas. The drying gas was heated to 350˚C and introduced to the capillary region at a flow rate of 36 arbitrary units. The capillary was heated to 320˚C and the capillary potential was set at −40 V. Spray voltage was 4500 V and the corona current was 25 mA. Full data acquisition was performed for mass spectroscopy, scanning from m/z range 100 - 1500 in centroid mode in negative polarity. The activation type was collision-induced dissociation. The normalized collision energy was 35 with an activation time of 30.00 ms.

2.3.3. Identification of Biochemicals in the Biofilm-EPS

The biochemicals in the biofilm-EPS were identified by analyzing LC-MS chromatograms using NIST Mass Spectral Library (National Institute of Standards and Technology, Gaithersburg, MD, USA). The molecules which were identified with 100% and 90% - 100% probabilities were considered as compounds with confirmed and predicted structures, respectively.

2.4. Statistical Analysis

The data were analyzed using the statistical software Minitab version 17. FTIR absorbance data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's HSD test. Probability ≤ 0.05 was used as the threshold for significance.

3. Results and Discussion

3.1. Morphology of Biofilms

In the FBB, fungal filaments acted as the biotic surface for bacterial cells to co-

lonize (Figure 1), as was also observed in previous studies [16] [17]. The attachment of bacterial cells to fungal filaments initiated within the first 48 hours of the biofilm formation and reached to its maximum attachment by 96 hours. The accumulation of EPS was clearly observed during the maturation of the FBB, and the highest quantity of EPS was observed on day 7 (Figure 1).

The same observations were made in CBBs. In the FCBs, fungal filaments were intertwined with cyanobacterial threads and they were surrounded by EPS. In the FCBBs, bacterial cells attached to fungal and cyanobacterial filaments surround by an extracellular matrix.

3.2. Characterization of the Biofilm-EPS

Production of mainly lipids, polysaccharides and proteins was observed in the biofilm-EPS (Figure 2). The highest amounts of lipids, proteins and polysaccharides were produced by the FCBSα biofilm, whereas the lowest amounts were produced by the CBBα, CBSβ and FCα, respectively (Figure 2).

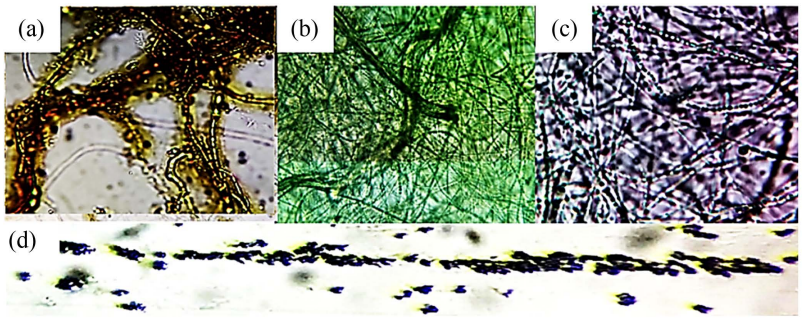


Figure 1. Morphological characteristics of (a) Fungal-bacterial, (b) Cyanobacterial-fungal, (c) Cyanobacterial-bacterial-fungal, and (d) Cyanobacterial-bacterial biofilms. Magnification 400×.

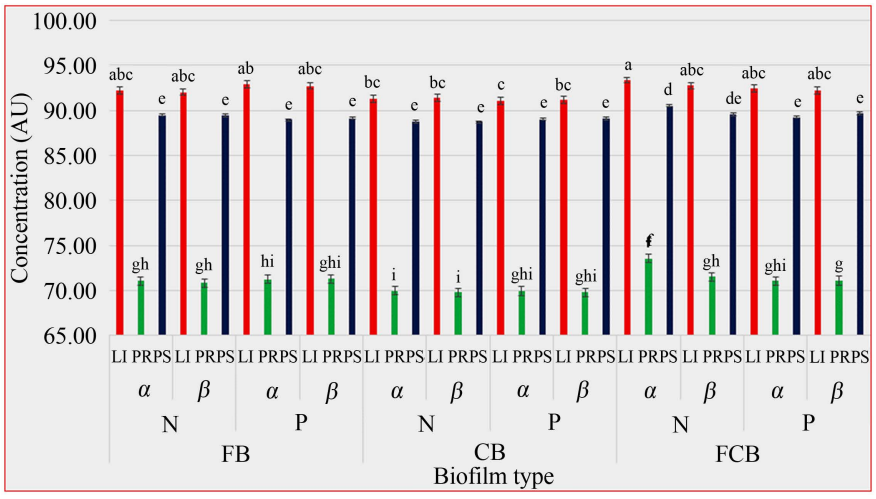


Figure 2. EPS biochemicals produced by different biofilms (C = *Nostoc* sp., F = *Aspergillus niger*, N = gram negative *Stenotrophomonas maltophilia*, P = gram positive *Bacillus subtilis*, α = inoculation ratio of F: C: B = 1:12:3, β = inoculation ratio of F:C:B = 12:1:4, LI = lipids, PR = proteins, PS = polysaccharides).

In FCBBs, *S. maltophilia* performed better than *B. subtilis* in producing lipids, proteins and carbohydrates (Figure 2). In FBBs, *B. subtilis* did better than *S. maltophilia* in producing lipids (Figure 2), and *S. maltophilia* and *B. subtilis* performed similarly in producing proteins (Figure 2). Moreover, *S. maltophilia* produced higher amounts of carbohydrates than *B. subtilis* (Figure 2). In CBBs, *S. maltophilia* and *B. subtilis* performed similarly in producing lipids and proteins (Figure 2), but *B. subtilis* produced a higher amount of polysaccharides than *S. maltophilia* (Figure 2).

In FCBBs, the ratio α performed better than the ratio β in producing lipids, proteins and carbohydrates (Figure 2). In FCBs, the ratio β produced a higher amount of carbohydrates than the ratio of α (Figure 2). In FBBs and CBBs, both ratios performed similarly in producing lipids, proteins and carbohydrates (Figure 2).

Compound diversity of the fungal-bacterial biofilms (here the *A. niger-S. maltophilia* biofilm) was higher than that of the other biofilms (Figure 3). Possibly, it is the reason for the success of fungal-bacterial biofilms in industry [18]. Therefore, the *A. niger-S. maltophilia* biofilm was characterized further in order to identify the composition of its biofilm-EPS.

3.3. Characterization of *A. niger-S. maltophilia* Biofilm-EPS

Production of mainly lipids, polysaccharides and proteins was observed in the biofilm-EPS (Figure 4). They are reported to play a crucial role in gene regulatory, protein and signaling networks which govern functioning and stability of biofilms [3]. Moreover, they also contribute to enhance functioning of ecosystems like soil and human body [7].

In the FBB, number of high molecular weight metabolites (HMWMs) was significantly higher than that of low molecular weight metabolites (LMWMs) ($P < 0.05$, Figure 5). In natural systems, the HMWMs like multifunctional enzymes

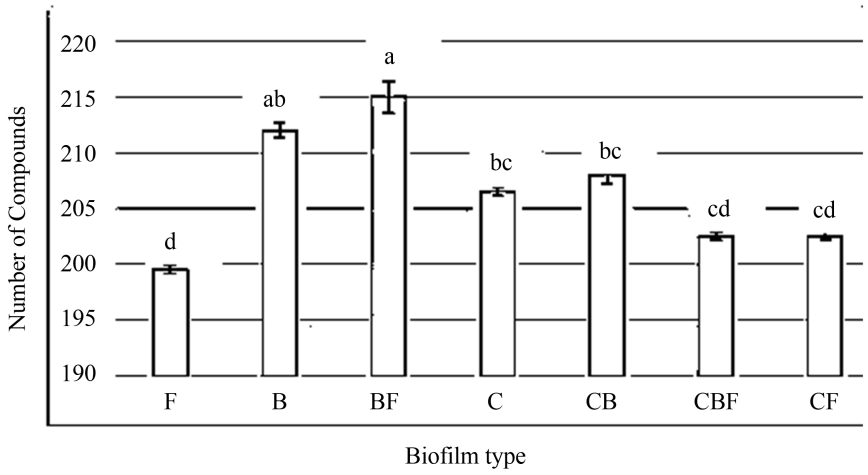


Figure 3. Total number of compounds of the EPS produced by different mono and mixed culture biofilms. B—bacterial, F—fungal, C—cyanobacterial, BF—bacterial-fungal, CB—cyanobacterial-bacterial, CF—cyanobacterial-fungal, CBF—cyanobacterial-bacterial-fungal.

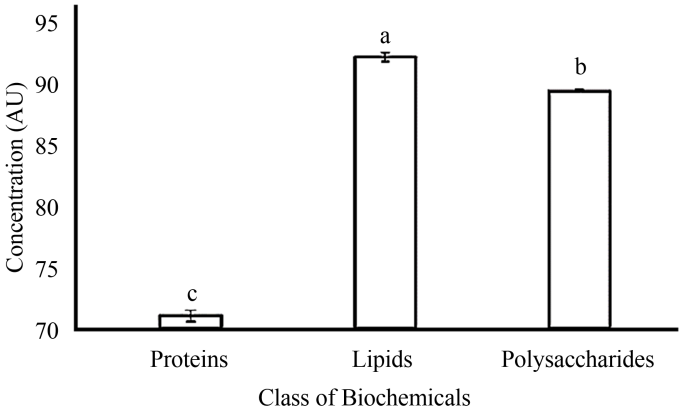


Figure 4. Biochemicals in the EPS of the developed *Aspergillus niger-Stenotrophomonas maltophilia* biofilm.

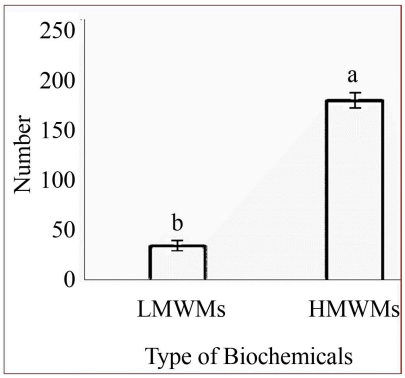


Figure 5. Number of biochemicals in the EPS of the developed *Aspergillus niger-Stenotrophomonas maltophilia* biofilm characterized by its molecular weight. Low molecular weight metabolites (LMWMs, <900 kDa), High molecular weight metabolites (HMWMs, >900 kDa).

work for the generation of diverse chemistry. The LMWMs participate in globally important processes which determine the physiological characteristics of Prokaryotes, Archaea and Eukaryotes, and also regulation of species-species relations [19]. Those processes influence biomineralization and even the ozone level in the stratosphere [20].

Identification of Biochemicals in Biofilm-EPS

In the *A. niger-S. maltophilia* biofilm, 214 biochemicals were detected from LC-MS, and only 5 of them were fully identified. In addition, 14 biochemicals were predicted with 90.3% - 98.9% probability. In all, only 9% of the biochemicals of the LC-MS spectrum was characterized.

Most of the biochemicals identified belong to compounds related to medicines, and the rest to agrochemicals, bioremediating agents and industrially important chemicals which are used individually in our society (**Table 1** and **Table 2**). However, they collectively carry out many physicochemical and biological functions via nodes of the complex interactive biochemical networks in the natural environment. For example, plant growth promoting rhizobacteria (PGPR)

Table 1. Biochemicals identified from the *Aspergillus niger-Stenotrophomonas maltophilia* biofilm-EPS.

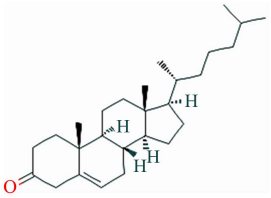
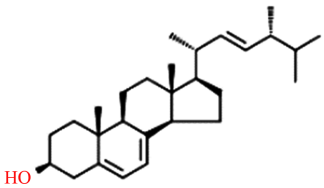
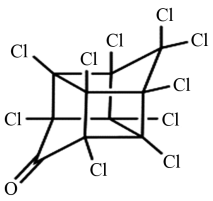
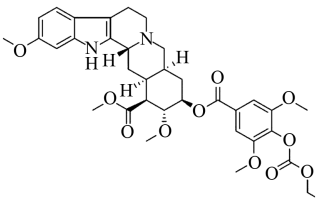
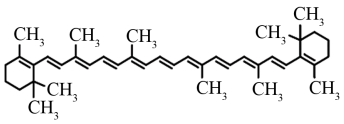
Biochemical molecule	Structure	Action/Use
Cholest-5-en-3-one		Steroid drug that has positive uses against obesity, liver disease, and keratinization [21]
Ergosterol		The precursor of vitamin D_2 [22]
Kepone		Used as an insecticide [23]
Syrosingopine		An antihypertensive agent related to reserpine that was found to potentiate the anticancer effects of the antidiabetic agent metformin and phenformin unaccompanied by harmful effects on normal cells [24]
β Carotene		The precursor of vitamin A [25]

Table 2. Predicted biochemicals in the *Aspergillus niger-Stenotrophomonas maltophilia* biofilm-EPS.

Biomolecule	Action/Use	Probability (%)
1,2-Benzenedicarboxylic acid, dinonyl ester	Prevention of oxidative stress-induced neurodegenerative disorders [26]	93.0
9,10-Anthracenedione, 2-ethyl-	Anticancer compound [27]	96.4
Bufotalin	Anticancer compound [28] [29]	90.3

Continued

Digitoxin	Drug for treating heart failure [30]	91.1
Erbon	Herbicide [31]	96.2
Hydrazine, tetraphenyl-	Catalyzing hydroamination reactions [32]	92.4
Lincomycin	Antibiotic [33]	94.3
Methoserpidine	Antihypertensive drug (used to treat high blood pressure) related to reserpine [34]	94.3
N-Benzyloxycarbonyl-L-tyrosine	Used as a substrate for testing microbial protease production [35]	92.0
Phosphoramidic acid	Produce nanodots with fluorescence [36]	98.9
Phthalylsulfathiazole	Antibiotic [37]	96.0
tert-Butyl 2,4,5-trichlorophenyl carbonate	Used for the preparation of Tezacaftor, a drug used for the treatment of cystic fibrosis [38]	98.8
Testosterone cypionate	Drug for treating hypogonadism [39]	98.4
Vobassan-17-oic acid, 4-demethyl-3-oxo-, methyl ester	Anti-depressant and Anti-inflammatory effects [40] [41]	96.5

exert their action in the rhizosphere by producing a wide array of extracellular molecules for communication and defense, which play a crucial role in biochemical networks for beneficial plant-microbe interactions [42]. A similar functioning has been observed in metabolic networks of human gut microbiome [43].

Microbial society of a complex biofilm is comparable to human society. The biofilm microbes produce the biochemicals (goods) they require for their functioning and health, which lead to sustenance. The biochemicals used by the biofilms are as same as those which are utilized by the human society. The striking difference is that humans use them individually with some adverse side effects and relatively low efficacy whereas the biofilm microbes utilize them simultaneously to improve the network interactions, which leads to better functioning in the environment, as has already been exemplified in agriculture [6]. This striking difference in utilization of the biochemicals and their enhanced effect when they act simultaneously needs further investigations. Moreover, some behavioral patterns and intelligence of microbes are analogous to those of humans [44] [45]. These similar features clearly show the historical evolution of life starting from microbes on the earth.

At present, the ecologically important biochemicals are depleted in the nature due to degradation caused by anthropogenic activities, and it leads to deteriorate ecosystem health [46]. When biochemicals are added to any degraded ecosystem,

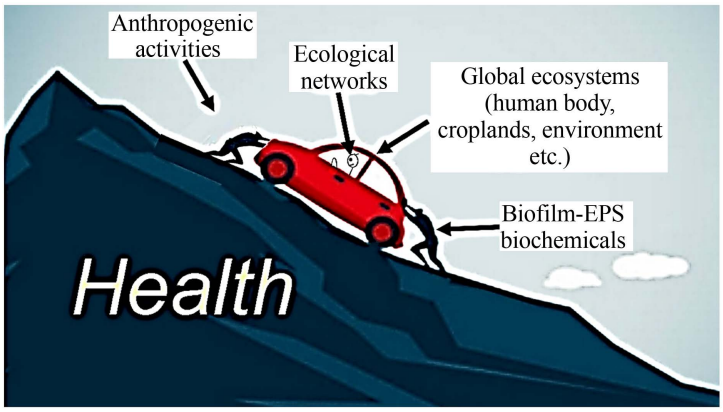


Figure 6. A cartoon showing the action of biofilm-EPS biochemicals in reinstating the health of global ecosystems.

its biodiversity will increase with simultaneous improvement of ecosystem functioning that leads to reinstate ecosystem health. The biofilm-EPS is a good candidate in supplying the biochemicals to any degraded ecosystem for its recovery, as depicted in the cartoon in **Figure 6**. This concept is valid for managed as well as natural ecosystems, including even human body ecosystem [7] [46].

4. Conclusion

The diverse biofilms used in the present study showed distinct morphological characteristics during their formation. Among them, fungal-bacterial-cyanobacterial biofilms and fungal-bacterial biofilms are in the forefront when considering the quantity of EPS produced and their biochemical diversity, respectively. Moreover, numerous important biochemicals are generated in the biofilm-EPS suggesting a huge potential of reinstating degraded ecosystems, including human body ecosystem. However, the current practice of utilizing the biochemicals by the human society needs revisiting, if we are to reduce their adverse side effects and also to further improve their efficacy of action in various applications.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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