RESEARCH ARTICLE

EVALUATION OF CELLULOLYTIC FUNGAL-BACTERIAL BIOFILMS FOR THE ENHANCEMENT OF BIOETHANOL PRODUCTION

A. P. Henagamage

Department of Science and Technology, Faculty of Applied Sciences, Uva Wellassa University, Badulla, Sri Lanka

ABSTRACT

Biodegradation of lignocellulosic materials by cellulolytic microbial consortia would be beneficial in cellulosic biofuel production. This study aimed at investigating the cellulose degradation potential of fungal-bacterial biofilms (FBBs) followed by bioethanol production. Fungi and bacteria were isolated from soil samples and their cellulolytic activity was analyzed in carboxy methylcellulose medium. FBBs were developed from the selected fungi and bacteria and their cellulolytic activity was evaluated using reducing sugar production through 3,5-Dinitro Salicylic acid method. Cellulolytic digestion followed by fermentation was performed with the selected biofilms by co-culturing *Saccharomyces cerevisiae* suspension. Ethanol content was quantified by dichromate method and confirmed through FTIR analysis. The highest significant ($P < 0.05$) cellulolytic activity was observed in *Aspergillus niger* (F6) and *Pseudomonas aeruginosa* (B7). Significantly ($P < 0.05$) the highest cellulolytic activity was recorded by *A. niger-P. aeruginosa biofilm (FB2).* Fermentation study confirmed higher production of ethanol by the biofilms co-cultured with *S. cerevisiae* over their single cultures and the highest was recorded by *A. niger* - *Bacillus subtilis* biofilm (FB1) co-cultured with *S. cerevisiae*. In this context, FBB can be considered as an important agent for bioethanol production from cellulosic materials through cellulolytic digestion followed by fermentation.

Keywords: *Cellulose, Cellulolytic activity, Fungal-bacterial biofilms, Bioethanol*

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1. INTRODUCTION

Bioethanol, which is made from lignocellulosic biomaterials, is being developed as a possible alternative fuel for traditional energy sources [17]. Cellulose is the major compound in lignocellulosic materials and the hydrolyzing of crystalline cellulose into simple and fermentable sugars is the major hurdle in bioethanol production from lignocellulosic materials [32]. Cellulase enzymes produced by different bacteria viz.,

*Corresponding author: *amila@uwu.ac.lk*

Pseudomonas sp., *Bacillus* sp., and *Micrococcus* sp., and fungi *viz.*, *Tricoderma sp*. and *Aspergillus sp*., may hydrolyze cellulosic material to soluble sugars [26]. Although many microbes are capable of degrading cellulose, only a few of them produce sufficient amounts of enzyme capable of complete cellulose hydrolysis in vitro.

Microbial biofilms have emerged as new sources for biocatalysts in recent years due to their potential of synthesizing added-value compounds such as organic acids, enzymes and alcohols [29]. Biofilms are aggregates of microorganisms usually attached to a solid surface, which are protected by a matrix of extracellular polymeric substances (EPS) [2]. Biofilms can have significantly different properties from their free-living individuals and can be used to increase the efficiency of biotransformation processes particularly through the production of extracellular enzymes [35]. Numerous of these enzymes participate in the breakdown of both soluble and solid biopolymers, such as cellulose. Comparing microbial biofilm-derived cellulases to their pure cultures and various mixtures, they seem to have higher enzyme activity [2]. However, the function of biofilm in cellulose degradation and its underlying mechanism remains poorly studied.

Another anticipated benefit of biofilm technology in the biofuels sector is that it could boost product tolerance to inhibition caused by the presence of EPS [7]. Therefore, biofilms reactors are used in alcohol production, in order to overcome cell washout and low productivity in continuous fermentation. Biofilms were also shown to have higher ethanol tolerance in yeast and bacteria than planktonic equivalents [38]. Although the biofilm technology is applied for enhancing delignification and saccharification [33], there are no records of utilizing a single biofilm-based system in bioethanol production processes. Hence this study aimed at developing a biofilm-based system for bioethanol production through microbial lignocellulosic digestion followed by fermentation.

2. MATERIAL AND METHODS

2.1 Isolation and screening of cellulolytic fungi and bacteria

Coir retting water and soil samples collected from three different places a near coir mill located at Kuliyapitiya, Kurunegala district, Sri Lanka, were subjected to a serial dilution (10-fold) followed by the isolation of fungi on potato dextrose agar (PDA) and bacteria on Nutrient Agar (NA). Isolated bacterial colonies and fungi were differentiated

according to the colony morphology and the colonies were sub cultured to prepare pure isolations. Pure bacterial and fungal isolates were individually inoculated on Carboxy Methyl Cellulose (CMC) agar plates and incubated at 28 ± 1^0C for 48 hours for bacteria and incubated at 28 ± 1^0C for 72 hours for fungi. The plates were flooded with 0.1% Congo red for 20 min and washed with 1 M NaCl for 15 min. Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial and fungal isolates. The diameter of the clear zone was measured to calculate the cellulolytic index (clear zone/ colony diameter).

2.2 Formation of fungal-bacterial biofilms

The most cellulolytic fungal and bacterial isolates were combined in all possible ways in 25 mL of Yeast Mannitol Broth (YMB) medium and cultured at room temperature with continuous shaking for eight days to create biofilms. The adhesion of bacterial cells to fungal filaments was observed continuously under an optical microscope (model BX43F) by staining with lactophenol cotton blue [35]. The Biofilms with the best attachments and single cultures were chosen to study their cellulose breakdown efficiency in liquid medium.

2.3 Cellulose degradation assay

Cellulase activity of the selected biofilms and their single cultures was determined by estimating the reducing sugar produced during enzymatic reaction by 3, 5-dinitrosalicylic (3, 5-DNS) acid method [19]. The selected biofilms combinations (5 mL) and 5 mL of their single cultures (10^6 cfu/ mL) were inoculated into 15 mL of YMB medium incorporated with 5 g of sterilized cellulose powder separately. The initial reducing sugar content was measured using 3, 5-DNS acid method before the inoculation of biofilms and their single cultures. Subsequently, the cultures were incubated at room temperature with continuous shaking for eight days. Then, the amount of reducing sugar produced with the time was measured by withdrawing samples of cell-free culture supernatant at 2 days intervals using 3, 5-DNS acid method. The reaction was terminated by adding 1.5 ml of DNS reagent followed by boiling the mixture for 5 min. Sugars liberated were determined by measuring absorbance at 540 nm using UV spectroscopy. Cellulase production was estimated by using glucose calibration curve. All the experiments were

carried out in triplicates. The best cellulolytic biofilm combinations were subjected for the fermentation with continuous mixing.

2.4 Bioethanol production through fermentation

Two grams of active yeast (*Saccharomyces cerevisiae*) was added to YMB medium and incubated at room temperature for 24 hours with continuous shaking. The best cellulolytic biofilms and their single cultures (10^6 cfu/mL) were inoculated to YMB medium incorporated with 5 g of sterilized cellulose powder and incubated at room temperature under continuous mixing for 72 hours. After 72 hours of incubation, 5 ml of *S. cerevisiae* (10^5 cells/mL) suspension was co-cultured with each biofilm combinations/ single cultures (1: 1 ratio) and allowed for the fermentation of the produced reducing sugars during the initial incubation into bioethanol under anaerobic conditions for 10 days in a closed system (pH 5.5, 28 ± 1^0C). Biofilm combinations and single cultures of the biofilms without co-culturing *S. cerevisiae* were considered as negative controls. Further, *S. cerevisiae* only was considered as the positive control. Ethanol content was quantified by dichromate method and the presence of ethanol was confirmed through FTIR analysis in the frequency range of 400 to 4,000 cm^{-1}. All experiments were run in triplicate to determine the ethanol production.

2.5 Molecular identification of microbial components in the cellulolytic biofilm

The fungal component of the biofilm's genomic DNA was isolated [33]. Universal primers, ITS1 (5'–TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'–TCC TCC GCT TAT TGA TAT GC3') were used to amplify fungal DNA [34]. PCR products were purified, sent for sequencing, compared with the other related sequences using BLAST search in GenBank (NCBI) [16]. Identification of the bacterial component was done through 16S rRNA sequence analysis. The genomic DNA of each biofilm-forming bacterial isolate was extracted using Gene Jet DNA purification Kit according to the manufacturer's protocol. Amplification of 16S rRNA gene was carried out by means of 16S rRNA pair of primer named as 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5' GGTTACCTTGTTACGACTT 3') [18]. The amplified products were sequenced at the Macrogen Sequencing facility in Korea and compared using BLAST search in GenBank (NCBI).

2.6 Statistical analysis

Each experiment was performed in triplicate and standard deviation for each experiment was calculated. The effects of different monocultures and biofilm amended samples were analyzed using the analysis of variance (ANOVA) and least significance difference (LSD) was tested using Turkeys comparison at 5% confidence level using Minitab 17 software.

3. RESULTS AND DISCUSSION

3.1 Isolation and preliminary screening of microorganisms in coir retting water and soil

Twelve bacterial strains and five fungal strains were isolated from coir retting water and eight bacterial strains and six fungal strains were isolated from coir retting soil and coded for convenience. For the preliminary screening, all the bacterial and fungal strains were evaluated for the capability of degradation of CMC. Out of the 20 bacterial isolates and 06 fungal isolates, 08 bacterial and all 06 fungal isolates exhibited the capability of growing in CMC medium in which the sole source of carbon was cellulose. Out of the isolates, 03 bacterial (B7, B5 and B6) and 02 fungal (F6 and F3) isolates showed high cellulolytic activity over the other isolates (Fig. 1). Out of those, bacterial isolate B7 and fungal isolate F6 showed the highest significant ($P< 0.05$) cellulolytic activities. Therefore, the isolates showing the highest cellulolytic activities (F6, F3, B7, B5 and B6) were selected to form fungal- bacterial biofilms.

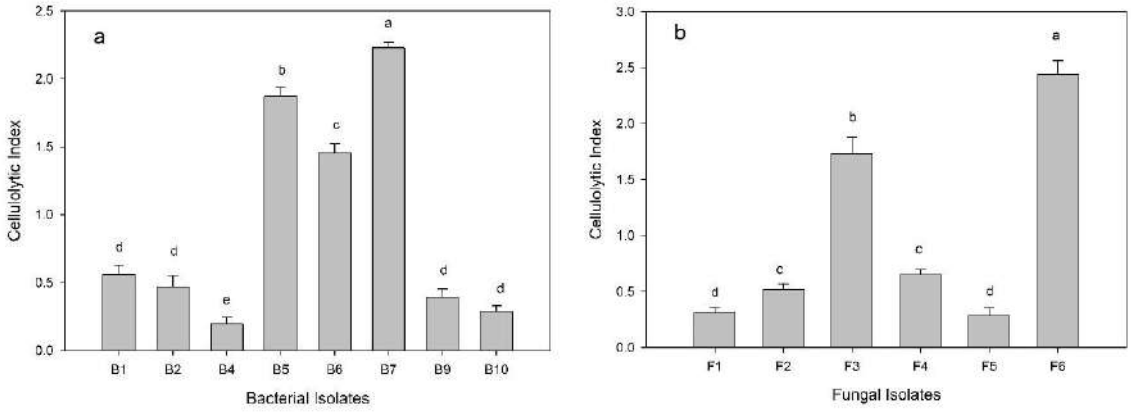

Figure 1: Cellulolytic index of isolated microorganisms (a) Bacterial cellulolytic index (b) fungal cellulolytic index. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations

3.2 Formation of Fungal-bacterial biofilms

The fungal filaments in Fungal- Bacterial Biofilms (FBBs) served as a surface for bacterial cells to colonize. Strength of the attachment varied depending on the microbial composition. Out of all FBB combinations, the highest attachment strength between bacterial cells and the fungal filament was observed in the combination of *A. niger* (F6) and *P. aeruginosa* (B7) (Fig. 2 a-d). Fungal isolate F3 did not contribute to develop FBBs with any other bacterial isolate. Based on the attachment strength, FBBs with two bipartite associations (*A. niger - P. aeruginosa and A. niger - B. subtilis*) and a FBB with one tripartite association (*A. niger - P. aeruginosa - B. subtilis*) were evaluated for their cellulolytic capacity with their individual fungal and bacterial isolates.

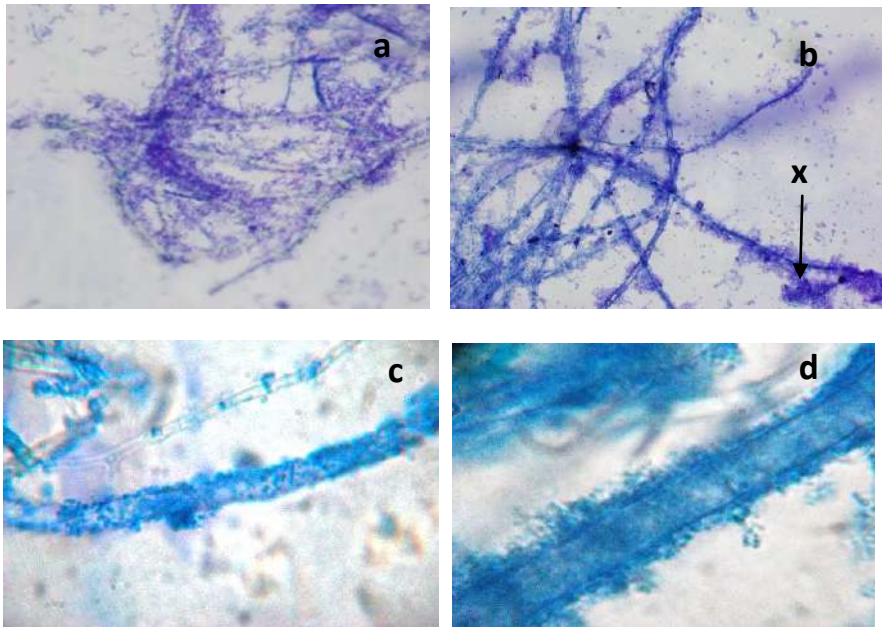

Figure 2: Bacterial colonization on *A. niger* mycelium in FBBs (a) colonization of *B. subtilis*, (b) *P. aeruginosa*, on *A. niger* mycelium in FBBs at x 400 magnification. (c) *B. subtilis* (d) *P. aeruginosa*, on *A. niger* mycelium in FBBs at x 1000 magnification. Darkness (x) is due to lactophenol cotton blue stain absorbed by EPS produced by the biofilms

3.3 Cellulolytic activity of the biofilms and their single cultures

Cellulolytic activity of the biofilms and their single cultures during eight days of incubation is expressed as the reducing sugar production (Fig. 3). Sugar production was significantly higher ($P < 0.05$) in all the biofilm combinations over the single cultures at each sampling time from day 2 to day 8 (Fig.3 and b). Out of all the microbial treatments,

the highest sugar production was observed in the biofilm combination FB2 (*A. niger – P. aeruginosa) and it was not significantly different from FB1 (A. niger - B. subtilis*). Fungal isolate F6 (*A. niger*) showed the highest sugar production over the other single cultures. Although, a sharp increment was observed in the sugar production up to day 4 by the biofilm combinations FB1 and FB2, the rate of increment was gradually decreased with the time until day 8. After day 6, it was clear that the sugar production rate by the bacterial and fungal isolates became constant. However, this pattern was not observed from the biofilm combination FB1 and FB2 where the rate showed continuous increment even after day 6 until day 8 and the increment rate showed high compared to biofilm combination FB3. Out of all the biofilms, FB2 showed the highest cellulolytic activity at every time intervals up to day 8.

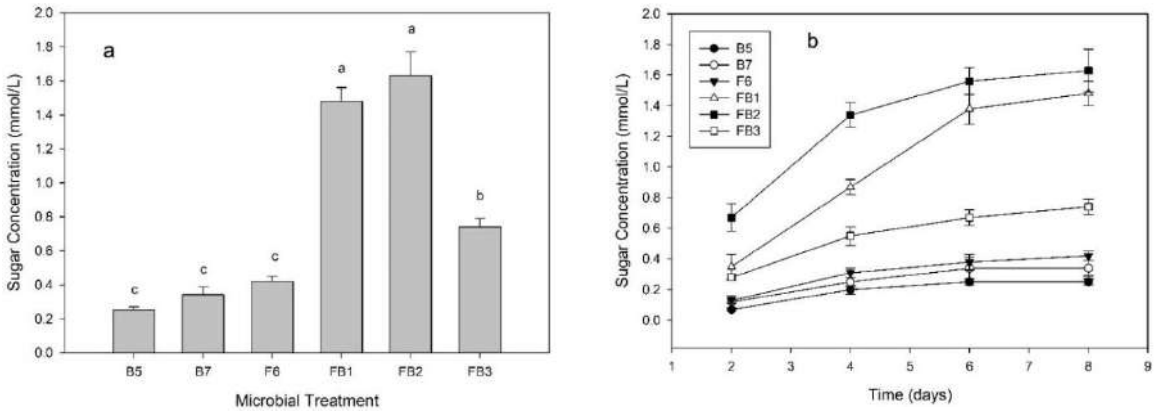

Figure 3: Cellulolytic activity in the form of sugar production by biofilms and their single cultures. Fig.3a shows sugar production after eight days of incubation. Fig.3b shows sugar production with the time. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations

3.4 Molecular identification of microbial components in the cellulolytic biofilm

Nucleotide sequence analysis of the responsive microbial components through GenBank search revealed that the isolates had high sequence similarity to the species B5-*Pseudomonas aeruginosa* (CP054591.1), B7-*Bacillus subtilis* (CP053102.1), F6-*Aspergillus niger* (AM270218.1), (Table 1) among the nucleotide sequences available in the NCBI database.

Table 1: Molecular identification of biofilm forming cellulolytic fungal and bacterial isolates

Sample identity	Length of the fragment (bp)	Closest Relative	Similarity (%)	Accession Number
B7	770	*Pseudomonas aeruginosa*	100%	CP054591.1
B5	516	*Bacillus subtilis*	100%	CP053102.1
F6	601	*Aspergillus niger*	100%	AM270218.1

3.5 Qualitative and Quantitative analysis of ethanol production by cellulolytic biofilms

Ethanol production of the biofilms and their single cultures with and without the addition of *S. cerevisiae* is shown in figure 4. All the biofilms co-cultured with *S. cerevisiae* showed higher ethanol production over their single cultures with *S. cerevisiae.* Although the highest cellulolytic activity was shown by the biofilm FB2 (*A. niger – P. aeruginosa),* the highest ethanol production was observed by the biofilm FB1 (*A. niger* and *B. subtilis*) co-cultured with *S. cerevisiae.* However, any significant difference was not observed in ethanol production between FB1*(146.2 ppm)* and FB2 (134.7 ppm) co-cultured with *S. cerevisiae*.

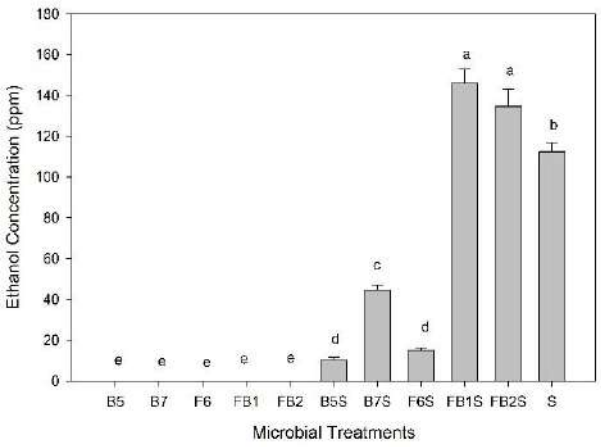

Figure 4: Ethanol production by different microbial treatments. B5, B7, F6, FB1 and FB2 are the treatments without co-culturing of *S. cerevisiae.* B5S, B7S, FB1S, FB2S are microbial treatments co-cultured with *S. cerevisiae.* S is only *S. cerevisiae* treatment. Columns with the same letter are not significantly different at 5% probability level. vertical bars show standard deviations

Interestingly, the ethanol production was enhanced in FB1 co-cultured with *S. cerevisiae* by 30% and by 20% in FB2 co-cultured with *S. cerevisiae* in comparison with ethanol

production by only *S. cerevisiae*. Further any ethanol productions were not recorded by the biofilms and the monocultures without the addition of *S. cerevisiae*.

The FTIR analysis confirmed the ethanol production by the Biofilm combinations FB1S and FB2S through the comparison of the standard FTIR chromatogram for pure ethanol (figure 5). Around 3,500–3,000 cm^{-1}, large absorption peaks representing OH bond were observed with maximum absorption at 3,328 cm^{-1} for pure ethanol [20]. The spectrum also displayed absorption peaks at 2,973 cm^{-1}, 2928 cm^{-1} and 2887 cm^{-1} for CH bond [18]. FT-IR spectra of all biofilms with *S. cerevisiae* revealed a slight shift. Clear peaks were observed at 1,380 cm^{-1}, 1369 cm^{-1} and 1326 cm^{-1} are representing CO bond of the ethanol. Further, the peak intensities for FB2 with *S. cerevisiae* in the region of 3400−2700 cm^{-1} and 1400 cm^{-1} to 1300 cm^{-1} were lower than that of the FB1 with *S. cerevisiae.*

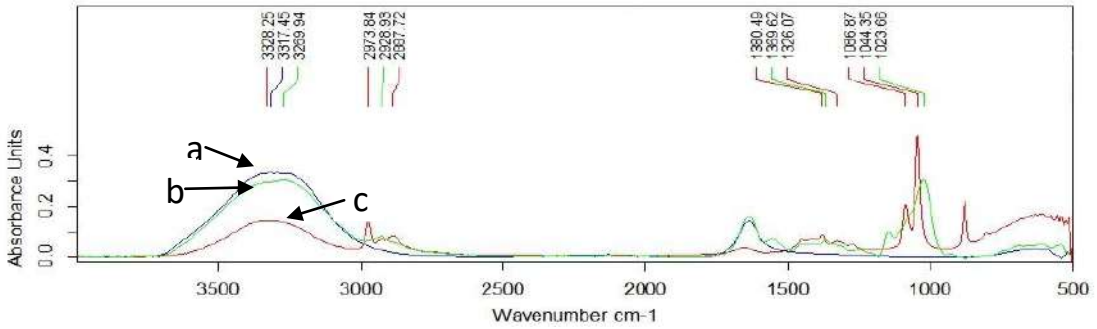

Figure 5: FTIR spectra for ethanol production by the responsive bio-films; (a) Bio-film FB1 with *S. cerevisiae.* (b) Bio-film FB2 with *S. cerevisiae.* (c) Pure ethanol.

Cellulose-degrading microorganisms co-exist in almost all natural environments, particularly in soil and water [15]. However, agro-industrial waste residues are used by cellulose-degrading microorganisms for the production of cellulolytic enzymes [26]. In the current study, microorganisms were isolated from the locations where the coir retting process is conducted. Retting is the first step in the production of coir because diverse microorganisms found in retting grounds release cellulolytic enzymes into the solution [13]. In the retting process, both an anaerobic and aerobic species of bacteria including *Pseudomonas sp, Micrococcus sp., Bacillus sp. and Aerobacter sp.* and several types of *Saccharomyces sp.* are active in different stages of decomposition. Habitats where these substrates are present are the best sources for isolation of cellulolytic microorganisms. Under submerged conditions, microorganisms dwelling with coir fibers secrete various

extra cellular enzymes which help to degrade building materials and it is resulted in the separation of coir fibers [27].

The production of cellulase has been reported from a wide variety of bacteria and fungi [12]. Several microorganisms have been discovered from coir retting water *viz.*, *Pseudomonas* sp., *Bacillus* sp., *Klebsiella* sp., etc. which have the capacity to convert cellulose into simple sugars [21]. In the current study, *A. niger, P. aeruginosa and B. subtilis* performed better in producing cellulase over the other isolates (Fig. 1). These results are in consistent with previous reports showing an extensive cellulolytic activity of filamentous fungi such as *A. niger* and bacteria such as *B. subtilis* and *Pseudomonas* sp. [11, 28]. It has been reported that the cellulolytic enzyme from *Bacillus* sp., and *Micrococcus* sp., isolated from the estuarine coir retting effluents [12].

Biofilms are formed when bacteria develop primarily adhering to surfaces in nature. The production of EPS begins after cell attachment to a surface. Filamentous fungi can be considered as biofilm-forming organisms as they naturally adapted to grow on surfaces [37]. The production of EPS by fungal mycelium has the potential to increase EPS production in FBBs [24,8]. In the current study, microscopic observation indicated high EPS secretion by FBBs (Fig. 2a-d). It has been reported that extracellular enzymes involved in lignocellulose decomposition are often bound to fungal EPS matrices. This association between extracellular enzymes and EPS matrices affects the activities of the enzymes [3]. Additional EPS-producing microbes *viz.*, *Bacillus* sp., *Pseudomonas* sp., and *Agrobacterium* sp. [24], are reported producing a high concentration of extracellular enzyme [9].

There have been instances of biofilms being used in paper and pulp industries and textile industries due the enhanced production of cellulase [14]. Biofilm has the ability to concentrate free cellulases from the medium and enhance cellulose degradation by keeping the enzyme with cells through the formation of bacteria-cellulase-cellulose complexes [4]. In the current study, FBBs had higher cellulolytic activity over their single cultures in the liquid medium. Further, two bipartite biofilm associations in the current study (*A. niger – B. subtilis* and *A. niger- P. aeruginosa) showed the highest cellulolytic activity among all microbial treatments* (Fig. 3 a, b). Biofilms with *A. niger* reported having high cellulase activity than freely suspended mycelial cultures [31]. This

is due to the different expression levels of some lignocellulolytic enzyme-encoding genes of *A. niger* in biofilms with the comparison of single mycelial pellets under submerged condition [30]. According to a study, *Aspergillus* sp. was stable and produced enzymes over longer periods of time with a roughly 4.5-fold increase in productivity compared to single suspended cells [10]. This observation suggests the possibility that the same environmental factors that cause the development of extracellular lignocellulose degrading enzyme may also cause the creation of EPS matrix material in the biofilm environment.

The microbial enzymatic conversion of starchy biomass into sugars and/or the fermentation of sugars with subsequent distillation of ethanol to fuel grade are required for the manufacture of lignocellulosic bioethanol. S. cerevisiae is the common microbe employed in ethanol production under anaerobic conditions due to its high ethanol productivity and high ethanol tolerance [1]. *S. cerevisiae* requires glucose to be catalyzed via the glycolysis or Embden-Meyerhof pathway in order to get pyruvate, which is then anaerobically transformed into ethanol by the activity of particular enzymes with a yield of 90-93% from the glucose consumed. [23]. The energy for growth of *S. cerevisiae* cells during ethanol fermentation is mostly provided by the glycolytic pathways [6]. However, the current study showed an extensive production of ethanol in the biofilm system (*A. niger – B. subtilis* and *A. niger- P. aeruginosa)* co-cultured with *S. cerevisiae* over the system with only *S. cerevisiae* (Fig. 4). Further, the same biofilm combinations showed higher cellulolytic activity over their single cultures (Fig. 3). These results are in consistent with previous reports showing the possibility of fungal biofilms for cellulose fermentation [25]. *S. cerevisiae* co-cultured biofilms in continuous packed-bed bioreactors can produce high amount of ethanol from molasses. Biofilm is known to improve a greater ethanol tolerance for yeast and bacteria than their planktonic counterparts [38]. It has been suggested that biofilm provides protection by enhancing the stability of the hydration layer around the cell [5]. The compact biofilm microenvironment holds highly structured water surrounding the yeast cells, which prevents such an ethanol dehydration to happen [5]. Further, it can be envisaged that for higher ethanol production, a continuous supply of sugars is needed. The FBBs keep on hydrolyzing cellulose which will eventually be converted to ethanol by the yeast. So there is a high synergistic effect between the FBB and the yeast. This might be the reason

of producing higher amount of ethanol by the microbial biofilm systems co-cultured with *S. cerevisiae* while maintaining a higher production of bioethanol.

CONCLUSION

Lignocellulosic bioethanol production through microbial biofilms is one of the best alternatives for petroleum-based fuels. The most efficient cellulose degrading bacterial and fungal isolates were *B. subtilis*, *P. aeruginosa* and *A. niger* respectively. The cellulolytic activity of all the microbial biofilms was higher than the single cultures. The highest sugar formation was observed from *A. niger – P. aeruginosa biofilm. High ethanol productions were observed from* biofilm system (*A. niger – B. subtilis* and *A. niger- P. aeruginosa)* co-cultured with *S. cerevisiae* over the microbial system with only *S. cerevisiae. This confirms that c*ellulose biomass can be directly converted to bioethanol by microbial biofilms co-cultured with *S. cerevisiae* due to their dual ability to reduce cellulose and ferment reducing sugar ethanol together. However, the experiment was conducted with pure cellulose powder and hence testing with agriculture waste/lignocellulosic biomass is needed for a better conclusion.

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