AIMS Microbiology

AIMS Microbiology, 1(1): 1-10.
DOI: 10.3934/microbiol.2015.1.1
Received date 01 July 2015,
Accepted date 30 August 2015,
Published date 08 September 2015

http://www.aimspress.com/

Research article

Hydrophobic nature and effects of culture conditions on biofilm formation by the cellulolytic actinomycete *Thermobifida fusca*

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Abstract: *Thermobifida fusca* produces a firmly attached biofilm on nutritive and non-nutritive surfaces, such as cellulose, glass, plastic, metal and Teflon®. The ability to bind to surfaces has been suggested as a competitive advantage for microbes in soil environments. Results of previous investigations indicated that a Gram-positive cellulolytic soil bacteria, *Cellulomonas uda*, a facultative aerobe, specifically adhered to nutritive surfaces forming biofilms, but cells did not colonize non-nutritive surfaces. Cell surface hydrophobicity has been implicated in the interactions between bacteria and the adhesion to surfaces. It was recently described that the cellulolytic actinomycete *T. fusca* cells hydrophobicity was measured and compared to the cellulolytic soil bacteria *C. uda.* Also, *T. fusca* biofilm formation on non-nutritive surface, such as polyvinyl chloride, was examined by testing various culture ingredients to determine a possible trigger mechanism for biofilm formation. Experimental results showed that partitioning of bacterial cells to various hydrocarbons was higher in *T. fusca* cells than in *C. uda*. The results of this study suggest that the attachment to multiple surfaces by *T. fusca* could depend on nutrient availability, pH, salt concentrations, and the higher hydrophobic nature of bacterial cells. Possibly, these characteristics may confer *T. fusca* a selective advantage to compete and survive among the many environments it thrives.

Keywords: biofilm; *Thermobifida fusca*; *Cellulomonas uda;* dialysis tubing; hydrophobicity; SEM; PVC

1. Introduction

Thermobifida fusca strain YX is a Gram-positive, aerobic, moderate thermophilic soil actinomycete that is able to grow and degrade cellulose at 55 °C [10]. It is a major degrader of plant cell walls in heated organic materials such as compost heaps, rotting hay, manure piles or mushroom growth medium and has been used with an enormous success in industrial processes for cellulose degradation [10]. It was recently published that *T. fusca* produce a biofilm phenotype on nutritive surfaces such as cellulose, and on non-nutritive surfaces, such as glass, plastic, metal, Teflon® and can also grow non-attached to surfaces is cellulose and glucose media [10]. In addition, it was described that *T. fusca* biofilm matrix and non-attached mycelial pellets structures were composed of carbohydrates, a hallmark of biofilm formation, and DNase 1disruptsbiofilm formed by this cellulolytic actinomycete [1].

Biofilm development is a multi-step process in which individual free-floating (planktonic) cells approach the surface, attach, form micro-colonies in the surface, move along the surface, and associate with one another [3]. It has been noticed that the primary adhesion between bacteria and surfaces (e.g. nutritive or non-nutritive) is mediated by nonspecific interactions [7], by the hydrophobic nature of the bacterial cells [17] or a trigger mechanism such as depleted nutrients (e.g. nutritive signal) [15], pH [5,9,14], and osmolality of the media [14]. However, it is known that not all microbes are capable of developing a biofilms on multiple surfaces, which allows microbes to compete and survive in soil environments when nutrients are scarce. Previous investigations indicated that a Gram-positive facultative aerobe and cellulolytic soil bacterium, *Cellulomonasuda*, specifically adhered to nutritive surfaces under low nitrogen conditions, but cells did not colonize non-nutritive surfaces [15].

In the present study we examine the ability of *T. fusca* to adhere to surfaces and correlate this adhesion with the cell surface hydrophobicity as compared to other microbes incapable of binding to multiple surfaces. In addition, this study will evaluate cellulose degradation by *T. fusca*, as compared to other cellulolytic microbe, and search for mechanisms that triggers *T. fusca* biofilm formation by increasing and depleting culture media components on 96 wells polyvinyl chloride (PVC) microtiter plates.

2. Materials and Methods

2.1. Bacterial strain, media, and culture conditions

T. fusca strain YX (wild type) was obtained from Diana Irwin and David Wilson (Cornell University). Cultures were grown in flasks of Hägerdal medium [15], a minimal medium, at 53 to 55 °C on a 66133-shaker incubator (New Brunswick Scientific Co.) to provide aeration. Stock cultures were grown using cellobiose as substrate and were maintained at −20 °C in 15% glycerol. An insoluble substrate such as Spectra/Por regenerated cellulose (dialysis tubing) (Spectrum Laboratories Inc., Rancho Dominguez, CA) was used as carbon source (cellulose) as nutritive surface for biofilm formation. In order to obtain *T. fusca* aleuriospores, cells were cultured on Hägerdal agar medium using 1.2% Bacto agar (Sigma) and 1% filter-sterilized cellobiose as carbon source. One mL of *T. fusca* culture was spread on Hägerdal agar cellobiose medium and cultured for 48 h at 55 ºC. *T. fusca* spores were isolated using 5 mL filter sterilized phosphate-urea-magnesium

(PUM) buffer [17] on Hägerdal agar sucrose medium. Spores were collected using a sterile Pasteur pipette, filtered through glass wool, and the filtrate was collected and stored.

Cellulomonas uda ATCC 21399 was obtained from the American Type Culture collection. *C. uda* was cultured aerobically in GS-2 medium [15] adding 0.2% cellobiose and incubated for one day. Then *C. uda* was transferred from previous culture to *GS-2 medium [15] a nitrogen-limited medium, which was the same as GS-2 medium except that it lacked urea and contained 0.01% (w/v) yeast extract with 0.1% cellobioseor dialysis tubing membrane as carbon source and incubated under aerobic conditions for 2 days.

2.2. Microscopy

For scanning electron microscopy (SEM), *T. fusca* biofilms formed on dialysis tubing membrane were rinsed to remove any planktonic or non-attached cells and fixed for 24 h in a solution containing 2% glutaraldehyde, 0.025 M sodium phosphate at pH 7.2, followed by three washes in 0.1 M sodium phosphate buffer. Samples were washed in sterile water and dehydrated in ethanol for 24 h. The samples were gold-palladium coated (Au/Pd 60/40) for 2 min and visualized by using a scanning electron microscope model JSM-5410 (JEOL, Tokyo, Japan).

For fluorescence microscopy analyses of biofilms formed by *T. fusca* and *C. uda* on dialysis tubing membrane, the strains were cultivated under culture conditions and incubated as previously described under section 2.1 with dialysis tubing as carbon source. Biofilms formed on dialysis tubing were rinsed five times with sterile ultrapure water to remove non-attached cells, then stained with methylene blue, and rinsed again. Samples were examines macroscopically using a Kodak EasyShare DX4900 digital camera and were microscopically examined by using a Zeiss Axioskop 20 microscope equipped with a DC290 Kodak digital camera.

2.3. Hydrophobicity

Bacterial cell-surface hydrophobicity was estimated by the Microbial Adhesion To Hydrocarbon (MATH) test, as described by Rosenberg *et al.* 1984. The test is based on the partitioning of bacterial cells between an aqueous phase and a hydrocarbon, where the more hydrophobic the bacterial cells, the greater their affinity for the hydrocarbon. Samples of *T. fusca* aleuriospores were prepared as described above. *T. fusca* and *C. uda* cells were cultured with cellobiose and centrifuged at 12,000 × g for 10 min, washed twice with PUM buffer [17], and resuspended in 5 mL of PUM buffer. Bacterial cell suspension samples were normalized to an A_{400} of 0.50 transferred to tubes to which 600 μL of hexadecane or xylene was added (hydrocarbons were individually tested). Tubes were vigorously vortexed for 60 s and allowed to stand for 15 min, after which the A_{400} of the aqueous phase was measured. Cell hydrophobicity percentages were calculated as follows: [(A_{400} before mixing − A_{400} aqueous phase after mixing) / A_{400} before mixing] × 100.

2.4. Microtiter plate biofilm assay

Biofilm formation in polyvinyl chloride (PVC) microtiter plates was measured as described previously [14]. *T. fusca* spore suspensions were prepared as previously described. Biofilm production assays were performed using Hägerdal medium, with cellobiose as the sole carbon source.

Samples (15 mL) of the spore suspension were transferred to 135 mL of ultrapure sterile water to create a 1:10 dilution. Then, for each sample 10 μL were transferred into 16 sterile wells of PVC microtiter plates (Cellstar, Sigma-Aldrich) each, containing 90μLto create a dilution 1:100.The conditions tested in the plates were: sterile Hägerdal medium, Hägerdal medium without $(NH_4)_2SO_4$, Hägerdal medium with NH_4Cl substituted for $(NH_4)_2SO_4$, Hägerdal medium with an excess (50%) of $FeSO_4$, a lack of $FeSO_4$, an excess (50%) of NaCl or without NaCl. Also, the effect of pH was examined in Hägerdal media at various pH values. Each plate included 16 wells of uninoculated Hägerdal medium as control wells. The average OD from the control wells was subtracted from the OD of all test wells. On each test day microtiter plate wells were washed five times with ultrapure water to remove loosely associated bacteria. Plates were air dried for 45 min and each well was stained with 150 μL of 1% filter sterilized crystal violet solution for 30 min. After staining, plates were washed with ultrapure water five times. At this point, biofilms were visible as purple clumps formed on the bottom of each well. The quantitative analysis of biofilm production was performed by adding 200 μL of 95% ethanol to destain the wells as previously described by O'toole and Kolter, 1998. One hundred microliters from each well was transferred to a new microtiter plate and the optical density of the crystal violet present in the destaining solution (ethanol) was measured at 595 nm. Experiments were carried out in duplicate with 16 wells in each plate for each treatment, and evaluated every 24 h during a 72 h period.

2.5. Statistical analyses

Tests were run in triplicates three times, 96 well plate assays where run in triplicates 16 wells per sample. Statistical analyses of the hydrophobicity tests were determined by using Student *t*-test comparisons performed with Sigma Plot Statistical Software. Differences were considered to be significant at $P \leq 0.05$.

3. Results

3.1. Biofilm formation on cellulose membrane examined by SEM

T. fusca cellulose degradation was examined using SEM (Figure 1). Dialysis tubing, appearing under a biofilm (Figure 1a ← arrow), was degraded when *T. fusca* colonized the cellulosic substrate (Figure 1a). A higher magnification view of dialysis tubing membrane (Figure 1b) revealed that dialysis tubing membrane was shredded in a region that apparently had been covered by a biofilm (area magnified from Figure 1a ← arrow).

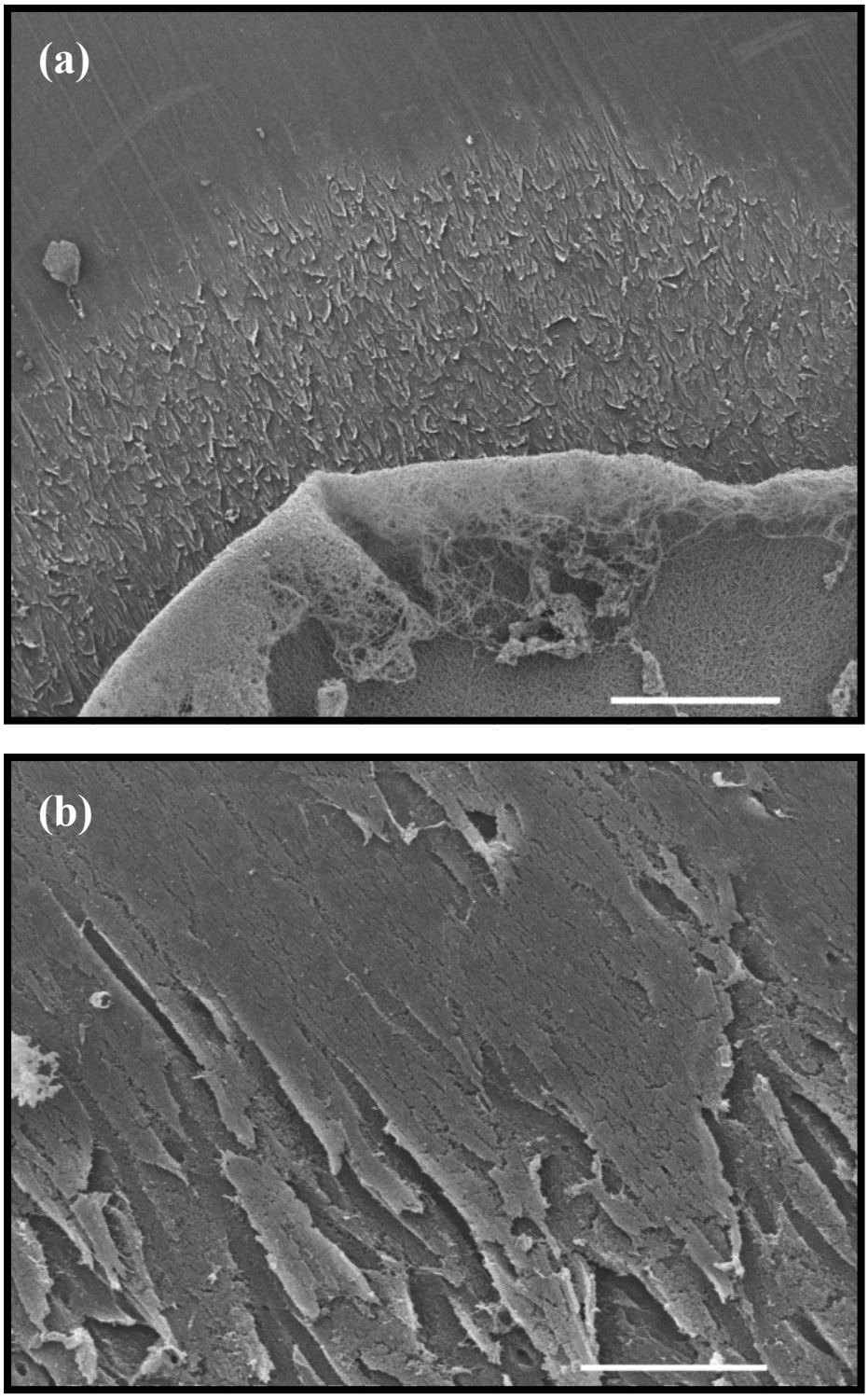

Figure 1. Legend of the figure. SEM of dialysis tubing degraded by *T. fusca* biofilm. Figure (1a) shows *T. fusca* biofilm growth folded back and dialysis tubing membrane degraded. Scale bar 100 µm. Figure (1b) shows a higher magnification view of dialysis tubing degraded by *T. fusca* biofilm growth (magnified from arrow area showed in Figure 1a). Scale bar 10µm.

3.2. Determination of bacterial cell surface hydrophobicity

Attachment of bacteria to surfaces may be related to cell surface hydrophobicity [17]. The hydrophobic nature of this microbe was estimated using the MATH test [17], which is based on the partitioning of bacterial cells between hydrocarbon and aqueous phases. The hydrophobicity percentages of *T. fusca* cells were higher than *C. uda* (Table 1). When tested against *n*-Hexadecane and Xylene the percentages of *T. fusca* MPs (95.0 and 99.0%) and aleuriospores (99.0 and 89.0%) were significantly higher, than the hydrophobicity of *C. uda* cells (77.0 and 57.0%), which selectively attach only to nutritive surfaces [15]. The hydrophobic nature of *T. fusca* cells might facilitate their binding to multiple surfaces. Also, when using dialysis tubing membrane, *T. fusca* biofilms degraded the cellulosic substrate faster (less than 15 days) than biofilms formed by *C. uda* (less than 60 days) (Table 1).

Table 1. Quantitative analyses of cell hydrophobicity using the MATH test.

Cellulolytic organisms	Source	Hydrophobicity percentage		Cellulose degradation [a]
		n-Hexadecane	Xylene	
Obligate aerobe *Thermobifida fusca*	mycelial pellets	95.0	99.1	+++
	Aleuriospores	99.0	89.0	
	biofilm			+++
Facultative aerobe *Cellulomonas uda*	Cells[*]	77.6	57.0	++
	biofilm			++

[a] Relative activity determined as the amount of time to completely degrade the dialysis tubing membrane. Less than 15 days (+++), less than 60 days (++).

[*] $P \leq 0.05$

3.3. PVC 96 well microtiter plate assay

T. fusca biofilm formation was maximal when all the components were present in Hägerdal medium (Figure 2). However, biofilm production was inhibited when certain components of Hägerdal medium were removed or increased in concentration (Figure 2). *T. fusca* developed a biofilm when cultured without $(NH_4)_2SO_4$ (Figure 2, -N) but when $(NH_4)_2SO_4$ was substituted by NH_4Cl (Figure 2, NH_4Cl), biofilm production was reduced ($P \leq 0.05$). *T. fusca* biofilms were also reduced by a lack (-Fe) or excess (+Fe) of iron (Figure 2) ($P \leq 0.05$). Also, *T. fusca* developed biofilms when cultured without NaCl (-NaCl). However, when using an excess of NaCl (+NaCl) (Figure 2), *T. fusca* biofilm formation was reduced ($P \leq 0.05$). *T. fusca* developed robust biofilms at pH 7 and 9, whereas biofilm formation at pH 3 and 11 was minimal.

4. Discussion

It is known that the hydrophobicity of microbial cells has been implicated in the adherence of various bacteria to surfaces [17]. The microbial adhesion to hydrocarbons or MATH assay as an

effective measure of bacterial hydrophobicity, which implicates a microbe's ability to bind to surfaces [17]. In our study, the hydrophobicity of *T. fusca* cells using *n*-hexadecane and xylene was compared with that of *C. uda* cells. Based on the MATH test using *n*-hexadecane and xylene, hydrophobicity of both *T. fusca* vegetative cells and aleuriospores was statistically higher than that of *C. uda*. It is known that *C. uda* exclusively binds to nutritive surfaces such as cellulose and chitin [15]. Thus, when observed under fluorescence microscopy, degradation of dialysis tubing membrane by *C. uda* cells occurs after 15 days and *T. fusca* attaches to any surface in 72 h and degrades the cellulosic membrane in less than 15 days, highly faster than *C. uda* (60 days) ($P \leq 0.05$) (Table 1). This capability may be an advantage for this actinomycete in cellulosic environments when competing for nutritive sources against other cellulose degrader microbes or *T. fusca* would be a suitable microbe for industrial processes that requires higher cellulose degradation strategies.

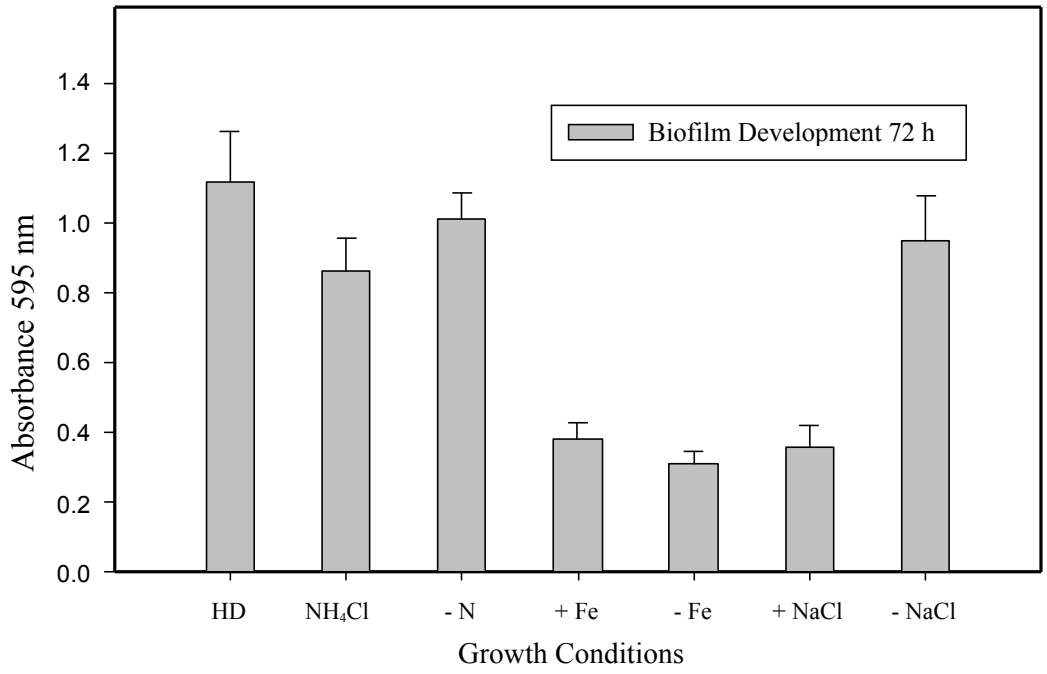

Figure 2. Legend of the figure. Effect of nutrients on *T. fusca* biofilm formation during a 72 h growth period. Conditions tested: Hägerdal (HD) medium, substituting ammonium sulfate ($(NH_4)_2SO_4$) with NH_4Cl, no ammonium sulfate (-N), higher $FeSO_4$ concentration (+Fe), no $FeSO_4$ (-Fe), higher salt concentration (+NaCl) and no salt (-NaCl). *n: 48* per strain.

Our hydrophobic percentages and cellulose degradation results (Table 1) show that *T. fusca* cells may bind to nutritive surfaces due to their higher cell hydrophobic nature as it has been suggested for other microbes [6]. This cell surface-binding potential and faster cellulose degradation may confer an ecological advantage for *T. fusca* to compete for food and survive under lack of nutrients conditions, as compared to other microbes in soil environments that do not have an equivalent capability.

On this study using PVC 96 microtiter plate as non-nutritive surface, *T. fusca* biofilm production was reduced, but never inhibited, when certain components of Hägerdal medium were

removed or increased in concentration (Figure 2). *T. fusca* developed a biofilm when cultured without $(NH_4)_2SO_4$ (Figure 2, -N) but when $(NH_4)_2SO_4$ was substituted by NH_4Cl (Figure 2, NH_4Cl), biofilm production was reduced. Reguera 2001 reported that nitrogen depletion triggers *C. uda* biofilm formation, however, this study showed that *T. fusca* produced biofilms regardless of nitrogen availability. *T. fusca* biofilms were also reduced by a lack or excess of iron. Similarly, it has been reported that *P. aeruginosa* biofilm formation requires iron [2,8] and under some growth conditions, biofilm formation by *Actinobacillus actinomycetemcomitans*, a pathogen associated with oral and extra-oral infections, requires iron [16]. Although, *T. fusca* developed biofilms when cultured without NaCl, when using an excess of NaCl, *T. fusca* biofilm formation was reduced, but never inhibited as it happened when grown as planktonic cells (results not shown). Salt-mediated effects are usually attributed to changes in the ionic strength of the medium, possibly because the addition of ions also impacts osmolarity [14]. Similarly, it has been reported that the salt concentration of the medium affects biofilm formation by *P. fluorescens* [14], *Salmonella enterica* [9] and *A. actinomycetemcomitans* [16]. Our results showed that the nutritional ingredients of the culture medium can influence biofilm formation by *T. fusca*.

T. fusca biofilm formation was examined at various pHs. *T. fusca* developed robust biofilms at pH 7 and 9, whereas biofilm formation at pH 3 and 11 was minimal. Our results correlate with *S. epidermidis* studies, where the slime production depended on the pH value of the medium [5]. When *S. epidermidis* was culture with highly acidic (pH 3) and alkaline (pH 12) levels, biofilm formation on PVC microtiter plates were lower, while when using neutral (pH 7) levels the adhesion was moderate [5]. Our results showed that stress conditions, such as changes in pH or nutrients, may triggers biofilm formation by allowing or reducing biofilm formation to surfaces. Nevertheless, under all the conditions tested in this study, *T. fusca* was capable of forming biofilms. The extent of biofilm formation however was dependant on nutrient, salt concentrations, pH and the higher hydrophobic nature of *T. fusca* cells.

T. fusca biofilm studies could serve as a model for understanding the role of biofilm formation by filamentous, cellulolytic microorganisms. *T. fusca* is a major cellulose degrader, but a deficiency in nutrients in soil environments may be a challenge for many microorganisms to compete and survive. Thus, the ability of *T. fusca* to develop as biofilms on multiple surfaces, regardless of nutrient availability might provide a selective advantage among cellulolytic microbes, enabling to secure nutrients, and as survival strategy when resources are limited.

5. Conclusion

The present research study has highlighted the cellulose degradation rate and hydrophobic microbial adhesion to hydrocarbons by two cellulolytic microbes *Thermobifida fusca* and *Cellulomonas uda*. Degradation of cellulose (dialysis tubing membrane) by *C. uda* cells occurs after 15 days and *T. fusca* degrades the cellulosic membrane in less than 15 days, highly faster than *C. uda* (60 days). Culture conditions factors are now known to be capable of influencing biofilm development by *T. fusca* in non-nutritive surface such as, PVC. *T. fusca* developed a biofilm when cultured without ammonium sulfate but when substituted by NH_4Cl biofilm production was reduced. *T. fusca* developed biofilms when cultured without NaCl, when using an excess of NaCl, *T. fusca* biofilm formation was reduced, but never inhibited as it happened when grown as planktonic cells. Also, robust biofilms were developed at pHs 7–9. Further research is required to improve the

understanding all aspects of biofilms formation by cellulose degrader microbes, specifically filamentous microbes such as *T. fusca*, including their significance in soil environments when microorganisms compete and resources are limited.

Acknowledgments

T. fusca strain YX was kindly provided by David Wilson and Diana Irwin of Cornell University. We gratefully thank Josephine Morello of the University of Chicago, for critical readings and helpful comments on the manuscript. Also, we thank Dale Callahan at the University of Massachusetts Microscopy facility. This work was conducted at the University of Massachusetts Amherst and was supported by US-Department of Energy under grant DE-FG02-02ER155330.A. N. Alonso currently works for the Food and Drug Administration, Center for Tobacco Products-Office of Science. The findings and conclusions of this project do not necessarily reflect the views of the US Food and Drug Administration Agency or the US-FDA Center for Tobacco Products, Office of Science.

Conflict of Interest

Author declares no conflict of interests in this paper.

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