

RESEARCH ARTICLE

Evaluation of cellulose degrading efficiency of some fungi and bacteria and their biofilms

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Abstract: In industrial applications like cellulosic biofuel production, efficient degradation of cellulose is one of the major concerns. But the efficiencies of cellulose degradation by different types of biofilms are not yet explored. This study was carried out to determine whether the community actions of fungal-bacterial biofilms may increase the rates of cellulose bio-degradation. Cellulolytic fungi and bacteria were isolated from soils, composts and leaf litter and screened for simple sugar production. To identify efficient sugar producing communities, combinations of these isolates were tested in batch cultures of cellulose broth prepared with commercial cellulose. Three different communities; fungal and bacterial monocultures, fungal-bacterial biofilms and fungal mixed cultures were tested. The monoculture of *Acremonium* sp. was the highest sugar yielder among all tested microorganisms. Among fungal mixed cultures, *Acremonium* sp. with *Fusarium* sp. was an effective sugar yielder. *Acremonium* sp. with a *Bacillus* sp. was effective among fungal-bacterial biofilms. These three cultures were also inoculated into broth media prepared with *Eupatorium odoratum* or *Panicum maximum* or *Lantana camara* or *Mimosa pigra* to assess their simple sugar production from plant materials. The highest sugar production was by *Acremonium* sp. monoculture and the lowest was by the *Acremonium* sp. with *Bacillus* sp. biofilm. This was true for all four weeds. Thus, all the fungal-bacterial biofilms tested were comparatively less effective sugar producers.

Keywords: Bio-degradation, cellulose, fungal-bacterial biofilms, mixed-cultures, monocultures.

INTRODUCTION

The ever increasing demand for energy is one of the greatest challenges for humans. Fossil fuels, which have been the major energy source since the industrial revolution are dwindling rapidly and the increased use

of fossil fuels has caused greenhouse gas emissions and created undesirable damage to the environment. The current instability of oil supplies and the continuous fluctuation of prices have further ignited widespread interest in alternative energy sources. Biofuels are among the promising transportation green energy sources for the future.

The extensive range of organic materials used for biofuel production include starch from cereal plants, lignocellulosic materials and algae etc. Biofuels are classified based upon the substrate used in their production. First generation biofuels are manufactured using starch from food-based cereal plants like corn and wheat; sugar from sugar cane; oil from the seeds of rape; soya bean and jatropha; vegetable oils and animal fats (Antizar-Ladislao & Turrion-Gomez, 2008). Second generation biofuels also known as cellulosic biofuels, are produced using lignocellulosic materials like forest and crop residues, straw, municipal and construction waste (Antizar-Ladislao & Turrion-Gomez, 2008). Both cyanobacteria and eukaryotic algae are extensively used in the production of third generation biofuels called algal biofuels and are becoming increasingly popular because their yields per unit area of land surpass those from all other feed stocks (Aristidou & Penttila, 2000; Hu *et al.*, 2008; Dellomonaco *et al.*, 2010). Organic wastes are also used in biogas and biofuel production. However, the public opinion on producing biofuels from edible sources is not favourable. Hence, there is more interest on research into the development of less expensive methodologies to produce biofuels from non-edible sources like lignocellulosic biomasses of plants and organic wastes.

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Invasive weeds are probable raw materials for cellulosic biofuel production. These are non-indigenous or non-native plants, which economically, environmentally, and ecologically affect the habitats and bioregions they invade (Westbrooks, 1998). Several types of invasive weeds are found in Sri Lanka. Among them *Lantana camara* L. and *Mimosa pigra* L. are two species present in abundance in the Central Province (Marambe, 2001). *Panicum maximum* L. and *Eupatorium odoratum* L. are also among the common invasive weeds (Gunasekera, 2008). If effective technologies can be developed to derive commercial products from these weeds, it will be beneficial both economically and ecologically, i.e. the constant pruning could improve the aesthetic appearance of their habitats. For industrial scale biofuel production, a continuous supply of raw materials should be assured. The fast growing nature of these weeds can provide raw materials in abundance for biofuel industries.

The hardwood structure of plant biomass consists of biopolymers like cellulose, hemicelluloses and lignin. Cellulose is the major component in the rigid cell walls of plants and is a linear polysaccharide polymer with many glucose monosaccharide units. An important feature of cellulose, relatively unusual in the polysaccharide world, is its crystalline structure. The difficulty of hydrolyzing this crystalline structure of cellulose into simple sugars is one of the major problems in the biofuel industry. Bioconversion, particularly enzymatic hydrolysis of these cellulosic materials into simple sugars has been a subject of intensive research. Cellulose degrading micro-fungi and biofilm technology are quite interesting fields to be researched for their potential applicability in biofuel industry.

Cellulase production by different cellulolytic microfungi is being vigorously studied for cost reduction strategies. Although a large number of microorganisms (fungi, bacteria and actinomycetes) are capable of degrading cellulose, only a few of them produce significant quantities of cell-free enzyme fractions capable of complete hydrolysis of cellulose *in vitro*. Cellulases obtained from compatible mixed cultures of fungi appear to have more enzyme activity as compared to their pure cultures and other combinations (Jayant *et al.*, 2011). Therefore, extensive studies into bio-degradation by fungal mixed cultures and the monocultures of their constituent species would be beneficial in cellulosic biofuel production.

There is a possibility that microbial biofilms could also be used as agents of cellulose degradation due to their favourable properties. A biofilm is a complex aggregation of microorganisms, which is characterized

by structural heterogeneity, genetic diversity, complex community interactions, and an extracellular matrix of polymeric substances (Flemming & Wingender, 2001; Seneviratne *et al.*, 2008; Wang & Chen, 2009). They are usually found on solid substrates, submerged in or exposed to an aqueous solution (Sadashivaiah & Mysore, 2010). Bacteria living in a biofilm can have significantly different properties from the same species that are free-living (Seneviratne *et al.*, 2008). In the perspective of biodegradation, the extracellular matrix of biofilms provides a confined microenvironment, keeping substrate, enzymes, and microbes in close proximity (Flemming & Wingender, 2001; Wang & Chen, 2009). The extra-cellular polysaccharides in this matrix have a variety of binding sites for extracellular enzymes and substrate macromolecules, facilitating the extracellular enzymatic reactions (Sharon & Lis, 1993; Stryer, 1995; Liu & Tay, 2002; Wang & Chen, 2009). There are several different types of biofilms depending on the organisms involved in biofilm formation. One common type of biofilm is fungal-bacterial biofilm in which the fungi act as the biotic surface to which the bacteria adhere (Seneviratne *et al.*, 2008).

The advantages of biofilms include concentration of cell-associated hydrolytic enzymes at the biofilm-substrate interface to increase the reaction rates, and the physiological synergy between fungi and bacteria offers the possibility of completing delignification and saccharification in one piece of biofilm. This type of synergy actually takes place in natural ecosystems (Sinsabaugh & Linkins, 1990; Wang & Chen, 2009) and in the rumen environment (Mcsweeney *et al.*, 1994; Gordon & Phillips, 1998; Wang & Chen, 2009), facilitating the recycling of lignocellulosic materials. Symbiosis promotes maintenance of fungal delignification activity since bacteria digest the fungal delignification products that could have caused feedback inhibition (Rainey *et al.*, 1990; Wang & Chen, 2009). Fungal delignification benefits hydrolytic bacteria by increasing the accessibility of cellulose and hemicellulose wrapped inside the lignin structure (Mcsweeney *et al.*, 1994; Gordon & Phillips, 1998; Boer *et al.* 2005; Wang & Chen, 2009). The use of bacterial-fungal synergy in a biofilm should be explored as a next step in delignification and saccharification integrated process development (Wang & Chen, 2009). Another expected advantage of biofilm technology in biofuel industry is that it may increase the tolerance towards product inhibition. This is an important aspect as it has been shown that during saccharification, the increased simple sugar concentration can inhibit cellulase production (Krisch & Szajani, 1997; Ciesarova *et al.*, 1998; Jirku, 1999; Desimone *et al.*, 2002; Zhou *et al.*, 2008; Wang & Chen, 2009).

Although there are no records of utilizing biofilm based bioreactors in biofuel production processes like delignification, saccharification or to combine these two processes with fermentation, the production of enzymes, which are useful in cellulosic biofuel production has been accelerated by biofilm technology i.e. cellulase production by *Trichoderma viride* in spouted-bed reactor (Webb *et al.*, 1986). The objective of this study was to compare the efficiency of fungal and bacterial monocultures with fungal-bacterial biofilms in the delignification and saccharification of plant substrates in order to subject the hydrolysate into biofuel production.

METHODS AND MATERIALS

Isolation and screening of cellulose degrading microorganisms

Cellulose degrading fungi and bacteria were isolated from soils, composts and leaf litter layers using 1 % cellulose agar medium broth [1 g of cellulose powder (Fluka, analytical grade) + 1.5 g agar in 100 mL of mineral salt solution, pH ~ 7] in which cellulose is the sole source of carbon. Mineral salts solution of following composition (g/L) ($\text{NH}_4)_2\text{SO}_4$ (2.0), K_2HPO_4 (1.0), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.0), NaCl (1.0), CaCO_3 (0.25), and 1.0 mL of trace elements solution per litre was used. The isolates were screened for the most efficient cellulose degrading bacteria and fungi by inoculating each isolate in three replicates, into 1 % cellulose broth (1 g of cellulose in 100 mL of mineral salt medium, pH ~ 7) in which the initial simple sugar content has been measured. A set of replicates of the same medium was left without inoculating and was used as the control. After incubating for 20 d at room temperature (~ 25 °C), the final sugar content was measured by the phenol-sulphuric method as described by Sadasivam & Manickam (1996). The cellulose broth was kept still to enable cellulose particles to settle on the bottom of the flask. 1.0 mL from the supernatant was decanted into an eppendorf tube under sterile conditions. It was centrifuged at 6000 r.p.m (Harrier 18/80) to sediment the cellulose particles. 0.5 mL from the supernatant was decanted into a test tube and subjected to phenol sulphuric method (Sadasivam & Manickam, 1996) by adding 0.5 mL of 5 % phenol in 0.1 M HCl into 0.5 mL of sample. 2.5 mL of conc. H_2SO_4 was added and mixed thoroughly. The mixture was kept to cool and the absorbance was measured at 490 nm. The final sugar yield was calculated by taking the difference between final and initial sugar concentrations. More efficient organisms (bacteria and fungi), which were able to produce high

concentrations of monosaccharides were selected for further biofilm production (Table 1). The efficient fungi were also inoculated in lignin agar medium [1 g of lignin powder (Sigma, analytical grade) + 1.5 g agar in 100 mL of mineral salt, pH ~ 7] in order to assess their capability of utilizing lignin as the sole source of carbon.

Identification of cellulolytic micro-organisms

The bacteria were presumptively identified by colony morphology, Grams stain, motility and biochemical tests according to the Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Fungi were tentatively identified using macroscopic and microscopic morphological characteristics. Slide culture technique was used to aid the fungal identifications.

Biofilm development

Each isolate of efficient cellulose degrading bacteria (selected after efficiency screening) was grown in 25 mL of nutrient broth (standard nutrient broth from Himedia) for 2 d. Isolates of efficient cellulose degrading fungi were grown separately in 25 mL of potato dextrose broth for 1 wk. The bacteria and fungi were mixed with each other into desired combinations. Those mixed cultures were incubated for 21 d and observed under a light microscope once in every 2 d for fungal-bacterial film formation. Bacterial attachments along the fungal mycelium were taken as fungal-bacterial biofilms according to the definition by Seneviratne *et al.* (2008).

Screening of different types of cultures

The developed fungal-bacterial biofilms, combinations of several biofilms and single microbial cultures were screened for better simple sugar producers by inoculating into 1 % cellulose broth (mineral salt solution 100 mL + cellulose powder 1.0 g, pH ~ 7) in three replicates. The initial simple sugar content of the cellulose broth was measured before inoculation. The cultures were incubated at room temperature (~ 25 °C) for 5–6 wks. In order to aerate the cultures during incubation, regular shaking (3–5 times per day) of the cultures was done manually. The simple sugar content in each culture was measured as described earlier. The cultures employed in screening were batch cultures. Therefore, negative feedbacks were also observed. In order to calculate the average sugar yield per week, harmonic mean was used. The highest sugar yielders among fungal monocultures, fungal mixed cultures and fungal-bacterial biofilms were selected to inoculate into plant substrates.

Table 1: Genera of 10 most efficient cellulose degrading fungi and bacteria with their simple sugar yields during the initial screening

Type of microorganism	Source	Generic name	Code no	Simple sugar concentration (mmol/L)
Fungi	Garden soil	<i>Acremonium</i> sp.	F23	0.2998
	Compost	<i>Fusarium</i> sp.	F12	0.2674
	Compost	<i>Aspergillus</i> sp.	F15	0.2502
	Compost	<i>Mucor</i> sp.	F5	0.2286
	Compost	<i>Aspergillus</i> sp.	F2	0.2204
	Compost	<i>Aspergillus</i> sp.	F9	0.2071
	Compost	<i>Trichoderma</i> sp.	F8	0.1601
	Leaf litter	<i>Penicillium</i> sp.	F52	0.1345
	Compost	<i>Fusarium</i> sp.	F97	0.0366
	Compost	<i>Graphium</i> sp.	F99	0.0324
Bacteria	Leaf litter	<i>Listeria</i> sp.	B90	0.1108
	Leaf litter	<i>Bacillus</i> sp.	B91	0.0793
	Leaf litter	<i>Bacillus</i> sp.	B89	0.0696
	Compost	<i>Alcaligenes</i> sp.	B16	0.0623
	Compost	<i>Alcaligenes</i> sp.	B5	0.0438
	Leaf litter	<i>Neisseria</i> spp.	B97	0.0397
	Garden soil	<i>Alcaligenes</i> sp.	B36	0.0275
	Compost	<i>Neisseria</i> sp.	B3	0.0257
	Garden soil	<i>Listeria</i> sp.	B43	0.0217
	Leaf litter	<i>Streptococcus</i> sp.	B99	0.0214

Biodegradation of selected weed material

Four invasive weeds, *E. odoratum*, *P. maximum*, *L. camara* and *M. pigra* were selected for the degradation studies because of their abundance in Sri Lanka. The above ground parts of the weeds were collected as the substrate. The washed, dried and ground plant substrate of each weed was prepared separately into 5 % weed media by mixing with distilled water. The media were sterilized and their pH and initial simple sugar concentrations were measured. The weed media were slightly acidic with pH ~ 5.5 – 6. Each weed medium was inoculated separately with broth cultures of microorganisms selected

after screening in three replicates (Table 4). They were incubated at room temperature for 15 wks with regular manual shaking (3–5 times per day). The simple sugar yield in each culture was measured weekly. Once a week, half of the liquid in the medium was removed and replaced with sterile distilled water. It was done after keeping the culture still to sediment the substrate. Removal of the substrate and aggregated fungal mycelium was avoided as much as possible to minimize negative feed backs due to accumulation of sugar products from breakdown of cellulose. At the end of the incubation period, cumulative sugar yields were calculated. Statistical analyses were done using Minitab 14 statistical analysis software.

RESULTS AND DISCUSSION

All the isolates exhibited the capability of growing in cellulose agar medium in which the sole source of carbon was cellulose. However, the screening revealed that some organisms were more efficient in simple sugar production than others. The amount of simple sugar produced and released to the medium can depend on the activities of cellulase systems and physiological characteristics of the particular species. The genera of the cellulose degrading fungi which were found to be efficient were *Acremonium*, *Fusarium*, *Aspergillus*, *Mucor*, *Trichoderma*, *Penicillium* and *Graphium* (Table 1). The efficient cellulose degrading bacterial isolates were *Bacillus*, *Listeria*, *Alcaligenes*, *Neisseria* and *Streptococcus* (Table 1).

Acremonium sp. (F23) was the isolate, which produced the highest simple sugar concentration (Table 2). The analysis of variance (culture type vs harmonic mean) showed that the differences in sugar yields (alpha was set at 0.05) were significant ($F = 11.90$ and $p = 0.000$). Post hoc comparison with Hsu's MCB (multiple comparisons with the best) revealed that the *Acremonium* sp. (F23 fungal monoculture) was significantly higher in its sugar yield. Among fungal mixed cultures also a similar situation was observed (Table 3). The analysis of variance of tested fungal mixed cultures (no. of fungi in the culture vs harmonic mean) showed that the differences in sugar yields (alpha was set at 0.05) were significant ($F = 67.01$, $p = 0.000$). Post hoc comparison with Hsu's MCB indicated that the *Acremonium* spp. (F23 fungal

Table 2: Sugar production from commercial cellulose by the most efficient cellulose degrading fungi, three efficient bacteria and biofilms made by combinations of these organisms

Microbial combination	Culture type	Sugar concentration (Weekly)						Harmonic mean
		At start	2 nd Week	3 rd Week	4 th Week	5 th Week	6 th Week	
Control	C	0.0143	0.0147	0.0145	0.0145	0.0135	0.0126	0.0139 (0.00)
B89	B	0.0143	0.0323	0.0394	0.0437	0.0439	0.0292	0.0367 (0.00)
B91	B	0.0143	0.0642	0.0373	0.0426	0.0298	0.0721	0.0441 (0.00)
B90	B	0.0143	0.0534	0.0333	0.0872	0.0956	0.0772	0.0598 (0.01)
F23	F*	0.0143	0.1042	0.0886	0.1034	0.1901	0.1826	0.1211 (0.02)
F23 B89 B90	FBB	0.0143	0.0665	0.0827	0.0952	0.0986	0.0520	0.0746 (0.01)
F23 B91 B89 B90	FBB	0.0143	0.0529	0.0601	0.0638	0.0691	0.0706	0.0626 (0.00)
B91 B89 B90	BC	0.0143	0.0360	0.0352	0.0374	0.0403	0.0600	0.0402 (0.00)
F23 B91	FBB	0.0143	0.0906	0.0461	0.0633	0.0700	0.1260	0.0707 (0.01)
F23 B90	FBB	0.0143	0.0738	0.0353	0.0590	0.0893	0.1346	0.0646 (0.01)
F23 B89	FBB	0.0143	0.0630	0.0773	0.0933	0.0911	0.1001	0.0827 (0.01)
F15 B89	FBB	0.0143	0.0525	0.0499	0.0751	0.0778	0.0410	0.0558 (0.00)
F12 B89	FBB	0.0143	0.0458	0.0570	0.0600	0.0850	0.0358	0.0522 (0.00)
F15 B90	FBB	0.0143	0.0498	0.0720	0.0828	0.0858	0.0452	0.0627 (0.01)
F12 B90	FBB	0.0143	0.0395	0.0744	0.0890	0.0524	0.0699	0.0600 (0.01)
F23 B89 & F12 B90	2*FBB	0.0143	0.0590	0.0810	0.1001	0.0533	0.0790	0.0707 (0.01)
F23 B89 & F12 B90 & F15 B89	3*FBB	0.0143	0.0500	0.0599	0.0884	0.0810	0.0706	0.0671 (0.01)

FBB: Fungal bacterial biofilm; 2*FBB: Two biofilms; 3*FBB: Three biofilms; BC: Bacterial mixed culture;

F*- *Acremonium* sp. fungal monoculture; B: Bacterial monoculture; C: Control; Values within parentheses are standard errors

monoculture) and the mixed culture of three fungi (F23 F12 F15) were superior to all the other monocultures and mixed cultures. These two were not significantly different from each other in their effectiveness. The highest recorded simple sugar concentration within a week was observed in co-cultures having two fungal species, F23 F12 and F23 F15; in the third week a slightly higher sugar concentration than that of F23 monoculture was observed although the increase was not statistically significant ($F = 0.64$, $p = 0.571$).

Most of the genera examined in prior research have been reported for their cellulose degrading ability. Sirisena and Manamendra (1995) have reported cellulolytic activities of *Listeria* sp. (along with *Enterobacteria* and *Pseudomonas* isolates). Vazquez-Arista et al. (1997) have reported *Alcaligenes* spp. among cellulolytic bacteria isolated from the digestive system of a Coleopteran. Although direct evidence on cellulolytic activities

of *Neisseria* were not found in literature, an enzyme catalyzing the hydrolysis of beta-1,4 bonds in cellulose acetate has been purified by Moriyoshi et al. (2002) from *Neisseria sicca*. Oyeleke and Okusanmi (2008) have isolated *P. aeruginosa*, *Streptococcus*, *Bacillus*, *Penicillium*, *Aspergillus*, *Mucor* and *Fusarium* species, which were able to hydrolyze cellulose. The filamentous fungi *Acremonium cellulolyticus* and *Trichoderma reesei* are well known to be potential cellulase producers (Fujii et al., 2009).

According to the preliminary screening, the fungal isolates F23, F12 and F15 and the bacterial isolates B90, B91 and B89 produced high sugar yields during the screening (Table 1). These organisms were subjected to fungal-bacterial biofilm formation and the isolate combinations F23-B91, F23-B90, F23-B89, F12-B89, F12-B90, F15-B89 and F15-B90 were successful in producing biofilms.

Table 3: Sugar production from commercial cellulose by cultures of efficient cellulose degrading fungi

Fungal combination (code no.)	No. of fungi included	Sugar concentration (mmol/L)						Harmonic mean
		At start	2 nd Week	3 rd Week	4 th Week	5 th Week	6 th Week	
F23	F*	0.0114	0.0704	0.0890	0.2032	0.1926	0.1083	0.1116
F12	1	0.0114	0.0390	0.0587	0.1861	0.0743	0.0923	0.0691
F15	1	0.0114	0.0418	0.0587	0.1685	0.0891	0.0829	0.0712
F52	1	0.0114	0.0331	0.0476	0.1489	0.1059	0.1051	0.0650
F23 F12	2	0.0114	0.0441	0.0769	0.2138	0.1685	0.1128	0.0907
F23 F12 F15	3	0.0114	0.0658	0.1123	0.1858	0.0909	0.1295	0.1037
F23 F12 F15 F5	4	0.0114	0.0693	0.0587	0.0893	0.1346	0.1175	0.0853
F23 F12 F15 F5 F2	5	0.0114	0.0311	0.0406	0.0797	0.1159	0.1018	0.0570
F23 F12 F15 F5 F2 F52	6	0.0114	0.0331	0.0418	0.0839	0.1207	0.0977	0.0591
F23 F12 F15 F5 F2 F52 F8	7	0.0114	0.0362	0.0423	0.0863	0.1127	0.0877	0.0601
F23 F12 F15 F5 F2 F52 F8 F9	8	0.0114	0.0443	0.0455	0.0914	0.1161	0.0944	0.0670
F23 F12 F15 F52	4	0.0114	0.0612	0.0506	0.0867	0.1312	0.0883	0.0751
F23 F15	2	0.0114	0.0697	0.0637	0.2049	0.0796	0.1391	0.0915
Control	C	0.0114	0.0100	0.0106	0.0116	0.0104	0.0107	0.0106

F*: *Acremonium* sp. fungal monoculture; C: Control

The cellulose degrading ability and sugar producing pattern of these fungal-bacterial biofilms, fungal mixed cultures and some fungal/bacterial monocultures in commercial cellulose were tested and the results are presented in Tables 2 and 3. Unlike the fungal-bacterial biofilms, fungal-fungal biofilms are not easily identifiable under the light microscope. Hence random combinations of fungal mixed cultures were employed in this study.

According to the results, the recorded highest sugar concentration was observed with *Acremonium* sp. (F23) monoculture. The biofilms and fungal mixed cultures

tested in this study were unable to exhibit increased sugar production rates than *Acremonium* sp. (F23). All the bacterial isolates were significantly less efficient in saccharification of cellulose compared to the most efficient fungi, which ranked from 1st – 8th in the initial screening (Table 1). Therefore, the incorporation of these bacteria into more efficient fungi may have reduced the sugar yields of fungal monocultures by simply utilizing the sugars available in the cultures. In fungal mixed cultures also less sugar yields were observed compared to *Acremonium* sp. monoculture, probably due to the less efficient organisms utilizing the simple sugars liberated into the medium.

Table 4: Cultures selected for inoculation into weed substrates

Selected culture	Organisms involved	Reasons for the selection
F 23	<i>Acremonium</i> sp. monoculture	Highest harmonic mean of sugar yield Highest recorded sugar concentration in a week
F23 B89	<i>Acremonium</i> sp. and <i>Bacillus</i> sp. biofilm	Highest harmonic mean of sugar yield Sugar yield gradually increased rather than fluctuated
F23 F12	<i>Acremonium</i> sp. and <i>Fusarium</i> sp. mixed culture	Highest recorded sugar concentration in a week Third highest harmonic mean of sugar yield

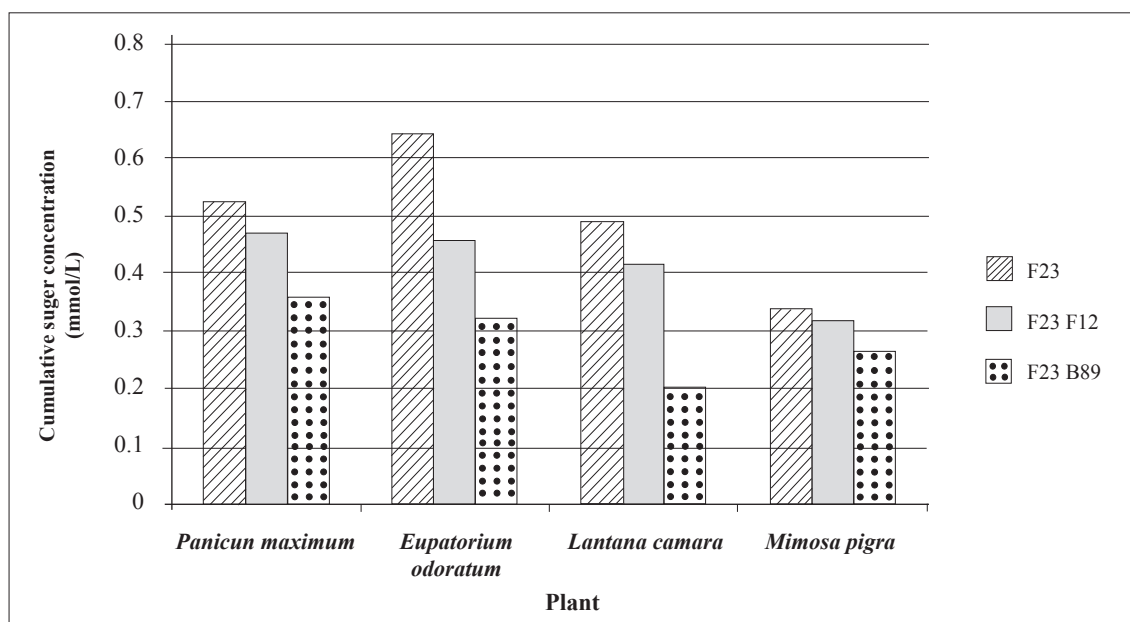


Figure 1: Cumulative sugar yield in all four weed substrates by the three selected microbial cultures
Statistical analysis of above data with two way analysis of variance (cumulative yields vs plant, culture; alpha set at 0.05) indicates that the differences were significant with $F = 12.03$ and $p = 0.008$. No significant difference among plant substrates due to one way ANOVA (cumulative yields vs plant) with $F = 1.24$ and $p = 0.357$.

Considering all the observations from simple sugar analysis in commercial cellulose, three different cultures were selected for the inoculation of weed substrates. The selected culture, nature of the culture and the reasons for theselectionaresummarizedinTable4. These cultures were inoculated into weed substrates; *Eupatorium*, *Panicum*, *Lantana* and *Mimosa* in submerged cultures. These weeds were selected as raw material for degradation studies due to their abundance and harmful effects on the Sri Lankan environment. Weed substrates were not chemically pretreated for delignification. Yet *Acremonium* sp. and *Fusarium* sp. had the ability to degrade lignin as both of them were capable of growing in lignin medium in the absence of any other carbon source. In contrast to the batch culturing method employed with commercial cellulose, manually maintained continuous cultures were used with weed substrates in order to get the maximum sugar yield by reducing the negative feed backs due to accumulation of the product. The cumulative sugar yield of each plant and each microbial culture inoculated into plant substrates was calculated and analyzed (Figure 1). One way ANOVA showed that the differences of the cumulative yields of different cultures were significant with $F = 5.41$ and $p = 0.029$. Hsu's MCB revealed that the tested biofilm was significantly less effective but the cumulative yields of the fungal mixed culture and F23 monoculture were not significantly different from each other (Figure 1).

In plant substrates both cellulose and hemicelluloses are present. Cellulase systems are capable of degrading hemicelluloses as well as cellulose (Lynd *et al.*, 2002). Therefore, the simple sugar yield during the plant substrate degradation can be attributed to degradation of both cellulose and hemicelluloses. The highest sugar yield was provided by the *Acremonium* sp. (F23) monoculture in all four plant substrates. The sugar yields of *E. odoratum* and *P. maximum* were higher than that of *M. pigra* and *L. camara* probably due to their less lignified soft nature of the tissues. Hence, for biofuel production *E. odoratum* and *P. maximum* can serve as better raw material sources than the other two species. All the microbial communities that were tested during this research were unable to increase the rate of cellulose degradation through community actions. The biofilms and the fungal combinations tried were perhaps not showing any synergistic effect towards saccharification process and hence further research is required in this field.

CONCLUSION

The screening of fungal and bacterial isolates for effective organisms revealed that the ten most efficient

cellulose degrading fungal isolates were *Acremonium* sp., two *Fusarium* spp., three *Aspergillus* spp., *Mucor* sp., *Trichoderma* sp., *Penicillium* sp. and *Graphium* sp. The most efficient cellulose degrading bacteria included two *Listeria* spp., two *Bacillus* spp., three *Alcaligenes* spp., two *Neisseria* spp. and a *Streptococcus* sp. Among them cellulose degrading fungal species (ranked 1st – 8th) were more effective than the bacteria (i.e. B90, B91 and B89).

Acremonium sp. (F23) monoculture was the most efficient simple sugar yielder compared to other single and mixed microbial cultures. Its efficiency did not increase when incorporated into fungal-bacterial biofilms or fungal mixed cultures. All the microbial communities that were tested during this study were unable to increase the rate of cellulose degradation through community actions.

In the simple sugar production by microbial cellulose degradation, *E. odoratum* and *P. maximum* produced higher sugar yields than the other two weeds. Therefore, *E. odoratum* and *P. maximum* are better raw materials than *L. camara* and *M. pigra* for use in the production of cellulosic biofuels.

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REFERENCES

1. Antizar-Ladislao B. & Turrion-Gomez J.L. (2008). Second-generation biofuels and local bioenergy systems. *Biofuels, Bioproducts and Biorefining* **2**(05): 455 – 469.
2. Aristidou A. & Penttila M. (2000). Metabolic engineering applications to renewable resource utilization. *Current Opinion on Biotechnology* **11**(02):187–198.
3. Boer W.D, Folman L.B., Summerbell R.C. & Boddy L. (2005). Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiology Reviews* **29**(04): 795 – 811.
4. Ciesarova Z., Domeny Z., Smogrovicova D., Patkova J. & Sturdik E. (1998). Comparison of ethanol tolerance of free and immobilized *Saccharomyces uvarum* yeasts. *Folia Microbiologica* **43**(01): 55 – 58.
5. Dellomonaco C., Fava F. & Gonzalez R. (2010). The path to next generation biofuels: successes and challenges in the era of synthetic biology. Review. *Microbial Cell Factories* **9**: 3.
6. Desimone M.F., Degrossi J., D'Aquino M. & Diaz L.E. (2002). Ethanol tolerance in free and sol-gel immobilized

- Saccharomyces cerevisiae*. *Biotechnology Letters* **24**(19):1557 – 1559.
7. Flemming H.C. & Wingender J. (2001). Relevance of microbial extracellular polymeric substances (EPSs) – Part I: structural and ecological aspects. *Water Science and Technology* **43**(06): 1 – 8.
 8. Fujii T., Fang X., Inoue H., Murakami K. & Sawayama S. (2009). Enzymatic hydrolyzing performance of *Acremonium cellulolyticus* and *Trichoderma reesei* against three lignocellulosic materials. *Biotechnology for Biofuels* **2**(1): 24.
 9. Gordon G.L.R. & Phillips M.W. (1998). The role of anaerobic gut fungi in ruminants. *Nutrition Research Reviews* **11**(01): 133 – 168.
 10. Gunasekera L. (2008). *Identification guide for invasive plants in Sri Lanka*, 1st edition, pp. 125. Print & Print graphics (Pvt.) Ltd., Colombo 10.
 11. Holt J.G., Krieg N.R., Sneath P.H.A., Staley J.T. & Williams S.T. (1994). *Bergey's Manual of Determinative Bacteriology*, 9th edition, pp. 787. Williams and Williams, Baltimore, USA.
 12. Hu Q., Sommerfeld M., Jarvis E., Ghirardi M., Posewitz M., Seibert M. & Darzins A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. *The Plant Journal* **54** (04): 621– 639.
 13. Jayant M., Rashmi J., Shailendra M. & Deepesh Y. (2011). Production of cellulase by different co-culture of *Aspergillus niger* and *Penicillium chrysogenum* from waste paper, cotton waste and baggase. *Journal of Yeast and Fungal Research* **2**(02): 24 – 27.
 14. Jirku V. (1999). Whole cell immobilization as a means of enhancing ethanol tolerance. *Journal of Industrial Microbiology and Biotechnology* **22**(03):147 – 151.
 15. Krisch J. & Szajani B. (1997). Ethanol and acetic acid tolerance in free and immobilized cells of *Saccharomyces cerevisiae* and *Acetobacter aceti*. *Biotechnology Letters* **19**(06):525 – 528.
 16. Liu Y. & Tay J.H. (2002). The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. *Water Research* **36**(07):1653–1665.
 17. Lynd L.R., Weimer P.J., Zyl W.H.V. & Pretorius I.S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews* **66**(03): 506 – 577.
 18. Marambe B. (2001) Alien invasive plants in the central province of Sri Lanka. *Sri Lankan Biodiversity Review* **1**: 21 – 30.
 19. Mcsweeney C.S., Dulieu A., Katayama Y. & Lowry J.B. (1994). Solubilization of lignin by the ruminal anaerobic fungus *Neocallimastix patriciarum*. *Applied Environmental Microbiology* **60**(08): 2985 – 2989.
 20. Moriyoshi K., Ohmoto T., Ohe T. & Sakai K. (2002). Purification and characterization of an endo-1,4-beta-glucanase from *Neisseria sicca* SB that hydrolyzes beta-1,4 linkages in cellulose acetate. *Bioscience, Biotechnology & Biochemistry* **66**(3): 508 – 515.
 21. Oyeleke S.B. & Okusanmi T.A. (2008). Isolation and characterization of cellulose hydrolyzing microorganism from the rumen of ruminants. *African Journal of Biotechnology* **7**(10): 1503 – 1504.
 22. Rainey P.B., Cole A.L.J., Fermor T.R. & Wood D.A. (1990). A model system for examining involvement of bacteria in basidiome initiation of *Agaricus-bisporus*. *Mycological Research* **94**(02):191 – 195.
 23. Sadashivaiah A.B. & Mysore V. (2010). Biofilms: Their role in dermal fillers. *Journal of Cutaneous and Aesthetic Surgery* **3**(01): 20 – 22.
 24. Sadasivam S. & Manickam A. (1996). *Biochemical Methods*, 1st edition, p. 10. New Age International Limited Publishers, New Delhi, India.
 25. Seneviratne G., Zavahir J.S., Bandara W.M.M.S. & Weerasekara M.L.M.A.W. (2008). Fungal-bacterial biofilms: their development for novel biotechnological applications. *World Journal of Microbiology and Biotechnology* **24**(06): 739 – 743.
 26. Sharon N. & Lis H. (1993). Carbohydrates in cell recognition. *Scientific American* **268**(01): 82 – 89.
 27. Sinsabaugh R.L. & Linkins A.E. (1990). Enzymatic and chemical-analysis of particulate organic-matter from a boreal river. *Freshwater Biology* **23**(02): 301–309.
 28. Sirisena D.M. & Manamendra T.P. (1995). Isolation and characterization of cellulolytic bacteria from decomposing rice straw. *Journal of National Science Council Sri Lanka* **23**(1): 25 – 30.
 29. Stryer L. (1995). *Biochemistry*, 4th edition, pp. 189 – 191. Freeman, New York, USA.
 30. Vazquez-Arista M., Smith R.H., Olalde-Portugal V., Hinojosa R. E., Hernandez-Delgadillo R. & Blanco-Labra A. (1997). Cellulolytic bacteria in the digestive system of *Prostephanus truncatus* (Coleoptera: Bostrichidae). *Journal of Economic Entomology* **90**(5): 1371–1376.
 31. Wang Z.W. & Chen S. (2009). Potential of biofilm-based biofuel production. *Applied Microbiology and Biotechnology* **83**(01): 1–18.
 32. Webb C., Fukuda H. & Atkinson B. (1986). The production of cellulase in a spouted bed fermentor using cells immobilized in biomass support particles. *Biotechnology and Bioengineering* **28**(1): 41–50.
 33. Westbrooks R. (1998). *Invasive Plants, Changing the Landscape of America: Fact Book*, p. 3. Federal Interagency Committee for the Management of Noxious and Exotic Weeds (FICMNEW), Washington D.C., USA. Available at <http://digitalcommons.usu.edu/cgi/viewcontent.cgi?article=1489&context=govdocs>
 34. Zhou B., Martin G.J.O. & Pamment N.B. (2008). Increased phenotypic stability and ethanol tolerance of recombinant *Escherichia coli* KO11 when immobilized in continuous fluidized bed culture. *Biotechnology and Bioengineering* **100**(04): 627 – 633.