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Characterization of a Defined Cellulolytic and Xylanolytic Bacterial Consortium for Bioprocessing of Cellulose and Hemicelluloses

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Abstract Diminishing fossil fuel reserve and increasing cost of fossil hydrocarbon products have rekindled worldwide effort on conversion of lignocelllloloses (plant biomass) to renewable fuel. Inedible plant materials such as grass, agricultural, and logging residues are abundant renewable natural resources that can be converted to biofuel. In an effort to mimic natural cellulolytic–xylanolytic microbial community in bioprocessing of lignocelluloses, we enriched cellulolytic–xylanolytic microorganisms, purified 19 monocultures and evaluated their cellulolytic–xylanolytic potential. Five selected isolates (DB1, DB2, DB7, DB8, and DB13) were used to compose a defined consortium and characterized by 16S ribosomal RNA gene sequence analysis. Nucleotide sequence blast analysis revealed that DB1, DB2, DB7, DB8, and DB13 were respectively similar to *Pseudoxanthomonas byssovorax* (99%), *Microbacterium oxydans* (99%), *Bacillus* sp. (99%), *Ochrobactrum anthropi* (98%), and *Klebsiella trevisanii* (99%). The isolates produced an array of cellulolytic–xylanolytic enzymes (filter paper cellulase, β-glucosidase, xylanase, and β-xylosidase), and significant activities were recorded in 30 min. Isolates DB1 and DB2 displayed the highest filter paper cellulase: 27.83 and 31.22 Umg^{-1}, respectively. The highest β-glucosidase activity (18.07 Umg^{-1}) was detected in the culture of isolate DB1. Isolate DB2 produced the highest xylanase activity (103.05 Umg^{-1}), while the highest β-xylosidase activity (7.72 Umg^{-1}) was observed with DB13. Use of microbial consortium in bioprocessing of lignocelluloses could reduce problems such as incomplete synergistic enzymes, end-product inhibition, adsorption, and requirement for high amounts of enzymes in direct use of enzymes.

Keywords Bacterial consortium · rRNA gene sequence analysis · Cellulolytic–xylanolytic enzymes · Cellulose · Hemicelluloses · Bioprocessing

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Introduction

Hydrocarbon oil reserves are fast diminishing [1]. Fossil oil is a nonrenewable source of fuel, and costs of fossil oil products are high. These factors necessitated the current worldwide efforts to develop renewable alternatives to fossil fuels. Development of liquid fuel through enzymatic hydrolysis of carbohydrate polymers in lignocelluloses of nonfood biomass to sugars and fermenting them to ethanol is one approach toward fossil fuel independence [2]. Lignocelluloses (cellulose, hemicelluloses, and lignin) are the major constituents of plant fiber. Lignocellulosic biomass is a renewable biological resource that is constantly replenished through photosynthesis [3]. Biosynthesis of cellulose by both terrestrial plants and marine algae has been estimated to occur at a rate of 0.85×10^{11} ton per annum [4].

Celllulose is the most abundant constituent of plant biomass, and invariably the most abundant biopolymer on earth [5]. It is a high-molecular-weight linear polymer of glucose joined by β-D-(1,4) linkages [6]. Enzymes of the cellulase complex (exoglucanase, endoglucanase, and β-glucosidase) act in concert to degrade biomass cellulose to soluble sugars [7]. Microorganisms including fungi [8–13] are the primary sources of lignocellulose hydrolyzing enzymes. Members of the genera—*Cellulomonas*, *Clostridium*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces*—are some bacteria reported to produce cellulases [14–16].

Hemicelluloses are the second most plentiful component of plant biomass. Xylan is the major hemicellulose in plant matter [17]. It is generally composed of a linear 1,4-β-linked D-xylose backbone and branches of other sugars. Xylan is a heterogeneous natural polymer, and thus requires diverse xylanases [18] for complete breakdown to monomers. Xylan degrading enzymes include those that degrade the main chain which are endo-β-1,4-xylanase and β-xylosidase; and side chain-cleaving enzymes that include α-glucuronidase, α-L-arabinofuranosidase, acetylxylan esterase, and feruloyl esterase [19–21]. Xylanases are produced by bacteria, fungi, and actinomycetes [22, 23]. Reported xylan degrading bacteria include [24] strains of *Aeromonas*, *Bacillus*, *Bacteroides*, *Cellulomonas*, *Microbacterium*, *Paenibacillus*, *Ruminococcus*, and *Streptomyces*.

Efficient bioconversion of biomass polysaccharides to fermentable sugars is central to the development of competitive biological processes for making nonfood fiber ethanol [25, 26]. Existing commercial enzymes were mainly developed for pulp and paper and food industries [27]. Although the commercial enzymes can effectively degrade lignocelluloses to monomers, problems of using cell-free enzymes include end-product inhibition, adsorption of enzymes, and requirement for high amounts of enzymes [28–31]. The high concentrations of enzyme needed make the process very expensive [32–34]. Furthermore, commercial preparations do not contain all synergistic enzymes including ligninolytic enzymes required to make cellulose more accessible to hydrolysis [34, 35]. Whole-cell saccharification and consolidated bioprocessing (utilization of microorganisms that can convert biomass to fermentable sugars and to ethanol) [36] are alternative routes that can reduce the aforesaid problems associated with cell-free enzyme processes. In an effort to mimic whole-cell bioprocessing of cellulose and hemicelluloses by natural cellulolytic–xylanolytic microbial communities, we enriched cellulolytic–xylanolytic microorganisms, purified monocultures, evaluated their cellulolytic–xylanolytic potential, and composed a defined five-member bacterial consortium (FMBC). We then employed 16S rRNA gene sequence analysis to characterize the monocultures of the consortium.

Methods

Enrichment and Selection of Cellulolytic and Xylanolytic Bacteria

Inoculum for each enrichment culture was one of the following: grassland soil, hardwood compost, pine needle compost, mulched flowerbed soil, paper processing wood waste, and paper mill waste water. The enrichment basal medium was FTW mineral salts medium [37] with 1 ml of trace elements solution [38]. Initial pH was adjusted to 7.4 before autoclaving (121°C, 20 min) using aliquots of 1 M NaOH. Sterilized substrate mixture (pine wood chips, 0.25 g, and lawn grass, 0.25 g) and 0.5 g of each inoculum were aseptically transferred to 50-ml centrifuge tubes and the volume was adjusted to 50 ml using the enrichment basal medium. Cultures were incubated with orbital shaking at about 30°C for 5 days. Potential cellulose and xylan-degrading bacteria were plated on carboxymethylcellulose (CMC)-FTW agar containing 0.1% CMC, and incubated at 30°C for 3 days. Discrete colonies were purified by repeated streaking on CMC-FTW agar. Nineteen isolates capable of growth on CMC as the sole source of carbon were isolated.

Screening of Isolates for Production Cellulolytic, and Xylanolytic Enzymes

Inoculum was prepared by transferring five loopfuls of each bacterial isolate to 5 ml sterile FTW mineral elements solution in a 15-ml centrifuge tube. The cell suspensions were vortex mixed to disperse the bacterial cells. The medium for screening isolates for cellulolytic and xylanolytic enzymes was 5 ml FTW mineral salts solution placed in Fisher brand 18×50 mm borosilicate glass culture tubes equipped with polypropylene caps. Pulverized dry Bermuda grass was added to a final concentration of 0.5% (0.025 g/5 ml). The medium was sterilized by autoclaving (121°C for 20 min). At ambient temperature, the medium was inoculated with 200 μl of each inoculum in triplicate and incubated with orbital shaking at 30°C for 6 days.

Determination of Activities of Enzymes

Filter paper cellulase assay [39] was adapted except that filter paper disks were used [40]. Each filter paper disk (7 mm in diameter) weighed approximately 33.8 mg. The reaction mixture comprised 0.5 ml of enzyme, 0.5 ml sodium acetate buffer (pH 5.0), and ten disks of Whatman No. 1 filter paper. The tubes were incubated for 30 min in a 50°C water bath. Thereafter, sugar was determined by DNS method [41]. In brief, 1 ml of dinitrosalicylic acid reagent (DNS) and 0.5 ml of 50% sodium-potassium tartrate were added to each reaction mixture. Each tube was then heated in boiling water for 8 min and cooled to ambient temperature before the absorbance was read at λ_{575} against water blank. Enzyme activity (μmol or units ml^{-1} 30 min^{-1}) was read from a glucose standard curve. Xylanase activity was measured using the cellulase procedure except that the reaction mixture contained 0.5% oat spelts xylan. β-Glucosidase activity reaction mixture comprised 100 μl of enzyme, 800 μl of 100 mM sodium acetate buffer (pH 5.0), and 100 μl of 40 mM *p*-nitrophenol B-D-glucoside in 100 mM sodium acetate buffer (pH 5.0). The reaction mixture was incubated for 30 min at 50°C. Thereafter, the reaction was immediately terminated by the addition of cold 500 mM sodium carbonate [42]. Light yellow color development due to liberation of *p*-nitrophenol from the substrate was determined by measuring absorbance at λ_{405}. One unit of β-glucosidase activity was defined as 1 μmol of *p*-nitrophenol released by

1 ml of enzyme in a 30-min assay. β-Xylosidase activity was measured as described for β-glucosidase except that the substrate was 20 mM *p*-nitrophenol β-D-xyloside.

Protein Determination

Protein concentration was quantified by the Bradford method [43] using Coomassie® protein assay reagent (Pierce, Rockford, IL). Briefly, 25 μl of supernatant sample was added to a cuvette and 750 μl of Coomassie reagent was added, at room temperature. Each cuvette was incubated at ambient temperature for 10 min and read at λ_{595} using water blank. Protein concentration was then quantified using regression equation of bovine serum albumin standard curve.

Analysis of Anaerobic Growth Capability of Isolates in Liquid and Surface Cultures

In liquid culture, the isolates were grown in sterile enrichment mineral medium containing CMC and 0.03% yeast extract in sealed 15-ml Falcon tubes. The media were each aseptically purged with nitrogen before they were inoculated and incubated at 30°C for 3 days. For surface cultures, cells were streaked on tryptic soy agar amended with 0.1% CMC. Cultures were incubated under anaerobic condition in a BBL gas pack anaerobic jar equipped with palladium catalyst, at 30°C for 3 days.

Extraction of DNA for Identification of Selected Isolates

Purity of isolates was assessed by streaking on tryptic soy agar and incubated at 30°C for 24 h. Cells were suspended in nuclease-free water (Promega Corporation, Madison, WI) and recovered by centrifugation. DNA was extracted from cells using Promega wizard genomic DNA purification kit with slight modification. Nucleic acid lysis solution (600 μl) was used to resuspend cells and incubated at 80°C for 5 min and then cooled. RNase solution (3 μl) was added followed by incubation at 37°C for 20 min. Protein precipitation solution (200 μl) was added, and the mixture was placed on ice for 5 min. After centrifugation, the supernatant was transferred to a tube and 600 μl of ice-cold 95% ethanol was added. The precipitate recovered by centrifugation was washed with 70% ethanol at ambient temperature and DNA pellet was resuspended in Promega rehydration solution.

Polymerase Chain Reaction Amplification of 16S Rrna Gene

Eubacteria 16S rRNA gene universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) were used for polymerase chain reaction (PCR) amplification of the 16S rRNA gene [44]. The PCR reaction consisted of 25 μl of PCR master mix (Promega), 2 μl of genomic DNA template, 5 μl of primer 27f (25 pmol), 5 μl of primer 1492r (25 pmol) and 13 μl of nuclease-free water. Thirty-five cycles of PCR (initial denaturation at 95°C for 5 min; subsequent denaturation at 95°C for 0.5 min; annealing at 50°C for 1 min; extension at 72°C for 1 min) and final extension at 72°C for 5 min were used for amplification. PCR amplicons were purified using Montage-PCR filter units (Millipore Corp, Billerica, MA).

DNA Cycle Sequencing

Sanger (BigDye) terminator kit (Applied Biosystems, Foster City, CA) was employed for cycle sequencing. The reaction mixture for amplification of DNA templates included

dideoxynucleotide triphosphates of A, G, C, and T, fluorescently labeled dideoxynucleotide triphosphates and primer 519r (5′-GWATTACCGCGGCKGCTG-3′). PCR extension products were separated and detected by capillary electrophoresis using Applied Biosystems 3730-XL DNA sequencer according to the manufacturers' instructions, at the Institute for Integrative Genome Biology, UC Riverside, CA.

DNA Sequence Similarity and Phylogenetic Analysis

Nucleotide sequence of 16S rRNA gene of monocultures of the consortium were deposited to the Genbank database under accession numbers HM197761 (DB1), HM197762 (DB2), HM197763 (DB7), HM197764 (DB8), and HM197765 (DB13). Nucleotide sequence similarity searches were analyzed by Genbank Blast [45]. Nucleotide sequence employed included the V3 hypervariable region [46]. Evolutionary position among members of the consortium and related organisms was analyzed by RDP beta 10 (Fig. 1) [47].

Time Course of Cellulase and Xylanase Production by Defined Bacterial Consortium

A five-member defined bacterial consortium (DB1, DB2, DB7, DB8, and DB13) was composed on the basis of their enzyme activities and anoxic growth capability. The medium comprised 0.2 g of each substrate (Bermuda grass, switch grass, and corn stover) and 48.5 ml of FTW mineral elements solution in 50-ml centrifuge tubes. Each medium was inoculated with 1.5 ml of the bacterial consortium prepared by mixing equal volumes of the five bacteria after OD_{600} of each bacterial suspension was normalized to approximately 1.0 by dilution. Sealed culture tubes were incubated without shaking for 6 days. Culture supernatants were recovered after centrifugation. Viability of individual bacterial members of the defined consortium in the medium was assessed after 6 days of incubation by standard plate count on tryptic soy agar plates.

Results

Cellulolytic and Xylanolytic Activities of Monoculture Isolates

Filter paper cellulase, β-glucosidase, xylanase, and β-xylosidase activities in culture supernatants of the bacterial isolates are presented in Table 1. Isolates DB1 and DB2 displayed the highest filter paper cellulase activity, 27.83 and 31.22 Umg^{-1} protein, respectively. The highest β-glucosidase activity (18.07 Umg^{-1} protein) was detected in the culture of isolate DB1. Substantial levels of β-glucosidase was also detected in cultures of isolates DB4, DB8, DB9, and other isolates (Table 1). β-Glucosidase activity was very low in culture supernatants of isolate DB19 and not detected in cultures of isolates DB20, DB17, and DB11. Xylanase activities of many isolates were substantial (Table 1). Isolate DB2 displayed the highest xylanase activity (103.05 Umg $protein^{-1}$). The highest β-xylosidase activity was detected in culture supernatant of isolate DB13 (7.72 Umg $protein^{-1}$). β-Glucosidase and β-xylosidase activities were, nonetheless, not detected in some cultures.

Evaluation of Anoxic Growth in Liquid Submerged and Surface Culture

Substantial growth was observed with isolates DB5, DB6, DB7, DB8, DB10, DB11, DB12, DB13, and DB14 in tryptic soy broth anoxic culture. Luxuriant growth on surface agar

DB1 Lineage: Bacteria; "Proteobacteria"; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Pseudoxanthomonas

agagtttgatcatggctcagagtgaacgctggcggtaggcctaacacatgcaagtcgaacggcagcgggtagggagcttg
ctccctatgccggcgagtggcggacgggtgaggaatacatcggaatctactctgtcgtgggggataacgtagggaaactt
acgctaataccgcatacgacctacgggtgaaagtgggggaccgcaaggcctcacgcgatagaatgagccgatgtccgatt
agctagttggcggggtaaaggcccaccaaggcgacgatcggtagctggtctgagaggatgatcagccacactgggactga
gacacggcccagactcctacgggaggcagcagtggggaatattggacaatgggcgcaagcctgatccagccataccgcgt
gagtgaagaaggccctcgggttgtaaagntcttttgttgggaaagaaatcctgttggctaatacccggcagggatgacga
cccaaga

DB2 Lineage: Bacteria; "Actinobacteria"; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Microbacterium

agagtttggatcatggctcaggatgaacgctggcggcgtgcttaacacatgcaagtcgaacggtgaacacggagcttgct
ctgtgggatcagtggcgaacgggtgagtaacacgtgagcaacctgcccctgactctgggataagcgctggaaacggcgtc
taatactggatatgtgacgtgatcgcatggtctgcgtctggaaagaatttcggttggggatgggctcgcggcctatcagc
ttgttggtgaggtaatggctcaccaaggcgtcgacgggtagccggcctgagagggtgaccggccacactgggactgagac
acggcccagactcctacgggaggcagcagtggggaatattgcacaatgggcgcaagcctgatgcagcaacgccgcgtgag
ggatganggccttcgggttgtaaacctcttttagcagggaagaagcgaaagtgacgacctc

DB7 Lineage: Bacteria; "Firmicutes"; "Bacilli"; Bacillales; Bacillaceae; Bacillus

catggctcaggatgaacgctggcggcgtgcctaatacatgcaagtcgagcgaatggattaagagcttgctcttatgaagt
tagcggcggacgggtgagtaacacgtgggtaacctgcccataagactgggataactccgggaaaccggggctaataccgg
ataacattttgaaccgcatggttcgaaattgaaaggcggcttcggctgtcacttatggatggacccgcgtcgcattagct
agttggtgaggtaacggctcaccaaggcaacgatgcgtagccgacctgagagggtgatcggccacactgggactgagaca
cggcccagactcctacgggaggcagcagtagggaatcttccgcaatggacgaaagtctgacggagcaacgccgcgtgagt
gatgaaggctttcgggtcgtaaaactctgtgttagggaagaacaagtgctagttgaataagctggcacctgacgtcc

DB8 Lineage: Root; Bacteria; "Proteobacteria"; Alphaproteobacteria; Rhizobiales; Brucellaceae; unclassified_Brucellaceae

agagtttgatcatggctcagaacgaacgctggcggcaggcttaacacatgcaagtcgagcgccccgcaaggggagcggca
gacgggtgagtaacgcgtgggaacgtaccatttgctacggaataactcagggaaacttgtgctaataccgtatgagcccg
aaaggggaaagatttatcggcaaatgatcggcccgcgttggattagctagttggtggggtaaaggcctaccaaggcgacg
atccatagctggtctgagaggatgatcagccacactgggactgagacacggcccagactcctacgggaggcagcagtggg
gaatattggacaatgggcgcaagcctgatccagccatgccgcgtgagagatgaaggccctagggttgtaaagctctttca
cccggtgaagataatgaccgtacccgagag

DB13 Lineage: Root; Bacteria; "Proteobacteria"; Gammaproteobacteria; "Enterobacteriales"; Enterobacteriaceae; Raoultella

agagtttgctcatggctcagattgaacgctggcggcaggcntaacacatgcaagtcgagcggtagcacagagagcttgct
ctcgggtgacgagcggcggacgggtgagtaatgtctgggaaactgcctgatggagggggataactactggaaacggtagc
taataccgcataacgtcgcaagaccaaagtgggggaccttcgggcctcatgccatcagatgtgcccagatgggattagct
agtaggtggggtaatggctcacctaggcgacgatccctagctggtctgagaggatgaccagccacactggaactgagaca
cggtccagactcctacgggaggcagcagtggggaatattgcacaatgggcgcaagcctgatgcagccatgccgcgtgtat
gaagaaggccttcgggttgtaaagtactttcagcgaggaggaaggcgttaaggttaataaccctgncgattgacgtact
cgcga

Fig. 1 Taxonomic lineage and nucleotide sequence of members of the defined bacterial consortium. Taxonomic lineage was determined using RDP beta 10 [47]

culture was observed with isolates DB5, DB6, DB7, DB8, DB10, DB11, DB12, DB13, and DB14. Other isolates (DB1, DB2, DB16, and DB17) displayed slight to moderate growth in gas pack anaerobic surface culture. Isolates DB3, DB4, DB9, DB15, and DB19 did not grow in cultures incubated under anaerobic condition.

Composition of Defined Bacterial Consortium

Based on cellulolytic and xylanolytic enzymes produced by the isolates and anoxic growth capacity (desirable for coculture with yeast), five isolates were selected and used to compose a defined bacterial consortium. The FMBC comprised the isolates DB1, DB2, DB7, DB8, and DB13.

Table 1 Cellulolytic and xylanolytic enzymes in cell-free culture supernatant of isolates

Isolate	Source	Cellulase (U/mg protein)	β-Glucosidase (U/mg protein)	Xylanase (U/mg protein)	β-Xylosidase (U/mg protein)
DB1	PMWW	27.83	18.07	17.78	1.25
BD2	Grass Land Soil	31.22	7.14	103.05	1.95
DB3	PPWW	17.89	4.52	12.32	0.86
DB4	PMWW	23.78	10.00	22.73	6.89
DB5	Pine Needle Compost	19.22	3.70	14.47	0
DB6	Sawdust	21.21	5.51	14.10	5.51
DB7	Sawdust	19.38	8.47	13.68	6.61
DB8	Flower Bed Soil	20.69	10.15	15.02	5.38
DB9	Pine Needle Compost	25.82	10.21	14.60	3.14
DB10	Grass Land Soil	20.92	3.9	12.39	1.16
DB11	Sawdust	21.92	0	18.59	0
DB12	Pine Needle Compost	25.51	3.09	18.14	2.65
DB13	Hardwood Compost	21.34	2.30	12.62	7.72
DB14	Flower Bed Soil	17.88	5.17	12.32	3.28
DB15	Hardwood Compost	17.56	5.47	12.61	3.65
DB16	PPWW	20.73	7.65	14.34	3.92
DB17	Grass Soil	24.75	0	14.00	0
DB19	Pine Needle Compost	15.67	0.109	10.20	0
DB20	Flower Bed Soil	18.80	0	9.55	0.38

PMWW paper mill water waste; *PPWW* paper processing wood waste

Morphological Characteristics of Monocultures of the Bacterial Consortium

Microscopic examination of monoculture isolates revealed that isolate DB1 was a Gram-positive rod, and colonies were yellow on tryptic soy agar. Isolate DB2 was a Gram-positive rod and produced light yellow colonies on tryptic soy agar. Isolate DB7 was a Gram-positive rod, and colonies did not form pigments on tryptic soy agar. Isolate DB8 was a Gram-negative rod, and showed no pigmentation on tryptic soy agar. Isolate DB13 was a Gram-negative rod, and colonies displayed no pigmentation on tryptic soy agar.

Ribosomal RNA Gene Sequence Similarity and Phylogenetic Analysis

Isolates DB1, DB2, DB7, DB8, and DB13 were respectively identified as *Pseudoxanthomonas* sp. DB1, *Microbacterium* sp. DB2, *Bacillus* sp DB7, *Ochrabactrum* sp. DB8, and *Klebsiella* sp. DB13 by Genbank blast analysis of 16S rRNA gene sequence (Table 2). Blast results revealed that isolate DB1 was 99% identical to *Pseudoxanthomonas byssovorax* and 96% identical to *Pseudoxanthomonas taiwanensis*. Isolate DB2 was 99% identical to *Microbacterium oxydans* and 96% identical to *Microbacterium maritypicum*. DB7 was 99% identical to *Bacillus cereus* and *Bacillus thurigiensis*. Isolate DB8 was 99% identical to *Ochrobactrum anthropi* and 98% identical to *Ochrobactrum intermedium*. DB13 was 99% identical to *Klebsiella trevisanii* and 98% identical to *Pantoea agglomerans*. Figure 2 presents a phylogenetic tree showing the evolutionary position of members of the defined

Table 2 DNA-based identification of monocultures of the defined bacterial consortium

Isolate	16S rDNA nucleotides[a]	GenBank submission	GenBank match	Most probable organism	Similarity (%)
DB1	487	HM197761	AY928806	*Pseudoxanthomonas byssvorax*	99
DB2	461	HM197762	EU373400	*Microbacterium oxydans*	99
DB7	477	HM197763	GQ360073	*Bacillus* sp.	99
DB8	430	HM197764	AB120120	*Ochrabactum anthropi*	99
DB13	485	HM197765	AF129444	*Klebsiella trevisanii*	99

[a] Includes hypervariable regions [46]

bacterial consortium among related organisms using *Deinococcus radiodurans* as the outgroup organism.

Time Course of Cellulolytic and Xylanolytic Enzymes Development in Anoxic Cultures of Defined Bacterial Consortium

Time course of filter paper cellulase development in anoxic cultures using switch grass, corn stover, and Bermuda grass as the carbon source is shown in Fig. 3. Filter paper cellulase activity was detectable in cultures after 2 days of incubation and was maximal after 3 days of incubation with 37.16, 10.80, and 30.48 Umg^{-1} protein recorded in switch grass, corn stover, and Bermuda grass cultures, respectively. Thereafter, filter paper cellulase activity levels declined significantly. The highest filter paper cellulase activity was observed in cultures with switch grass and Bermuda grass as substrates. Figure 4 depicts xylanase development in switch grass, corn stover, and Bermuda grass cultures. After 1 day of incubation, xylanase activity was not detected in cultures containing switch grass and corn stover. Contrarily, xylanase activity was detected in cultures containing Bermuda grass as the substrate within 1 day of incubation and reached maximum (54.18 Umg^{-1} protein) in 3 days. Thereafter, xylanase activity levels in culture decreased. Xylanase activity was maximal on day 5 and day 4 in corn stover and switch grass cultures, respectively. Figure 5 depicts visible decomposition of Bermuda grass by the FMBC. Microbial consumption of sugars upon saccharification of biomass, nonetheless, resulted to low remaining sugar in

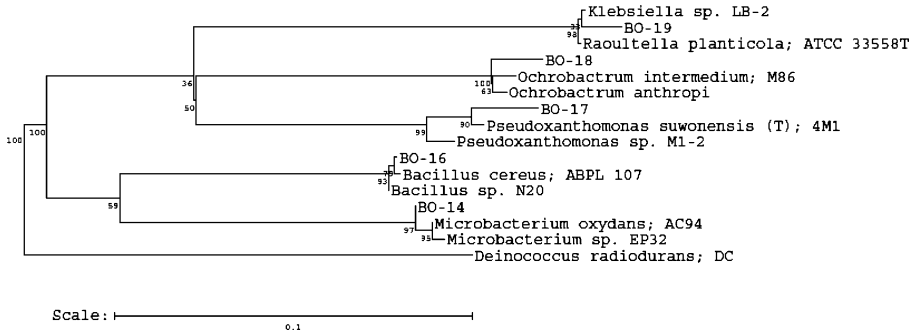

Fig. 2 Phylogenetic tree showing genetic distance between mococultures of the bacterial consortium and related organisms. *Deinococcus radiodurans* was the outgroup organism. The scale is the evolutionary distance value. The number at each node is the bootstrap from 100 analyses [47]

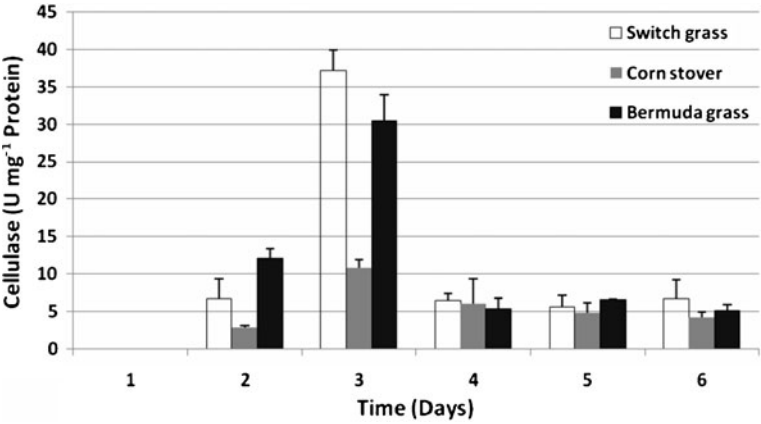

Fig. 3 Time course of cellulase production by bacterial consortium in static anoxic culture

cultures. In Bermuda grass, corn stover, and switch grass cultures, approximately 203.64, 239.94, and 263.16 mgl^{-1}, were respectively observed. No significant changes in culture pH were observed. In switch grass cultures, pH of 5.57±0.04 and 5.71±0.21 were recorded on day 1 and day 6, respectively. In corn stover cultures, pH of 5.83±0.04 and 6.16±0.17 were respectively recorded on the first and sixth day of incubation. Analysis of predominance of members of the defined bacterial consortium after 6 days of incubation revealed that isolate DB7 was the most predominant organism in all cultures followed by DB2 and DB1. These three isolates were therefore used to compose a defined three-member bacterial consortium for further studies.

Discussion

Worldwide concern on cost and depletion of fossil oil reserves has spurred research into development of processes for bioenergy [7, 16]. Lignocellulosic materials are cost-efficient

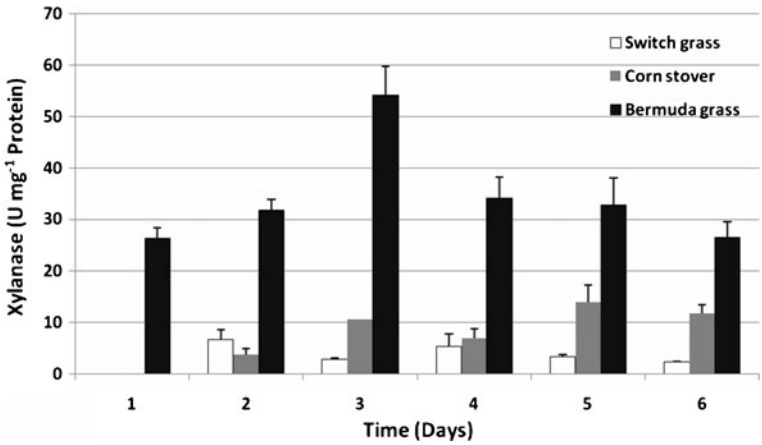

Fig. 4 Time course of xylanase production by bacterial consortium in static anoxic culture

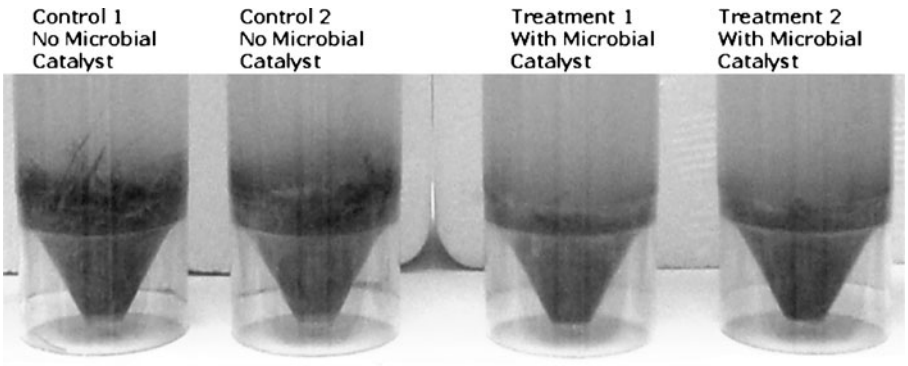

Fig. 5 Disappearance of Bermuda grass in culture of the bacterial consortium

sources of biomass fuel. Biodegradation of lignocelluloses under natural conditions involve a concerted action of a mixture of microorganisms. This study presents the development and characterization of a defined microbial consortium for bioprocessing of cellulose and hemicelluloses to mimic the process in natural environments. Nineteen cellulolytic and xylanolytic bacteria purified from cultures inoculated with paper mill waste water, wood processing waste, and soil produced an array of cellulolytic and xylanolytic enzymes.

Consolidated bioprocessing (CBP), the utilization of microorganisms that can simultaneously convert biomass to sugars and to ethanol is an alternative route to production of bioethanol [36]. This strategy would not be efficient without the use of a combination of microorganisms because mixed cultures have several advantages over monocultures (pure cultures) [48]. Such advantages include increased resistance to contamination by unwanted microorganisms, better adaptation to changing conditions, enhanced substrate utilization, and improved productivity [49]. Mixed culture system will also reduce the cost of commercial enzymes. A defined microbial consortium can be efficiently employed in CBP without need for gene manipulation. Moreover microbial synergism may help overcome nutritional limitations through synergetic interactions between microbes [50, 51] that may negatively impact the efficiency and productivity of a monoculture system. In a study [48], a mixed culture of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* LMA displayed a highly significant increase in the production of volumetric enzyme activity (98.4 $Ul^{-1}h^{-1}$), filter paper activity (7.1 Uml^{-1}), carboxymethyl cellulase activity (4.7 Uml^{-1}), soluble proteins (2.1 $mgml^{-1}$), dry biomass (21.4 $g^{-1}l^{-1}$), and percentage of utilized cellulose (89.4%) compared with monoculture of *A. niger*. Degradation of cellulose by pure cultures of *Cellulomonas flavigena* was less efficient than in mixed cultures [52].

Low lignocelluloses bioconversion rates are a well-known phenomenon and a major hurdle in biological production of sugars from plant biomass [53]. Although members of the FMBC produced an array of cellulolytic and xylanolytic enzymes, remaining sugar levels in cultures were low. This was, however, not surprising because microorganisms degrade lignocellulosic substrates to monomers and oligomers for uptake for carbon and energy. A possible solution will be continuous removal of sugars from the cultures and fermentation to ethanol. Besides microbial consumption of the sugars upon hydrolysis of lignocelluloses, inefficient production of fermentable sugars by microbial catalysis of plant fiber is largely due to incomplete synergistic enzymes, end-product inhibition [54], enzyme inactivation [30], requirement for high amounts of enzymes, recalcitrance due to highly ordered structure and crystallinity of cellulose [55], and the heterogeneous nature of a

variety of plant fiber [18, 31]. These factors necessitate use of enzyme mixtures or microbial consortium producing a variety of cellulolytic and xylanolytic enzymes as well as ligninases [34, 35] for complete lignocellulose saccharification.

β-Glucosidases are extremely important enzymes in enzymatic hydrolysis of lignocelluloses to sugars in that cellobiose, the principal product in most microbial hydrolysis of cellulose inhibits endo- and exoglucanases which significantly slows down the process [56]. Thus, a high level of β-glucosidase is necessary to avoid the accumulation of cellobiose [57]. Interestingly, β-glucosidase was produced by the microbial isolates. In a microbial coculture, the inhibitory effect of cellobiose was significantly diminished by better conversion into glucose by β-glucosidase produced by the culture [58]. β-Xylosidases are similarly important in that they convert xylobiose, a nonfermentable intermediate product, to xylose which can be fermented to ethanol.

Database comparison of nucleotide sequence of monocultures used to compose FMBC revealed the genera of the isolates to be *Pseudoxanthomonas* sp. DB1, *Microbacterium* sp. DB2, *Bacillus* sp DB7, *Ochrabactrum* sp. DB8, and *Klebsiella* sp. DB13. A cellulose-decomposing bacterial association included *Alcaligenes* sp., *Ochrobactrum* sp., *Sphingomonas* sp., *Achromobacter* sp., *Pseudomonas* sp., and *Sporocytophaga* [59]. A study [60] reported xylanase production from *Bacillus thermoleovorans* strain K-3D and *Bacillus flavothermus* strain LB3A. Xylanase production capability was also reported in *Bacillus*, *Cupriavidus*, *Microbacterium*, *Pseudoxanthomonas*, and *Rhodococcus* [61]. *Microbacterium* species produced enzymes involved in cellulose and xylan degradation [62–65]. Recombinant strains of *Klebsiella oxytoca* have been employed in cellulose degradation and fermentation [66, 67]. However, there is paucity of information on lignocellulose biodegradation by natural isolates of *Klebsiella*. The capacity of the *Klebsiella* isolate to degrade or produce cellulolytic and xylanolytic enzymes can be attributed to the possibility of acquisition of plasmids encoding cellulolytic and xylanolytic enzymes from the environment. Nevertheless, noncellulolytic microorganisms exist in association with cellulolytic microorganisms by using their breakdown products. Exchange of growth factors between a community of cellulose-degrading bacteria and other microorganisms supported growth and higher rates of cellulose degradation [68]. In a stable consortium of aerobic and anaerobic cellulolytic bacteria it was estimated that two strains of aerobic bacteria introduced anaerobic conditions and an anaerobic bacterium supplied metabolites, acetate, and glucose [69].

Bermuda grass (*Cynodon* species) is an enduring grass, widely used as lawn grass and as forage for livestock, has potential as an energy crop for production of fuel [70, 71]. Large quantities are generated annually from lawn mowing. Interestingly, extensive degradation of Bermuda grass was observed with the mixed microbial catalysts. Efficient recovery of sugar, nonetheless, is a major hurdle to scale in whole cell saccharification of Bermuda grass and other plant biomass.

Conclusions

In an effort to develop a defined microbial consortium to mimic natural biomass fiber degradation processes, this study enriched, purified, and characterized monocultures of cellulolytic and xylanolytic bacteria. A defined bacterial consortium was then developed using selected isolates. The study highlighted an important process for composition of a defined microbial consortium to supply the necessary array of enzymes for saccharification of plant fiber. Results suggest potential application of this process in bioconversion of lignocelluloses. On the other hand, it requires further studies for efficient recovery of

sugars. Efficient utilization of plant biomass will not only diminish overdependence on fossil fuels but could solve modern biomass waste disposal problems [60].

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