



Research article

Characterization of a microbial consortium with potential for biological degradation of cactus pear biomass for biofuel production



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HIGHLIGHTS

- Ethanol yields from cactus pear are not commercially viable due to poor release of fermentable carbohydrates.
- A soil consortium consisting of 14 genera of eubacteria and four genera of fungi was characterized.
- *Pectobacterium cacticida* degraded cladodes most effectively among all axenic isolates evaluated.
- *P. cacticida* holds potential promise to promote fermentable sugar release and ethanol yields.

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ABSTRACT

Cactus pear (*Opuntia ficus-indica*) is a crassulacean acid metabolism (CAM) species that serves as a food, feed, and bioenergy crop. *O. ficus-indica* is an attractive alternative biofuel feedstock due to its low water demand and high biomass productivity. Current ethanol yields from *O. ficus-indica* are not commercially viable due to low concentrations of released fermentable carbohydrates. Axenic strains of bacteria and fungi were isolated and characterized from a soil microbial community consortium that effectively degrades cladodes into soluble components. The consortium consisted of species representing 14 genera of eubacteria and four genera of fungi. The digestion efficiency of each axenic isolate was evaluated by measuring the release of soluble material after aerobic digestion of cladodes and direct measurement of cellulase and pectinase activities in the culture supernatants. *Pectobacterium cacticida* was the most effective eubacterial species identified for degrading cladodes among all isolates evaluated. Thus, *P. cacticida* holds great promise for increasing the release of fermentable sugars and improving overall ethanol yields.

1. Introduction

Cactus pear (*Opuntia ficus-indica*) and related *Opuntia* spp. have the potential to become important bioenergy feedstocks for ethanol or biogas production on marginal, abandoned, semi-arid, and arid lands around the world (Cushman et al., 2015; Davis et al., 2019; do Nascimento Santos et al., 2016; Krümpel et al., 2020; Lueangwattanapong et al., 2020; Mason et al., 2015). Cactus pear species exhibit water-use efficiencies that are three-to six-fold greater than C₄ or C₃ photosynthesis crops, respectively, due to their ability to perform crassulacean acid metabolism (CAM) (Borland et al., 2009; Cushman et al., 2015). *Opuntia* spp. also possess associated co-adaptive traits, such as tissue succulence to attenuate drought, thick cuticles and epicuticular wax layers to limit water loss, and roots that can maximize uptake under wet conditions and

minimize water losses under dry conditions (Dubrovsky et al., 1998; Niechayev et al., 2019; North and Nobel, 1997). In addition to drought tolerance, *Opuntia* spp. can tolerate high temperatures, a critically important trait for climate resilience (Chetti and Nobel, 1988; Nobel and de la Barrera, 2003).

The annual biomass productivity potential of *Opuntia* spp. meets or exceeds that of C₃ and C₄ photosynthesis crops, but with dramatically lower crop water demands (Borland et al., 2009; Cushman et al., 2015; Neupane et al., 2021; Nobel, 1991, 1996). Without supplemental irrigation, average annual biomass production typically ranges from 3–15 Mg DM ha⁻¹ yr⁻¹ depending upon the ambient conditions and location (Consoli et al., 2013; de Queiroz et al., 2015; do Nascimento Santos et al., 2016; Grünwaldt et al., 2015; Krümpel et al., 2020; Mason et al., 2015; Reis et al., 2016; Sánchez et al., 2012). However, with supplemental

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Table 1. Primer sets used for the amplification of 16S rDNA target genes for eubacterial isolates. A) Forward and reverse primers for the BacUni1055 16S rRNA consensus sequence spanning from 1055 bp to 1406 bp obtained from Ferris et al. (1996). B) Forward and reverse primers for the BacUni335 16S rRNA consensus sequence spanning from 335 bp to 788 bp obtained from Dorn-In et al. (2015).

Consensus Sequence	Forward Primer	Reverse Primer
A) 16S rRNA	ATGGCTGTCGTACAGCT	ACGGGCGGTGTGTAC
B) 16S rRNA	CADACTCTACGGGAGGC	ATCTGTTTGMTCCCVCRG

Table 2. Primer sets used for the amplification of EF1- α and 28S rDNA target genes for fungal isolates. A) Forward and reverse primers for the EF1- α consensus sequence spanning from 1018 bp to 1620 bp obtained from Stielow et al. (2015). B) Forward and Reverse Primers for the nuclear large subunit (LSU) 28S rRNA consensus sequence spanning from 200 bp to 476 bp obtained from Asemaninejad et al. (2016).

Consensus Sequence	Forward Primer	Reverse Primer
A) Elongation Factor 1-alpha (EF1- α)	GAYTTCATCAAGAATGAT	GACGTTGAADCCRACRTTGTGTC
B) Nuclear Large Subunit- 28S rRNA	AACKGCGAGTGAAGCRGYA	CSATCACTSTACTTGTGKCGC

irrigation and high-density cultivation with fertilizer application, annual productivities of 30–50 Mg DM ha⁻¹ yr⁻¹ are possible (Dubeux et al., 2006; Flores-Hernández et al., 2004; Garcia de Cortázar & Nobel, 1991, 1992; Lima et al., 2016; Neupane et al., 2021; Nobel et al., 1992). In addition to their high productivity, *Opuntia* spp. can be clonally propagated easily and are suitable for coppicing (Cushman et al., 2015; Mason et al., 2015).

As an herbaceous feedstock *O. ficus-indica* has a relatively low lignin content compared with more woody feedstocks, thereby reducing its recalcitrance to hydrolysis and saccharification, which might also reduce processing costs (Yang et al., 2015). Pretreatment steps such as physical chopping, thermal degradation, enzymatic, and acid pretreatment have been used to process *O. ficus-indica* biomass for bioethanol (Kuloyo et al., 2014; Pérez-Cadena et al., 2018; Retamal et al., 1987) or biogas production (Calabrò et al., 2018; Krümpel et al., 2020; Lueangwattanapong et al., 2020; Myovela et al., 2019; Ramírez-Arpide et al., 2018). Chemical pretreatment, such as alkaline hydrolysis of *O. ficus-indica* and *O. (Nopalea) cochenillifera* fruit combined with enzymatic saccharification can improve bioethanol production using a simultaneous saccharification and fermentation process (de Souza Filho et al., 2016). Various pretreatments have been attempted with acidic pretreatment of the biomass improving methane yields (Calabrò et al., 2018).

Microbial digestion can potentially improve ethanol or biogas production from *Opuntia* spp. by hydrolyzing pectin and other carbohydrates not easily degraded by chemical acidification (Myovela et al., 2019; Pérez-Cadena et al., 2018). Necrotrophic species, such as *Pectobacterium* and *Dickeya*, cause soft rot disease in many plant species including cacti by secreting a broad set of cell wall degrading enzymes via the type II secretion pathway (Hugouvieux-Cotte-Pattat, 2016; Motyka et al., 2017; Valenzuela-Soto et al., 2015; Varvaro et al., 1993). Ethanol production from *O. ficus-indica* cladodes or fruits of 2.5–34.9 g L⁻¹ have been reported (Alencar et al., 2018; de Souza Filho et al., 2016; do Nascimento Santos et al., 2016; Kuloyo et al., 2014; Pérez-Cadena et al., 2018; Retamal et al., 1987). Biogas (methane) yields of 233–325 L kg⁻¹ have been reported from the anaerobic digestion of *O. ficus-indica* cladodes (Calabrò et al., 2018; Lueangwattanapong et al., 2020; Mason et al., 2015; Ramírez-Arpide et al., 2018; Ramos-Suárez et al., 2014; Valenti et al., 2018).

One of the major approaches to improve the utility of *Opuntia* spp. as a biofuel feedstock is to increase the release of fermentable sugars and subsequent conversion of these sugars into ethanol (Kuloyo et al., 2014) or biogas (Myovela et al., 2019). *O. ficus-indica* cladodes consist of about 25% soluble extractives (Yang et al., 2015). Pectins account for 6–14% of the dry-weight mass of *O. ficus-indica* cladodes and consist of galactose, arabinose, glucuronic and galacturonic acids, xylose, rhamnose, and

glucose (Ginestra et al., 2009; Goycoolea and Cárdenas, 2003; Peña-Valdivia et al., 2012). *O. ficus-indica* cladodes consist of about 13% cellulose and about 18% hemicellulose (Yang et al., 2015). Degradation of both cell-wall pectins and mucilage pectins from *O. ficus-indica* cladodes yields arabinose, galactose, glucose, rhamnose, and xylose (Ginestra et al., 2009; Goycoolea and Cárdenas, 2003; Peña-Valdivia et al., 2012). Arabinose and xylose can be utilized by genetically modified fermentation microbes like *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* to produce ethanol (Becker and Boles, 2003; Kuloyo et al., 2014; Matsushika et al., 2009). However, there is a clear need to improve the release of fermentable sugars of *O. ficus-indica* cladodes to improve overall ethanol or methane yields. Here, we have isolated and characterized by molecular barcoding a microbial soil consortium from a commercial *O. ficus-indica* orchard and provide evidence for the ability of the consortium and individual axenic isolates to degrade *O. ficus-indica* cladodes and to excrete detectable pectinase activity.

2. Materials and methods

2.1. Materials

A microbial soil sample was obtained from a mixture of cladodes and soil from a commercial *O. ficus-indica* production field in Salinas, California (approximately 36° 32' 47" N, 121° 24' 69" W, and altitude of ~118 m). This sampling site was selected because cladode trimmings were routinely tilled into the soil to recycle crop residues and was likely to contain soil microbes with the ability to degrade cactus pear cladodes. The microbial soil sample was incubated for 22 days at room temperature in a sealed one-gallon Ziploc bag. The resulting slurry of microbes and degraded plant material was mixed well and divided into 30 ml aliquots and transferred to 50 ml conical tubes (Argos Technologies) and 40% (v/v) sterile glycerol was added to a final concentration of 15% (v/v). Soil samples were then flash frozen in liquid nitrogen and stored at -80 °C until use. To increase the sample volume for biomass degradation assays, 250 ml of the sample + glycerol mix were grown in 750 ml of M9 minimal media +100 mM MES buffer (pH = 6.0) to promote bacterial growth or grown in 750 ml of Vogel's minimal media +100 mM MES buffer (pH = 6.0) to promote fungal growth at 27 °C for 48 h with shaking at 90 RPM on an incubator shaker (New Brunswick Scientific). M9 minimal media consisted of 0.1 μ M CaCl₂, 2 μ M MgSO₄•7 H₂O, and 11.28 g L⁻¹ of 5x M9 salts, containing 33.9 g L⁻¹ Na₂HPO₄, 15 g L⁻¹ KH₂PO₄, 5 g L⁻¹ NH₄Cl, and 2.5 g L⁻¹ NaCl. Vogel's minimal media consisted of 8.8 mM NaCitrate•2 H₂O, 25 mM KNO₃, 19 mM (NH₄)₂PO₄, 12 mM KH₂PO₄, 0.81 mM MgSO₄•7 H₂O, 0.9 mM CaCl₂, 17 μ M citric acid, 17 μ M ZnSO₄•7 H₂O μ M, 3.3 μ M FeSO₄, 3.8 μ M (NH₄)₂SO₄, 1 μ M CuSO₄ •5

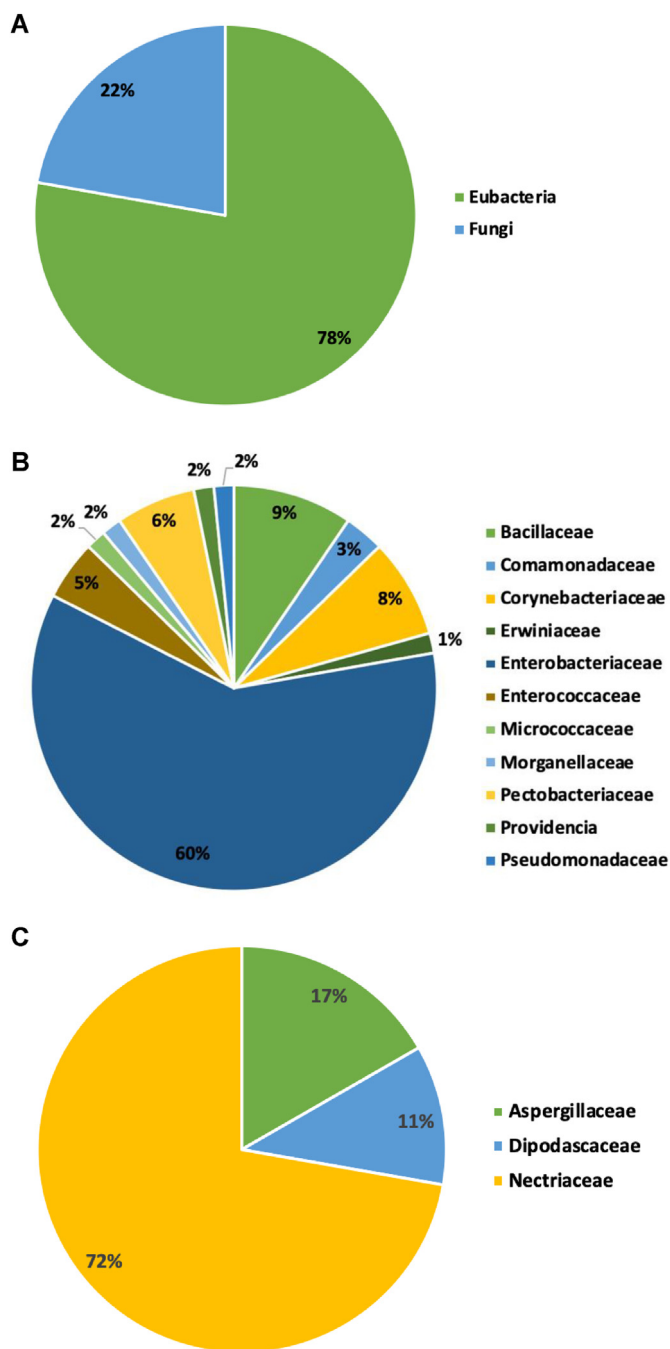


Figure 1. Distribution of species occurrence with cactus pear (*Opuntia ficus-indica*) soil microbial consortium. A) Major groups of microbial species identified within the soil consortium based on their distribution into eubacterial or fungal clades and their relative discovery frequency. B) Relative distribution of eubacterial genera based upon their relative discovery frequency. C) Relative distribution of fungal genera based upon their relative discovery frequency.

H₂O, 3 μM MnSO₄•H₂O, 0.8 μM H₃BO₃, and 0.2 μM Na₂MoO₄•2 H₂O. The resulting liter of each type of mixed sample was adjusted to 15% v/v glycerol, flash frozen in liquid nitrogen and stored at -80 °C until use.

2.2. Microbial isolation

Thawed sample was diluted using sterile, deionized water in a ten-fold dilution process where 100 μl of the 10⁻⁷ to 10⁻⁹ dilutions were pipetted onto one of four growth plates containing 1.5% (w/v) biological

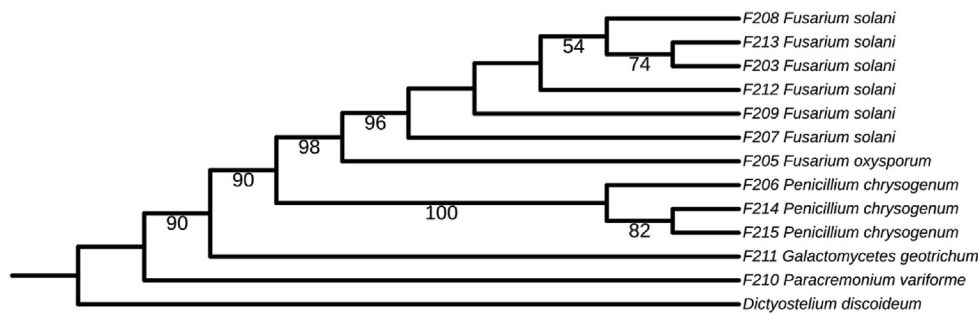


Figure 2. Maximum likelihood phylogenetic tree based upon sequences of the eubacterial 16S rRNA gene sequences representing the diversity of isolates obtained from the cactus pear (*Opuntia ficus-indica*) soil microbial consortium. The bootstrap support values indicated on individual nodes are based on maximum likelihood estimates. Only bootstrap values higher than 45 are indicated. The outgroup used was *Escherichia coli*.

grade agar and (1) M9 minimal media with 1% (w/v) dried *O. ficus-indica* cladode powder, (2) M9 minimal media with 1% (w/v) pectin from orange peel (Sigma-Aldrich), (3) Vogel's minimal media with 1% (w/v) *O. ficus-indica* dried cladode powder, or (4) Vogel's minimal media with 1% (w/v) pectin from orange peel. *O. ficus-indica* dried cladode powder was prepared by lyophilizing sliced, fresh *O. ficus-indica* cladodes, grinding the dried *O. ficus-indica* biomass into a fine powder using a mortar and pestle or a blender (NutriBullet mixer/blender system), and sieving the powder through two layers of cheese cloth to remove large particles.

Eubacterial plates were grown at 27 °C for 24–48 h, whereas the fungal plates were grown at 27 °C for 48–96 h. Individual isolates were sampled from initial sample plates and restructured two or more times to the corresponding media types described above until axenic colonies were obtained. Single, axenic isolates were then regrown in the corresponding liquid media types and replated using 10⁻³ through 10⁻⁵ serial dilutions to confirm that axenic cultures had been obtained. Liquid culture stocks were prepared for each axenic isolate, with sterile glycerol added to a final concentration of 15% (v/v) and stored at -80 °C until use. Each axenic strain was then genotyped and used for *O. ficus-indica* cladode digestion trials.

A



B

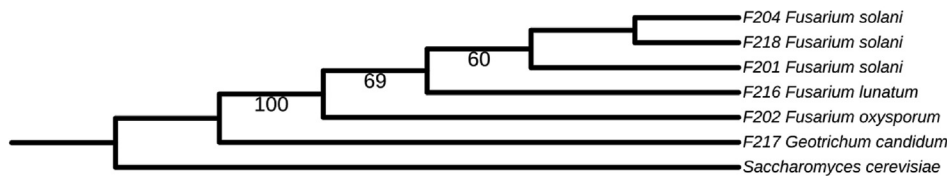


Figure 3. Maximum likelihood phylogenetic tree based upon sequences of the eukaryotic (fungal) A) EF-1 α genes or B) nuclear large subunit (LSU) 28S rRNA sequences representing the diversity of isolates obtained from the cactus pear (*Opuntia ficus-indica*) soil microbial consortium. The bootstrap support values indicated on individual nodes are based upon maximum likelihood estimates. The outgroups used were A) *Dictyostelium discoideum* for the fungal EF-1 α sequences and B) *Saccharomyces cerevisiae* for nuclear large subunit 28S rRNA sequences, respectively.

2.3. Genotyping of axenic strains

Single colonies of the axenic strains were cultured for 24 h in liquid M9 or Vogel's minimal media with 0.5% (w/v) ground, dry *Opuntia* cladode powder or 0.5% (w/v) pectin. The DNA was then extracted using a MO BIO Microbial DNA Isolation Kit (Qiagen). PCR amplification was performed using the extracted DNA and primers targeting the highly conserved 16S rRNA region in eubacteria (Dorn-In et al., 2015; Ferris et al., 1996) and the highly conserved elongation factor 1 alpha (EF1 α) (Stielow et al., 2015) and large subunit (LSU) 28S rRNA in fungi (Asemaninejad et al., 2016) (Tables 1 and 2). Empirical temperature gradient trials indicated that the optimum annealing temperature for the primers was 47 °C and 53 °C for the eubacterial and fungal primers, respectively. The amplified DNA products were extracted using a QIAquick Gel Extraction Kit (Qiagen) and quantified with a Qubit 4 fluorometer (ThermoFisher Scientific). The amplification products were then sequenced using Sanger sequencing on an Applied Biosystems 3730 automated DNA Analyzer at the Nevada Genomics Center using the same forward bacterial or fungal primers used for amplification. Sequences were compared to known sequences using the National Center for Biotechnology Information's (NCBI) Basic Alignment Search Tool (BLAST) using default settings to identify the genus (and species) designations for each axenic strain from the top hit of each search.

2.4. Phylogenetic analysis

The 16S rRNA, nuclear large subunit (LSU) 28S rRNA, and EF-1 α sequences were trimmed manually to remove low-quality end sequences and aligned initially using the MUSCLE multiple sequence aligner (Edgar, 2004). Alignment iterations were run until convergence was achieved or up to 20 iterations. The alignments results were polished manually to remove any gaps present in more than 50% of the sequences using Gblocks (Talavera and Castresana, 2007). Best-fit evolutionary models for the alignments were selected using IQ-Tree based upon testing Akaike's Information Criterion (AIC), corrected AIC (AICc) and Bayesian Information Criterion (BIC) criterion (Nguyen et al., 2015). Maximum likelihood (ML) gene trees were estimated using RAxML v7.3.0 (Stamatakis, 2015) with the best-fit models K2P + G4, K2P and TNe + G4 for the 16S rRNA, LSU 28S rRNA, and EF-1 α sequence alignments, respectively.

SH-aLRT branch support calculations were performed at 1000 iterations with IQ-TREE (Nguyen et al., 2015). Trees were drawn using Interactive Tree of Life (iTOL) ver. 4.0 (Letunic and Bork, 2019). The phylogenetic trees were rooted with an outgroup selected from taxa outside the relationship of interest. For eubacterial 16S rRNA sequences, the outgroup was *Escherichia coli*. For the fungal 28S rRNA sequences, the outgroup was *Saccharomyces cerevisiae*. For the fungal EF-1 α sequences, the outgroup was *Dictyostelium discoideum*.

2.5. Cactus pear growth

O. ficus-indica plants were grown in 7.6-liter pots containing a 3:1 ratio of Sunshine MVP soil mix (Sun Gro Horticulture) and decomposed granite. Plants were grown at the Nevada Agricultural Experiment Station (Reno, NV) greenhouse complex under natural light (1,100–1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and temperature (28–32 °C day/17–18 °C night) conditions. From October through March, plants were watered once per week, whereas from April–September, plants were watered twice per week. All plants were fertilized monthly with Miracle Gro® fertilizer (Scott's MiracleGro) according to manufacturer's instructions. Systemic insecticide treatment (Marathon® 1% Granular, OHP) was applied every three months according to manufacturer's instructions.

2.6. Digestion assays

The uppermost, mature, >1-year old *O. ficus-indica* cladodes were harvested from plants containing 2–4 cladodes. Cladodes were cut into 11.5 cm by 1.5 cm transverse sections weighing approximately 7.5 g each and autoclaved at 121 °C for 45 min in glass Pyrex 9820 culture tubes. For eubacterial and fungal digestion trials, 15 ml of M9 or Vogel's minimal media, respectively, was added to each autoclaved section so that each was submerged completely. Autoclaving was not only essential for obtaining sterile cladode section, but also likely aided in the loosening of cell wall components and might have helped promote digestion by the microbial consortium or isolates. For the eubacterial digestion trials, the M9 minimal media +100 mM MES (pH = 6.0) + mixed sample was used as a positive control, M9 minimal media +100 mM MES (pH = 6.0) was used as a negative control, and 100 μl of isolated, axenic eubacterial strain +14.9 ml of M9 minimal media +100 mM MES (pH = 6.0) was

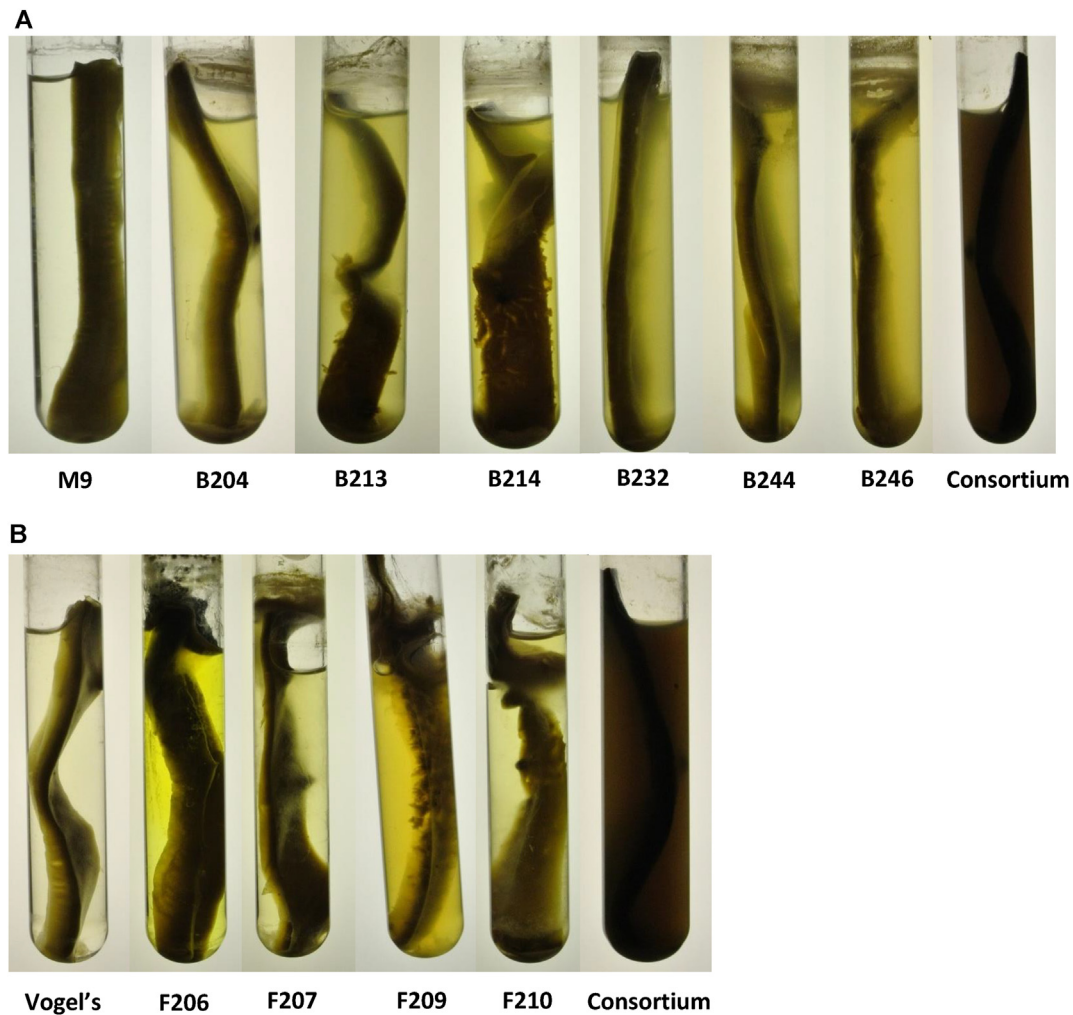


Figure 4. Representative images of cactus pear cladode transverse sections undergoing digestion. Representative images of cladode transverse sections from *Opuntia ficus-indica* incubated with A) Eubacterial isolates: M9 minimal media (negative control), and various eubacterial isolates (B204, B213, B214, B232, B244, and B246, and soil microbial consortium (positive control) after 30 days or B) Fungal isolates: Vogel's minimal media (negative control), F206, F207, F209, and F210, and soil microbial consortium (positive control) after 30 days.

used as experimental treatments. For the fungal trials, the Vogel's minimal media +100 mM MES (pH = 6.0) + mixed sample was used as a positive control, Vogel's minimal media +100 mM MES (pH = 6.0) was used as a negative control, and 100 μ l of isolated, axenic fungal strain +14.9 ml of Vogel's minimal media +100 mM MES (pH = 6.0) was used as experimental treatments. Cultures used for inoculation were grown for five days on respective minimal media containing 0.5% (w/v) *Opuntia* cladode powder. The optimal growth pH for the microbial consortium sample was determined to be pH = 6, maintained by the use of MES buffer in the media. All digestion assays were conducted in triplicate using 64 axenic eubacterial and 18 axenic fungal isolates. Transverse cladode sections were digested for 5 or 30 days at 28 °C, with shaking at 90 RPM on an I2400 incubator shaker (New Brunswick Scientific). Five days was selected because visible changes in the integrity of cladode segments were evident at this time. Thirty days was selected to provide clear visible contrasts in microbial growth among the different isolates. After the digestion period, the digested cladodes were frozen at -80 °C and stored until use.

To analyze the ability of each microbial strain to convert insoluble cladodes into soluble material, the percent mass of the soluble fraction was measured. The frozen digested samples were thawed at room temperature and then separated by vacuum filtration into soluble and insoluble fractions. Individual samples were filtered through a 47 mm

nitrocellulose membrane filters (0.45 μ m) (MF-Millipore, HAWP04700) using a 150 ml Magnetic Filter Funnel (Pall). Vacuum was applied using a GAST air vacuum pump (DOA-P704) or an Oakton water vacuum pump (WP-25). The insoluble fraction retained by the filter was washed three times with 10 ml deionized water. The soluble fraction that flowed through the filter was collected in TC5000 50 ml conical centrifuge tubes (Argos Technologies). Both the soluble and insoluble fractions were lyophilized using a LabConCo Freeze Dry System (Freezone 18). After five days, only the digested microbial consortium sample remained viscous and were dried further by heating at 200 °C for 1 week using an Isotemp Oven (ThermoFisher Scientific) to drive off residual water. This step was necessary because the soluble fraction of the soil consortium digestate was extremely hydrophilic. The dried soluble and insoluble fractions were weighed using a 2524T (AE Adam) electronic scale. Percent mass in the soluble fractions was obtained using the sum of the insoluble and soluble fractions as total weight. All digestion assays were performed in triplicate and expressed as the ratio of mass in the supernatant (soluble fraction) compared with the total biomass of the digested cladode. Data visualization and statistical analysis was performed using Prism 9 software (GraphPad Software). Statistical significance was determined using one-way Analysis of Variance (ANOVA) with Dunnett's multiple comparison test at the 95% confidence interval with alpha = 0.5.

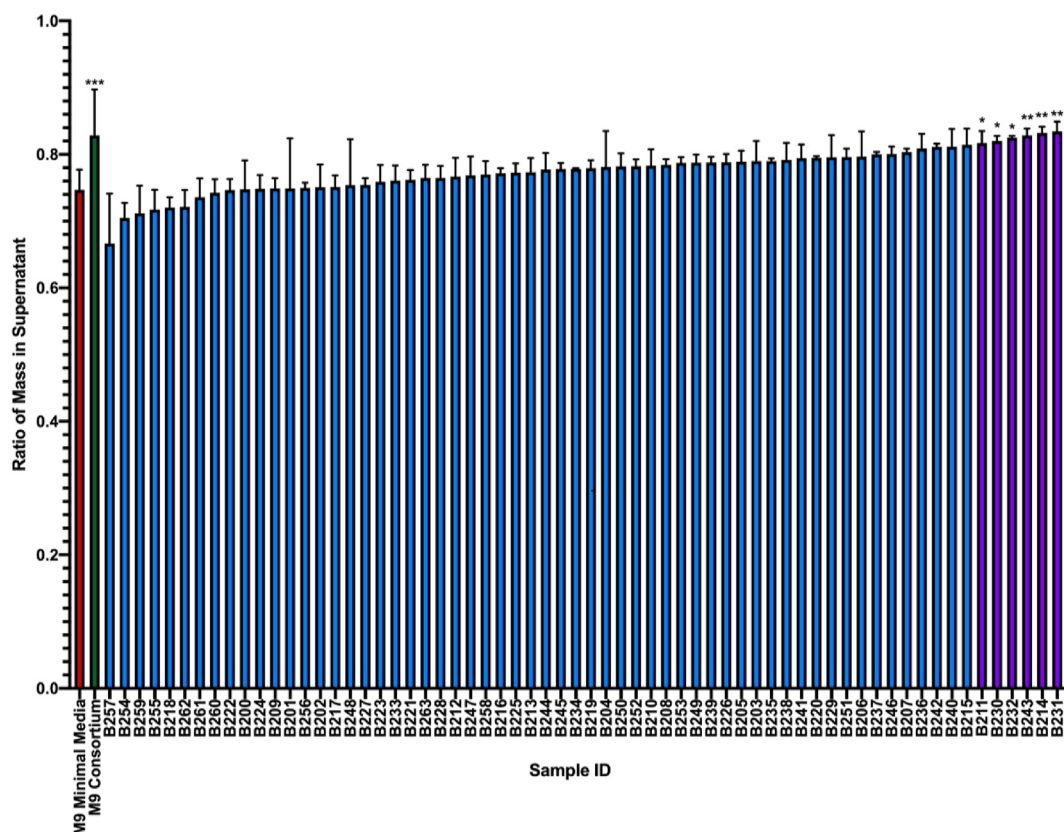


Figure 5. Quantitative eubacterial degradation of cactus pear cladode transverse sections. The ratio of mass in the supernatant (soluble section) is shown for each eubacterial isolate after five days of digestion of *O. ficus-indica* transverse sections. The red bar represents the M9 minimal media (negative control), the green bar represents the soil microbial consortium in M9 minimal media (positive control), the blue bars represent eubacterial species exhibiting weak digestion activity, and the purple bars represent the eubacterial species with strong digestion activity. Values represent means \pm standard error of the mean (SEM) ($n = 3$). * $p < 0.05$, ** $p < 0.001$, and *** $p < 0.0001$, one-way ANOVA with Dunnett's multiple comparison test ($\alpha = 0.05$).

2.7. Enzyme assays

To provide direct evidence that the microbial consortium or individual isolates contain enzymatic activities for cladode digestion, the cellulase and pectinase activities of the culture supernatants were investigated. Three eubacterial (i.e., *Pectobacterium cacticida*, *Enterobacter* sp.) and fungal (i.e., *Fusarium solani*, *Penicillium chrysogenum*) isolates with the highest biomass degrading activities were selected for enzyme activity testing. Cladode transverse segments were prepared as described above and inoculated with 200 μ l of a five-day-old culture of the top three eubacterial and fungal isolates determined by the digestion assays. Controls samples consisted of cladode transfer segments incubated in their respective media to which neither microbial consortium nor axenic strains were added. After five days, the liquid fraction of the culture was recovered and centrifuged for 5,000 \times g for 15 min to remove cells. The supernatant was then concentrated and desalted to remove low molecular weight salts and sugars using a centrifugal filtration device (Amicon® ultra-15 centrifugal filter unit – 10 kDa MWCO) at 4,000 \times g for 30 min using an equal volume of 20 mM citrate buffer (pH = 6.5) until a final volume of ~1 ml was obtained.

A modified, phenol-free, colorimetric assay was used for the detection of cellulase and pectinase activities using 3, 5-dinitrosalicylic acid (DNS) as the reagent (Miller, 1959) with 0.5% carboxymethyl cellulose (CMS, Sigma-Aldrich) and 0.5% soluble citrus pectin (Sigma-Aldrich) as substrates (Deshavath et al., 2020; Vatanparast et al., 2012). DNS reagent was prepared by adding 1.6 g NaOH to 75 ml of deionized water until dissolved with stirring, followed by 1 g (1% w/v) DNS (Sigma-Aldrich) with continuous stirring. Lastly, 3 g (3% w/v) sodium potassium nitrate (Sigma-Aldrich) was added, and the volume was brought to 100 ml.

Reactions consisted of 1 ml of 0.5% w/v CMC or 0.5% w/v pectin prepared in 20 mM sodium citrate buffer (pH 6.5) and 20 μ l of concentrated culture supernatants or cellulase or pectinase standards incubated for 1 h at 37 °C. Then, 120 μ l of DNS reagent was added and the samples were heated at 100 °C for 10 min and the absorbance of each sample was read at 540 nm using a NanoDrop™ 2000 UV-Vis spectrophotometer (ThermoFisher Scientific) using a 1 mm pathlength disposable, polystyrene cuvette (ThermoFisher Scientific). Standard curves for cellulase (RPI Research Products) and pectinase (Sigma-Aldrich) were created and activities from the culture supernatants were determined by interpolation of values from the standards. One unit (U) of enzyme activity was defined as 1 μ mol of reducing sugar released per min. All enzymatic assays were performed in triplicate. Data visualization and statistical analysis was performed using Prism 9 software (GraphPad Software). Statistical significance was determined using one-way Analysis of Variance (ANOVA) with Dunnett's multiple comparison test at the 95% confidence interval with $\alpha = 0.5$.

3. Results and discussion

3.1. Isolation and genotyping of microbial strains

Using serial dilution and repeated restreaking and replating of well isolated colonies, axenic cultures of 64 bacterial isolates and 18 fungal isolates were obtained from the original microbial consortium sample isolated from field soil containing decomposing *O. ficus-indica* cladodes. Of the bacterial isolates, 63 of the 64 axenic strains were genotyped successfully using conserved primers targeting the Bacterial Universal (BacUni) 16S rRNA and amplicon sequencing. Of the fungal isolates, all

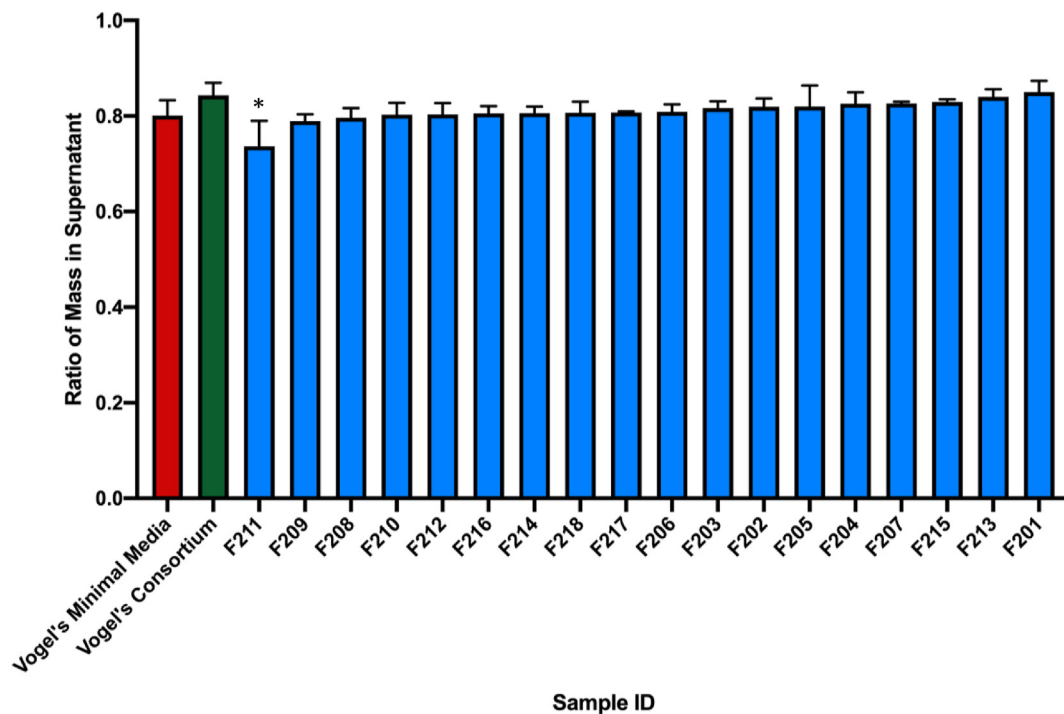


Figure 6. Quantitative fungal degradation of cactus pear cladode transverse sections. The ratio of mass in the supernatant (soluble fraction) is shown for each fungal isolate after five days of digestion of *O. ficus-indica* cladodes transverse sections. The red bar represents the Vogel's minimal media (negative control), the green bar represents the soil microbial consortium in Vogel's minimal media (positive control), and blue bars represent fungal species exhibiting weak digestion activity. Values represent means \pm standard error of the mean (SEM) ($n = 3$). * $p < 0.05$, one-way ANOVA with Dunnett's multiple comparison test ($\alpha = 0.05$).

18 axenic isolates were genotyped successfully using conserved primers targeting the 28S rRNA or the large subunit of elongation factor 1 alpha (LS-1 α) and amplicon sequencing. The primers used are shown in Tables 1 and 2.

The soil microbial community consortium was comprised of 81 isolates, of which 78% were eubacterial and 22% were fungal strains (Figure 1A). The 63 identified eubacterial isolates were identified to the genus or in some cases, the species level. A total of 11 different genera were identified including representatives from the Bacillaceae, Comamonadaceae, Corynebacteriaceae, Erwiniaceae, Enterobacteriaceae, Enterococcaceae, Micrococcaceae, Morganellaceae, Pectobacteriaceae, Providencia, and the Pseudomonadaceae (Figure 1B). The most common eubacterial family was the Enterobacteriaceae, which was represented by 38 isolates (60%) within the *Enterococcus*, *Enterobacter*, *Cronobacter*, *Citrobacter*, and *Klebsiella* genera. The next most common families included the Bacillaceae with six isolates (*Bacillus* sp., *Bacillus safanis*), the Pectobacteriaceae with six isolates of *Pectobacterium cacticida*, the Corynebacteriaceae with five isolates (*Corynebacterium* sp.), and the Enterococcaceae with three isolates each (*Enterococcus* sp., *E. saccharolyticus*). A complete listing of all eubacterial isolate descriptions and sequences is presented in Supplemental Table 1.

In contrast, the 18 identified fungal isolates were identified to the species level and included representatives of the Aspergillaceae, Dipodascaceae, and Nectriaceae (Figure 1C). The most common fungal family was the Nectriaceae, which was represented by 13 isolates (72%) within the *Fusarium* (*Fusarium solani*, *F. oxysporum*, *F. lunatum*) and *Paracremonium* (*P. variforme*) genera. The next most common families included the Aspergillaceae with three isolates (*Penicillium chrysogenum*) and the Dipodascaceae with two isolates (*Galactomyces geotrichum*, *Geotrichum candidum*). A complete listing of all fungal isolate descriptions and sequences is presented in Supplemental Table 2. The composition of the microbial sample defined here likely reflected the long-term cultivation of *O. ficus-indica* and the practice of tilling the cladode and fruit trimmings back into the soil in order to dispose of this material on site

and return nutrients to the soil. While the soil sample represents a rich source of cladode-degrading microbes, this practice might also represent a risk for the introduction and spread of bacterial and fungal diseases particularly in regions with ample rainfall. However, such spread was deemed a low risk due to the relatively dry conditions in Salina, CA, which received an average annual rainfall of only 326 mm per year (Arguez et al., 2010).

3.2. Phylogenetic analyses

Maximum likelihood (ML) phylogenetic relationship trees were generated using nucleotide sequences of the 16S rRNA sequences derived from each of the eubacterial isolates or the LSU 28S rRNA and EF-1 α sequences derived from each of the fungal isolates. The eubacterial phylogenetic tree was rooted with the 16S rRNA sequence from *Escherichia coli* (Figure 2). The fungal phylogenetic trees were rooted with the LSU 28S rRNA sequence from *Saccharomyces cerevisiae* (Figure 3A) or with an EF-1 α sequence from *Dictyostelium discoideum* (Figure 3B) as appropriate to the target barcode gene. The resulting eubacterial tree revealed several major well resolved clades including those for *Enterobacter* spp., *Pectobacterium cacticida*, *Corynebacterium* spp., and *Bacillus* spp. (Figure 2). For the vast majority of isolates, the species identifiers were consistent with their placement along the relationship tree. However, there were a few exceptions including *Micrococcus* sp., *Proteus* sp., and *Citrobacter* sp.. The resulting fungal trees revealed clades structures that were entirely consistent with the species designations (e.g., *Fusarium* spp. and *Penicillium chrysogenum*) (Figure 3).

3.3. Digestion assays

All eubacterial species from the soil consortium showed some obvious degradation and solubilization of the *O. ficus-indica* cladodes transverse sections upon visual inspection (Figure 4A). In order to quantify the extent to which isolates were effective in digesting the

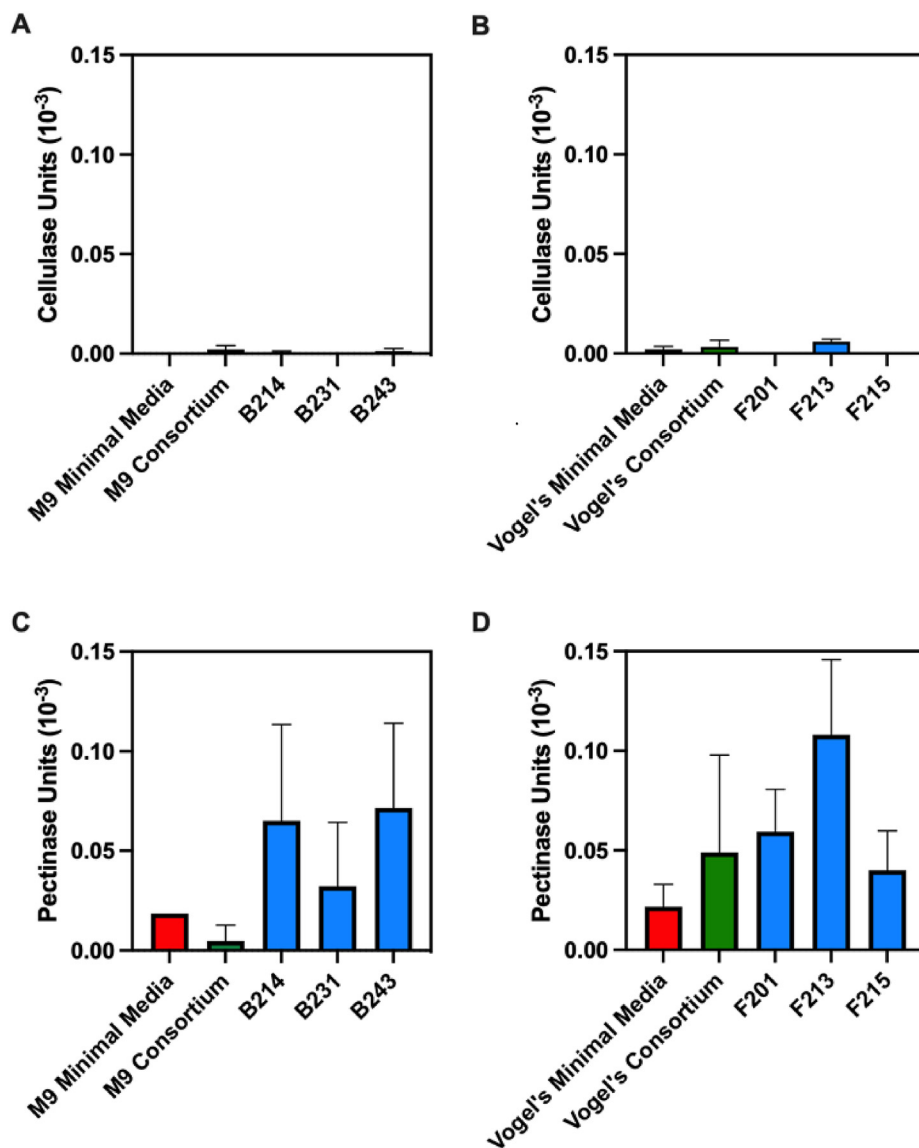


Figure 7. Evaluation of enzymatic activities in cactus pear cladode transverse sections culture supernatants. Cellulase (A, B) and pectinase (C, D) activities present in culture supernatants are shown for selected eubacterial (A, C) and fungal (B, D) axenic isolates after five days of digestion of *O. ficus-indica* transverse sections. The red bar represents the M9 or Vogel's minimal media (negative control), the green bar represents the soil microbial consortium in M9 or Vogel's minimal media (positive control), the blue bars represent selected eubacterial or fungal axenic isolates exhibiting enzymatic activity. Values represent means \pm standard error of the mean (SEM) ($n = 3$) and were tested using one-way ANOVA with Dunnett's multiple comparison test ($\alpha = 0.05$).

cladode into soluble material, a filtration-based assay was developed to separate insoluble from soluble fractions of the digested cladode. This assay was selected instead of the more traditional Brix measurements as the cladodes contained, in addition to glucose, large amounts of galacturonic acid, arabinose, galactose, xylose, mannose, and rhamnose due to their high pectin contents (Ginestra et al., 2009; Goycoolea and Cárdenas, 2003). The insoluble and soluble fractions were then dried and the ratio of soluble mass in the supernatant fraction was determined gravimetrically. Incubation of the cladode transverse sections in M9 minimal media alone served as a negative control and resulted in a mean ratio of 0.747 (Figure 5). In contrast, the soil consortium in M9 minimal media served as a positive control and resulted in a mean ratio of 0.828 (Figure 5). Six axenic isolates showed significantly higher digestions than the M9 minimal media control including B211 (0.817), B230 (0.820), B232 (0.825), B243 (0.828), B214 (0.832), and B231 (0.834) as determined using a one-way Analysis of Variance (ANOVA) with Dunnett's multiple comparison test at the 95% confidence interval.

All of the top digesting isolates belonged to either the *Enterobacter* genus (e.g., B230, B231, B232) or *Pectobacterium cacticida* (e.g., B211, B214, B243). The primary species of interest was *P. cacticida* (previously named *Erwinia cacticida*) (Motyka et al., 2017), a Gram-negative

facultative anaerobic, infectious plant pathogen that causes soft-rot in a broad plant host range that includes *O. ficus-indica* and *Agave* spp. (Alcorn et al., 1991; Jiménez-Hidalgo et al., 2004; Valenzuela-Soto et al., 2015; Varvaro et al., 1993). *P. cacticida* (*E. cacticida*) is known to cause significant disease damage in intensive cultivation settings of *O. ficus-indica* (Varvaro et al., 1993). Furthermore, *P. cacticida* and related species are known to secrete pectinases and other cell wall degrading enzymes during the progression of disease to degrade plant cell walls (Hugouvieux-Cotte-Pattat, 2016; Motyka et al., 2017). *Enterobacter* spp. are also Gram-negative, facultative anaerobic plant pathogens that cause soft-rot in a broad range of plant species including cacti (e.g., *Hylocereus* spp.) (Masayahit et al., 2009).

As top-digesting eubacterial species, both *P. cacticida* and *Enterobacter* spp. represent a useful starting point for optimization of digestion and deserve further study for their ability to efficiently digest *O. ficus-indica* biomass or as sources of digestive enzymes (e.g., pectinases, polygalacturonidases, methylesterases, acetylerases, feruloyl esterases, and xylanase) for ethanol or methane production. *P. cacticida* and *Enterobacter* utilize different enzymes to degrade biomass and may be combined to create a new, more effective mixed consortium. Isolated strains could also be subjected to selection on pectin growth media to improve their digestion efficiency.

In contrast to the eubacterial isolates, none of the fungal strains, nor microbial consortium grown on Vogel's minimal media to favor fungal growth, degraded the *O. ficus-indica* cladodes significantly better than minimal media (Figure 6). Incubation of the cladode transverse sections in Vogel's minimal media alone served as a negative control and resulted in a mean ratio of 0.801 (Figure 6). In contrast, the soil consortium in Vogel's minimal media served as a positive control and resulted in a mean ratio of 0.815 (Figure 6). The top four fungal strains, F201, F207, F213, and F215 had soluble ratios ranging from 0.826 to 0.85, which were not statistically significant from either the Vogel's minimal media or the Vogel's consortium as determined using a one-way Analysis of Variance (ANOVA) with Dunnett's multiple comparison test at the 95% confidence interval.

All of the top digesting isolates were either *Fusarium solani* (e.g., F201, F207, F213) or *Penicillium chrysogenum* (e.g., F215). *Fusarium solani* is a soil-borne plant pathogen that causes *Fusarium* wilting disease and typically attacks plant roots (Okungbowa and Shittu, 2012). *Fusarium* spp. have been documented to infect *O. ficus-indica* (e.g., *F. solani*, *F. oxysporum* f. sp. *opuntiarum*) (Ammar et al., 2004; de Souza et al., 2010). *P. chrysogenum* is commonly found on decaying vegetable matter and typically causes spoilage of pectinaceous fruits and is a well-known industrial source of Penicillin (van den Berg et al., 2007). However, *P. chrysogenum* is also a rich source of pectinase enzymes (Banu et al., 2010). The five-day digestion period might have limited the degradation and subsequent release of soluble sugars by these fungal isolates. Given the relatively slower digestion by the fungal species and low concentrations of released soluble material, these fungal isolates should be reevaluated using longer digestion times in future degradation trials.

3.4. Enzyme assays

In order to reinforce our working hypothesis that the microbial consortium and isolated strains excreted enzymes involved in cladode digestion, we assayed directly the culture supernatants from eubacterial and fungal isolates that showed the highest digestion activities for both cellulase and pectinase using a modified 3,5-dinitrosalicylic acid (DNS) reagent (Miller, 1959) and 0.5% w/v carboxymethylcellulose or 0.5% w/v citrus pectin as substrates (Deshavath et al., 2020; Vatanparast et al., 2012). Whereas only trace amounts of cellulase activity were detected in the culture supernatants of either the five-day-old, eubacterial or fungal consortia or individual axenic strains (Figure 7A, B), detectable pectinase activities were clearly present (Figure 7C, D). Compared with the undigested control samples, the eubacterial consortium or axenic isolates showed a 0.2–3.7-fold increase in pectinase activity relative to the control media supernatants. Similarly, the fungal consortium or axenic isolates showed a 1.84–5-fold increase in pectinase activity relative to the control media supernatants. While these detectable enzyme activities were not significant different as determined using a one-way Analysis of Variance (ANOVA) with Dunnett's multiple comparison test at the 95% confidence interval, the microbial cultures showed clearly detectable pectinase activities. Given the relatively slow rates of digestion, these activity differences should be reevaluated using longer digestion times in future enzymatic assays for cellulase and pectinase activities.

4. Conclusions

Previous studies have shown that biological pretreatment of *O. ficus-indica* cladodes using non-specific microbial consortia can improve methane yields from *O. ficus-indica* biomass, but targeted degradation of cladodes with defined microbial consortia or individual axenic strains has not been previously investigated. This study showed that pretreatment of *O. ficus-indica* cladodes with soil microbes improves the released soluble content from *O. ficus-indica* cladodes. Furthermore, the culture supernatants of the eubacterial and fungal consortia and isolated axenic strains contained measurable pectinase activity. Strains of *Pectobacterium cacticida* and *Enterobacter* sp. were identified as the most effective isolates

to degrade the cladodes into soluble components and are expected to provide a useful resource for future biological digestion trials of *O. ficus-indica* biomass.

Declarations

Author contribution statement

Brittany B. Blair; John C. Cushman: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Won Cheol Yim: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplemental Data Isolate number and genus/species identifiers and associated primer sequences are listed in Supplemental Tables 1 and 2.

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