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Inhibitors of biofilm formation by biofuel fermentation contaminants

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HIGHLIGHTS

- Biofilm-forming contaminants were isolated from a fuel ethanol plant.
- A variety of potential biofilm inhibitors were tested.
- Enzymes and *Bacillus* sp. culture supernatants were promising biofilm inhibitors.
- MALDI-TOF mass spectra showed lipopeptide inhibitors produced by *Bacillus* sp.
- *Bacillus* sp. merit further study as potential inhibitors of biofilm formation.

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ABSTRACT

Biofuel fermentation contaminants such as *Lactobacillus* sp. may persist in production facilities by forming recalcitrant biofilms. In this study, biofilm-forming strains of *Lactobacillus brevis*, *Lactobacillus fermentum*, and *Lactobacillus plantarum* were isolated and characterized from a dry-grind fuel ethanol plant. A variety of potential biofilm inhibitors were tested, including microbial polysaccharides, commercial enzymes, ferric ammonium citrate, liamocins, phage endolysin, xylitol, and culture supernatants from *Bacillus* sp. A commercial enzyme mixture (Novozyme 188) and culture supernatants from *Bacillus subtilis* strains ALT3A and RPT-82412 were identified as the most promising biofilm inhibitors. In biofilm flow cells, these inhibitors reduced the density of viable biofilm cells by 0.8–0.9 log cfu/cm^2. Unlike *B. subtilis* strain RPT-82412, *B. subtilis* strain ALT3A and Novozyme 188 did not inhibit planktonic growth of *Lactobacillus* sp. MALDI-TOF mass spectra showed the production of surfactin-like molecules by both *B. subtilis* strains, and the coproduction of iturin-like molecules by strain RPT-82412.

1. Introduction

Commercial biofuel fermentations are not conducted under pure culture conditions and chronic bacterial contamination is expected (Becker et al., 2011). Contaminants compete with yeast for carbon and growth factors and also produce inhibitory byproducts, such as lactic and acetic acids (Narendranath et al., 2001). Acute infections occur unpredictably, resulting in "stuck" fermentations that require costly shut-downs for cleaning and disinfection. A variety of Gram positive and Gram negative bacteria (Lushia and Heist, 2005) have been isolated from biofuel fermentations. However, lactic acid bacteria, particularly *Lactobacillus* sp., have been identified as major contaminants of both conventional and cellulosic fermentations (Skinner and Leathers, 2004; Schell et al., 2007). Biofuel production facilities often treat chronic contamination problems with antibiotics, either prophylactically or in response to elevated levels of organic acids (Bayrock et al., 2003). While the effectiveness of this approach is unclear, evidence suggests that the use of antibiotics selects for resistance among contaminants (Bischoff et al., 2007; Lushia and Heist, 2005). Furthermore, regulatory constraints may limit the future use of antibiotics in biofuel production.

In a longitudinal study, three individual biofuel production facilities exhibited characteristic profiles of lactic acid bacteria over a nine month period, suggesting persistent endemic infections typical of biofilms (Skinner and Leathers, 2004). Subsequently, biofuel

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contaminants were shown to form biofilms under laboratory conditions (Skinner-Nemec et al., 2007). Pure cultures of biofuel contaminants showed strain-specific capacities to form biofilms (Rich et al., 2011). Biofilms are difficult to remove by cleaning and are generally considered to be resistant to antimicrobial agents (Stewart et al., 2004). In biofuel production facilities, biofilms might occur in saccharification tanks or continuous yeast propagation systems, and persist in heat exchangers or dead-end pipes.

Research has identified a number of potential biofilm control approaches, including novel antimicrobial agents, physicochemical approaches, enzymatic treatments, and inhibitors of quorum sensing (Yang et al., 2012). Nevertheless, biofilms continue to be a major problem in industrial and clinical settings. Furthermore, little is known about biofilms involving *Lactobacillus* sp., and particularly those strains contaminating biofuel fermentations. Biofilm control solutions for the biofuel industry will have to be relatively inexpensive, stable under fermentation conditions, and suitable for large-scale operations. In the current study, strains of *Lactobacillus* sp. were isolated as biofilm-forming contaminants of biofuel fermentations, and a variety of potential inhibitors were tested for their ability to reduce biofilm formation by these contaminants.

2. Methods

2.1. Bacterial strains used in this study

Biofuel contaminant strains (*L. fermentum* strains BR0909#5.10 and BR0909#6.24; *L. plantarum* strains BR0909#5.11 and BR0909#5.38; and *L. brevis* strains BR0909#5.19 and BR0909#5.37) were isolated for this study as described below. *Bacillus cereus* strain MR1 and *B. subtilis* strain ALT3A were previously isolated from environmental sources in Thailand and described as producing antibacterial activities against some strains of lactic acid bacteria that contaminate fuel ethanol plants (Manitchotpisit et al., 2013). *B. subtilis* strain RPT-82412 was isolated as a laboratory contaminant (Peoria, IL) of *Schizophyllum commune* cultures that exhibited a zone of growth inhibition on solid medium (Table 1).

2.2. Isolation of biofuel contaminants

More than one thousand contaminants were collected from a Midwestern dry-grind biofuel ethanol facility over a two-year period. Samples were collected from the combined liquefaction tank, prop tank, early fermentor, and late fermentor. Bacterial contaminants were isolated as previously described (Skinner and Leathers, 2004). Samples were serially diluted in phosphate buffered saline and spread on deMan-Rogosa-Sharpe (Difco MRS, Becton Dickinson, Sparks, MD) solid medium with 0.001% (w/v) cycloheximide to suppress yeast growth. Plates were incubated anaerobically at 37 °C using the Mitsubishi AnaeroPack system (Thermo Fisher Scientific, Pittsburg PA) or the GasPak EZ System (Becton Dickinson) and strains were purified twice as single colonies and stored at −80 °C in 40% (v/v) glycerol in 96-well microtiter plates.

2.3. Identification of bacterial strains

Strains were characterized by the sequences of their 16S rRNA genes as previously described (Bischoff et al., 2007). Primers were U1 (5′-CCAGCAGCCGCGGTAATACG-3′, corresponding to nucleotides 518–537 of the *Escherichia coli* 16S rRNA gene) and U2 (5′-ATCGGYTACCTTGTTACGACTTC-3′, corresponding to nucleotides 1513–1491 of the same gene), as described by Lu et al. (2000). PCR utilized the HotMaster Taq DNA Polymerase kit (5 Prime, Gaithersburg, MD). Buffer contained 25 mM TAPS, pH 9.3, 2.5 mM $MgCl_2$, 50 mM KCl, 1.0 mM β-mercaptoethanol, and 0.2 mM dNTPs. Denaturation was at 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 s, 45 °C for 10 s, and 65 °C for 50 s. The resulting products (about 1000 bp) were sequenced by standard methods using the U1 primer. GenBank accession numbers for the strains used in this study are reported in Table 1. Sequences were compared with those in GenBank using BLASTn (Altschul et al., 1997) available at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Putative species identifications were based on similar strains with the highest BLAST scores, which in all cases shared greater than 98% identity to a known species.

2.4. Assays for inhibition of yeast fermentation of corn mash

Biofuel contaminants were assayed for inhibition of yeast fermentations of corn mash using a model system as previously described (Bischoff et al., 2009). Cultures were 40 mL in 50 mL flasks, containing corn mash (33% solids) from a commercial dry-grind ethanol facility and supplemented with 0.12% (w/v) ammonium sulfate and 0.05% (v/v) glucoamylase (Optidex L-400, Genencor International Inc., Rochester, NY). Preinocula of *Saccharomyces cerevisiae* strain NRRL Y-2034 (ARS Culture Collection, Peoria, IL) and *Lactobacillus* sp. strains were harvested by centrifugation and resuspended in phosphate buffered saline to an OD_{600} of 80 and 8 for the yeast and bacteria, respectively. Cultures were inoculated with 0.5 mL of each dilution, equivalent to final concentrations of 6×10^7 yeast cells/mL of and 1×10^7 bacterial cells/mL. Flasks were capped with a vented rubber stopper and incubated at 32 °C and 100 rpm for 72 h. Ethanol, glucose, lactic acid, and acetic acid were determined by HPLC using a 300 mm Aminex HPX 87H column (Bio-Rad Laboratories, Inc., Hercules, CA) on a HP 1100 Series HPLC system equipped with a refractive index detector (Agilent Technologies, Santa Clara, CA).

2.5. Potential inhibitors of biofilm formation tested in this study

Virginiamycin was purchased from Research Products International Corp., Mt. Prospect, IL. Alternan was prepared as previously

Table 1
Strains of *Bacillus* and *Lactobacillus* spp. used in this study.

Strain number	Species	Isolation site and date	GenBank accession number
ALT3A	*B. subtilis*	Mangrove leaf, Thailand, June 2010[a]	KJ685547
MR1	*B. cereus*	Mangrove leaf, Thailand, June 2010[a]	KJ685546
RPT-82412	*B. subtilis*	Laboratory contaminant, USA, August 2012[b]	KJ685545
BR0909#5.10	*L. fermentum*	Combined liquefaction tank, USA, September 2009[b]	KJ685539
BR0909#5.11	*L. plantarum*	Combined liquefaction tank, USA, September 2009[b]	KJ685540
BR0909#5.19	*L. brevis*	Combined liquefaction tank, USA, September 2009[b]	KJ685541
BR0909#6.24	*L. fermentum*	Early fermentation tank, USA, September 2009[b]	KJ685542
BR0909#5.38	*L. plantarum*	Combined liquefaction tank, USA, September 2009[b]	KJ685543
BR0909#5.37	*L. brevis*	Combined liquefaction tank, USA, September 2009[b]	KJ685544

[a] Manitchotpisit et al. (2013).
[b] This study.