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Resolving bacterial contamination of fuel ethanol fermentations with beneficial bacteria – An alternative to antibiotic treatment☆

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ABSTRACT

Fuel ethanol fermentations are not performed under aseptic conditions and microbial contamination reduces yields and can lead to costly "stuck fermentations". Antibiotics are commonly used to combat contaminants, but these may persist in the distillers grains co-product. Among contaminants, it is known that certain strains of lactic acid bacteria are capable of causing stuck fermentations, while other strains appear to be harmless. However, it was not previously known whether or how these strains interact one with another. In this study, more than 500 harmless strains of lactic acid bacteria were tested in a model system in combination with strains that cause stuck fermentations. Among these harmless strains, a group of beneficial strains was identified that restored ethanol production to near normal levels. Such beneficial strains may serve as an alternative approach to the use of antibiotics in fuel ethanol production.

1. Introduction

Nearly 15 billion gallons of fuel ethanol are produced each year in the US from the yeast fermentation of corn starch (ethanolrfa.org and US Department of Energy, Energy Information Administration). However, a variety of factors dictate that fuel ethanol fermentations are not produced under aseptic conditions, and chronic and acute infections are commonplace (Connolly, 1999; Beckner et al., 2011; Brexó and Sant'Ana, 2017). A wide variety of bacterial and fungal contaminants have been identified using both culture-dependent and independent approaches (Skinner-Nemec et al., 2007; Beckner et al., 2011; Rich et al., 2015; Li et al., 2016). Among these contaminating microorganisms in the corn mash-to-ethanol process, the lactic acid bacteria (LAB) are widely considered to be the most problematic (Skinner and Leathers, 2004; Bischoff et al., 2009; Rich et al., 2015). Indeed, the production of acetic acid by heterofermentative LAB is problematic for yeast production of ethanol from corn mash (Rich et al., 2015). Ethanol production from sugarcane is also plagued by chronic microbial contamination (Brexó and Sant'Ana, 2017).

Chronic bacterial contamination reduces both the sugar available for conversion to ethanol and the essential micronutrients required for optimal yeast growth, resulting in reduced ethanol production. Acute infections occur unpredictably, and the accumulation of bacterial by-products, such as acetic and lactic acids, inhibits yeast growth and may result in "stuck" fermentations that require costly shut-downs of facilities for cleaning (Makanjuola et al., 1992; Narendranath et al., 1997). Despite efforts to prevent contamination with extensive cleaning and disinfecting procedures, numerous factors can impede these efforts, such as contaminated saccharification tanks or continuous yeast propagation systems which can act as reservoirs of bacteria to continually reintroduce contaminants (Skinner and Leathers, 2004; Skinner-Nemec et al., 2007). Similarly, contaminants that form biofilms in fermentor tanks and pipes are costly and difficult to remove (Skinner-Nemec et al., 2007; Rich et al., 2015).

A variety of antimicrobial agents are used to treat chronic and acute infections, including antibiotics used in clinical and veterinary medicine (e.g., erythromycin, penicillin and tetracycline). The most commonly used antibiotics used in fuel ethanol production in the US are virginiamycin, penicillin and erythromycin (Connolly, 1999; Lushia and Heist, 2005). Regular dosing with antibiotics in industrial fermentations is known to lead to bacterial antimicrobial resistance (Bischoff et al., 2007). The problems with antimicrobial resistance are well-known (Lutgring et al., 2017), and are further complicated by the presence of biologically active virginiamycin in the distillers grains

Abbreviations: Lb., Lactobacillus; LAB, lactic acid bacteria

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. USDA is an equal opportunity provider and employer.

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coproduct of fuel ethanol production (Benjamin, 2008; Luther, 2012; Bischoff et al., 2016).

Other commercially available chemical-based products include hop acids and chlorine dioxide. Since treatment for contamination is often prophylactic, necessitating the addition of antibiotics to each fermentation batch, control and treatment of bacterial contamination is a critical control point for decreasing costs and improving efficiency of ethanol production. We describe herein an alternative to antibiotics for resolving the deleterious effects of bacterial contamination on yeast-production of fuel ethanol.

2. Materials and methods

2.1. Materials

Corn mash was obtained from a commercial dry-grind ethanol facility and stored at −20 °C and used as previously described (Bischoff et al., 2009; Rich et al., 2015). Although the corn mash was not sterilized, plating of corn mash samples on MRS agar did not detect bacteria in the mash (< 10^2 CFU/ml) (Bischoff et al., 2009). Microbial strains were isolated from a Midwestern dry-grind fuel ethanol plant and selected from a previous screen (Rich et al., 2015). Specifically, 516 strains that did not inhibit *Saccharomyces cerevisiae* production of ethanol from the previous screen and three additional strains were employed as potentially beneficial strains against a model inhibitory strain, *Lactobacillus fermentum* 0315-1 (Bischoff et al., 2009). *Saccharomyces cerevisiae* (NRRL Y-2034), *Lb. paracasei* (B-50314) and *Oenococcus oeni* (B-3472 and B-3473) strains were obtained from the ARS (NRRL) Culture Collection (Peoria, IL).

2.2. Stuck fermentation model

Saccharomyces cerevisiae NRRL Y-2034 pre-inocula were grown in 300 mL Erlenmeyer flasks with 50 mL yeast peptone (YP) media supplemented with 5% (w/v) dextrose at 28 °C and 200 rpm for 24 h. Bacterial isolates were grown in 15 mL conical tubes containing 10 mL MRS liquid medium and incubated statically at 37 °C for 24 h. Cells were harvested by centrifugation at 3,220*g* for 15 min at 4 °C and inocula prepared by resuspending cells in phosphate buffered saline to a density of OD600 equivalent to 400 and 80 for yeast and bacteria, respectively. One OD600 of *S. cerevisiae* corresponds to ca. 6 × 10^7 CFU/mL, and one OD600 of *Lactobacillus* sp. is equivalent to ca. 1 × 10^8 CFU/mL.

Model fermentations were in duplicate 40 mL cultures in 50 mL flasks containing corn mash (33% solids) supplemented with 0.12% (w/v) ammonium sulfate and 0.05% (v/v) glucoamylase (Optidex L-400, Genencor International Inc., Rochester, NY). Cultures were inoculated with 50 μL of diluted potentially beneficial bacterial cells, equivalent to 1 × 10^7 bacterial cells/mL. Sterile saline (50 μL) served as a negative control in the place of the potentially beneficial bacteria. Flasks were capped with a vented rubber stopper and incubated at 32 °C and 100 rpm. After 3 h, 50 μL of deleterious bacterial challenge, *Lb. fermentum* 0315-1 that causes a stuck fermentation (Bischoff et al., 2009), was added to each flask resulting in a 1 × 10^7 cell/mL initial concentration. Sterile saline (50 μL) served as a negative control in the place of the bacterial challenge for unchallenged control flasks. All flasks were then inoculated with 100 μL of the diluted yeast, equivalent to final concentration of 6 × 10^7 yeast cells/mL. The flask cultures with vented rubber stoppers were incubated at 32 °C and 100 rpm for 72 h.

2.3. Preparation of mixtures of challenge and beneficial strains

Model fermentations with mixtures of either beneficial or challenge strains were conducted as described above with inocula that contained 0.2 × 10^7 cells/mL of each strain to yield a total bacterial load equivalent to 1 × 10^7 bacterial cells/mL. When a mixture of bacterial strains were used against an individual strain (either challenge or beneficial), the mixture and the individual strain were inoculated with 1 × 10^7 bacterial cells/mL. The mixture of beneficial strains (Mix B) was made by mixing equal amounts of *Lb. plantarum* (1101 5.22 and 1010 5.22), *Lb. casei* (1004 6.20), *Lb. pontis* (1004 5.35) and *Lb. amylovorus* (1101 7.24). The mixture of challenge strains (Mix C) was made by mixing equal amounts of *Lb. fermentum* (1001 5.32), *Lb. plantarum* (1101 7.25), *Lb. brevis* (1101 5.1), *Lb. mucosae* (1101 7.7) and *Lb. casei* (091009 7.3).

Table 1
Impact of beneficial bacterial treatment on ethanol production by yeast challenged with *Lactobacillus fermentum* 0315-1.

Species	> 20 g/L	10–20 g/L	0–10 g/L	< 0 g/L	Total
Lactobacillus amylolyticus	2	2	3	3	10
Lb. amylovorus	1	1	1	2	5
Lb. brevis	0	1	0	0	1
Lb. casei	5	34	25	20	84
Lb. diolivorans	0	0	2	0	2
Lb. fermentum	0	3	2	12	17
Lb. hamsteri	1	6	7	7	21
Lb. harbinensis	0	1	1	1	3
Lb. helveticus	0	2	0	0	2
Lb. johnsonii	0	0	0	2	2
Lb. mucosae	0	0	6	6	12
Lb. panis	0	0	2	2	4
Lb. paracasei	0	2	1	0	3
Lb. paralimentaris	0	1	0	0	1
Lb. plantarum	29	98	81	58	266
Lb. pontis	1	16	12	5	34
Lb. rossiae	0	0	0	2	2
Lb. vaginalis	0	1	1	2	4
Lactococcus lactis	0	0	1	0	1
Oenococcus oeni	0	0	1	1	2
Staphylococcus epidermidis	0	0	2	11	13
Weissella confusa	0	1	1	0	2
mixed	2	1	4	8	15
unknown	1	5	4	3	13
Total	42	175	157	145	519

2.4. Identification of contaminant species

Bacteria were isolated from a variety of sampling points and identified by sequencing 16S rDNA as previously described (Rich et al., 2015). Of the 768 strains previously identified, we selected 516 strains (or mixtures of strains) for further examination (Table 1). Among these 516 strains, 471 (91%) were identified as one of 18 different *Lactobacillus* species, with *Lb. plantarum* and *Lb.* casei being the most commonly tested species (51% and 16%, respectively). Fifteen (3%) mixed cultures and 13 (3%) unidentified strains were also included. Three other strains from the ARS culture collection were also screened, *Lb. paracasei* (B-50314) and *Oenococcus oeni* (B-3472 and B-3473).

2.5. HPLC analysis

Samples (72 h) were harvested and clarified by centrifugation. Supernatants were diluted 10-fold into ddH_2O. Ethanol, residual glucose, lactic acid, and acetic acid concentrations were determined by high performance liquid chromatography using a 300 mm Aminex HPX-87H column (Bio-Rad) and a Shimadzu Prominence HPLC system equipped with a refractive index detector and quantified using standard curves of each compound. Samples (10 μL) were injected onto a column at 65 °C and eluted with 5 mM H_2SO_4 mobile phase at 0.6 mL/min.

3. Results and discussion

LAB are Gram-positive, non-spore forming bacteria that are aerotolerant anaerobes (da Silva Sabo et al., 2014) and have a centuries-old