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Bacterial contaminants of fuel ethanol production

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Abstract Bacterial contamination is an ongoing problem for commercial fuel ethanol production facilities. Both chronic and acute infections are of concern, due to the fact that bacteria compete with the ethanol-producing yeast for sugar substrates and micronutrients. Lactic acid levels often rise during bouts of contamination, suggesting that the most common contaminants are lactic acid bacteria. However, quantitative surveys of commercial corn-based fuel ethanol facilities are lacking. For this study, samples were collected from one wet mill and two dry grind fuel ethanol facilities over a 9 month period at strategic time points and locations along the production lines, and bacterial contaminants were isolated and identified. Contamination in the wet mill facility consistently reached 10^6 bacteria/ml. Titers from dry grind facilities were more variable but often reached 10^8/ml. Antibiotics were not used in the wet mill operation. One dry grind facility added antibiotic to the yeast propagation tank only, while the second facility dosed the fermentation with antibiotic every 4 h. Neither dosing procedure appeared to reliably reduce overall contamination, although the second facility showed less diversity among contaminants. *Lactobacillus* species were the most abundant isolates from all three plants, averaging 51, 38, and 77% of total isolates from the wet mill and the first and second dry grind facilities, respectively. Although populations varied over time, individual facilities tended to exhibit characteristic bacterial profiles, suggesting the occurrence of persistent endemic infections.

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.

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Introduction

The United States fuel ethanol industry has doubled in the last 10 years, with more than 2 billion gallons of ethanol having been produced in 2002 [1]. Fuel ethanol production has a significant impact on American agriculture, utilizing 900 million bushels of corn annually, representing 9% of the total crop [2]. Production capacity is expected to grow to more than 4 billion gallons by the year 2006 [18].

Nevertheless, the profitability of fuel ethanol production is still dependent on favorable corn prices, tax policies, and other factors. A great deal of research is underway to improve the economics of ethanol production, particularly by developing value-added coproducts and replacing corn with low cost biomass substrates [9, 17, 31]. Much less work is being done on the problem of microbial contamination of fuel ethanol fermentations.

Unlike most beverage alcohol operations, fuel ethanol fermentations are not designed to be carried out under pure culture conditions. Chronic infections are expected and tolerated, although they are generally believed to be deleterious to ethanol production. Contaminants create a constant drain on carbon available for conversion to ethanol and compete for growth factors needed by yeast. They also produce byproducts that are inhibitory to yeast, particularly lactic and acetic acids. Acute infections occur unpredictably and can lead to "stuck" fermentations, requiring that facilities be shut down for cleaning, resulting in expensive down times.

It is generally believed that lactic acid bacteria are the primary bacterial contaminants of fuel ethanol fermentations [8, 12, 14, 27]. Production facilities routinely monitor lactic and acetic acid concentrations as a practical means to judge the level of contamination.

In one survey of a commercial ethanol plant in Korea using tapioca and barley as feedstocks, "almost all" bacteria were judged to be lactic acid bacteria, with *Lactobacillus fermentum*, *L. salivarius*, and *L. casei* predominating [5].

Most recent studies have employed model laboratory systems using pure cultures of yeast and specific bacteria or their inhibitory products. Pure cultures of *Saccharomyces cerevisiae* are clearly stressed by added acetic and lactic acids, resulting in decreased rates of growth and ethanol production [19, 24, 30]. The effects of contaminating organisms are less clear.

Artificial infection of a simulated malt whiskey fermentation by *L. brevis*, *L. plantarum*, or *Leuconostoc* sp. resulted in increased acidity due to lactic acid, and reduced ethanol production and yeast growth [21]. *Lactobacillus casei* var. *pseudoplantarum* proliferated in a model beet molasses fermentation, producing lactic and acetic acids and inhibiting ethanol production rates [10]. In a model fed-batch molasses fermentation, *L. fermentum* produced lactic acid and strongly inhibited yeast fermentation after a few cell recycles [26]. When 10^5–10^9 colony forming units (CFU) of *L. plantarum*, *L. paracasei*, *Lactobacillus* #3, *L. rhamnosus*, or *L. fermentum* were introduced into wheat mash fermentations, ethanol reductions of up to 7.6% resulted, depending on the bacterial species and inoculum size [22]. On the other hand, Chin and Ingledew found that wheat mashes artificially infected with *L. fermentum* or *L. delbruekii* at 10^8 CFU/ml were not seriously impaired in ethanol productivity [7]. Thomas et al. [29] found that *L. collinoides*, *L. fermentum*, *L. plantarum*, and *L. paracasei* subsp. *paracasei* inoculated at 10^7 cells/ml did not affect fermentation rates or yeast viability in corn mashes; however, if bacteria were precultured in the mash for 24 h to $>10^9$ cells/ml, ethanol production was reduced by up to 22%. *Lactobacillus paracasei* introduced into a very high gravity multistage continuous system, even at high inoculation ratios, did not effect ethanol or lactic acid levels [4]. Thus, the deleterious effects of contamination may depend on the specific contaminants present and culture conditions employed.

Various agents have been tested for control of bacterial contaminants under laboratory conditions, including antiseptics such as hydrogen peroxide, potassium metabisulfite, and 3,4,4′-trichlorocarbanilide [6, 11, 23, 25], and antibiotics such as penicillin, tetracycline, monensin, and virginiamycin [3, 13, 28]. All of these agents differentially inhibited bacteria over yeast, although variability was found in the minimum inhibitory concentration among bacterial isolates, even of the same species. Penicillin and virginiamycin are commercially sold today to treat bacterial infections of fuel ethanol fermentations, and some facilities use these antibiotics prophylactically.

To further complicate the issue, fuel ethanol is produced by a variety of different methods. Corn generally is processed by either a wet milling or dry grind process. In wet milling, corn is initially steeped in water and treated with SO_2 at 52°C for 20–40 h [9, 15, 16]. Lactic acid bacteria proliferate under these conditions, and in fact are believed to facilitate the process. Dry grinding is a simpler process in which a crude corn mash is directly treated and fermented [9, 20]. Since dry grind operations lack a prolonged steeping period, it might be imagined that lactic acid bacteria would be less prevalent, although this is generally not believed to be the case by fuel ethanol producers (personal communications). Fermentations may be either batch or continuous. Depending on the facility design and operating practices, numerous opportunities may exist for contaminants to persist or thrive. Most operations require that yeast be propagated or conditioned before use, another potential point of contamination.

To our knowledge, no quantitative studies are available on the natural occurrence of bacteria in commercial corn-based fuel ethanol production. Consequently, an initial survey of representative facilities was performed in an effort to better define the problem.

Materials and methods

Experimental conditions

In a preliminary set of experiments, different incubation conditions were tested using seven control strains chosen from the ARS Culture Collection at the National Center for Agricultural Utilization Research (Peoria, Ill.). These were *L. brevis* strain NRRL B-4527, *L. casei* subsp. *casei* strain NRRL B-1922, *L. delbrueckii* strain NRRL B-763, *L. fermentum* strain NRRL B-4524, *L. paracasei* subsp. *paracasei* strain NRRL B-4564, *L. plantarum* strain NRRL B-4496, and *L. rhamnosus* strain NRRL B-442. The bacteria were all plated onto deMan-Rogosa-Sharpe (Difco MRS) broth (Becton Dickinson, Sparks, Md.) with 1.5% Bacto Agar (Becton Dickinson) and incubated either anaerobically using the BBL GasPak Anaerobic System (Becton Dickinson), microaerobically using the BBL Campy Pouch Microaerophilic System (Becton Dickinson) or aerobically at either 28, 30, or 37°C. In all cases, best growth was obtained under anaerobic conditions at 37°C.

The identification test kits were also tested preliminarily on these seven known strains. Both API and Biolog assays gave the most consistent and accurate results when incubated at 37°C. As suggested by the manufacturer, the Biolog system proved most consistent and accurate when organisms were subcultured twice on special blood agar plates.

Treatment of samples

One wet mill and two dry grind facilities were studied. The continuous wet mill operation employed two parallel sets of five consecutive fermentation tanks that merged into two final fermentation tanks. Dry grind fuel

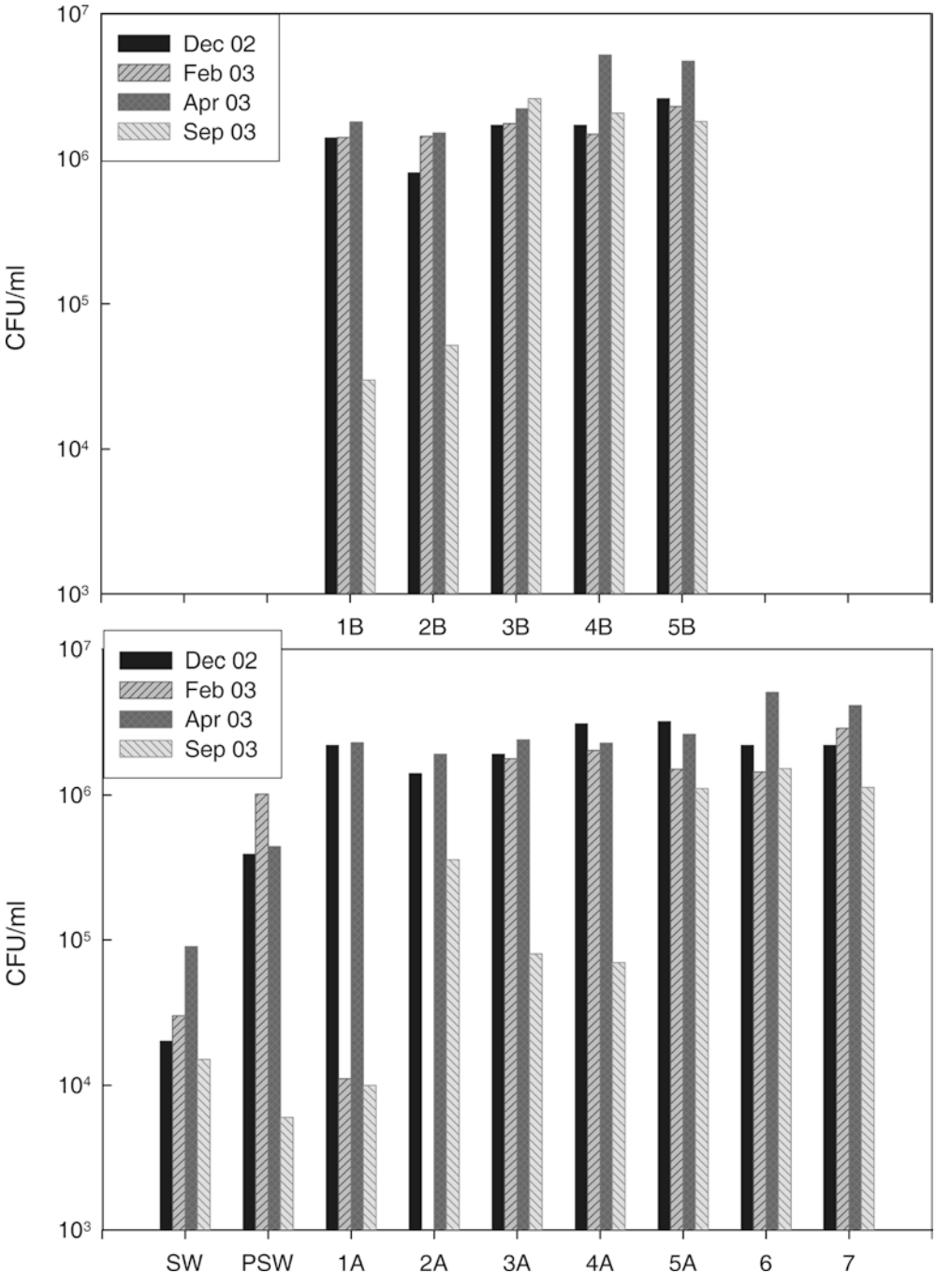

Fig. 1 Total viable bacteria [colony forming units (CFU)] from a continuous wet mill fuel ethanol facility. Corn steep water destined for the fermentation was tested before (SW) and after (PSW) pasteurization. The fermentation employed a parallel series of five fermentation tanks (1A–5A, *lower panel*; and 1B–5B, *upper panel*), which merged into sequential tanks 6 and 7 (*lower panel*). Samples were obtained over a 9-month period on the dates indicated

ethanol facility #1 used three independent batch fermentation tanks, and dry grind facility #2 had a single batch fermentation tank. Small samples (50–100 ml) from strategic points along the production lines (such as yeast prop tanks, fermentation tanks, and steep water supplies) were shipped on wet ice and stored at 4°C. Within 24 h of arrival, a series of dilutions was made in MRS and at least three dilutions plated in duplicate onto MRS plates supplemented with 0.001% cycloheximide (Sigma, St. Louis, Mo.) to suppress yeast growth. Appropriate dilutions were counted for total CFU, and random colonies were single-colony isolated three times before being tested with the identification kits.

API tests

Isolates were grown on solid MRS medium and identified to the species level and numbered biotype with the API 50 CHL test kit (bioMerieux, Montreal, QC, Canada) as per company directions. Briefly, bacteria were harvested with a sterile cotton swab into the API 50 CHL medium to a density of 2 McFarland units using an ATB Densimat densitometer (bioMerieux). The bacterial suspension was then distributed into each of the 50 wells on the API test strips, covered with a few drops of mineral oil and incubated at 37°C for 48–72 h. The color change was then graded on a scale of 0–5, with

Table 1 Bacterial species identified from a continuous wet mill fuel ethanol facility using no antibiotics. All values are rounded to the nearest percent

	February 2003	April 2003	September 2003
Bifidobacterium sp.	20[a]	2	0
B. adolescentis	(56)[b]		
B. angulatum	(22)	(100)	
Unidentified *Bifidobacterium* sp.	(22)		
Clostridium sp.	7	9	0
C. aerotolerans	(67)		
C. clostridiiforme	(33)	(100)	
Eubacterium biforme	0	0	2
Lactobacillus sp.	44	48	60
L. acidophilus 1	(10)	(4)	(11)
L. acidophilus 3			(14)
L. brevis 3			(4)
L. buchneri		(8)	
L. casei			(4)
L. crispatus	(15)	(8)	(18)
L. delbrueckii subsp. *delbrueckii*	(45)	(39)	(21)
L. delbrueckii subsp. *lactis*			(11)
L. fermentum	(15)		
L. hilgardii	(5)		(4)
L. lindneri			(4)
L. paracasei subsp. *paracasei*			(7)
L. pentosus		(4)	
L. reuteri		(19)	
Unidentified *Lactobacillus* sp.	(10)	(19)	(4)
Lactococcus lactis subsp. *lactis*	0	4	0
Leuconostoc sp.	0	6	4
L. carnosum		(33)	
L. citreum		(67)	(50)
L. lactis subsp. *lactis* 1			(50)
Pediococcus sp.	2	0	6
P. damnosus 2			(67)
P. parvulus			(33)
Unidentified *Pediococcus* sp.	(100)		
Weisella paramesenteroides	0	0	2
Unidentified	28	32	26
Sample size	46	54	47

[a]Values represent occurrence of the genus within the sample (percent of total isolates)
[b]Values in parentheses are percent of species within the genus

3–5 counting as positive, 2 as doubtful, and 0–1 as negative. The data were then entered into the APILab Plus computer program version 3.3.3 (bioMerieux) and the isolate identified.

Biolog tests

Isolates were also identified using the Biolog system (Biolog, Hayward, Calif.) as per company directions. Briefly, single colonies from MRS plates were subcultured twice on Biolog Universal Anaerobe (BUA) blood agar plates (Oxyrase, Mansfield, Ohio) before being harvested for identification. Each anaerobic inoculating fluid (AN-IF) tube was blanked individually on a turbidimeter (590 nm) before cells were introduced. Cells were harvested from the BUA plates with a sterile cotton swab and used to make a suspension of transmittance level 65%. This cell suspension then was used to inoculate the wells of AN microplates (Biolog). The microplates were held under aerobic conditions for 10–15 min and then incubated under anaerobic conditions using a Mitsubishi Anaero-pack system with rectangular jar and indicator (Mitsubishi Gas Chemical, Tokyo, Japan) at 37°C for 20–24 h. The optical densities of the wells were read on a MicroStation Universal Microbiology Workstation dual channel plate reader (Biolog) at 590 and 750 nm and analyzed using the Biolog MicroLog 3 release 4.20 program and AN database software, version 6.01 (Biolog).

Choice of tests

It was necessary to use both the API strip and Biolog methods of identification. These two identification systems differed primarily in their data base sets. For instance, the Biolog system identified some species that are not present in the API data base, such as *Weisella confusa* and *L. amylovorus*. On the other hand, the API assay provided subspecies identification of certain species, such as *L. brevis* and *L. acidophilus*. When identifications based on two test methods did not agree, the identification with the higher confidence value was chosen. Overall, the API assay was used for 33% of identifications, the Biolog assay was used for 41% of identifications, and the tests agreed in 25% of cases.

Results and discussion

Continuous wet mill

Samples were obtained from a continuous wet mill facility in December 2002 and in February, April, and September of 2003, representing a 9 month span of weather conditions and processing variables. Total viable bacteria (CFU) were determined as described from samples taken from steep water before and after pasteurization, and from two parallel sets of consecutive fermentation tanks (1–5) that merged into one common tank (6) before ending in a final tank (7) (Fig. 1). Steep water provides yeast nutrients essential for the ethanol fermentation process. Before pasteurization, steep water contained from 10^4–10^5 CFU/ml. Surprisingly, on three of four sampling dates, steep water showed higher bacterial counts after pasteurization than before. This may suggest an early point of contamination in the process, for example by cross-contamination through heat exchangers. All fermentations contained on the order of 10^6 bacteria/ml by the time the fermenting mash reached tanks 5A and 5B, and many reached this level more quickly.

Isolates were purified and identified from each of the samples (Table 1). Lactic acid bacteria commonly associated with food products made up the majority of identified isolates. *Lactobacillus* sp. made up 44–60% of

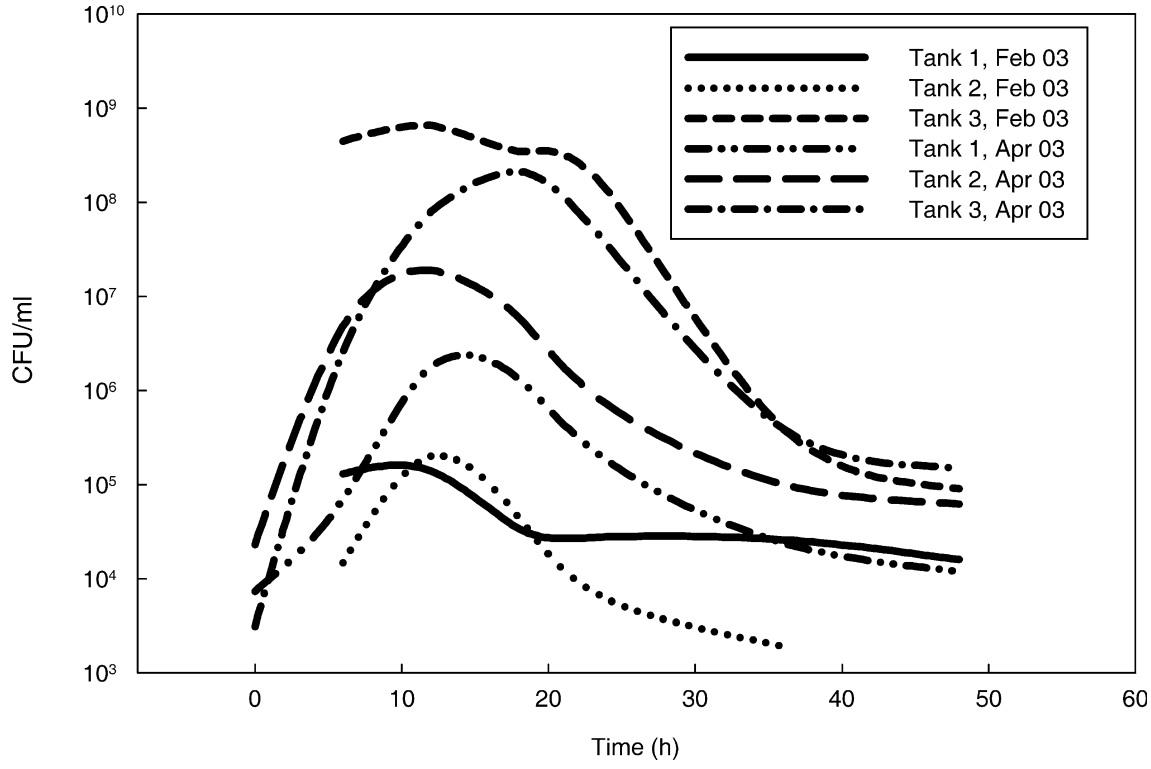

Fig. 2 Total viable bacteria (CFU) from batch dry mill fuel ethanol facility #1. Three independent batch fermentation tanks were sampled over the course of the fermentation on the dates indicated

the total isolates. Among *Lactobacillus* isolates identified to species, *L. delbrueckii* subsp. *delbrueckii* was the most abundant, consistently comprising 21–45% of *Lactobacillus* isolates. This species is homofermentative, producing lactic acid as the major product from glucose. *L. acidophilus* and *L. crispatus* were also commonly found. *Bifidobacterium* isolates were abundant only in samples from February 2003, perhaps reflecting a seasonal or random aberration. Other isolates present at lower frequencies included species of *Clostridium*, *Eubacterium*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weisella* (Table 1).

Batch dry grind facility #1

A more limited number of sampling dates (February and April 2003) were available from a batch dry grind facility that employed three independent fermentation tanks. A single dose of antibiotic (virginiamycin) was added to the yeast propagation tank, but no additional antibiotic was added to any of the fermentation tanks. Samples were obtained over the course of 48 h batch fermentations (Fig. 2). Available samples from the yeast propagation tank (shown as 0 h) were relatively low in total bacterial counts, from 3×10^3 to 2×10^4 CFU/ml. Bacteria grew during the fermentation, reaching peak levels of 10^5–10^8 CFU/ml at 12–18 h before falling to lower levels by the end of the process. As shown, indi-

Table 2 Bacterial species (percent of total) identified from a batch dry grind fuel ethanol facility #1. All values are rounded to the nearest percent

	February 2003	April 2003
Bacteroides forsythus	1[a]	0
Bifidobacterium sp.	1	2
Lactobacillus sp.	37	39
L. acidophilus 1		(8)[b]
L. acidophilus 3	(4)	
L. amylovorus		(3)
L. brevis 2		(8)
L. brevis 3		(8)
L. buchneri		(3)
L. casei	(8)	
L. delbrueckii subsp. *lactis*	(31)	(19)
L. gasseri	(4)	(28)
L. paracasei subsp. *paracasei*	(19)	(3)
L. plantarum	(4)	(8)
L. rhamnosus	(12)	
Unidentified *Lactobacillus* sp.	(19)	(11)
Lactococcus sp.	6	0
L. lactis subsp. lactis	(50)	
L. raffinolactis	(50)	
Leuconostoc sp.	1	8
L. citreum		(71)
L. mesenteroides subsp. *cremoris*	(100)	(29)
Pediococcus sp.	24	19
P. acidilactici	(18)	(18)
P. pentosaceus 1	(82)	(71)
Weisella confusa	18	24
Unidentified	11	9
Sample size	71	92

[a]Values represent occurrence of the genus within the sample (percent of total isolates)
[b]Values in parentheses are percent of species within the genus

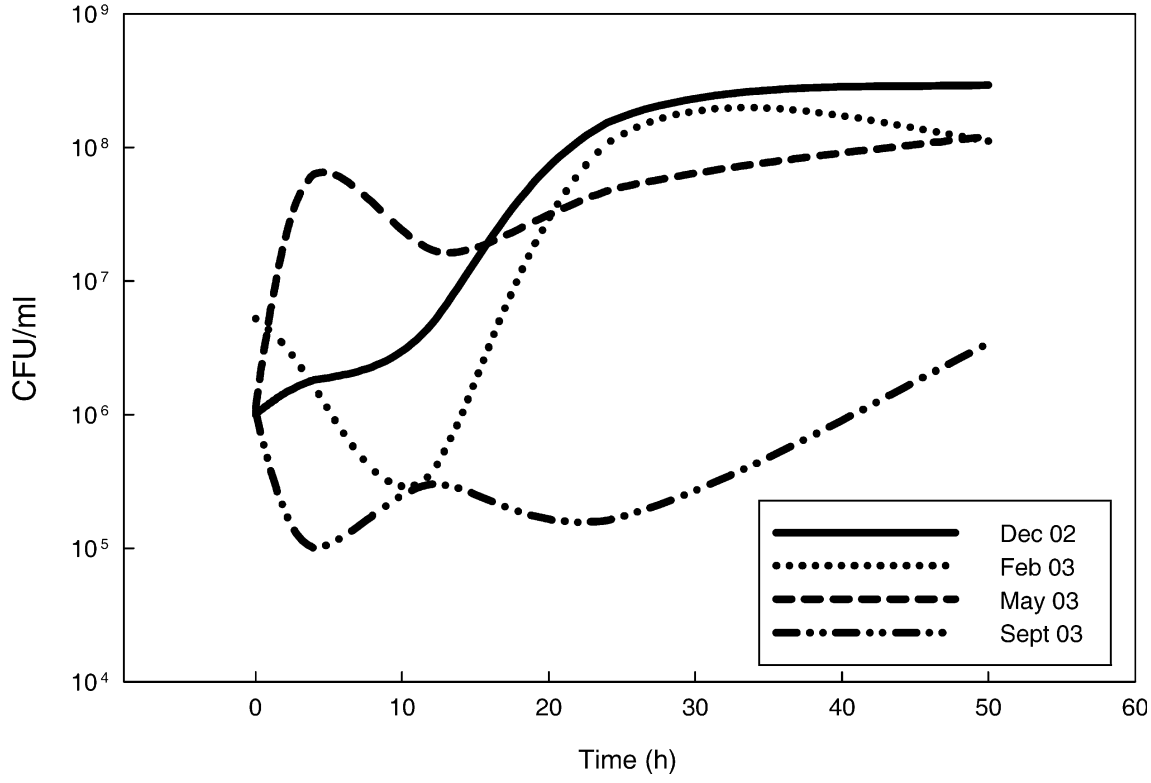

Fig. 3 Total viable bacteria (CFU) from batch dry mill fuel ethanol facility #2. The single batch fermentation tank was sampled over the course of the fermentation on the dates indicated

vidual fermentation tanks varied considerably in the level of contamination, although tank 3 showed the highest levels of bacteria in separate runs, suggesting a possible systemic problem.

Isolates were purified and identified from each of the samples (Table 2). Despite the variability in total contamination levels, the composition of contaminants was similar between sampling dates. *Lactobacillus* sp. were once again most abundant at 37–39% of isolates. Interestingly, *L. delbrueckii* subsp. *lactis* was the most commonly identified species, while the similar

Table 3 Bacterial species identified from batch dry grind fuel ethanol facility #2. All values are rounded to the nearest percent

	December 2002	February 2003	May 2003	September 2003
Fusobacterium nucleatum subsp. *nucleatum*	0[a]	0	6.3	0
Lactobacillus sp.	87	69	69	87
L. acidophilus 3	(15)[b]	(44)		(15)
L. amylovorus				(10)
L. brevis			(9)	(5)
L. brevis 2		(11)		
L. brevis 3		(11)	(36)	(15)
L. buchneri	(5)			
L. crispatus	(35)		(18)	(10)
L. delbrueckii subsp. *delbrueckii*	(10)	(11)	(18)	(5)
L. delbrueckii subsp. *lactis*	(5)			(10)
L. fermentum	(5)	(22)		(5)
L. helveticus				(5)
L. hilgardii	(5)			(10)
L. plantarum 1				(5)
L. reuteri	(5)			
Unidentified *Lactobacillus* sp.	(15)		(18)	(5)
Leuconostoc lactis	0	8	0	0
Pediococcus parvulus	0	0	0	4
Propionibacterium granulosum	0	0	0	4
Weisella viridescens	0	0	6	0
Unidentified	13	23	19	4
Sample Size	23	13	16	23

[a]Values represent occurrence of the genus within the sample (percent of total isolates)
[b]Values in parentheses are percent of species within the genus

L. delbrueckii subsp. *delbrueckii*, the most common isolate from the wet mill plant, was not found. Furthermore, the dry grind facility also differed in having larger populations of *Pediococcus* sp. and *W. confusa* (each 18–24% of total isolates). This suggests that even batch operations may have signature bacterial populations, perhaps representing long-term endemic infections.

Batch dry grind facility #2

Samples were obtained over a 9 month period, in December 2002 and February, May, and September 2003, from a second batch dry grind facility that periodically dosed the fermentation tank with antibiotic (virginiamycin) throughout the fermentation. This facility employed single tank batch fermentations (50–55 h). As shown in Fig. 3, initial bacterial populations were on the order of 10^6 CFU/ml, and grew through variable kinetics to reach maxima of approximately 10^8 CFU/ml. Unlike batch dry grind facility #1, populations were highest at the end of the fermentation. However, maximal contamination levels were similar in both cases.

Isolates were purified and identified from each of the samples (Table 3). *Lactobacillus* sp. made up from 69% to 87% of total isolates, with no other genus being strongly represented. This is the highest percentage of *Lactobacillus* sp. isolates found in the three facilities examined. *L. delbrueckii* subsp. *delbrueckii* was the most consistently identified species, although several others were common. This may suggest that periodic dosing with antibiotics selects for a less complex population of contaminants. On the other hand, the characteristic bacterial profile found at this facility may be due to other factors such as endemic infections.

Conclusions

Overall, the continuous wet mill facility exhibited the most consistent and lowest maximal levels of bacterial contamination, while the batch dry grind facility #1, which used antibiotic only in the yeast propagation tank, showed the greatest variability in contamination. Surprisingly, batch dry grind facility #2, which dosed the fermentation tank with antibiotic throughout the fermentation, typically reached high levels of bacteria, although growth kinetics differed and the diversity of genera was substantially reduced. In all cases, it is unclear whether bacterial contamination affected ethanol productivity. The three facilities examined were all considered to be ''healthy'' and performing satisfactorily. In this sense, this survey provides a baseline for normal contamination levels. Results support the generally accepted belief that lactic acid bacteria, particularly *Lactobacillus* sp., are the most prevalent contaminants of corn-based fuel ethanol production. Individual production facilities also appeared to have characteristic bacterial flora, possibly due to persistent endemic infections. Less variability was found as a function of sampling time and season, also suggesting the stability of bacterial populations.

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